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Acute/chronic pathogen infections: roles of resident, probiotic, and live biotherapeutic microbes in limiting or preventing disease

Edited by
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Acute/chronic pathogen infections: roles of resident, probiotic, and live biotherapeutic microbes in limiting or preventing disease

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Diversity recovery and probiotic shift of gastric microbiota in functional dyspepsia patients after *Helicobacter pylori* eradication therapy

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The effects of *Helicobacter pylori* eradication on gastric mucosa-colonizing microbes in patients with functional dyspepsia (FD) remain unclear. Here, we explored microbial variation induced by *H. pylori* infection and eradication treatment in FD patients. Gastric microbial abundance and diversity were significantly reduced in the *H. pylori*-infected FD patients. Eradication treatment increased alpha and beta diversity of gastric mucosa-colonizing microbes, and promoted the expansion of several probiotic microbes, such as *Leuconostoc mesenteroides*, which exhibited a matched antagonistic performance against *H. pylori*. Significant variation was observed in gastric mucosa-colonizing microbes between *H. pylori*-positive and *H. pylori*-negative FD patients. Eradication treatment induced microbial diversity recovery and may provide sufficient nutrition and space for probiotic microbes, such as *Leuconostoc mesenteroides*.

KEYWORDS

functional dyspepsia, gastric microbe, microbial diversity, *Helicobacter pylori*, eradication therapy

Introduction

Helicobacter pylori infects more than 50% of the world's population, and its histological detection in gastric tissue has a sensitivity and specificity of over 95% (Crowe, 2019). *Helicobacter pylori* community flourishing not only induces gastric microbial imbalance, but also subsequent alimentary diseases. For example, *Helicobacter pylori* infection is an important contributor of *H. pylori* associated dyspepsia, and therefore eradication is recommended as first-line therapy in infected FD patients (Talley and Ford, 2015). The recent meta-analysis, conducted by Ayesha and colleagues, further showed that postinfectious FD patients had increased duodenal eosinophils, compared with control and *H. pylori*-negative FD patients. This study also suggested

further studies were required to elevate the quality of evidences (Shah et al., 2022).

Another recent meta-analysis, conducted by Alexander and colleagues, indicates that *H. pylori* eradication therapy leads to both cure and improvement in FD symptoms (Ford et al., 2022). But complete eradication of *H. pylori* is extremely difficult, even with the use of combined and repeated medications (Chey et al., 2022; Hawkey et al., 2022; Malfertheiner et al., 2023). Correcting microbial imbalance appears to be more effective against recurrent *H. pylori* infection and overgrowth (Ford et al., 2020), although the underlying mechanism remains largely unknown. Therefore, it is crucial to study the structural characteristics of gastric microbiota in FD patients before and after eradication treatment.

As *H. pylori* infection is an important contributor of *H. pylori* associated dyspepsia, *H. pylori*-positive and *H. pylori*-negative FD patients are often prescribed different treatments (Suzuki and Moayyedi, 2013). In current clinical guidelines, sustained dyspepsia control after successful eradication identifies *H. pylori* as the organic cause, thus *H. pylori*-associated dyspepsia is considered as a separate clinical entity. Meanwhile, patients with *H. pylori* gastritis and persistent dyspepsia despite eradication therapy eliminating the infection were identified as having FD (Moayyedi et al., 2017; Miwa et al., 2022). Therefore, comparing the gastric microbiota in FD patients with and without *H. pylori* infection may provide valuable clues for overcoming recurrent *H. pylori* infection.

In addition, Moniente et al. reviewed histamine-producing bacteria (HPB), most of which colonize the human digestive tract (Moniente et al., 2021). Dual blockade of histamine receptors can be a highly effective treatment for *H. pylori*-positive FD patients, although its microbial mechanism is still unclear (Potter et al., 2020). Furthermore, while eradication drugs can induce dysbiosis of gut commensal bacteria, including HPB, the effects of *H. pylori* eradication on bacterial community structure and HPB in FD patients remain largely unknown.

This study aims to explore gastric microbial variation between *H. pylori*-negative and *H. pylori*-positive FD patients, and these *H. pylori*-positive FD patients before and after *H. pylori* eradication therapy. Our study contributes more evidences of gastric microbial shift induced by *H. pylori* infection and eradication therapy, that may profoundly influence FD progression.

Materials and methods

Subjects

The FD patients were recruited from the First People's Hospital of Yunnan Province and Third People's Hospital of Yunnan Province, China, from May 2019 to August 2020. Each FD patient received FD therapy but no use of antibiotics (within 3 months) and PPI/H2 inhibitors/NSIADs. All study protocols and procedures were approved by the Medical Ethics Board of the First People's Hospital of Yunnan Province (GXBSC-2021001, 2021 updated), China, and were carried out in accordance with all relevant provincial, national, and international guidelines, including the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their inclusion in the study.

Diagnosis of FD and *Helicobacter pylori* eradication

We used clinical feature inquiry, laboratory examination, and imaging analysis (e.g., ultrasound or computed tomography (CT)) to diagnose FD according to clinical practice guidelines (Moayyedi et al., 2017; The Committee of Digestive System Diseases, Chinese Association of Integrative Medicine, 2017; Miwa et al., 2022). Based on ROME IV- criteria, the patients were diagnosed as FD when they showed one or more the following symptoms including postprandial fullness, early satiation, epigastric pain and epigastric burning via above tests, and had no evidence of structural disease meanwhile. The symptoms had been present for the 3 months before diagnosis and symptom onset have preceded diagnosis by at least 6 months (Stanghellini et al., 2016). Gastroscopy was performed prior to eradication, and a mucosal biopsy was taken from the gastric antrum in FD patients. The C-urea breath test was applied to identify FD patients infected with *H. pylori*, with positively infected patients given bismuth quadruple therapy consisting of rabeprazole (10 mg bid), clarithromycin (250 mg bid), amoxicillin (500 mg bid), and colloidal bismuth pectin (300 mg bid) for 14 days (Liu et al., 2018; Zhou et al., 2022). For the patients with an allergy to penicillin, omeprazole was given. Twelve months later, patients underwent gastroscopy again for mucosal collection of gastric antrum and disease progression investigation. All samples were collected from May 2019 to August 2020. Upon collection, the mucosal samples were immediately placed on ice and frozen at −80°C within 1 h for microbiome analyses.

DNA extraction and Illumina MiSeq sequencing

Microbial community genomic DNA was extracted from gastric mucosa using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable V3–V4 region of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5′-ACTCCTACGGG AGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) using an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed in triplicate. Purified amplicons were pooled in equimolar concentrations and paired-end sequenced on the Illumina MiSeq PE300 platform (Illumina, San Diego, USA) using standard protocols.

Processing of sequencing data

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp v0.20.0 (Chen et al., 2018), and merged

Abbreviations: ANOSIM, Analysis of similarities; FD, Functional dyspepsia; FDR, False discovery rate; *H. pylori*, *Helicobacter pylori*; HP, *Helicobacter pylori*; LDA, Linear discriminant analysis; LEfSe, Linear discriminant analysis effect size; LM, *Leuconostoc mesenteroides*; NMDS, Non-metric multidimensional scaling; OTUs, Operational taxonomic units.

using FLASH v1.2.7 (Magoč and Salzberg, 2011). Operational taxonomic units (OTUs) with 97% similarity cutoff (Stackebrandt and Goebel, 1994; Edgar, 2013) were clustered using UPARSE v7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed using RDP Classifier v2.2 (Wang et al., 2007) against the 16S rRNA database (Silva v132) with a confidence threshold of 0.7. To remove possible contamination, we sequenced three samples of pure water as a negative control with the same procedures, including DNA extraction, PCR amplification, cDNA library construction, and final sequencing.

Bioinformatics and statistical analysis

Alpha diversity analysis

Community richness (Chao) and diversity indices (Shannon) were used to estimate alpha diversity, which was checked by Student's *t*-test and displayed in an estimation plot.

Abundance difference analysis

Significant difference of bacteria abundance uses Wilcoxon rank-sum test to apply hypothesis on genus (top 21) and phylum (top 7 or 8) levels of the microbial community of different groups of FD patients according to the community abundance data. False discovery rate (FDR) and Tukey–Kramer methods (CI = 0.95) were used for multiple testing corrections and *post-hoc* tests, respectively.

Phenotypic prediction of gastric mucosa-colonizing bacteria

Gastric mucosa-colonizing bacterial phenotypes were predicted and compared with BugBase (Ward et al., 2017) using the Kruskal-Wallis H test. Briefly, BugBase used the OTU table as an input file. The predicted 16S copy number was used to normalize the OTU table, with the preprocessed database and BugBase tool then used to automatically select thresholds to predict bacterial phenotypes.

LEfSe analysis

The nonparametric factorial Kruskal-Wallis sum-rank test was applied to detect features with significant differential abundance with respect to the class of interest. Biological significance was subsequently investigated using a set of pairwise tests among subclasses based on the Mann–Whitney-Wilcoxon test. Lastly, LEfSe analysis was applied to estimate the effect size of each differentially abundant feature and perform dimensionality reduction (Segata et al., 2011). The threshold of the logarithmic linear discriminant analysis (LDA) score for distinguishing features was set to 3.0.

Non-metric multidimensional scaling analysis

To reveal differences in the gastric mucosal bacterial communities in FD patients, UniFrac-weighted NMDS was performed based on the Bray–Curtis dissimilarity matrix using R v3.1.1 software.

Analysis of similarities

The vegan package in R was used for analysis and python was used for plotting. The R2 value obtained by ANOSIM represents the interpretation degree of sample differences between groups, i.e., ratio of group variance to total variance. Larger R2 values indicate that grouping has a higher interpretation degree to the difference, and $p < 0.05$ indicates high test reliability.

Results

Helicobacter pylori enrichment significantly decreased abundance of other gastric microbes in FD patients

Of the 98 recruited FD patients, 54 (55%) were *H. pylori*-positive based on the C-13 urea breath test and the numbers needed to treat (NNT) with eradication therapy was 44 (95% CI 39 to 62). The basic characteristics, occupations, and chief complaints of these patients are shown in Table 1. *LEfSe* analysis revealed that gram-negative proteobacterium *Neisseria subflava* was a taxonomic biomarker of *H. pylori*-positive FD patients. Analysis also indicated that *Lactobacillus* (including *Lactobacillus murinus* and *Lactobacillus iners*) and *Ochrobactrum* were taxonomic biomarkers of *H. pylori*-negative FD patients (Figure 1A). BugBase phenotypic analysis showed that *H. pylori*-positive FD patients had decreased total gram-positive bacterial abundance and increased total gram-negative bacterial abundance compared to *H. pylori*-negative patients (Figures 1C,D).

In addition to *H. pylori* and *Neisseria subflava*, other gram-negative bacteria also showed increased abundances in the *H. pylori*-positive FD patients, including *Escherichia* and *Veillonella*. Gram-positive bacteria, such as *Lactobacillus*, *Bacillus*, *Corynebacterium*, and *Bifidobacterium*, showed decreased abundances in the *H. pylori*-positive FD patients (Figure 1B). Of note, most of the gram-positive bacteria (*Lactobacillus*, *Bacillus*, and *Bifidobacterium*) exhibit probiotic potential, and *H. pylori* overgrowth may deprive these bacteria of essential nutrients and space.

TABLE 1 Basic characteristics of functional dyspepsia patients.

Index	*H. pylori* positive (*n* = 44)	*H. pylori* negative (*n* = 44)
Gender (male: female)	25:19	17:27
Age (mean ± SD)	48.74 ± 12.28	46.14 ± 12.46
Smoking (yes: no)	15:29	10:34
Antibiotic use (within 1 month)	No	No
Occupation		
Farmer	11	13
Factory worker	25	21
Office staff	2	8
Others	6	2
Chief complaint		
Epigastric pain	19	21
Epigastric burning	11	13
Postprandial fullness	5	1
Early satiety	4	2
Others	5	7

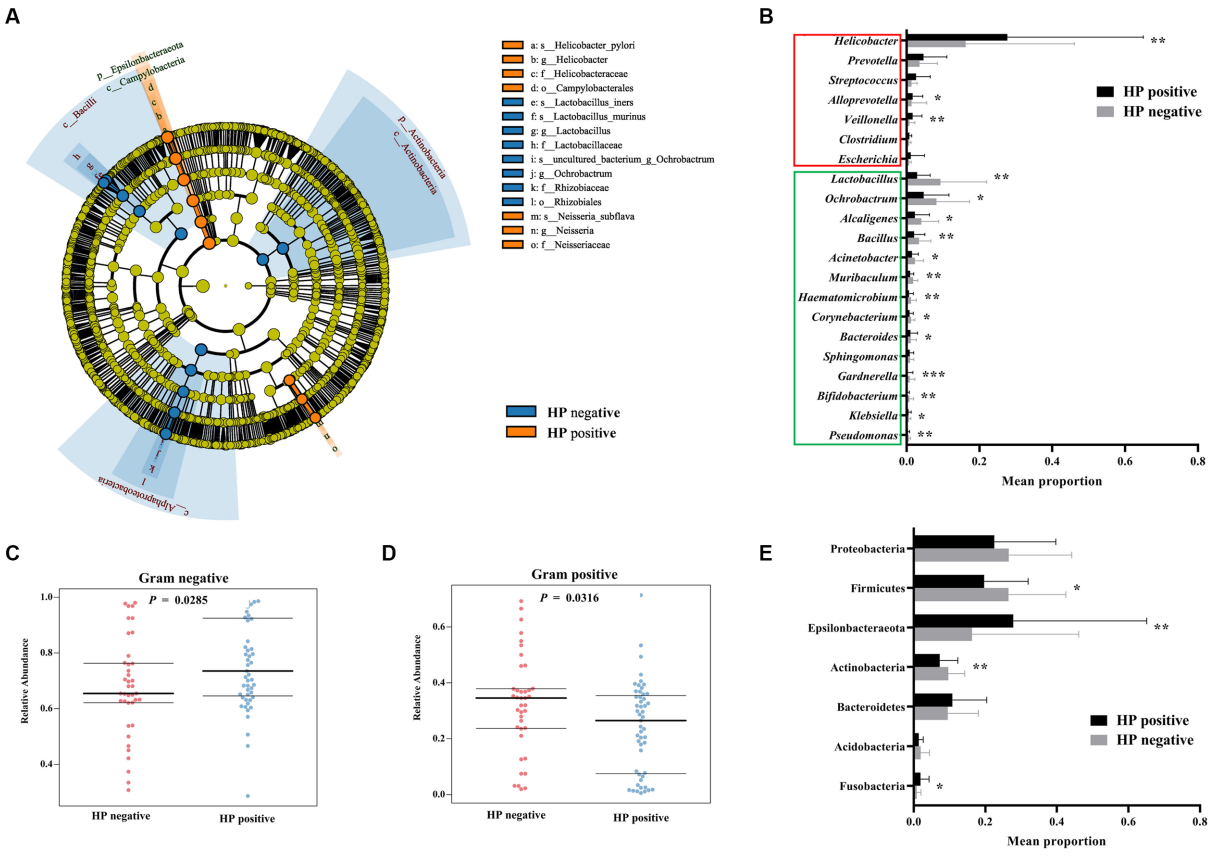


FIGURE 1
Differences in gastric bacterial abundances between *H. pylori*-positive and *H. pylori*-negative FD patients. LEfSe cladogram of potential biomarkers between *H. pylori*-positive and negative FD patients **(A)**. Wilcoxon rank-sum test comparing average composition of gastric mucosa-colonizing bacteria at genus **(B)** and phylum **(E)** level, CI = 0.95. *$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$. BugBase predicted gram negative **(C)** and positive **(D)** phenotypes of gastric mucosa-colonizing bacteria, CI = 0.95. *H. pylori* negative: n = 45; *H. pylori* positive: $n = 54$.

Most bacterial phyla (e.g., Proteobacteria, Firmicutes, Actinobacteria, and Acidobacteria) showed decreased abundances in the *H. pylori*-positive FD patients, except for Fusobacterium and Epsilonbacteraeota (Figures 1A,E).

Helicobacter pylori eradication increased gastric microbial alpha and beta diversity in FD patients

The *H. pylori*-positive FD patients were treated with bismuth quadruple therapy. Twelve months after treatment, gastric mucosa were collected by endoscopy for microbiota diversity analysis. After eradication therapy, these patients were identified to be *H. pylori*-negative via C-urea breath test, and patient's complaints were notably relieved (epigastric pain: 68% (13/19); epigastric burning: 64% (7/11); postprandial fullness: 80% (4/5); early satiety: 50% (2/4)). Alpha diversity analysis indicated that *H. pylori* eradication significantly increased the Chao (765.40 ± 188.10 to 1108.10 ± 95.55, $p < 0.0001$) and Shannon indices (5.34 ± 2.17 to 7.23 ± 1.03, $p = 0.0043$) of gastric mucosa-colonizing bacteria in the FD patients (Figures 2A,B). NMDS analysis showed that *H. pylori* eradication significantly homogenized the structure of the gastric mucosa-colonizing microbiota in the FD patients (Stress = 0.0065; Figure 2C). ANOSIM further confirmed that *H. pylori* eradication markedly transformed the structure of the gastric mucosa-colonizing microbiota in the FD patients ($R = 0.823$, $p = 0.001$; Figure 2D).

Helicobacter pylori eradication differentiated species and their potential functions

In the *H. pylori*-positive FD patients, only *H. pylori* exhibited a significant LDA score. After *H. pylori* eradication by bismuth quadruple therapy, the taxonomic biomarkers changed to *Lactobacillus* (*Lactobacillus plantarum*), *Leuconostoc* (*Leuconostoc mesenteroides*), *Muribaculum*, *Shewanella* (*Shewanella amazonensis*), and *Pantoea* (*Pantoea agglomerans*), indicating complete alteration in the structure of the gastric microbiota after *H. pylori* eradication (Figure 3A).

Specifically, *H. pylori* eradication significantly decreased the abundances of *Weissella* and *Prevotella*, which all possess pathogenic potential. Eradication significantly increased multiple bacteria, including *Pantoea*, *Shewanella*, *Leuconostoc*, *Muribaculum*, *Klebsiella*, *Serratia*, *Formosa*, *Pontibaca*, *Rouxiella*, *Akkermansia*, *Celeribacter*, *Kineothrix*, *Shimia*, *Clostridium*, and *Rahnella*. Fortunately, the abundances of bacteria known to belong to probiotic genera *Bifidobacterium* and *Lactobacillus* were not significantly changed by

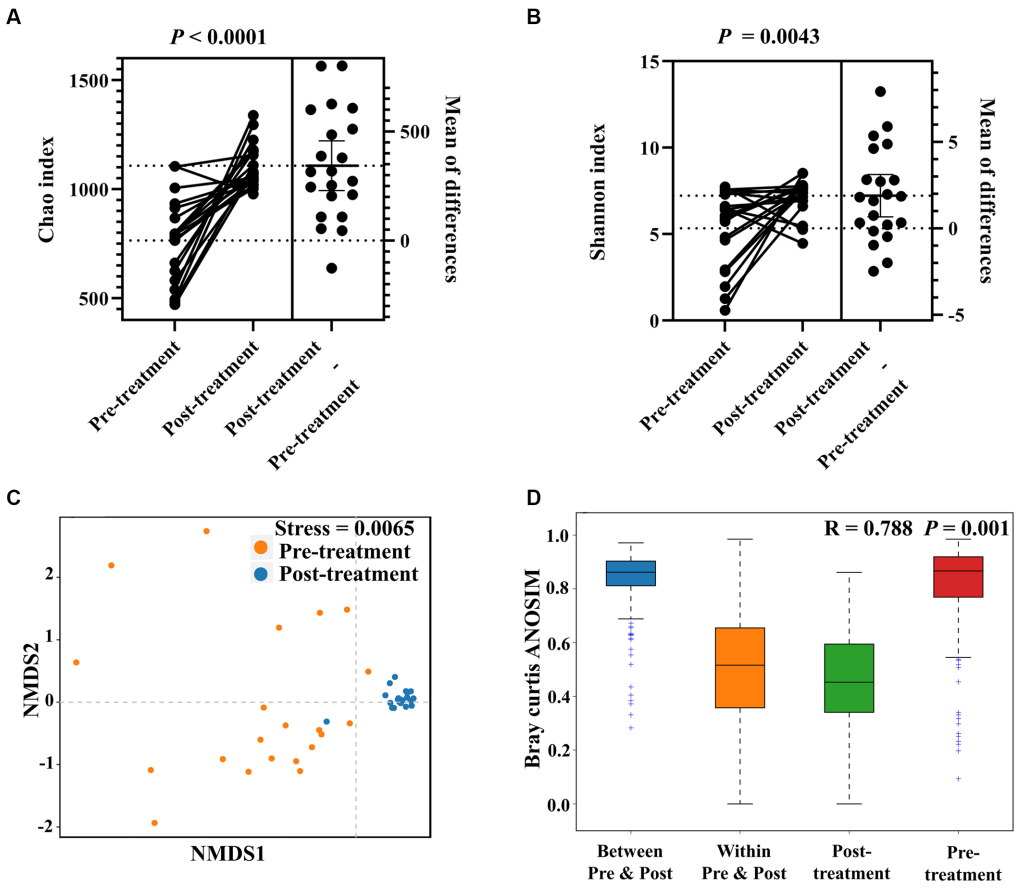


FIGURE 2
Effects of *H. pylori* eradication on gastric microbial diversity in FD patients. Alpha diversity of gastric mucosa-colonizing microbiota based on Chao index **(A)** and Shannon index **(B)**. Unweighted UniFrac NMDS of gastric mucosa-colonizing microbiota **(C)**. ANOSIM box plot based on unweighted UniFrac distance showing significant differences between pre-treatment (before *H. pylori* eradication) and post-treatment (after HP eradication) groups **(D)**.

H. pylori eradication (Figure 3B). At the phylum level, Proteobacteria, Acidobacteria, and Verrucomicrobia showed increased abundances (Figure 3C).

Furthermore, *H. pylori* eradication appears to increase the abundances of non-anaerobic bacteria. Functional prediction analysis indicated that the abundances of both aerobic and facultatively anaerobic bacteria increased significantly (aerobic: 0.25 ± 0.17 to 0.34 ± 0.09, $p = 0.0358$; aerobic: 0.09 ± 0.08 to 0.23 ± 0.26, $p < 0.0001$) after eradication treatment (Figures 3D,E). However, treatment also increased the abundances of biofilm-forming bacteria (0.35 ± 0.33 to 0.52 ± 0.54, $p = 0.0037$), which may impact biofilm-based antibiotic resistance and tolerance (Figure 3F).

Gastric bacterium of FD patients after *Helicobacter pylori* eradication can be divided into two subgroups in *Leuconostoc mesenteroides*-dependent manner

We further explored changes in gastric bacterial composition induced by eradication treatment. Results showed that *H. pylori* abundance was markedly decreased (Figure 4A), while *Leuconostoc mesenteroides* abundance was markedly increased (Figure 4B) after eradication treatment. This change in abundance of core bacteria indicated a fundamental shift in the gastric microbiota. Therefore, we divided the FD patients into two subgroups according to *Leuconostoc mesenteroides* abundance after eradication. Interestingly, nine core genera (i.e., *Pantoea*, *Serratia*, *Klebsiella*, *Rouxiella*, *Formosa*, *Celeribacter*, *Rahnella*, *Luteolibacter*, and *Gluconobacter*) showed a significant increase in abundance with the increase in *Leuconostoc mesenteroides* (Figure 4D), while 10 core genera (i.e., *Helicobacter*, *Akkermansia*, *Kineothrix*, *Clostridium*, *Alloprevotella*, *Bacteroides*, *Sphingomonas*, *Leptotrichia*, *Fusobacterium*, and *Neisseria*) showed a significant decreased in abundance (Figure 4C).

Discussion

Modulation of the microbiome in the stomach or small intestine is an important strategy for the management of *H. pylori*-positive FD patients (Ford et al., 2020). In the current study, *H. pylori*-positive FD patients showed increased levels of gram-negative bacteria, whereas *H. pylori*-negative FD patients showed increased levels of gram-positive bacteria, including probiotics such as *Lactobacillus* and *Bifidobacterium*. *Helicobacter pylori*-induced overgrowth of

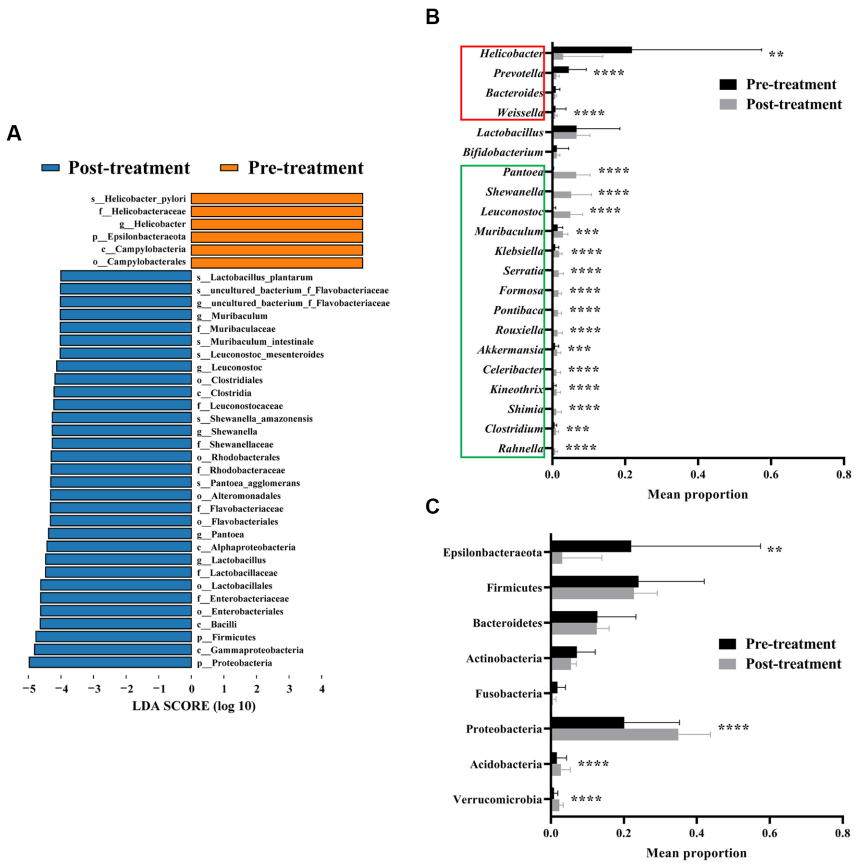


FIGURE 3
Increased gastric bacterial abundance after *H. pylori* eradication in FD patients. LEfSe histogram of LDA scores for differentially abundant features between pre-treatment (before HP eradication) and post-treatment (after *H. pylori* eradication) FD patients **(A)**. Threshold of logarithmic LDA score for discriminative features was set to 4.0. Wilcoxon rank-sum test comparing average composition of gastric mucosa-colonizing bacteria at genus **(B)** and phylum **(C)** level, CI = 0.95. *$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. BugBase predicted aerobic **(D)**, facultatively anaerobic **(E)**, and biofilm-forming **(F)** phenotypes of gastric mucosa-colonizing bacteria, CI = 0.95.

gram-negative bacteria may reduce space for probiotics, indirectly leading to dyspeptic symptoms. However, the initial drivers of gram-negative bacterial overgrowth remain to be explored.

Although *H. pylori* eradication has clear benefits in regard to improving FD symptoms (Ford et al., 2022), microbial diversity variation after treatment remains controversial. In our study, the Chao and Shannon indices of gastric mucosa-colonizing microorganisms were significantly increased in FD patients 12 months after eradication therapy. Most chief complaints of these patients were significantly released, including epigastric pain, epigastric burning and postprandial fullness. Companying these phenotypes, *H. pylori*'s dominance was replaced by *Lactobacillus* and *Leuconostoc*. Even there is no evidences of this dominance-replacing is a direct contributor of complaint relief, we recommend they are connected directly or indirectly.

Moreover, microbes that colonize different vertical layers of the gastric mucosa may be affected differently by therapeutic drugs due to distinct drug exposure. When drug exposure eradicates most non-drug resistant microbes, *H. pylori* may obtain superior nutrition and space. Phenotype prediction analysis showed increased abundance of aerobic microbes (including facultatively anaerobic) after eradication treatment. However, it is unclear whether this was due to variation in oxygen concentration of different vertical layers of the gastric mucosa or eradication by drug-specific targeting. On the other hand, the limited effects of eradication treatment may originate from incomplete deletion of *H. pylori* that colonize different vertical layers of the gastric mucosa, partially at least.

Histamine contributes to FD progression (Moniente et al., 2021), and blockade of its receptors induces a marked improvement in FD patients (Potter et al., 2020). Previous research has shown that *Leuconostoc mesenteroides* can scavenge 40% of amine-containing compounds by producing exopolysaccharides (Li et al., 2020). *Leuconostoc mesenteroides* can also effectively degrade histamine by over 85% (Lee et al., 2021). Our study showed that *H. pylori* eradication led to a significant increase in *Leuconostoc mesenteroides*, which exhibited an obvious antagonistic effect on microbial abundance of *H. pylori*. Thus, the dominant microbes were basically divided into two subgroups, and their abundances were closely correlated with either *H. pylori* or *Leuconostoc mesenteroides*. These findings would be more convincing if there was no limitation on the absence of healthy population as a control group. However, our data still remind

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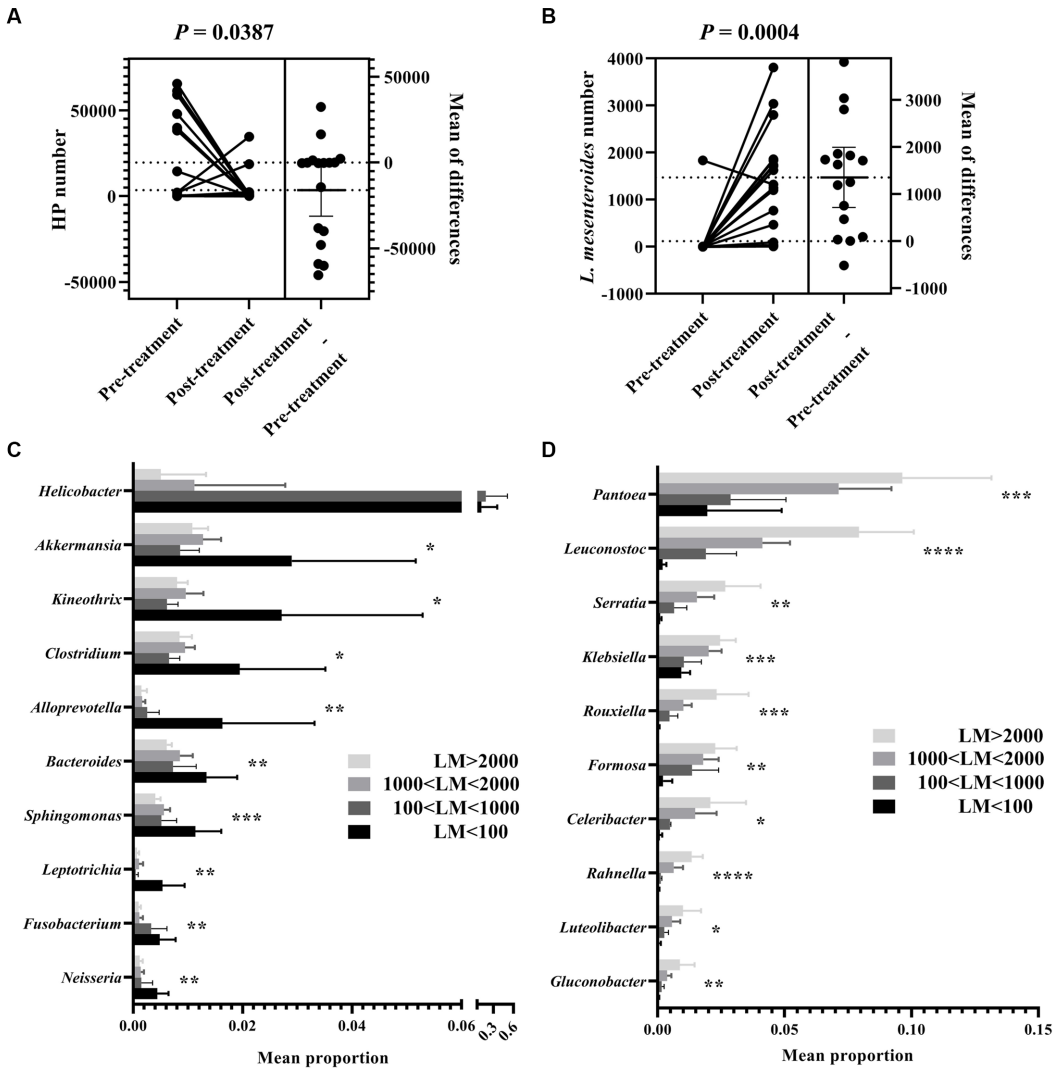


FIGURE 4
Leuconostoc mesenteroides abundance-dependent grouping of gastric bacteria in FD patients after *H. pylori* eradication. Abundance comparison of *H. pylori* **(A)** and *L. mesenteroides* **(B)** between pre-treated and post-treated FD patients. Wilcoxon rank-sum test comparing average composition of gastric mucosa-colonizing bacteria, divided into two subgroups, i.e., *H. pylori* l dominant and **(C)** and *L. mesenteroides* dominant **(D)** in FD patients after *H. pylori* eradication. LM < 100, *L. mesenteroides* number less than 100; 100 < LM < 1,000, *L. mesenteroides* number greater than 100, but less than 1,000; 1,000 < LM < 2,000, *L. mesenteroides* number greater than 1,000, but less than 2,000; LM > 2000, *L. mesenteroides* number lower than 2,000. CI = 0.95. *$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

physicians pay attention to *Leuconostoc mesenteroides* during FD patient recovery progress.

Meanwhile, our study has several limitations. Most FD patients were factory workers ($n = 46$) and farmers ($n = 24$) and came from same area, Kunming, China ($n = 83$). Their similar living and working environments may affect gastric microbiota components, leading to a microecosystem bias. The further study also should clarify the relationship between the characteristics of changes in gastric microbiota before and after *H. pylori* eradication and changes in FD symptoms.

In conclusion, our study revealed significant variation in gastric mucosa-colonizing microbes between *H. pylori*-positive and *H. pylori*-negative FD patients. Eradication treatment resulted in restoration of microbial diversity and improvement in dyspeptic symptoms, *Leuconostoc mesenteroides* may contribute to this improvement.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: https://www.ncbi.nlm.nih.gov/, PRJNA843681.

Ethics statement

The studies involving humans were approved by the Medical Ethics Board of the First People's Hospital of Yunnan Province. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

WW: Conceptualization, Funding acquisition, Investigation, Supervision, Validation, Writing – review & editing, Formal Analysis, Project administration, Software, Writing – original draft. ZL: Conceptualization, Formal Analysis, Funding acquisition, Software, Validation, Writing – review & editing. YZ: Validation, Writing – review & editing, Investigation, Methodology, Visualization. ZG: Investigation, Methodology, validation, Writing – review & editing, Resources. JL: Investigation, Methodology, Validation, Writing – review & editing, Project administration, Visualization. SL: Investigation, Methodology, Validation, Visualization, Writing – review & editing. JH: Investigation, Methodology, Validation, Visualization, Writing – review & editing. JG: Investigation, Validation, Writing – review & editing, Conceptualization, Funding acquisition, Supervision. FZ: Funding acquisition, Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing, Data curation. QG: Writing – original draft, Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Chey, W. D., Mégraud, F., Laine, L., López, L. J., Hunt, B. J., and Howden, C. W. (2022). Vonoprazan triple and dual therapy for *Helicobacter pylori* infection in the United States and Europe: randomized clinical trial. *Gastroenterology* 163, 608–619. doi: 10.1053/j.gastro.2022.05.055

Crowe, S. E. (2019). Infection. *N. Engl. J. Med.* 380, 1158–1165. doi: 10.1056/NEJMcp1710945

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Ford, A. C., Mahadeva, S., Carbone, M. F., Lacy, B. E., and Talley, N. J. (2020). Functional dyspepsia. *Lancet* 396, 1689–1702. doi: 10.1016/S0140-6736(20)30469-4

Ford, A. C., Tsipotis, E., Yuan, Y., Leontiadis, G. I., and Moayyedi, P. (2022). Efficacy of *Helicobacter pylori* eradication therapy for functional dyspepsia: updated systematic review and meta-analysis. *Gut* 71, 1967–1975. doi: 10.1136/gutjnl-2021-326583

Hawkey, C., Avery, A., Coupland, C. A. C., Crooks, C., Dumbleton, J., Hobbs, F. D. R., et al. (2022). *Helicobacter pylori* eradication for primary prevention of peptic ulcer bleeding in older patients prescribed aspirin in primary care (HEAT): a randomised, double-blind, placebo-controlled trial. *Lancet* 400, 1597–1606. doi: 10.1016/S0140-6736(22)01843-8

Lee, J., Jin, Y. H., Pawluk, A. M., and Mah, J. H. (2021). Reduction in biogenic amine content in Baechu (Napa cabbage) kimchi by biogenic amine-degrading lactic acid bacteria. *Microorganisms*. 9, 1–17. doi: 10.3390/microorganisms9122570

Li, Y., Liu, Y., Cao, C., Zhu, X., Wang, C., Wu, R., et al. (2020). Extraction and biological activity of exopolysaccharide produced by *Leuconostoc mesenteroides* SN-8. *Int. J. Biol. Macromol.* 157, 36–44. doi: 10.1016/j.ijbiomac.2020.04.150

Liu, W. Z., Xie, Y., Lu, H., Cheng, H., Zeng, Z. R., Zhou, L. Y., et al. (2018). Fifth Chinese National Consensus Report on the management of *Helicobacter pylori* infection. *Helicobacter* 23:e12475. doi: 10.1111/hel.12475

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Malfertheiner, P., Camargo, M. C., El-Omar, E., Liou, J. M., Peek, R., Schulz, C., et al. (2023). *Helicobacter pylori* infection. *Nat. Rev. Dis. Primers* 9:19. doi: 10.1038/s41572-023-00431-8

Miwa, H., Nagahara, A., Asakawa, A., Arai, M., Oshima, T., Kasugai, K., et al. (2022). Evidence-based clinical practice guidelines for functional dyspepsia 2021. *J. Gastroenterol.* 57, 47–61. doi: 10.1007/s00535-021-01843-7

Moayyedi, P., Lacy, B. E., Andrews, C. N., Enns, R. A., Howden, C. W., and Vakil, N. (2017). ACG and CAG clinical guideline: Management of Dyspepsia. *Am. J. Gastroenterol.* 112, 988–1013. doi: 10.1038/ajg.2017.154

Moniente, M., García-Gonzalo, D., Ontañón, I., Pagán, R., and Botello-Morte, L. (2021). Histamine accumulation in dairy products: microbial causes, techniques for the detection of histamine-producing microbiota, and potential solutions. *Compr. Rev. Food Sci. Food Saf.* 20, 1481–1523. doi: 10.1111/1541-4337.12704

Potter, M. D. E., Goodsall, T. M., Walker, M. M., and Talley, N. J. (2020). Dual histamine blockade for the treatment of adult functional dyspepsia: a single Centre experience. *Gut* 69:966. doi: 10.1136/gutjnl-2019-318412

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shah, A., Fairlie, T., Brown, G., Jones, M. P., Eslick, G. D., Duncanson, K., et al. (2022). Duodenal eosinophils and mast cells in functional dyspepsia: a systematic review and meta-analysis of case-control studies. *Clin. Gastroenterol. Hepatol.* 20, 2229–2242.e29. doi: 10.1016/j.cgh.2022.01.014

Stackebrandt, E., and Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* 44, 846–849. doi: 10.1099/00207713-44-4-846

Stanghellini, V., Chan, F. K., Hasler, W. L., Malagelada, J. R., Suzuki, H., Tack, J., et al. (2016). Gastroduodenal Disorders. *Gastroenterology* 150, 1380–1392. doi: 10.1053/j.gastro.2016.02.011

Suzuki, H., and Moayyedi, P. (2013). *Helicobacter pylori* infection in functional dyspepsia. *Nat. Rev. Gastroenterol. Hepatol.* 10, 168–174. doi: 10.1038/nrgastro.2013.9

Talley, N. J., and Ford, A. C. (2015). Functional dyspepsia. *N. Engl. J. Med.* 373, 1853–1863. doi: 10.1056/NEJMra1501505

The Committee of Digestive System Diseases, Chinese Association of Integrative Medicine (2017). Consensus opinion on diagnosis and treatment of functional dyspepsia with integrated traditional Chinese and Western medicine. *J. Chin. Digest.* 25, 889–894. doi: 10.3969/j.issn.1671-038X.2017.12.01

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Ward, T., Larson, J., Meulemans, J., Hillmann, B., Lynch, J., Sidiropoulos, D., et al. (2017) BugBase predicts organism-level microbiome phenotypes, 133462.

Zhou, L., Lu, H., Song, Z., Lyu, B., Chen, Y., Wang, J., et al. (2022). 2022 Chinese national clinical practice guideline on *Helicobacter pylori* eradication treatment. *Chin. Med. J.* 135, 2899–2910. doi: 10.1097/CM9.0000000000002546

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Gut microbiota and risk of lower respiratory tract infections: a bidirectional two-sample Mendelian randomization study

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Introduction: Observational studies have reported the association between gut microbiota and the risk of lower respiratory tract infections (LRTIs). However, whether the association reflects a causal relationship remains obscure.

Methods: A bidirectional twosample Mendelian randomization (MR) analysis was conducted by assessing genome-wide association study (GWAS) summary statistics for gut microbiota taxa and five common LRTIs. MR methods including inverse-variance-weighted (IVW), MR-Egger, weighted median, simple mode, and weighted mode were used to analyze the causality. Gene pleiotropy was tested using MR-Egger regression and MR-PRESSO methods. Cochran's Q test was used to check for heterogeneity. Leave-one-out analysis was used to assess the stability of effect sizes. Detected significant associations were validated by using an independent LRTI GWAS summary statistics dataset. An optional MR method of causal analysis using summary effect estimates (CAUSE) was further performed as a validation to avoid potential false-positive results.

Results: According to the MR-Egger estimates in forward MR analysis, a causal effect of gut *Blautia* on increased odds of bronchiectasis and pneumonia was suggested. MR-Egger regression pleiotropy intercept methods detected no significant horizontal pleiotropy between the instrumental variables of these associations. MR-PRESSO global test examined no potential horizontal pleiotropy. Cochran's Q test showed that no heterogeneity biased the results. The leave-one-out sensitivity analyses suggested robust causality results. These associations with consistent effect direction were successfully replicated in IVW analysis by using the validation GWAS dataset. However, these evidence of causality did not survive after applying strict Bonferroni correction or CAUSE analysis. The reverse MR analysis failed to achieve consistent results in the effect of LRTIs on gut microbiota through comprehensive discovery and validation processes.

Discussion: This study established no strong causality between genetically predicted gut microbiome and the risk of lower respiratory tract infections. However, specific subtypes of microbial genera, such as *Blautia*, were identified as potential influencers and require further investigation, particularly at the species or strain levels.

KEYWORDS

gut microbiota, lower respiratory tract infections, causal relationship, Mendelian randomization, *Blautia* genus

Introduction

Lower respiratory tract infections (LRTIs) remain a significant cause of morbidity and mortality worldwide, particularly affecting vulnerable populations such as the elderly, young children, and immunosuppressed individuals (Langelier et al., 2018). According to the Global Burden of Disease Study 2016, LRTIs were responsible for approximately 2,377,697 deaths globally in 2016, especially among people aged over 65 years (Collaborators, 2018). Despite substantial advances over the past decade, LRTIs still account for a fifth of all deaths worldwide, primarily in low- and middle-income countries. From an epidemiological point of view, definitions of LRTI mainly include bronchiectasis, pneumonia, influenza, acute bronchitis, and acute bronchiolitis (Collaborators, 2017). Treatment guidelines may vary among countries, but they generally focus on improving nutrition and hygiene, along with the distribution and usage of antimicrobial agents and vaccines (Kamata et al., 2022). Although early and appropriate antimicrobial treatment are available, many patients still succumb to LRTIs due to challenges such as drug resistance (Cazzola et al., 2017). Hence, there is a need for novel therapeutic strategies in addition to traditional antibiotics, making precision medicine for LRTIs an important approach.

In otherwise healthy individuals, the human gut is the most densely colonized organ, harboring an estimated 10^{14} bacteria from over 1,000 bacterial species (Bäckhed et al., 2005). Current studies have extensively explored the relationship between gastrointestinal microbiota and human diseases. Its importance in sustaining local and systemic tissue homeostasis has gained great recognition (Nenci et al., 2007; Gensollen et al., 2016). Furthermore, the metabolic by-products and ligands of gut commensal bacteria have the ability to modulate and fine-tune the development and function of the innate and adaptive immune system, which helps to protect against infections caused by diverse pathogens (Ichinohe et al., 2011; Abt et al., 2012). Studies taking advantage of germ-free mice and antibiotic-driven depletion of gut bacterial species have contributed significantly to the understanding of gut-lung axis in mediating a range of respiratory infectious diseases. For example, the disruption of gut microbiome development in infancy plays a role in increased susceptibility to pulmonary viral infections (Ichinohe et al., 2011) and the development of lung diseases including asthma and chronic obstructive pulmonary disease (Abrahamsson et al., 2014; Zeissig and Blumberg, 2014; Qu et al., 2022). Mechanically, gut microbes initiate the activation of the innate antiviral immune response via the interactions with pattern recognition receptors (ie., Toll like receptor) (Samuelson et al., 2015), and also contribute to the regulation of macrophage response and restoration of lung $CD4^+$ and $CD8^+$ T cells, which are crucial for improved survival in respiratory viral infections (Abt et al., 2012). Besides, imbalances in gut microbiome result in an exacerbation of cytokine-induced inflammation (Verdam et al., 2013), potentially resulting in lung morbidity. It is partially attributed to the reduced production of anti-inflammatory metabolites like short-chain fatty acids (SCFAs) (Trompette et al., 2014) and increased production of pro-inflammatory metabolites such as secondary bile acids (Duboc et al., 2013) from intestinal flora, which transported via circulation to play a role in gut-lung communication, thereby impacting respiratory health and infections. Probiotics and prebiotics have demonstrated effects in reducing the incidence of cystic fibrosis pulmonary exacerbations (Weiss et al., 2010), protecting against bacterial pneumonia (Vieira et al., 2016), and expediting recovery from respiratory viral infections (Kawahara et al., 2015; Samuelson et al., 2015). Understanding the role of gut microbiome and its influence on respiratory infections can bring further refinement to the discovery of biomarker and precision medicine for these diseases.

However, so far, it remains unclear whether there is a causal relationship between gut microbiome and LRTIs because of the confounding factors and possible reverse causation. Mendelian randomization (MR) is an emerging genetic epidemiological method that utilizes summary data from genome-wide association studies (GWASs) as instrumental variables to infer causality in exposure-outcome associations (Dan et al., 2021). It may help to identify specific causal microbe taxa to enhance the development of precision medicine in the treatment of LRTIs. Therefore, we employed a bidirectional two-sample MR analysis to investigate the causal effects of gut microbiome on the risk of five common LRTI diseases and vice versa.

Materials and methods

The study methods were compliant with the STROBE-MR checklist (Skrivankova et al., 2021). Our analysis used publicly available GWAS summary statistics. No new data were collected, and no additional ethical approval or informed consent was required. The flowchart of the study was shown in Figure 1.

Data sources

SNPs related to human 16S fecal microbiome composition were selected as instrumental variables from a large-scale multi-ethnic GWAS meta-analysis with 24 cohorts, comprising 18,340 individuals of different ethnicities and ages from the USA, Canada, Israel, South Korea, Germany, Denmark, the Netherlands, Belgium, Sweden, Finland, and the UK, most of whom had European ancestry ($n = 13,266$) (Kurilshikov et al., 2021). The microbial composition was profiled by targeting three distinct variable regions of the 16S rRNA gene, including V4 (10,413 samples, 13 cohorts), V3-V4 (4,211 samples, 6 cohorts), and V1-V2 (3,716 samples, 5 cohorts).

The discovery summary-level data of SNPs associated with the five common LRTI subtypes were obtained from the data of FinnGen Release 8 (released to the public on December 1, 2022),[1] including bronchiectasis, acute bronchiolitis, acute bronchitis, influenza, and pneumonia. Cases were defined as participants with at least one inpatient or outpatient ICD-9/10 code as a primary diagnosis for these LRTIs. We limited LRTI data to samples of European ancestry to avoid potential bias aroused by population stratification. This

1 https://finngen.gitbook.io/documentation/

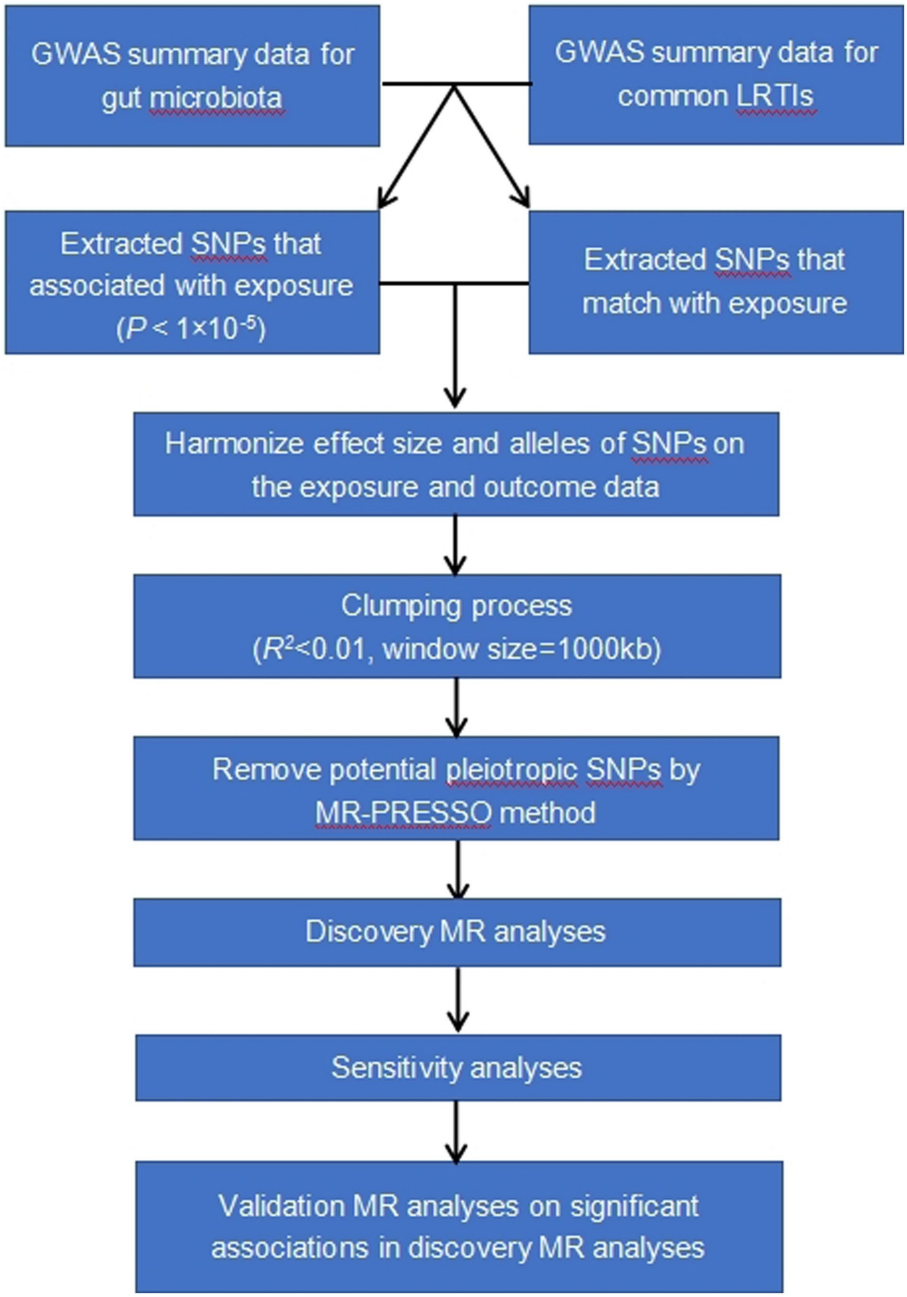


FIGURE 1
Study design and workflow.

GWAS consisted of 1,967 bronchiectasis cases (283,589 controls), 1,754 acute bronchiolitis cases (323,785 controls), 13,832 acute bronchitis cases (323,785 controls), 52,021 pneumonia cases (290,478 controls), and 7,580 influenza cases (286,619 controls).

The validation summary statistics of SNPs associated to the five common LRTI diseases were obtained from the GWAS reports measured in European participants from the UK Biobank by searching the GWAS catalog[2] (Jiang et al., 2021), which included a total of 583 bronchiectasis cases (455,765 controls) (accession number: GCST90044075), 172 acute bronchiolitis and bronchitis cases (456,176 controls) (accession number: GCST90044069), 2,842 pneumonia cases (453,506 controls) (accession number: GCST90044067), and 248 influenza cases (456,100 controls) (accession number: GCST90044068).

We also examined the possibility of reverse causality of LRTIs on gut microbiome, using the discovery datasets of GWASs related to gut microbiome and LRTIs.

Instrumental SNPs selection

The following selection criteria were used to choose the IVs: (1) SNPs associated with each genus were collected as potential IVs using a genome-wide significance threshold of $p < 1 \times 10^{-5}$ for their inclusion in the current study; (2) due to the presence of strong linkage

2 https://www.ebi.ac.uk/gwas/

disequilibrium (LD) among selected SNPs might bias the results, SNPs were clumped within study per bacterium genus using the PLINK (version 1.961) clumping procedure to eliminate the stringent LD between included IVs ($R^2 < 0.01$, with reference to the 1,000 Genomes Phase 3 CEU Project Panel (Abecasis et al., 2012), clumping window size = 1,000 kb) within a five megabase window; (3) to guarantee that the impact of SNPs on exposure corresponds to the same allele as the impact on the outcome, palindromic SNPs with intermediate allele frequencies were excluded; (4) when the exposure-related SNPs were not available in the outcome dataset, the proxy SNPs significantly correlated with the variants of interest were used ($R^2 > 0.8$); (5) selected SNPs with a minor allele frequency (MAF) ≤ 0.01 were excluded.

Statistical analysis

A bidirectional two-sample MR analysis was conducted to evaluate the causal relationship between the gut microbiome and common LRTI subtypes. When only a single SNP was available to construct the IV, the ratio of coefficients method was used to obtain MR estimates with first-order weights used to generate standard errors. Where more than one SNP was available to construct the IVs for a given genus, the random effect inverse variance weighted (IVW) MR approach was used as the principal analysis to acquire an overall estimate of the causal effect. In addition, complementary methods including MR-Egger regression, weighted median, weighted model, MR-pleiotropy residual sum and outlier (MR-PRESSO), and simple model were used to examine the causal association. The MR-Egger regression is used to assess the presence of pleiotropy, which occurs when a genetic variant affects multiple traits or outcomes, violating the instrumental variable assumption of MR. It provides a robust estimate of the causal effect by accounting for pleiotropy and can detect and quantify any directional pleiotropy bias (Bowden et al., 2016b). The weighted median estimator is a robust approach to estimate the causal effect when there is heterogeneity in the causal estimates from different genetic variants. It selects the median estimate as the overall causal effect, giving more weight to instruments with smaller variances and providing valid results as long as at least 50% of the weight comes from valid instruments (Bowden et al., 2016a). Similar to the weighted median, the weighted mode estimator is also designed to handle heterogeneity. It selects the mode estimate as the causal effect, which is the most frequent estimate among valid instruments. This method is useful when there is more pronounced heterogeneity in the causal estimates (Hartwig et al., 2017). MR-PRESSO is a method used to identify and correct for outliers or genetic variants that violate the no pleiotropy assumption. It detects outliers and removes them from the analysis, allowing for a more accurate estimation of the causal effect. Additionally, MR-PRESSO provides an adjusted causal estimate after removing outliers (Verbanck et al., 2018). To account for multiple testing, a conservative Bonferroni threshold for statistical significance was set to $p < 0.05 / 119 = 4 \times 10^{-4}$ for the forward MR analysis and $p < 0.05 / 5 = 1 \times 10^{-2}$ for the reverse MR analysis.

We also employed a Bayesian posterior probabilities-based MR method namely Causal Analysis Using Summary Effect Estimates (CAUSE) (Morrison et al., 2020), as a further validation analysis for the associations replicated in both discovery dataset and validation dataset. This approach demonstrates a reduced susceptibility to false positive associations resulting from correlated and uncorrelated horizontal pleiotropy by utilizing the maximum independent SNPs to increase detection power. To include a larger number of IVs, LD pruning was performed using a threshold of $r^2 < 0.1$ and $p < 1 \times 10^{-3}$ via a built-in function in the CAUSE R package, which utilized precomputed LD estimates.

Sensitivity analysis

To ensure the reliability of the conclusion, sensitivity analyses were performed to verify whether heterogeneity and pleiotropy within the genetic variables could bias the MR results. First, the MR-Egger regression was applied to detect and adjust for the underlying horizontal pleiotropic effects among the selected IVs through the assessment of the intercept. Second, the MR-PRESSO global test of heterogeneity was conducted to identify the underlying horizontal pleiotropy. Third, the Cochran' IVW Q statistics were used to quantify the heterogeneity across the selected IVs. With a consideration of possible significant heterogeneity between SNPs ($p < 0.05$), we applied a random-effect IVW model to perform the MR analysis. Fourth, the leave-one-out sensitivity analysis was implemented by omitting each instrumental SNP in turn to identify potential heterogeneous SNPs. Fifth, considering that various confounders including smoking, pulmonary comorbidities such as chronic obstructive pulmonary disease (COPD), asthma, lung fibrosis et al. were closely associated with the incidence of LRTIs, we conservatively queried each genus-related SNP used as the instrument in the PhenoScanner V2 database to identify SNPs that were significantly associated with GWAS traits potentially confounding LRTI phenotypes or might introduce horizontal pleiotropy at risk of affecting the five LRTI subtypes independent of gut microbiome at the genome-wide significance level. Whenever the GWAS p-value of the SNP was lower than the threshold ($p < 1 \times 10^{-5}$), we considered it to be correlated with the confounders (Kamat et al., 2019). We assessed the effect of gut microbiome after removing those SNPs from the MR estimates to exclude potential pleiotropic effects. This MR analysis was performed based on three assumptions: (1) The IV is closely associated with the exposure. (2) The IV is not associated with any potential confounders. (3) The IV can only influence the outcome via the exposure, and not by any other ways. The CAUSE method was used to detect the false positive error due to correlated horizontal pleiotropy. The SNPs selection assumptions and MR statistical analysis workflow were shown in Figure 2.

The strength of IVs was assessed by calculating the F-statistic using the formula $F = R^2 \times (N\text{-}1\text{-}K) / (1\text{-}R^2) \times K$, where R^2 represented the proportion of variance in the exposure explained by the genetic variants, N represented sample size, and K represented the number of instruments (Palmer et al., 2012). R^2 was calculated by using the formula $R^2 = 2 \times \text{MAF} \times (1 - \text{MAF}) \times \text{beta}^2$, where beta was the effect value of the genetic variant in the exposure, and MAF was the effect allele frequency of selected SNPs (Palmer et al., 2012; Burgess et al., 2016). If the corresponding F-statistic was >10, it was considered no significant weak instrumental bias was used. The power of the MR estimates was calculated using the mRnd MR power calculator[3] (Burgess et al., 2017). MR analyses were performed using R (version

3 https://cnsgenomics.com/shiny/mRnd/

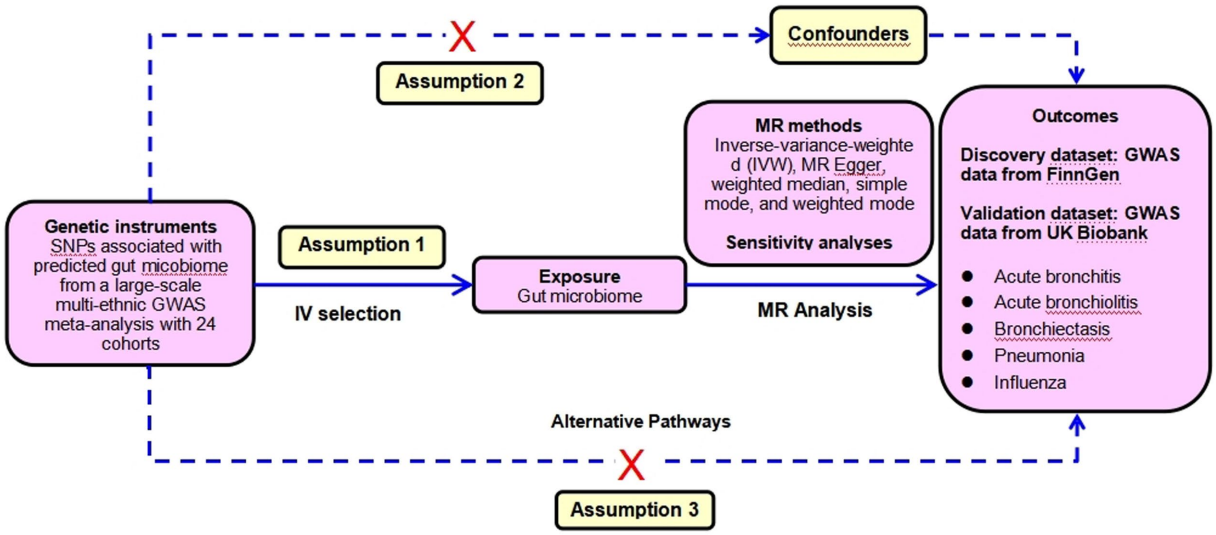


FIGURE 2
The SNP selection assumptions and forward MR analyses flowchart.

4.2.3), the TwoSampleMR package 0.5.6 (Hemani et al., 2018), MR-PRESSO (version 1.0) (Verbanck et al., 2018), and CAUSE (version 1.2.0) (Morrison et al., 2020).

Results

Forward MR analysis and sensitivity analyses

According to the selection criteria of IVs, a total of 1,517 SNPs associated with 119 bacterial genera were identified from the large-scale GWAS (Supplementary Table S1). After exclusion due to potential association to outcomes or outcome-related traits, unavailablility in outcome dataset (Supplementary Table S2), or palindrome, a total of 1,224 SNPs with four proxy SNPs were retained as IVs for the following forward MR analysis (Supplementary Table S3). The SNP number for bronchiectasis, acute bronchiolitis, acute bronchitis, influenza, and pneumonia in the discovery dataset was 1,220, 1,219, 1,221, 1,219, and 1,222, respectively. The main information of SNPs including effect allele, other allele, beta, SE, and *p* value were collected systematically for further analysis. The number of IVs associated with each bacterial genus varies from 3 to 20. The sum of F statistics for SNPs of individual bacterial genera was greater than the conventional threshold of 10, indicating that there was no significant bias from weak instrument variables (Table 1; Supplementary Table S4).

According to the five MR methods, the genetically determined gut microbiome, as a whole, had no causal relationship with LRTIs. The results of Cochran's IVW Q test showed no significant heterogeneity of these IVs. The discovery findings were confirmed in independent validation MR analysis (Table 1). Detailed information and strengths of IVs in validation dataset were presented in Supplementary Tables S5, S6.

However, the results of MR analyses for individual gut microbial genus in discovery datasets revealed that genetically predicted relative abundance of specific genera might be causally associated with an increased or decreased risk of LRTI (Supplementary Table S7). For example, based on the IVW estimates, *Bifidobacterium* was negatively associated to the risk of bronchiectasis [odds ratio (OR): 0.688, 95% confidence interval (CI): 0.520–0.910, $p = 0.009$], but positively associated to the risk of influenza (OR: 1.225, 95% CI: 1.061–1.415, $p = 0.006$). While according to the MR-Egger analysis, *Blautia* was consistently correlated with an increased risk of bronchiectasis (OR: 2.956, 95% CI: 1.143–7.648, $p = 0.049$), influenza (OR: 1.856, 95% CI: 1.140–3.022, $p = 0.032$), and pneumonia (OR: 1.336, 95% CI: 1.036–1.724, $p = 0.049$). In addition, abundance of genera *Erysipelatoclostridium* acted as a risk factor for acute bronchiolitis (OR: 3.483, 95% CI: 1.266–9.583, $p = 0.031$) and influenza (OR: 1.764, 95% CI: 1.089–2.860, $p = 0.038$), while *Oxalobacter* acted as a protective factor for acute bronchiolitis (OR: 0.799, 95% CI: 0.653–0.978, $p = 0.030$) and bronchitis (OR: 0.913, 95% CI: 0.842–0.989, $p = 0.027$) (Supplementary Table S8; Supplementary Figures S1, S2).

For these identified associations, the Cochran's Q test suggested that there was no significant heterogeneity across all these selected genetic instruments (Supplementary Table S8). The horizontal pleiotropy was evaluated by MR-Egger regression, and the results indicated that there was evidence of potential horizontal pleiotropy to skew the influence of *Anaerostipes* on bronchiectasis ($p = 0.002$), *Erysipelatoclostridium* on acute bronchiolitis ($p = 0.030$) and influenza ($p = 0.041$), *Holdemania* ($p = 0.010$) and *Olsenella*on ($p = 0.035$) on acute bronchiolitis, *Oscillospira* on acute bronchitis ($p = 0.038$), and *Ruminococcus_2* on pneumonia ($p = 0.039$) (Supplementary Table S8). The MR-Egger intercept test was sensitive to outliers and violations of INstrument Strength Independent of Direct Effect assumption, thus less efficient. Therefore, we also conducted MR-PRESSO global test, which was more robust to outliers. Nevertheless, no sign of horizontal pleiotropy was detected and no outliers were found by MR-PRESSO test (Supplementary Table S9). The leave-one-out analysis showed that no single SNP was driving these significant MR estimates, indicating that the results of the current MR analysis were robust (Supplementary Figure S3). However, after Bonferroni correction for

TABLE 1 MR results of causal effect of general gut microbiome on LRTI risk in forward analysis.

LRTIs	Datasets	Nsnp	Methods	F statistic	Beta	SE	*p*	OR (95% CI)	Horizontal pleiotropy			Heterogeneity	
									Intercept	SE	*p*	*Q*	*p*
Bronchiectasis	Discovery	1,220	MR-Egger	65.815	0.061	0.032	0.059	1.062 (0.998, 1.131)	−0.007	0.003	0.034	1215.693	0.624
			Weighted median		−0.005	0.019	0.786	0.995 (0.959, 1.032)					
			IVW		−0.002	0.012	0.864	0.998 (0.974, 1.022)					
			Weighted mode		−0.032	0.079	0.687	0.969 (0.829, 1.132)					
			Simple mode		−0.044	0.069	0.529	0.957 (0.835, 1.097)					
	Validation	1,076	MR-Egger	58.529	0.007	0.064	0.911	1.007 (0.888, 1.143)	0.004	0.006	0.519	1085.538	0.405
			Weighted median		0.081	0.038	0.031	1.084 (1.007, 1.167)					
			IVW		0.046	0.025	0.064	1.047 (0.997, 1.098)					
			Weighted mode		0.228	0.016	0.156	1.226 (0.917, 1.719)					
			Simple mode		0.228	0.136	0.093	1.226 (0.963, 1.638)					
Acute bronchiolitis	Discovery	1,219	MR-Egger	65.710	0.029	0.035	0.404	1.030 (0.961, 1.104)	−0.002	0.003	0.562	1268.436	0.236
			Weighted median		0.030	0.022	0.163	1.031 (0.988, 1.076)					
			IVW		0.011	0.014	0.436	1.011 (0.984, 1.038)					
			Weighted mode		0.091	0.082	0.271	1.095 (0.932, 1.287)					
			Simple mode		0.081	0.072	0.259	1.084 (0.942, 1.248)					
Acute bronchitis		1,221	MR-Egger	66.324	−0.020	0.013	0.105	0.980 (0.956, 1.004)	0.002	0.001	0.073	1288.878	0.131
			Weighted median		0.001	0.007	0.864	1.001 (0.987, 1.016)					
			IVW		0.000	0.005	0.937	1.000 (0.991, 1.010)					
			Weighted mode		0.022	0.029	0.466	1.022 (0.964, 1.083)					
			Simple mode		0.014	0.023	0.543	1.014 (0.969, 1.062)					
Acute bronchitis and bronchiolitis	Validation	1,233	MR-Egger	68.013	0.061	0.110	0.578	1.063 (0.857, 0.319)	−0.002	0.011	0.886	1256.368	0.308
			Weighted median		0.017	0.065	0.797	1.017 (0.895, 1.156)					
			IVW		0.047	0.042	0.270	1.048 (0.964, 1.138)					
			Weighted mode		−0.148	0.286	0.606	0.863 (0.492, 1.511)					
			Simple mode		−0.116	0.645	0.645	0.890 (0.544, 1.458)					

(Continued)

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TABLE 1 (Continued)

LRTIs	Datasets	Nsnp	Methods	F statistic	Beta	SE	*p*	OR (95% CI)	Horizontal pleiotropy			Heterogeneity	
									Intercept	SE	*p*	*Q*	*p*
Influenza	Discovery	1,219	MR-Egger	66.144	0.003	0.017	0.848	1.003 (0.971, 1.037)	−0.001	0.002	0.446	1296.740	0.105
			Weighted median		−0.005	0.010	0.603	0.995 (0.977, 1.014)					
			IVW		−0.009	0.007	0.186	0.991 (0.979, 1.004)					
			Weighted mode		−0.027	0.040	0.499	0.973 (0.900, 1.052)					
			Simple mode		−0.012	0.033	0.719	0.988 (0.926, 1.054)					
	Validation	1,233	MR-Egger	68.013	−0.024	0.091	0.788	0.976 (0.817, 1.165)	0.006	0.009	0.518	1180.687	0.850
			Weighted median		0.027	0.052	0.602	1.027 (0.928, 1.137)					
			IVW		0.030	0.035	0.396	1.030 (0.962, 1.103)					
			Weighted mode		0.031	0.230	0.894	1.031 (0.657, 1.617)					
			Simple mode		0.056	0.194	0.775	1.057 (0.723, 1.546)					
Pneumonia	Discovery	1,222	MR-Egger	66.324	0.001	0.007	0.897	1.001 (0.987, 1.015)	−0.000	0.001	0.754	1364.909	0.005
			Weighted median		−0.003	0.004	0.484	0.997 (0.989, 1.005)					
			IVW		−0.001	0.003	0.681	0.999 (0.993, 1.004)					
			Weighted mode		−0.005	0.015	0.733	0.995 (0.966, 1.025)					
			Simple mode		−0.005	0.015	0.733	0.995 (0.966, 1.025)					
	Validation	1,233	MR-Egger	68.013	−0.023	0.027	0.400	0.977 (0.927, 1.031)	0.003	0.003	0.236	1176.692	0.735
			Weighted median		0.007	0.015	0.664	1.007 (0.977, 1.037)					
			IVW		0.007	0.010	0.510	1.007 (0.987, 1.028)					
			Weighted mode		0.013	0.063	0.840	1.013 (0.895, 1.147)					
			Simple mode		−0.001	0.056	0.982	0.999 (0.895, 1.115)					

Nsnp is the number of SNPs used to estimate the causal effect size.

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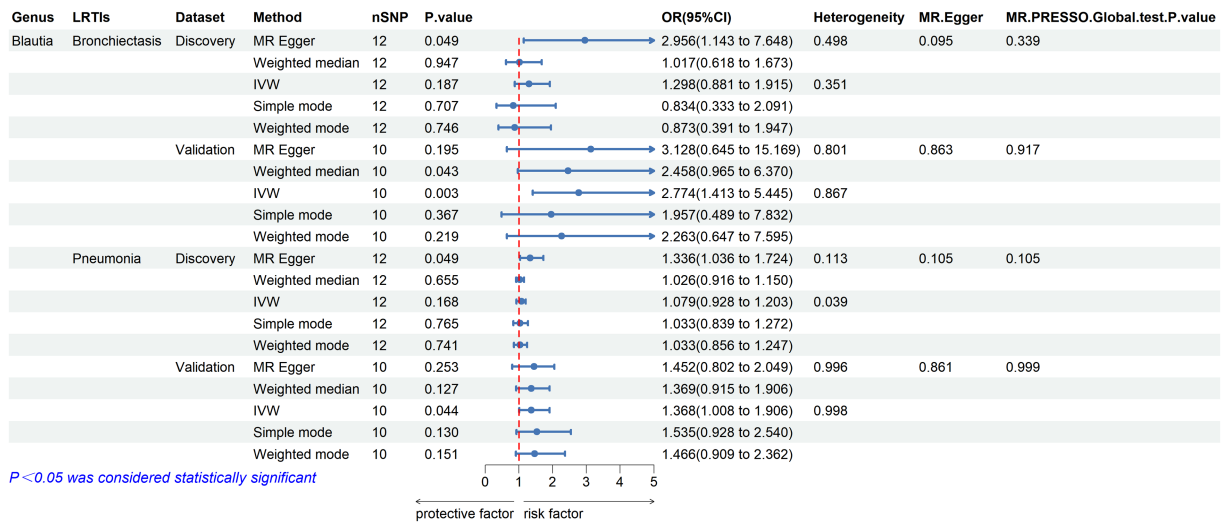


FIGURE 3
Validated casual links between specific gut microbial genus and LRTIs in forward MR analyses.

multiple tests, none of the significant associations presented between genetically predicted gut microbiome with LRTIs.

Furthermore, to validate the causal relationship identified in the discovery sample set, summary statistics from independent LRTI GWASs were employed. The validation MR analysis solely confirmed the causal association with the same direction between genetically predicted enrichment of microbial genus *Blautia* with bronchiectasis (IVW, OR: 2.774, 95% CI: 1.413–5.445, $p = 0.003$) and pneumonia (IVW, OR: 1.386, 95% CI: 1.008–1.906, $p = 0.044$) (Table 2; Figure 3). Still, application of the Bonferroni correction did not yield any statistically significant differences in validation. To further confirm the validated casual links between *Blautia* and LRTIs, CAUSE analysis was then performed. However, it indicated that the sharing model was better than the causal model, without providing consistent significant causality (Supplementary Table S10).

Reverse MR analysis and sensitivity analyses

We further examined the causal effect of LRTI diseases on gut microbiome by reverse MR analyses. The detailed information and predictive power of selected IVs were presented in Supplementary Tables S11–S13. The conventional MR methods resulted in multiple causal relationships between LRTI and gut microbiome (Supplementary Table S14). Specifically, according to the results of bidirectional MR analyses based on the discovery dataset, we found a bidirectional causal association between *Escherichia_Shigella* (Forward IVW, OR: 0.851, 95% CI: 0.737–0.982, $p = 0.028$; Reverse IVW, OR: 0.851, 95% CI: 0.763–0.950, $p = 0.004$) and acute bronchitis, as well as *Eubacterium_fissicatena_group* (Forward IVW, OR: 0.897, 95% CI: 0.816–0.987, $p = 0.025$; Reverse IVW, OR: 0.793, 95% CI: 0.647–0.971, $p = 0.025$) and acute bronchitis (Supplementary Tables S8, S15).

The Bonferroni correction was also performed in reverse MR analysis to account for five exposures, where $p < 0.01$ (0.05/5) was defined as a statistically significant difference. The causal effect of Bronchiectasis on *Corprobacter* (Reverse MR IVW, $p = 0.008$) and *Ruminiclostridium_6* (Reverse MR IVW, $p = 0.007$), acute bronchiolitis on *Corprobacter* (Reverse MR IVW, $p = 0.006$) and *Eggerthella* (Reverse MR IVW, $p = 0.003$), acute bronchitis on *Escherichia_Shigella* (Reverse MR IVW, $p = 0.004$), *Oscillibacter* (Reverse MR IVW, $p = 0.002$), and *Veillonella* (Reverse MR IVW, $p = 0.001$), as well as pneumonia on *Haemophilus* (Reverse MR IVW, $p = 0.003$) was still significant after Bonferroni correction. There was no evidence of heterogeneity between IV estimates with IVW methods from individual SNPs and no pleiotropy effect for these detected associations. No pleiotropic outliers were detected according to the MR-PRESSO tests (Supplementary Table S16). However, as there were not enough IVs used in the MR to undertake a sensitivity analysis for the causal effect of acute bronchiolitis on most microbial genera, the results should be interpreted with caution. To verify these causalities, we also conducted validation MR analysis by using independent LRTI GWAS data. Nevertheless, none of the associations were replicated in the validation dataset, indicating a poor causal effect of LRTIs on the gut microbiome.

Discussion

Colonization of the intestine with commensal bacteria is known to play a major role in the maintenance of the integrity of lung tissues against foreign infections. Intestinal flora has a powerful direct and indirect regulatory effect on the human immune system by increasing the number of immune cells, producing SCFAs and immunoglobulins, enhancing oral tolerance, and controlling inflammation (Samuelson et al., 2015). Consequently, an altered gut microbiome is always associated with various ensuing diseases including respiratory diseases. In this study, using the summary statistics of gut microbiota from the largest GWAS report and the summary statistics of LRTIs from the FinnGen consortium R8 release data and UK Biobank, we performed a bidirectional two-sample MR analysis to refer the

TABLE 2 MR results of significant causal effect of gut microbiome on LRTI risk in forwad analysis ($p < 0.05$).

Genus	LRTI	Dataset	N snp	Methods	Beta	SE	*p*	OR (95% CI)	Horizontal pleiotropy			Heterogeneity	
									Intercept	SE	*p*	*Q*	*p*
Blautia	Bronchiectasis	Discovery	12	MR-Egger	1.084	0.485	**0.049**	2.956 (1.143, 7.648)	−0.062	0.034	0.095	8.785	0.498
				Weighted median	0.017	0.254	0.947	1.017 (0.618, 1.673)					
				IVW	0.261	0.198	0.187	1.298 (0.881, 1.915)				12.175	0.351
				Simple mode	−0.181	0.469	0.707	0.834 (0.333, 2.091)					
				Weighted mode	−0.136	0.409	0.746	0.873 (0.391, 1.947)					
		Validation	10	MR-Egger	1.141	0.805	0.195	3.128 (0.645, 15.169)	−0.011	0.061	0.863	4.585	0.801
				Weighted median	0.908	0.482	0.043	2.458 (0.965, 6.370)					
				IVW	1.020	0.344	**0.003**	2.774 (1.413, 5.445)				4.613	0.867
				Simple mode	0.672	0.707	0.367	1.957 (0.489, 7.832)					
				Weighted mode	0.817	0.618	0.219	2.263 (0.674, 7.595)					
	Pneumonia	Discovery	12	MR-Egger	0.290	0.130	**0.049**	1.336 (1.036, 1.724)	−0.016	0.009	0.105	15.558	0.113
				Weighted median	0.026	0.058	0.655	1.026 (0.916, 1.150)					
				IVW	0.076	0.055	0.168	1.079 (0.928, 1.203)				20.517	0.039
				Simple mode	0.033	0.106	0.765	1.033 (0.839, 1.272)					
				Weighted mode	0.033	0.096	0.741	1.033 (0.856, 1.247)					
		Validation	10	MR-Egger	0.372	0.303	0.253	1.452 (0.802, 2.627)	−0.004	0.022	0.860	1.265	0.996
				Weighted median	0.314	0.206	0.127	1.369 (0.915, 2.049)					
				IVW	0.327	0.162	**0.044**	1.386 (1.008, 1.906)				1.298	0.998
				Simple mode	0.429	0.257	0.130	1.535 (0.928, 2.540)					
				Weighted mode	0.382	0.244	0.151	1.466 (0.909, 2.362)					

Nsnp is the number of SNPs used to estimate the causal effect size; Bold values denote statistical significance at the $p < 0.05$ level.

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causal association between gut microbiota and five common phenotypes of LRTI, which might be helpful to shed light on the impact of gut microbiota on airway immunity and the host's ability to defend against respiratory infections. Additionally, our findings may inspire the development of precision medicine for treating these scenarios.

Through a comprehensive discovery and validation approach, this study suggested *Blautia* as a potential risk factor for an increased risk of bronchiectasis and pneumonia. Despite the causality did not surpass the strict Bonferroni correction threshold and was not confirmed in CAUSE analysis, it provided suggestive evidence of a potential causal effect between genus *Blautia* and LRTI frequency and might stimulate further specialized studies to gain insights into its impact on respiratory health. As a dominant genus of anaerobic bacteria in the feces and intestines of mammals, the probiotic characteristics of *Blautia* and its protective role in various host physiological dysfunctions have been reported, such as obesity, diabetes, cancer, and inflammatory bowel diseases. *Blautia* contributes to maintaining environmental balance in the intestine, preventing inflammation by upregulating intestinal regulatory T cells, and producing SCFAs (Kim et al., 2014). Its ability to produce bacteriocin, one of the common secondary metabolites that possess antibacterial activity against pathogenic microorganisms such as *Listeria monocytogenes*, *Clostridium perfringens*, and *Escherichia coli* (Martinez et al., 2013), also gives *Blautia* the potential to inhibit the colonization of pathogenic bacteria in the intestine (Liu et al., 2021). Our results did not align with previous findings regarding the protective effect of *Blautia* in human diseases. However, higher abundance of *Blautia* was also reported in the fecal microbiota of patients with irritable bowel syndrome, ulcerative colitis, functional gastrointestinal symptoms (Rajilić-Stojanović et al., 2011; Nishino et al., 2018; Ohlsson, 2022), and breast cancer (Luu et al., 2017), suggested its association to the local or systemic inflammation. Indeed, *Blautia*, especially *Blautia coccoides*, activates the secretion of inflammatory cytokines such as tumor necrosis factor α (TNF-α) to an even greater extent than lipopolysaccharide (Tuovinen et al., 2013). TNF-α is a potent protective cytokine that contributing to anti-viral and anti-bacterial responses during the early phase of infection, however, excessive production causes heightened lung immunopathology and inflammation, particularly in the late phase of infection. Besides, in patients with rheumatoid arthritis, increased relative abundance of gut genus *Blautia* was related with lower levels of T cells, B cells, $CD4^+$ T cells, and Tregs (Li et al., 2021). The findings suggest that *Blautia* species act as culprits in the pathogenesis of infectious diseases potentially due to their pro-inflammatory and anti-immune properties. Mediterranean diet (MD) is plant-based and consistently considered to be benefit on human health (Barber et al., 2023). Studies demonstrated that administration of MD reduced *Blautia* within gut microbiota (Merra et al., 2020; Zhu et al., 2020). Evidence for the effects of dietary fiber within MD showed a direct suppressive effect on *Clostridium difficile* infection. As previous study has indicated a positive association of *Blautia* with key inflammatory cytokines such as TNF-α, the authors hypothesized that MD improves the inflammatory milieu and infection through modulation of gut microbiota, at least in part (Merra et al., 2020; Zhu et al., 2020). The conflicting conclusions may be related to the genomic difference within the genus, as there are 12 independent *Blautia* species with a total of 195 genome assemblies, nevertheless, most studies focused on the genus level and did not delve into investigations at species or even strain-levels (Liu et al., 2021). Therefore, drawing general conclusions at the genus level may lead to partial understandings and misinterpretations because different species of *Blautia* may exert different effects on human health. For example, *Blautia coccoides* was reported to be positively associated to the level of cytokines including TNF-α (Tuovinen et al., 2013), while *Blautia luti* and *B. wexlerae* in the gut microbiota of obese children was negatively related to those proinflammatory cytokines and chemokines (Benítez-Páez et al., 2020).

Although not consistently validated, our results implied that specific genera of gut commensal microbiota compositions might have protective effects against respiratory infections, highlighting their potential use as medications in the context of airway infections (Wolvers et al., 2010; Ozen et al., 2015). Based on the results from the discovery dataset, an increased abundance of *Oxalobacter* was consistently related to a lower risk of acute bronchiolitis and bronchitis, which are primarily caused by viral infections, particularly respiratory syncytial virus (RSV) (Kinkade and Long, 2016; Joseph and Edwards, 2019). *Oxalobacter* is a Gram-negative bacterium that degrades oxalate in the gut to decrease urinary oxalate excretion. Its probiotics preparation has been commercially available as a biotherapeutic agent in the management of calcium oxalate renal stones (Hiremath and Viswanathan, 2022). *Oxalobacter* was identified to be associated with intestinal virus infection (Gozalbo-Rovira et al., 2021) and infectious urinary stone (Bruyere et al., 2008). Our analyses gave a new clue that use of *Oxalobacter* strains might show beneficial effects on the host immunity and/or against pathogens in respiratory system. *Bifidobacterium* is a genus of Gram-positive, anaerobic bacteria that are commonly found in the human gut microbiome. These bacteria are known for their immunomodulatory properties and have been shown to have a beneficial impact on human health (Hidalgo-Cantabrana et al., 2017). In our study, *Bifidobacterium* was suggested as a protective factor for the risk of bronchiectasis, which is associated to bacteria-related recurrent respiratory tract infections (RRTIs) (Amati et al., 2019). Studies have revealed that RRTI patients suffer from intestinal flora imbalance (Ozen et al., 2015; Li et al., 2019), manifested as a significant reduction in the number of *Bifidobacteria* (Peng et al., 2016; Li et al., 2019). Restoring *Bifidobacteria* with oral *Bifidobaeterium tetravaccine* tablets (Live) effectively maintained the balance of intestinal micro-ecology and reduced average annual frequency of acute respiratory tract bacterial infection and use of antibiotics (Li et al., 2019). Thus, *Bifidobacterium* may be viewed as potential next-generation probiotic candidates in the treatment of bacterial lung infections. Actually, studies evaluating the use of *Bifidobacterium* as probiotics have already demonstrated their effect in the control of respiratory viral infections, such as COVID-19 (Taufer and Rampelotto, 2023) and H7N9 (Zhang et al., 2020). The potential mechanisms of action underlying the protective effects of *Bifidobacterium*, drawing from current knowledge, may involve the stimulation on immune system, reduction of inflammation, competitive advantage with pathogenic microbes, and maintenance of gut barrier function (Taufer and Rampelotto, 2023). A group of SCFAs-producing bacteria including *Parabacteroides* (Ahmed et al., 2019), *Anaerotruncus* (Yan et al., 2019), and *Lachnospiraceae_NC2004_group* (Egerton et al., 2022) were also implied to be protective in LRTIs by our results. SCFAs, mostly acetic acid, propionic acid, and butyric acid, are metabolites produced after gut microbial fermentation of dietary fiber. Accumulated evidence supported that SCFAs not only maintain local and systemic immune homeostasis but also boost host immunity to

pathogens in a range of airway inflammatory conditions (Antunes et al., 2023; Dang et al., 2023), through sophisticated modulations on the maturation, accumulation, and function of immune cells, activating the transmembrane G protein-coupled receptors, and inhibiting histone deacetylases, et al. (Tan et al., 2014).

Notably, the association between gut microbiome and LRTIs found in the observational studies may be influenced by reverse causation. To address it, we performed a reverse MR analysis to investigate the causation in the opposite direction. Although there were some initial indications of causal effects of LRTIs on the gut microbiome in the discovery sample set, no notable genetically predicted associations were observed in the validation dataset. This suggested that reverse causation is unlikely to explain the findings in forward MR analysis.

This study has several strengths. To the best of our knowledge, this is the first MR study to infer the causal relationship between the gut microbiota and LRTIs. Genetic variants used to represent the gut microbiota were sourced from the largest available GWAS analysis, ensuring the strength of instruments for MR approach. Horizontal pleiotropy was detected and excluded by using the MR-PRESSO and MR-Egger regression intercept term tests. Furthermore, we conducted a comprehensive validation process by using an independent sample data and the CAUSE method to facilitate robust causal inferences. Our study somehow deepens the understanding of the gut microbiome on human health and highlights the microbiome-related agents as potential precision therapeutics to ensure enhanced resistance toward respiratory infections.

There are several limitations that should be considered while interpreting the results of this study. First, a standard MR method assumed a linear relationship between exposure and outcome, so the non-linear association and threshold effect between gut microbiota and LRTIs could not be detected. Second, we only explored the causal links between gut microbiota and LRTIs at the taxonomic level of genus, thus their associations at the species level could not be revealed. Third, although most participants in the GWAS meta-analysis for gut microbiota data were of European descent and the outcome GWAS data were restricted only from European subjects, the interference from population stratification might still exist. Moreover, the extrapolation of the current findings to other ethnic groups was limited. Future MR studies on this topic should be considered in non-European populations to confirm the results. Fourth, although we measured LD among all selected SNPs using northern Europeans from Utah samples from the 1,000 Genomes Project, we could not exclude the possibility that our results might be affected by unmeasured confounders. Fifth, by selecting representative diseases for LRTIs, we aimed to capture a range of lower respiratory tract infections of clinical importance. The ambiguous and broad phenotype definition for LRTIs might induce the risk of non-differential mismatch between specific genera and individual diseases because high heterogeneities in LRTIs due to varied etiologies, pathogens, disease stages, clinical and radiology features, and physiology/lung functions might significantly influence the results. For example, the inclusion of influenza with a blurred boundary between upper and lower respiratory tract effects potentially introduced a potential limitation in the causal inferences drawn from our analysis. Sixth, since the data sources did not include individual level data, we failed to report the number of individuals at each stage of the study and the reasons why individuals were excluded from further study. This limitation might influence the adjustment of validity and generalisability of results by readers. Finally, the genetic instruments used in the MR analyses still might have pleiotropic effects, which could produce spurious findings. To limit this risk, we conducted multiple sensitivity analyses to provide statistical evidence of bias from pleiotropy or genetic confounding. But still, since the results failed to meet the stringent threshold in Bonferroni correction and CAUSE method, it is important to interpret our findings with caution, particularly when making specific causal inferences related to individual diseases within the LRTI group.

Conclusion

In summary, this study does not provide robust evidence for a causal relationship between genetically predicted gut microbiome genera and common phenotypes of lower respiratory tract infections. However, specific genera of gut commensal microbiota, such as *Blautia*, *Oxalobacter*, *Bifidobacterium*, and several SCFA-producing bacteria, are suggested to be potential indicators for respiratory infection susceptibility. Further specialized investigations, particularly studies focusing on the species or strain levels, are needed to gain more insights into their impact on lung health and to unlock the potential use of gut microbiota-based immune-regulatory therapies in respiratory infectious diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

WL: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing. XW: Formal analysis, Methodology, Writing – original draft. RF: Formal analysis, Methodology, Writing – original draft. CZ: Formal analysis, Methodology, Writing – original draft. JL: Data curation, Formal analysis, Methodology, Writing – original draft. XZ: Data curation, Formal analysis, Writing – original draft. XL: Data curation, Methodology, Software, Writing – original draft. MY: Formal analysis, Software, Writing – original draft. JM: Data curation, Methodology, Writing – original draft. BM: Formal analysis, Supervision, Writing – review & editing. HJ: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1276046/full#supplementary-material

References

Abecasis, G. R., Auton, A., Brooks, L. D., DePristo, M. A., Durbin, R. M., Handsaker, R. E., et al. (2012). An integrated map of genetic variation from 1,092 human genomes. *Nature* 491, 56–65. doi: 10.1038/nature11632

Abrahamsson, T. R., Jakobsson, H. E., Andersson, A. F., Björkstén, B., Engstrand, L., and Jenmalm, M. C. (2014). Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin. Exp. Allergy* 44, 842–850. doi: 10.1111/cea.12253

Abt, M. C., Osborne, L. C., Monticelli, L. A., Doering, T. A., Alenghat, T., Sonnenberg, G. F., et al. (2012). Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37, 158–170. doi: 10.1016/j.immuni.2012.04.011

Ahmed, S., Busetti, A., Fotiadou, P., Vincy Jose, N., Reid, S., Georgieva, M., et al. (2019). In vitro characterization of gut microbiota-derived bacterial strains with neuroprotective properties. *Front. Cell. Neurosci.* 13:402. doi: 10.3389/fncel.2019.00402

Amati, F., Simonetta, E., Gramegna, A., Tarsia, P., Contarini, M., Blasi, F., et al. (2019). The biology of pulmonary exacerbations in bronchiectasis. *Eur. Respir. Rev.* 28:190055. doi: 10.1183/16000617.0055-2019

Antunes, K. H., Singanayagam, A., Williams, L., Faiez, T. S., Farias, A., Jackson, M. M., et al. (2023). Airway-delivered short-chain fatty acid acetate boosts antiviral immunity during rhinovirus infection. *J. Allergy Clin. Immunol.* 151, 447–457.e5. doi: 10.1016/j.jaci.2022.09.026

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920. doi: 10.1126/science.1104816

Barber, T. M., Kabisch, S., Pfeiffer, A. F. H., and Weickert, M. O. (2023). The effects of the Mediterranean diet on health and gut microbiota. *Nutrients* 15:2150. doi: 10.3390/nu15092150

Benítez-Páez, A., Gómez Del Pugar, E. M., López-Almela, I., Moya-Pérez, Á., Codoñer-Franch, P., and Sanz, Y. (2020). Depletion of Blautia species in the microbiota of obese children relates to intestinal inflammation and metabolic phenotype worsening. *mSystems* 5, e00857–e00819. doi: 10.1128/mSystems.00857-19

Bowden, J., Davey Smith, G., Haycock, P. C., and Burgess, S. (2016a). Consistent estimation in Mendelian randomization with some invalid instruments using a weighted median estimator. *Genet. Epidemiol.* 40, 304–314. doi: 10.1002/gepi.21965

Bowden, J., Del Greco, M. F., Minelli, C., Davey Smith, G., Sheehan, N. A., and Thompson, J. R. (2016b). Assessing the suitability of summary data for two-sample Mendelian randomization analyses using MR-Egger regression: the role of the I2 statistic. *Int. J. Epidemiol.* 45, dyw220–dyw1974. doi: 10.1093/ije/dyw220

Bruyere, F., Traxer, O., Saussine, C., and Lechevallier, E. (2008). Infection and urinary lithiasis. *Prog. Urol.* 18, 1015–1020. doi: 10.1016/j.purol.2008.09.015

Burgess, S., Dudbridge, F., and Thompson, S. G. (2016). Combining information on multiple instrumental variables in Mendelian randomization: comparison of allele score and summarized data methods. *Stat. Med.* 35, 1880–1906. doi: 10.1002/sim.6835

Burgess, S., Small, D. S., and Thompson, S. G. (2017). A review of instrumental variable estimators for Mendelian randomization. *Stat. Methods Med. Res.* 26, 2333–2355. doi: 10.1177/0962280215597579

Cazzola, M., Rogliani, P., Aliberti, S., Blasi, F., and Matera, M. G. (2017). An update on the pharmacotherapeutic management of lower respiratory tract infections. *Expert. Opin. Pharmacother.* 18, 973–988. doi: 10.1080/14656566.2017.1328497

Collaborators, G. L. (2017). Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the global burden of disease study 2015. *Lancet Infect. Dis.* 17, 1133–1161. doi: 10.1016/s1473-3099(17)30396-1

Collaborators, G. L. R. I. (2018). Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990-2016: a systematic analysis for the global burden of disease study 2016. *Lancet Infect. Dis.* 18, 1191–1210. doi: 10.1016/s1473-3099(18)30310-4

Dan, Y. L., Wang, P., Cheng, Z., Wu, Q., Wang, X. R., Wang, D. G., et al. (2021). Circulating adiponectin levels and systemic lupus erythematosus: a two-sample Mendelian randomization study. *Rheumatology (Oxford)* 60, 940–946. doi: 10.1093/rheumatology/keaa506

Dang, A. T., Begka, C., Pattaroni, C., Caley, L. R., Floto, R. A., Peckham, D. G., et al. (2023). Butyrate regulates neutrophil homeostasis and impairs early antimicrobial activity in the lung. *Mucosal Immunol.* 16, 476–485. doi: 10.1016/j.mucimm.2023.05.005

Duboc, H., Rajca, S., Rainteau, D., Benarous, D., Maubert, M. A., Quervain, E., et al. (2013). Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. *Gut* 62, 531–539. doi: 10.1136/gutjnl-2012-302578

Egerton, S., Donoso, F., Fitzgerald, P., Gite, S., Fouhy, F., Whooley, J., et al. (2022). Investigating the potential of fish oil as a nutraceutical in an animal model of early life stress. *Nutr. Neurosci.* 25, 356–378. doi: 10.1080/1028415x.2020.1753322

Gensollen, T., Iyer, S. S., Kasper, D. L., and Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science* 352, 539–544. doi: 10.1126/science.aad9378

Gozalbo-Rovira, R., Rubio-Del-Campo, A., Santiso-Bellón, C., Vila-Vicent, S., Buesa, J., Delgado, S., et al. (2021). Interaction of intestinal bacteria with human rotavirus during infection in children. *Int. J. Mol. Sci.* 22:1010. doi: 10.3390/ijms22031010

Hartwig, F. P., Davey Smith, G., and Bowden, J. (2017). Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. *Int. J. Epidemiol.* 46, 1985–1998. doi: 10.1093/ije/dyx102

Hemani, G., Zheng, J., Elsworth, B., Wade, K. H., Haberland, V., Baird, D., et al. (2018). The MR-base platform supports systematic causal inference across the human phenome. *elife* 7:e34408. doi: 10.7554/eLife.34408

Hidalgo-Cantabrana, C., Delgado, S., Ruiz, L., Ruas-Madiedo, P., Sánchez, B., and Margolles, A. (2017). Bifidobacteria and their health-promoting effects. *Microbiol Spectr* 5. doi: 10.1128/microbiolspec.BAD-0010-2016

Hiremath, S., and Viswanathan, P. (2022). *Oxalobacter formigenes*: a new hope as a live biotherapeutic agent in the management of calcium oxalate renal stones. *Anaerobe* 75:102572. doi: 10.1016/j.anaerobe.2022.102572

Ichinohe, T., Pang, I. K., Kumamoto, Y., Peaper, D. R., Ho, J. H., Murray, T. S., et al. (2011). Microbiota regulates immune defense against respiratory tract influenza a virus infection. *Proc. Natl. Acad. Sci. U. S. A.* 108, 5354–5359. doi: 10.1073/pnas.1019378108

Jiang, L., Zheng, Z., Fang, H., and Yang, J. (2021). A generalized linear mixed model association tool for biobank-scale data. *Nat. Genet.* 53, 1616–1621. doi: 10.1038/s41588-021-00954-4

Joseph, M. M., and Edwards, A. (2019). Acute bronchiolitis: assessment and management in the emergency department. *Pediatr. Emerg. Med. Pract.* 16, 1–24.

Kamat, M. A., Blackshaw, J. A., Young, R., Surendran, P., Burgess, S., Danesh, J., et al. (2019). PhenoScanner V2: an expanded tool for searching human genotype-phenotype associations. *Bioinformatics* 35, 4851–4853. doi: 10.1093/bioinformatics/btz469

Kamata, K., Thein, K. N., Di Ja, L., Win, N. C., Win, S. M. K., Suzuki, Y., et al. (2022). Clinical manifestations and outcome of viral acute lower respiratory infection in hospitalised children in Myanmar. *BMC Infect. Dis.* 22:350. doi: 10.1186/s12879-022-07342-1

Kawahara, T., Takahashi, T., Oishi, K., Tanaka, H., Masuda, M., Takahashi, S., et al. (2015). Consecutive oral administration of *Bifidobacterium longum* MM-2 improves the

defense system against influenza virus infection by enhancing natural killer cell activity in a murine model. *Microbiol. Immunol.* 59, 1–12. doi: 10.1111/1348-0421.12210

Kim, C. H., Park, J., and Kim, M. (2014). Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. *Immune Netw* 14, 277–288. doi: 10.4110/in.2014.14.6.277

Kinkade, S., and Long, N. A. (2016). Acute bronchitis. *Am. Fam. Physician* 94, 560–565.

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., Demirkan, A., et al. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Langelier, C., Kalantar, K. L., Moazed, F., Wilson, M. R., Crawford, E. D., Deiss, T., et al. (2018). Integrating host response and unbiased microbe detection for lower respiratory tract infection diagnosis in critically ill adults. *Proc. Natl. Acad. Sci. U. S. A.* 115, E12353–e12362. doi: 10.1073/pnas.1809700115

Li, K. L., Wang, B. Z., Li, Z. P., Li, Y. L., and Liang, J. J. (2019). Alterations of intestinal flora and the effects of probiotics in children with recurrent respiratory tract infection. *World J. Pediatr.* 15, 255–261. doi: 10.1007/s12519-019-00248-0

Li, Y., Zhang, S. X., Yin, X. F., Zhang, M. X., Qiao, J., Xin, X. H., et al. (2021). The gut microbiota and its relevance to peripheral lymphocyte subpopulations and cytokines in patients with rheumatoid arthritis. *J Immunol Res* 2021:6665563. doi: 10.1155/2021/6665563

Liu, X., Mao, B., Gu, J., Wu, J., Cui, S., Wang, G., et al. (2021). Blautia-a new functional genus with potential probiotic properties? *Gut Microbes* 13, 1–21. doi: 10.1080/19490976.2021.1875796

Luu, T. H., Michel, C., Bard, J. M., Dravet, F., Nazih, H., and Bobin-Dubigeon, C. (2017). Intestinal proportion of Blautia sp. is associated with clinical stage and histoprognostic grade in patients with early-stage breast cancer. *Nutr. Cancer* 69, 267–275. doi: 10.1080/01635581.2017.1263750

Martinez, F. A., Balciunas, E. M., Converti, A., Cotter, P. D., and de Souza Oliveira, R. P. (2013). Bacteriocin production by Bifidobacterium spp. A review. *Biotechnol. Adv.* 31, 482–488. doi: 10.1016/j.biotechadv.2013.01.010

Merra, G., Noce, A., Marrone, G., Cintoni, M., Tarsitano, M. G., Capacci, A., et al. (2020). Influence of Mediterranean diet on human gut microbiota. *Nutrients* 13:7. doi: 10.3390/nu13010007

Morrison, J., Knoblauch, N., Marcus, J. H., Stephens, M., and He, X. (2020). Mendelian randomization accounting for correlated and uncorrelated pleiotropic effects using genome-wide summary statistics. *Nat. Genet.* 52, 740–747. doi: 10.1038/s41588-020-0631-4

Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., et al. (2007). Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* 446, 557–561. doi: 10.1038/nature05698

Nishino, K., Nishida, A., Inoue, R., Kawada, Y., Ohno, M., Sakai, S., et al. (2018). Analysis of endoscopic brush samples identified mucosa-associated dysbiosis in inflammatory bowel disease. *J. Gastroenterol.* 53, 95–106. doi: 10.1007/s00535-017-1384-4

Ohlsson, B. (2022). Functional bowel symptoms in the general population (review). *Mol. Med. Rep.* 26:226. doi: 10.3892/mmr.2022.12742

Ozen, M., Kocabas Sandal, G., and Dinleyici, E. C. (2015). Probiotics for the prevention of pediatric upper respiratory tract infections: a systematic review. *Expert. Opin. Biol. Ther.* 15, 9–20. doi: 10.1517/14712598.2015.980233

Palmer, T. M., Lawlor, D. A., Harbord, R. M., Sheehan, N. A., Tobias, J. H., Timpson, N. J., et al. (2012). Using multiple genetic variants as instrumental variables for modifiable risk factors. *Stat. Methods Med. Res.* 21, 223–242. doi: 10.1177/0962280210394459

Peng, S., Du, T. H., and Zhang, M. (2016). Changes in gut microbiota and serum D-lactate level and correlation analysis in children with recurrent pneumonia. *Zhongguo Dang Dai Er Ke Za Zhi* 18, 113–116. doi: 10.7499/j.issn.1008-8830.2016.02.004

Qu, L., Cheng, Q., Wang, Y., Mu, H., and Zhang, Y. (2022). COPD and gut-lung axis: how microbiota and host inflammasome influence COPD and related therapeutics. *Front. Microbiol.* 13:868086. doi: 10.3389/fmicb.2022.868086

Rajilić-Stojanović, M., Biagi, E., Heilig, H. G., Kajander, K., Kekkonen, R. A., Tims, S., et al. (2011). Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* 141, 1792–1801. doi: 10.1053/j.gastro.2011.07.043

Samuelson, D. R., Welsh, D. A., and Shellito, J. E. (2015). Regulation of lung immunity and host defense by the intestinal microbiota. *Front. Microbiol.* 6:1085. doi: 10.3389/fmicb.2015.01085

Skrivankova, V. W., Richmond, R. C., Woolf, B. A. R., Yarmolinsky, J., Davies, N. M., Swanson, S. A., et al. (2021). Strengthening the reporting of observational studies in epidemiology using Mendelian randomization: the STROBE-MR statement. *JAMA* 326, 1614–1621. doi: 10.1001/jama.2021.18236

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., and Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Adv. Immunol.* 121, 91–119. doi: 10.1016/b978-0-12-800100-4.00003-9

Taufer, C. R., and Rampelotto, P. H. (2023). The role of Bifidobacterium in COVID-19: a systematic review. *Life (Basel)* 13:1847. doi: 10.3390/life13091847

Trompette, A., Gollwitzer, E. S., Yadava, K., Sichelstiel, A. K., Sprenger, N., Ngom-Bru, C., et al. (2014). Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat. Med.* 20, 159–166. doi: 10.1038/nm.3444

Tuovinen, E., Keto, J., Nikkilä, J., Mättö, J., and Lähteenmäki, K. (2013). Cytokine response of human mononuclear cells induced by intestinal Clostridium species. *Anaerobe* 19, 70–76. doi: 10.1016/j.anaerobe.2012.11.002

Verbanck, M., Chen, C. Y., Neale, B., and Do, R. (2018). Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat. Genet.* 50, 693–698. doi: 10.1038/s41588-018-0099-7

Verdam, F. J., Fuentes, S., de Jonge, C., Zoetendal, E. G., Erbil, R., Greve, J. W., et al. (2013). Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. *Obesity (Silver Spring)* 21, E607–E615. doi: 10.1002/oby.20466

Vieira, A. T., Rocha, V. M., Tavares, L., Garcia, C. C., Teixeira, M. M., Oliveira, S. C., et al. (2016). Control of *Klebsiella pneumoniae* pulmonary infection and immunomodulation by oral treatment with the commensal probiotic *Bifidobacterium longum* 5(1A). *Microbes Infect.* 18, 180–189. doi: 10.1016/j.micinf.2015.10.008

Weiss, B., Bujanover, Y., Yahav, Y., Vilozni, D., Fireman, E., and Efrati, O. (2010). Probiotic supplementation affects pulmonary exacerbations in patients with cystic fibrosis: a pilot study. *Pediatr. Pulmonol.* 45, 536–540. doi: 10.1002/ppul.21138

Wolvers, D., Antoine, J. M., Myllyluoma, E., Schrezenmeir, J., Szajewska, H., and Rijkers, G. T. (2010). Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of infections by probiotics. *J. Nutr.* 140, 698S–712S. doi: 10.3945/jn.109.113753

Yan, F., Li, N., Shi, J., Li, H., Yue, Y., Jiao, W., et al. (2019). *Lactobacillus acidophilus* alleviates type 2 diabetes by regulating hepatic glucose, lipid metabolism and gut microbiota in mice. *Food Funct.* 10, 5804–5815. doi: 10.1039/c9fo01062a

Zeissig, S., and Blumberg, R. S. (2014). Life at the beginning: perturbation of the microbiota by antibiotics in early life and its role in health and disease. *Nat. Immunol.* 15, 307–310. doi: 10.1038/ni.2847

Zhang, Q., Hu, J., Feng, J. W., Hu, X. T., Wang, T., Gong, W. X., et al. (2020). Influenza infection elicits an expansion of gut population of endogenous *Bifidobacterium animalis* which protects mice against infection. *Genome Biol.* 21:99. doi: 10.1186/s13059-020-02007-1

Zhu, L., Sha, L., Li, K., Wang, Z., Wang, T., Li, Y., et al. (2020). Dietary flaxseed oil rich in omega-3 suppresses severity of type 2 diabetes mellitus via anti-inflammation and modulating gut microbiota in rats. *Lipids Health Dis.* 19:20. doi: 10.1186/s12944-019-1167-4

Glossary

AMPs	Antimicrobial peptides
CAUSE	Causal Analysis Using Summary Effect Estimates
COPD	Chronic obstructive pulmonary disease
CI	Confidence interval
GWAS	Genome-wide association study
IV	Instrumental variable
IVW	Inverse variance weighted
LD	Linkage disequilibrium
LRTIs	Lower respiratory tract infections
MR	Mendelian randomization
MD	Mediterranean diet
OR	Odds ratio
RRTIs	Recurrent respiratory tract infections
RSV	Respiratory syncytial virus
SCFAs	Short-chain fatty acids
SNP	Single nucleotide polymorphism
TNF-α	Tumor necrosis factor α

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Characterization of the probiotic properties of *Lacticaseibacillus rhamnosus* LR6 isolated from the vaginas of healthy Korean women against vaginal pathogens

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The human microbiome exhibits intricate populations across the body, with the vaginal tract serving as an ecosystem characterized by the prevalence of the genus *Lactobacillus*. Disruptions in the vaginal microbiota, which are frequently linked to variables such as sexual activity, hormonal fluctuations, and excessive use of antibiotics, can result in vaginal dysbiosis and the development of diseases such as bacterial vaginosis (BV) and candidiasis. *Lactobacillus* species, owing to their capacity to create an acidic environment through the production of lactic acid, have a key function within this complex microbial community: they inhibit the growth of harmful microorganisms. This study aimed to investigate the genomic characteristics of *L. rhamnosus* LR6, a newly discovered strain isolated from the vaginal microbiota of 20 healthy women to assess its potential as a vaginal probiotic. We performed a comparative investigation of the genetic traits of *L. rhamnosus* using 45 publicly available genomes from various sources. We evaluated the genetic characteristics related to carbohydrate utilization, adhesion to host cells, and the presence of bacteriocin clusters. A comprehensive study was conducted by integrating *in silico* evaluations with experimental techniques to authenticate the physiological characteristics of strain LR6. We further used a rat model to assess the impact of *L. rhamnosus* LR6 administration on the changes in the gastrointestinal tract and the vaginal microbiome. The assessments revealed a significantly high inhibitory activity against pathogens, enhanced adherence to host cells, and high lactic acid production. Rat experiments revealed changes in both the fecal and vaginal microbiota; in treated rats, *Firmicutes* increased in both; *Lactobacillaceae* increased in the fecal samples; and *Enterobacteriaceae* decreased but *Enterococcaceae*, *Streptococcaceae*, and *Morganellaceae* increased in the vaginal samples. The study results provide evidence of the genetic characteristics and probiotic properties of LR6, and suggest that oral

administration of *L. rhamnosus* LR6 can alter both gut and vaginal microbiome. Collectively, these findings establish *L. rhamnosus* LR6 as a highly promising candidate for improving vaginal health.

KEYWORDS

***Lacticaseibacillus rhamnosus*, vaginal probiotics, vaginal microbiome, bacterial vaginosis, vaginal candidiasis, bacteriocin, adhesion**

1 Introduction

The human microbiome presents complex patterns throughout the body; the vaginal tract is a particular ecosystem in which one genus, *Lactobacillus*, is predominant (Chee et al., 2020). Because of the presence of these lactic acid-producing bacteria, healthy vaginal tracts are regularly acidic, and this acidic environment can inhibit the overgrowth of potentially harmful bacteria. Although the exact reason for the disruption of the vaginal microbiota is unknown, dysbiosis is strongly associated with bacterial vaginosis (BV) caused by sexual activity, hormonal changes, or overuse of antibiotics (Abbe and Mitchell, 2023). The most prevalent bacteria that commonly cause BV include *Gadnerella vaginalis*, *Escherichia coli*, and several *Prevotella* species (Srinivasan et al., 2012). Similar to BV, candidiasis, is also a major cause of infection caused by *Candida* spp. (Pappas et al., 2018).

Several drugs, including metronidazole or clindamycin for BV, and fluconazole and clotrimazole for *Candidal vaginitis*, are efficient therapies for vaginal infections. However, these treatments can exacerbate vaginal dysbiosis, which is already present in vaginal infections, potentially disrupting the delicate balance of the vaginal microbiota by affecting beneficial microorganisms (Bradshaw et al., 2006; Schuyler et al., 2016). In contrast, probiotics can promote the recovery process by restoring the vaginal microbiota's environment (Liu et al., 2023). *Lactobacilli*, recognized for their capacity to maintain an acidic microenvironment through lactic acid production, play an essential role in this intricate microbial community by impeding the growth of detrimental microbes (Wu et al., 2022).

Lactobacillus rhamnosus, a species thoroughly studied within the *Lactobacillus* genus, has been isolated in many environments such as the human gastrointestinal tract and dairy products. This species is widely recognized for its potential to modulate the gut microbiota and enhance the immune system (Vélez et al., 2010; Martín et al., 2019). Therefore, it is frequently used as a probiotic supplement and has been integrated into many food products (Westerik et al., 2018). Not just in human gut system, it also has a great potential for probiotics for promoting vaginal health owing to antimicrobial characteristics, capacity to adhere to vaginal epithelial cells, and ability to produce hydrogen peroxide (Petrova et al., 2021). Increasing attention toward the therapeutic use of probiotics for improving vaginal health has inspired researchers to explore novel strains that exhibit enhanced beneficial properties (Liu et al., 2023).

This study aimed to explore the genomic attributes of *L. rhamnosus*, LR6, a newly isolated strain from the vaginal microbiota of a healthy female. We investigated the genetic characteristics of LR6, obtaining insights into its potential vaginal probiotic properties to adapt to the vaginal environment, by conducting a comparative analysis of its genomic properties with publicly accessible genomes of *L. rhamnosus* obtained from diverse origins. The inclusion of this substantial number of genomes serves to enhance the robustness of the comparative genomic analysis, facilitating a deeper understanding of the species' genetic variation and potential probiotic capabilities. In addition, we revalidated the physiological traits of the strains inferred from their genetic features by utilizing various vaginal pathogens and host cells, not only through *in silico* analysis but also through experimental approaches.

2 Materials and methods

2.1 Isolation of *Lacticaseibacillus* LR6 strain from the vaginal tracts of healthy women

Between November and December 2022, vaginal swab samples from 20 healthy Korean women were used to obtain the clinically isolated *L. rhamnosus* strain LR6. The subjects ranged in age from 19 to 50 years, had regular menstrual cycles, and had no vaginitis or cervicitis symptoms (Nugent score of 1–3 points for vaginal discharge). The study protocol was approved by the Institutional Review Board of the Konkuk University Medical Center (KUMC 2022-04-030). The vagina swab kit (Noblebio, South Korea) was used as a vaginal discharge sampling kit and collected through internal examination at the Department of Obstetrics and Gynecology using a vaginal swab kit (Noblebio, South Korea). The swabs were streaked on de Man, Rogosa, and Sharpe (MRS) agar plates (Difco, Detroit, MI, USA) supplemented with 0.01% sodium azide and incubated at 37°C under aerobic conditions for 24 h. Using the single colonies, colony PCR was performed using the universal 16S rDNA sequences primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTAGACTT-3').

2.2 DNA extraction and whole-genome sequencing

DNA from the bacterial resuspension of *L. rhamnosus* LR6 was with DNA Link (DNA Link Inc., Seoul, Korea) using the QIAGEN MagAttract HMW DNA kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Using Megaruptor v3, we generated 10 kb fragments by shearing genomic DNA according

to the manufacturer's recommended protocol. An AMpureXP bead purification system was used to remove small fragments. A total of 500 ng of each sample was used as input for library preparation. The SMRTbell library was constructed using the SMRTbell Express Template Preparation Kit v2.0 (101-685-400). Samples were pooled according to the volumes provided by the Microbial Multiplexing Calculator, and small fragments of less than 3 kb were removed using the AMpureXP bead purification system for the large-insert library. After the sequencing primer was annealed to the SMRTbell template, DNA polymerase was bound to the complex (Sequel II Binding kit 3.2). The complex was purified using AMPure to remove the excess primers and polymerase prior to sequencing. Library preparation and sequencing were performed using DNA Link (DNA Link Inc., Seoul, Korea). The SMRTbell library was sequenced using SMRT cells (Pacific Biosciences) with the Sequel II Sequencing Kit v2.0, and 1 × 15 h movies were captured for each SMRT Cell 8M using the Sequel II (Pacific Biosciences) sequencing platform.

2.3 Comparative genomics of *L. rhamnosus*

The 45 publicly available whole-genome sequences (status: complete, 2023.06) of *L. rhamnosus* were retrieved from the NCBI database. These genomes, including that of the newly isolated LR6 strain, were used for comparative genomic analysis. All genomes were converted to GFF format using the Roary pipeline (Page et al., 2015), and orthologs were clustered into the core genome, pan-genome, and gene sets with accessory and unique genes. Clustered gene sets and statistical information were subjected to the Pagoo pipeline, R package for pan-genome analysis (Ferrés and Iraola, 2021). Core gene sets were aligned using the Roary pipeline, and the Fasttree program was used to generate a phylogenetic tree. The constructed phylogenetic tree was visualized using the iTOL web tool (Letunic and Bork, 2021). The 45 genome sequences were used to annotate carbohydrate-active enzymes and substrates (CAZymes) using the dbCAN3 standalone version in the HMMER and dbCAN3 CAZyme databases (Zheng et al., 2023). Automatically annotated CAZyme sequences were reclassified into five classes: carbohydrate-binding protein module families (CBM), carbohydrate esterases (CEs), glycoside hydrolases (GHs), glycosyltransferases (GTs), and polysaccharide lyases (PLs). To observe the genetic distribution of LR6 mucosal adhesion genes associated with host interaction, spaCBA-strC1 (spaA:LGG_00442, spaB:LGG_00443, spaC:LGG_00444, srtC1:LGG_00441) spaFDE-strC2 (spaD:LGG_02370, spaE:LGG_02371, spaF:LGG_02372,srtC2:LGG_02369), mucus-binding factor (MBF) with Pfam-MucBP domain repeats (LGG_02337), adhesion modulator MabA (LGG_01865), and lectin-like protein-coding gene (LGR1_llp1) sequences were retrieved and used as query for blast analysis. Only genes with sequence similarity and coverage of >90% were used. Bacteriocin clusters were identified using BAGEL 4 standalone version (Van Heel et al., 2018). According to the area of interest, core and accessory genes were visualized using the gggenes package (ver. 0.5.1).

2.4 Growth conditions of bacterial strains

Three strains of *L. rhamnosus* were used in this study: one newly isolated from the human vagina (*L. rhamnosus* strain LR6) and two control strains (*L. rhamnosus* ATCC 7469T and LGG). All *L. rhamnosus* strains were incubated in de MRS broth for 18 h at 37°C. Three pathogens were used to determine the inhibitory effects of lactic acid bacteria on BV. *Candida albicans* ATCC 18804 and *Escherichia coli* ATCC 11775 were cultivated aerobically in yeast extract–peptone–dextrose (YPD) and Luria–Bertani (LB) broth media, and *Gardnerella vaginalis* ATCC 14018 was cultured in trypticase soy broth (TSB) with 2% of vitamin K1-hemin solution at 37°C under anaerobic conditions. To compare the potential survival in an acidic tolerance under anaerobic conditions, *L. rhamnosus* were cultured in pH3, 4, 5, and 6 in MRS media for 18h at 37°C under the aearobic conditions, and optical density (OD) at 600nm were measured.

2.5 Antibacterial and antifungal activities

To assess the antimicrobial activity of the three *L. rhamnosus* strains (LR6, L7469T, and LGG), *C. albicans* was inoculated into YPD media, *G. vaginalis* into TSB media, and *E. coli* into LB media. The supernatant from each *L. rhamnosus* strain was obtained by filtering bacterial cells (0.2 μm) after an 18-hour culture in MRS liquid medium. Following 18 hours of co-incubation with the respective pathogens, the inhibitory effect of the LR6, L7469T, and LGG supernatants was evaluated by measuring the optical density (OD) at 600 nm of the pathogen cultures.

2.6 Co-aggregation assay

Co-aggregation assay of *Lactobacillus* and vaginal pathogens was performed as previously described (Reuben et al., 2019). The culture media and conditions for *L. rhamnosus* LR6, *C. albicans*, *G. vaginalis*, and *E. coli* were the same as described in section "2.4. Growth conditions of bacterial strains" Cultures of *L. rhamnosus* LR6 and the pathogens were collected, washed with phosphate-buffered saline (PBS) twice, and each diluted to a standardized OD of 1.0 at 600 nm using PBS. A 2ml suspension of *L. rhamnosus* LR6 at OD 1.0 and a 2ml suspension of each pathogen at OD 1.0 were then co-incubated in round-bottom tubes at 37°C for 4 hours. Similarly, 4ml suspensions of *L. rhamnosus* and each of the pathogens were prepared individually under the same conditions. The OD of each single microbe suspension (ODLR and ODpathogen) and mixed suspension (ODmix) was measured at 600 nm, and the co-aggregation ratio was calculated as follows:

$$Coaggreation\ \% = \left[1 - \frac{2(ODmix)}{ODLR + ODpathogen}\right] \times 100$$

2.7 HeLa cell adhesion assay

HeLa cells used in this study were purchased from the Korean Cell Line Bank and cultivated on Park Memorial Institute

1640(RPMI 1640, Gibco, USA) supplemented with 10% fetal bovine serum(FBS, Gibco, USA) at 37°C for 2 days in 5% CO2. The HeLa cells were diluted to a concentration of 5×10^4 cells per well and incubated at 37°C and 5% CO_2 for 48 h. When the cells reached 80% confluence, dead cells were removed using PBS and distilled water (DW). Subsequently, each *L. rhamnosus* strain was inoculated at a concentration of 2×10^8 colony-forming units per milliliter (CFU/mL) and incubated for 1 h at 37 °C. Each well was washed with pre-PBS and DW and treated with trypsin and ethylenediaminetetraacetic acid (EDTA). Cells containing *L. rhamnosus* were quantified by colony counting on the MRS agar plates.

2.8 Measurement of metabolic acid-producing ability

The *L. rhamnosus* strains were cultured in MRS broth to confirm their acid-producing abilities. After centrifugation 15 m, 37 °C, 4000rpm, the supernatant was collected and sterilized by membrane filtration (0.2 μm). The concentrations of acetic, citric, and lactic acids were measured using high-performance liquid chromatography equipped with UV-DAD (HPLC Infinity 1290, Agilent Technologies). The separation of targeted metabolic acids was conducted on the reversed-phase analytical column CAPCELL PAK C18 MG (4.6 mm × 250 mm, 5 μm, Shiseido) maintained at 30°C. The binary mobile phase contained both A and B was 10 mM potassium dihydrogen phosphate (KH2PO4, pH 2.4 adjusted with phosphoric acid) and acetonitrile. The gradient of mobile phases was as follows: 0 min, A 100% B 0%; 7.5 min, A 100% B %; 8 min, A 85% B 15%; 16 min, A 85% B 15%; 17 min, A 0% B 100%; 18 min, A 0% B 100%; 19 min, A 100% B 0%; 20 min, A 100% B 0% for re-equilibrium. The flow rate was 1mL/min, UV wavelength was 210 nm and analytical time was 20 min per sample. An aliquot of 10 μL was injected and each sample was analyzed in triplicate.

2.9 Measurement of hydrogen peroxide producing ability

The hydrogen peroxide generating ability of the isolated strains was measured by streaking the strains on MRS–TMB agar (TMB: 3,3′,5,5′-tetramethylbenzidine), followed by their incubation at 37 °C for 48 h under anaerobic conditions. The plates were exposed to air for 30 min. Hydrogen peroxide-producing strains were identified by the conversion of white colonies to blue colonies owing to TMB oxidation.

2.10 Fecal and vaginal microbiota

Female 6-week-old Sprague-Dawley rats (n = 12) were purchased from Orient Bio. The objective was to assess the impact of *L. rhamnosus* LR6 administration on the changes in the microbiome of the gastrointestinal tract and vagina. The rats were housed in an environmentally controlled room (24 ± 2 °C, 40–60% relative humidity) under a 12 h light/dark cycle, and had free access to food and water during a 1 week adaptation period. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee board in the CellBiotech (IACUC, approval No: CBT-2023-09) based on guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The rats were randomly assigned to two groups (n = 6 per group). The negative control group was administered PBS, and the experimental group was administered *L. rhamnosus* LR6 (3.82×10^9 CFU/rat) for 2 weeks, daily basis for two weeks. The same volume of PBS (pH 7.4) was used as the negative control. Fecal pellets were collected by inducing defecation through gentle abdominal massage of the mice. Following this, 100 μL of sterile phosphate-buffered saline (PBS) was used to lavage the vaginal lumen, which allowed for the collection of vaginal content samples. Fecal samples were collected on days 0 and 14 and vaginal swabs were collected on day 14.

2.11 Microbiome analysis

The fecal and vaginal samples were vigorously agitated into to buffer to dislodge the cells. The SPINeasy DNA Pro kit for Soil (MP Biochemicals, Santa Ana, CA, USA) was used to extract DNA from the mouse vagina after it washed with PBS. The Illumina 16S Metagenomic Sequencing Library Preparation guide was followed during the processing of the extracted DNA to construct a sequencing library. We used a forward primer in the V4 region (CCA GCMGCC GCG GTA ATW C) and a reverse primer in the V5 region (CC GTC AAT TYY TTT RAG TTT) to target the V4–V5 region of the bacterial 16S rRNA gene for 16S rRNA gene sequencing. The Nextera XT v2 Index Kit was then employed to perform index PCR in order to generate a library. Using the MiSeq reagent kit V2, the NGS process involves sequencing 250-bp paired-end reads on the MiSeq platform (Illumina, San Diego, CA, USA). Qiime2 (QIIME2, v2023.5, viewed on June 26, 2023)[1] was used for data processing on the FASTQ file, and DADA2 was applied for sequence quality verification.

2.12 Statistical analysis

Unpaired two-tailed t-tests for single comparisons or Wilcoxon rank-sum tests were used to assess the significance of differences in *in vitro* experimental data. A value of $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Genomic characteristics of newly isolated vaginal Lactobacilli

To elucidate the genomic features of the recently isolated strain of *L. rhamnosus* LR6 from the vaginal microbiota of healthy females, a comparative genomics analysis was conducted. This analysis involved a comparison of the genomic sequences of the

1 http://qiime2.org

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TABLE 1 Forty five strains of *L. rhamnosus* used in this study.

Strain	Accession	Size (Mb)	GC%	CDS	Origin
Lacticaseibacillus rhamnosus LOCK900	GCA_000418475.1	2.88	46.80	2578	Feces
Lacticaseibacillus rhamnosus Pen	GCA_002076955.1	2.88	46.80	2573	Feces
Lacticaseibacillus rhamnosus VHProbi M14	GCA_024610975.1	2.90	46.70	2628	Fermented food
Lacticaseibacillus rhamnosus LV108	GCA_013167115.1	2.92	46.80	2630	Feces
Lacticaseibacillus rhamnosus B6	GCA_016599675.2	2.92	46.80	2628	Fermented food
Lacticaseibacillus rhamnosus AS	GCA_018286375.1	2.94	46.80	2583	Fermented food
Lacticaseibacillus rhamnosus CE1	GCA_018141205.1	2.94	46.80	2587	Fermented food
Lacticaseibacillus rhamnosus Strain 1.032	GCA_006151905.1	2.94	46.70	2622	Fermented food
Lacticaseibacillus rhamnosus ATCC 8530	GCA_000233755.1	2.96	46.80	2621	Feces
Lacticaseibacillus rhamnosus CLK 101	GCA_024442295.1	2.96	46.64	2635	Fermented food
Lacticaseibacillus rhamnosus GR-1	GCA_024665595.1	2.97	46.64	2638	Urethra
Lacticaseibacillus rhamnosus LR6	in this study	2.97	46.74	2782	Vagina
Lacticaseibacillus rhamnosus LR5	GCA_002286235.1	2.97	46.70	2659	Feces
Lacticaseibacillus rhamnosus NCTC13764	GCA_900636965.1	2.99	46.80	2654	Unknown
Lacticaseibacillus rhamnosus BIO5326	GCA_009720565.1	2.99	46.80	2655	Feces
Lacticaseibacillus rhamnosus SCT101060	GCA_002960215.1	2.99	46.80	2648	Feces
Lacticaseibacillus rhamnosus JCM1553	GCA_003433395.1	2.99	46.80	2641	Unknown
Lacticaseibacillus rhamnosus A5	GCA_029543065.1	2.99	46.80	2617	Fermented food
Lacticaseibacillus rhamnosus LOCK908	GCA_000418495.1	2.99	46.80	2665	Feces
Lacticaseibacillus rhamnosus CAU 1365	GCA_019967935.1	2.99	46.80	2667	Fermented food
Lacticaseibacillus rhamnosus NCTC13710	GCA_900636875.1	2.99	46.80	2651	Unknown
Lacticaseibacillus rhamnosus VHProbi Y39	GCA_022220485.1	2.99	46.80	2655	Feces
Lacticaseibacillus rhamnosus X253	GCA_018228745.1	2.99	46.80	2649	Fermented food
Lacticaseibacillus rhamnosus P118	GCA_023913535.1	2.99	46.80	2648	Fermented food
Lacticaseibacillus rhamnosus VSI33	GCA_029010255.1	2.99	46.80	2649	Vagina
Lacticaseibacillus rhamnosus PMC203	GCA_020826335.1	2.99	46.66	2647	Vagina
Lacticaseibacillus rhamnosus HN067	GCA_024397415.1	3.00	46.75	2666	Feces
Lacticaseibacillus rhamnosus ATCC 53103	GCA_000011045.1	3.01	46.70	2681	Feces
Lacticaseibacillus rhamnosus BIO6870	GCA_008831425.1	3.01	46.70	2691	Feces
Lacticaseibacillus rhamnosus GG	GCA_028475085.1	3.01	46.70	2699	Feces
Lacticaseibacillus rhamnosus LDTM7511	GCA_017795605.1	3.01	46.70	2665	Feces
Lacticaseibacillus rhamnosus LR-B1	GCA_004010975.1	3.01	46.70	2677	Blood
Lacticaseibacillus rhamnosus MGYG-HGUT-01293	GCA_902381635.1	3.01	46.70	2690	Feces
Lacticaseibacillus rhamnosus DSM 14870	GCA_002287945.1	3.01	46.70	2697	Vagina
Lacticaseibacillus rhamnosus DM065	GCA_024053515.2	3.02	46.59	2702	Oral
Lacticaseibacillus rhamnosus VHProbi F20	GCA_026427555.1	3.02	46.69	2717	Feces
Lacticaseibacillus rhamnosus BPL5	GCA_900070175.1	3.02	46.70	2704	Vagina
Lacticaseibacillus rhamnosus DM054	GCA_024158105.2	3.03	46.69	2704	Oral
Lacticaseibacillus rhamnosus Lc 705	GCA_000026525.1	3.03	46.63	2648	Dairy
Lacticaseibacillus rhamnosus 4B15	GCA_002158925.1	3.05	46.70	2734	Feces
Lacticaseibacillus rhamnosus VSI43	GCA_029011275.1	3.05	46.73	2692	Vagina
Lacticaseibacillus rhamnosus TK-F8B	GCA_015377485.1	3.06	46.65	2728	Fermented food
Lacticaseibacillus rhamnosus hsryfm 1301	GCA_008727835.1	3.07	46.75	2769	Feces
Lacticaseibacillus rhamnosus BFE5264	GCA_001988935.1	3.11	46.76	2785	Dairy
Lacticaseibacillus rhamnosus KF7	GCA_016653515.1	3.25	46.64	2902	Fermented food

newly isolated strain with those of 45 publicly accessible genomes of *L. rhamnosus*. This study included 45 strains of *L. rhamnosus*, which were obtained from various sources; these sources were feces (18 strains), fermented products (12 strains), dairy products (2 strains), the vagina (6 strains), urethra (1 strain), blood (1 strain), oral (2 strains), and unknown sources (3 strains). The genome sizes of these strains ranged from 2.88 Mb to 3.25 Mb, as shown in **Table 1**. Based on a study of the core and pan-genome, 1895 genes were identified and classified as the core gene set. Additionally, 1433 and 2373 out of the 5836 genes were estimated to belong to the shell and cloud gene categories, respectively (**Figures 1A, B**). To examine the evolutionary relationships among the sources of origin, 2373 cloud genes were annotated using the Clusters of Orthologous Genes (COG) database and subsequently classified based on their isolation sources (**Figure 1C**). Analysis of the 20 COG categories indicated that the functions of replication, recombination, and repair (L) were the most prevalent among the cloud genes derived from various sources, such as dairy, feces, fermented food, and oral and vaginal sources. Notably, the specific function of cloud genes originating from a second source remains unidentified, although they potentially play a role in the development of distinct genetic traits. In the case of the strains of vaginal origin, 29 genes were identified and annotated for carbohydrate transport and metabolism (G) and cell wall/membrane/envelope biogenesis (M), especially phosphotransferas (PTS) systems for various carbon sources and glycosyltransferase-like families. The principal component analysis (PCA) thoroughly differentiated genomes obtained from the vagina into three distinct phylogroups. However, no discernible pattern was identified based on the source of isolation, as shown in **Figure 1D**.

3.2 Genes associated with carbohydrate utilization enzymes and mucosal adhesion

The concatenated and aligned core genes were used to construct a phylogenetic tree of the 45 genomes, and four clades were identified (**Figure 2**). To explore the genetic basis of the four clades, the distribution of genes encoding carbohydrate utilization enzymes was analyzed using the CAZymes database and dbCAN3. Of the total 6759 genes, there were 836 CBM, 135 CEs, 3197 GHs, 2540 GTs, and 51 PLs. The CAZyme profiles showed that clades 1, 2, and 4 commonly possessed all types of CAZyme classes (CBM, CE, GH, GT, and PL), except for the AS and CE1 strains, whereas clade 3 displayed only four types of CAZyme profiles: CBM, CE, GH, and GT. The major CAZymes categories of LR6 were 13 genes of GH1 (β-glucosidase, EC 3.2.1.21) and 11 genes of GH13 (α-amylase, EC 3.2.1.1), which are important enzymes in the vagina environment that depolymerizes glycogen for producing lactic acid during fermentation process.

To link genetic diversity to probiotic features, mucosal adhesion genes associated with host interactions were analyzed (**Figure 2B**). Studies on *L. rhamnosus* GG, a well-documented reference strain, have shown several gene clusters associated with pathogens and vaginal colonization. spaCBA-strC1 is a proteinaceous pili coding operon previously observed in *L. rhamnosus GG* but not in GR-1 isolated from the vaginal tract. Comparably, those operons were absent from LR6 genomes also; alternatively, the spaFDE-strC2 operon was found. MBF with Pfam-MucBP domain repeats and the adhesion modulator MabA were also found upstream of the spaFDE-strC2 area in LR6. Putative lectin-like protein-coding genes (llp) are crucial factors for the adhesion ability of gastrointestinal and vaginal epithelial cells in *L. rhamnosus* GR-1. They are divided into three domains, and each region exhibits high sequence similarity with three genes from the LR6 genome. Taken together, the genes in LR6 that are linked to pilus-mediated mucosal adhesin have the potential for improved vaginal attachment and colonization as probiotics in the vagina.

3.3 Distribution of bacteriocin gene clusters

The BAGEL4 pipeline was used to determine the distribution of bacteriocin gene clusters among the 45 genomes of *L. rhamnosus*. Genes identified as open reading frames within bacteriocin biosynthetic gene clusters were categorized into three distinct functional groups: core peptide, transport/leader cleavage, and immunity/transport/modification. Considering that all strains exhibited Enterocin X chain beta and Carnocin_CP52 core peptides, with the exception of KF7, the most notable disparity between evolutionary clades was based on the presence of the LSEI_2386 domain from clades 1 or 3 (**Figure 3A**). Furthermore, it is worth noting that a particular core peptide, garvieacin Q, was detected only in certain strains within clade 3. Additionally, an absence of HlyD immune transporters in clade 1 was observed. However, these distinct genetic characteristics were not linked to the source of isolation. The distribution of the bacteriocin gene clusters, characterized by distinct patterns within each clade, is illustrated in **Figure 3B**. The prediction conducted for LR6 revealed the presence of HlyD and intA immunity transporters, in addition to the LanT exporter. Furthermore, three core peptide domains were identified: LSEI_2386, Enterocin, and Carnocin (**Figure 3B**). Taken together, the results indicate that LR6 possesses the genetic potential to synthesize class II bacteriocins through proper immune transporter and exporter genes, leading to an antimicrobial phenotype.

3.4 Antimicrobial activity of newly isolated LR6

To investigate the probiotic characteristics associated with antimicrobial activity against vaginal pathogens, we evaluated their inhibitory effects against three common pathogens, *E. coli*, *C. albicans*, and *G. vaginalis*, which cause uropathogenic and vaginal infections. These effects were compared with those of the two control strains, *L. rhamnosus* type strains ATCC7469 and GG (**Figure 4A**). The inhibitory activity of LR6 against *C. albicans* was significantly higher than that against both the type and GG strains (P-value = 0.0085 and 0.019, respectively). In addition, LR6 exhibited a significantly higher percentage of inhibition against *E. coli* than the LR-type strain (P-value = 0.0025). Moreover, LR6 had a more significant inhibitory effect on *G. vaginalis* than the LR-type strain (P-value = 0.00099). The antimicrobial

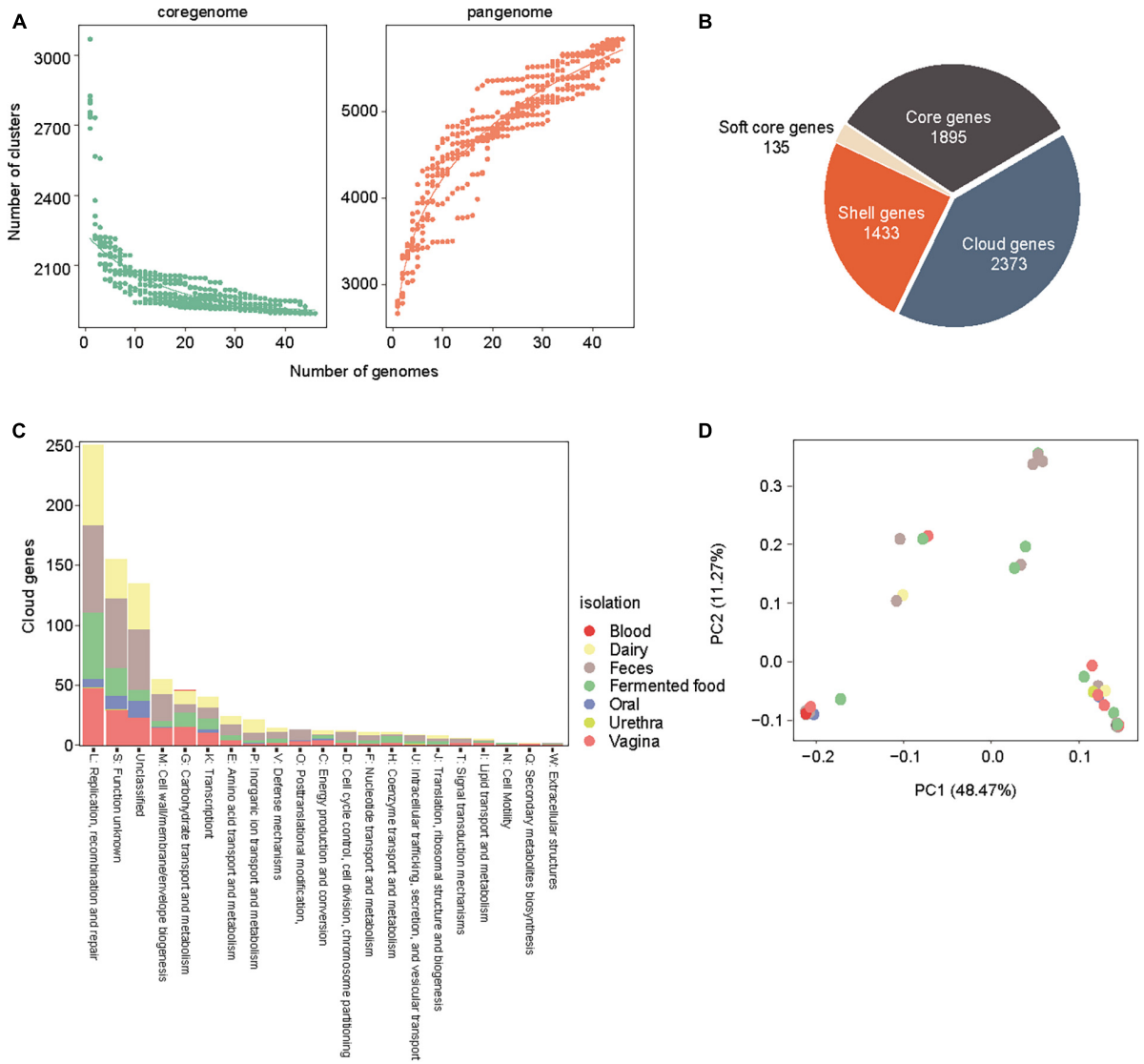


FIGURE 1
Core genome and pan genome analysis of the 45 *L.rhamnosus* strains. **(A)** Accumulation curves of core genome and pan genome of *L. rhamnosus* strains. **(B)** The number of core genes, cloud genes, shell genes and soft core genes are presented in pie chart. **(C)** COG category abundances in cloud genes of *L. rhamnosus* strains. Isolation sites were colored in the bar of COG category. **(D)** A principal components analysis generated from 45 *L. rhamnosus* genomes. Isolation sites were colored in the spots.

capability of these *L. rhamnosus* strains is well-documented to be primarily attributed to their hydrogen peroxide-producing characteristics. Therefore, we conducted an assessment of LR6's hydrogen peroxide production capacity (**Supplementary Figure 1**). As a result, LR6 was observed to produce hydrogen peroxide, and it is anticipated that this contributes to its antimicrobial abilities, likely in conjunction with the bacteriocins identified in the genome analysis.

In addition to inhibiting the growth of pathogens in the vaginal environment, the co-aggregation ability is essential because it can facilitate the formation of a barrier that prevents the colonization of pathogenic bacteria. We measured the co-aggregation ability of the LR6 strain using the same strains (**Figure 4B**). LR6 exhibited co-aggregation values of 28.59% with *C. albicans*, which were significantly higher than those of the LR-type strain (*P*-value = 0.036). When tested against uropathogenic *E. coli*, LR6 showed a co-aggregation value of 33.56%, which was significantly higher than those of both the LR-type and LGG strains (*P*-value = 0.019, 0.017, respectively). In the case of *G. vaginalis*, LR6 demonstrated a co-aggregation ability of 36.33%, similar to that of both the LR-type strain and LGG, indicating substantial co-aggregation with *G. vaginalis*.

3.5 Adhesive activity of *L. rhamnosus* LR6 on HeLa cells

In addition to co-aggregation with pathogens, adhesion to vaginal epithelial cells is a critical step in establishing colonization. Based on a previous genetic investigation of adhesin factors, the adhesion ability of HeLa cells was investigated (**Figure 4C**). Our results demonstrated a significant difference in adhesive

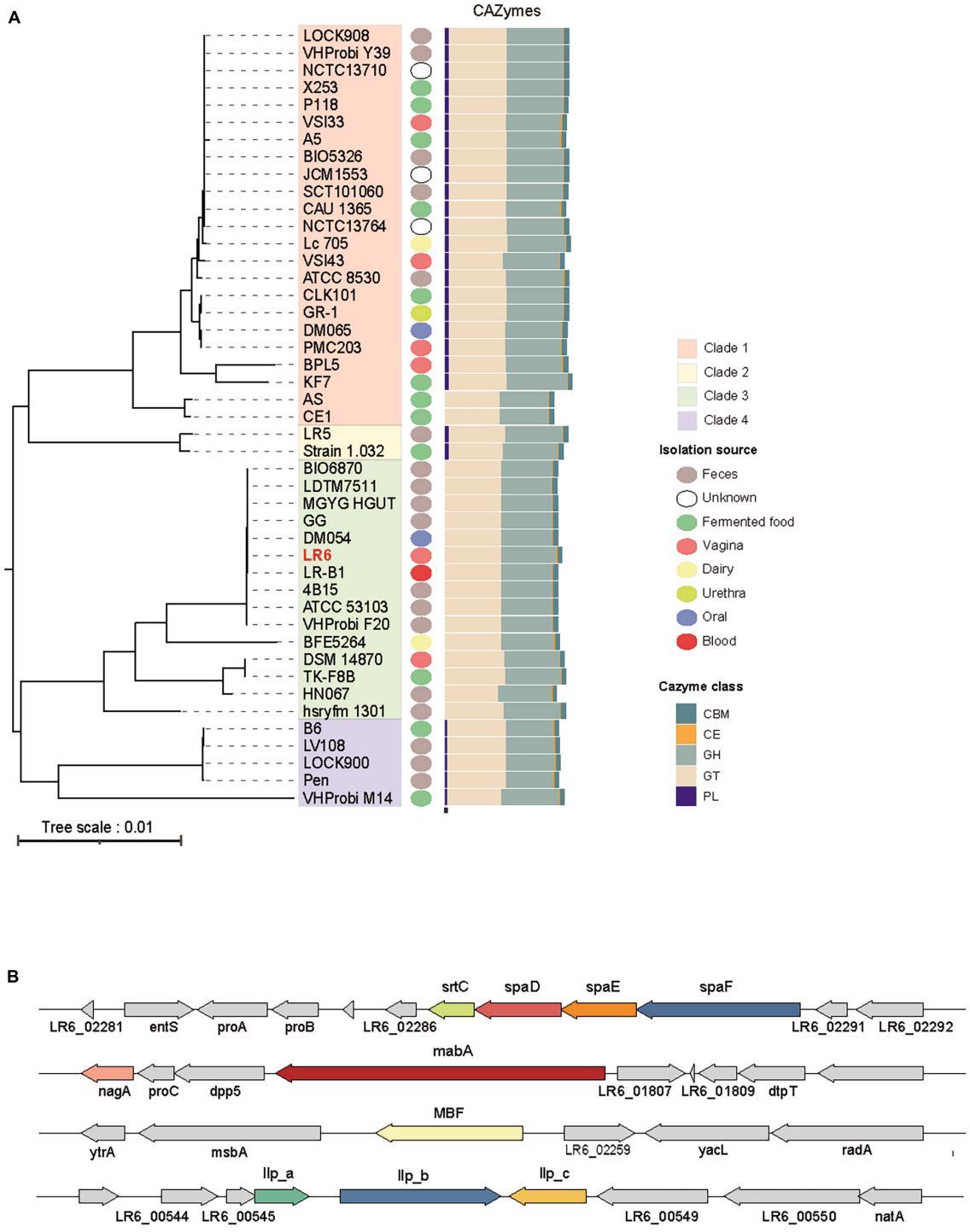


FIGURE 2
Phlyogenomic analysis of the 45 *L. rhamnosus* strains. **(A)** RAxML tree of 45 *L. rhamnosus* constructed from core-genome alignment. Branches are colored by four phylogroups. Each colored circle denoted isolation source and five classes of CAZymes utilization profiles are shown. **(B)** Mucosal adhesion genes associated with host interaction in *L. rhamnosus* LR6 were annotated through the blast search and four genomic regions are shown.

activity between *L. rhamnosus* LR6 and the type strain (*P*-value = 0.00079). The results demonstrated that LR6 showed significantly greater adhesion to HeLa cells, suggesting an enhanced capacity for interaction with vaginal epithelial cells. The adhesive capacity of LR6 was further supported by microscopic observations after Gram staining.

3.6 Organic acid production and acid tolerance

In general, a healthy vaginal environment is strongly associated with organic acids produced by the dominant genus *Lactobacillus*. To evaluate the acidification ability of the vaginal environment,

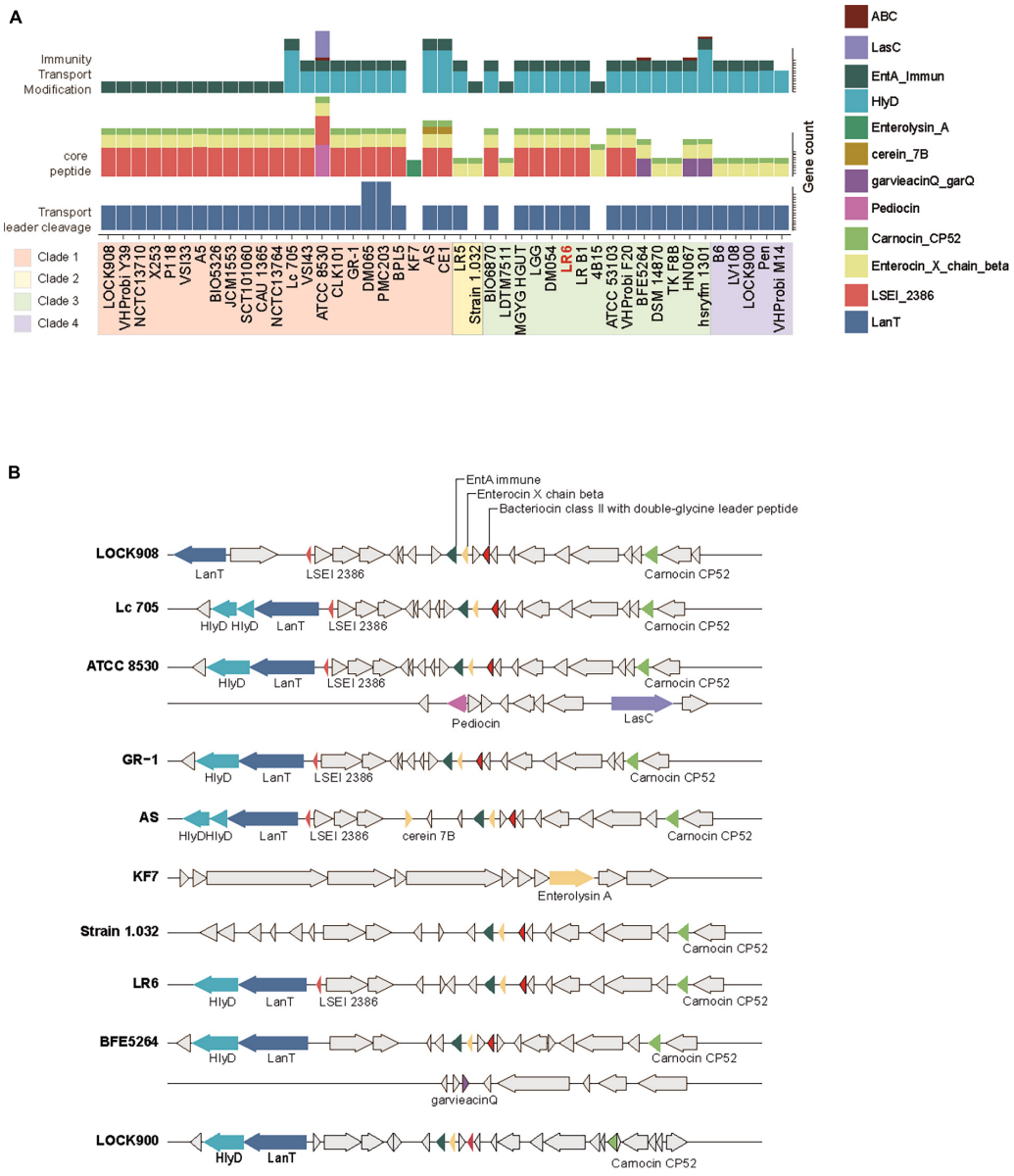


FIGURE 3
Distribution of biosynthetic gene cluster of bacteriocin genes of the 45 *L. rhamnosus* strains. **(A)** The number of gene count related to bacteriocin biosynthesis. Genes are colored based on their functions as indicate. Strains displayed with phylogenetic clade order. **(B)** Multiple sequence alignments of bacteriocin biosynthetic gene clusters among the representative strains from four phylogenetic clades. The color of gene arrow is filled in the same way as shown in panel **(A)**.

the concentrations of organic acids such as lactic acid, citric acid, and acetic acid produced by the three strains were measured (Figure 5A). When evaluating the organic acid production capabilities based on CFU quantities, it was noted that LR6 exhibited significantly elevated levels of lactic acid production than the ATCC7469 strain, measuring at 1.29 mg/10^9 CFU (P-value = 0.043). Furthermore, LR6 showed significantly higher production of acetic acid (0.35 mg/10^9 CFU). This value significantly surpassed the production levels of both the LR and LGG strains (P-value = 0.042 and p = 0.05, respectively). Regarding citric acid production, the LR6 strain had significantly better productivity than the LR-type strain (P-value = 0.043). To investigate the potential survival of the strains in a vagina-like environment, acidic tolerance under anaerobic conditions was also compared (Figure 5B). At pH 3, all three strains exhibited poor growth because of the harsh environmental conditions. However, under three different pH conditions (pH ranging from 4 to 6), particularly at pH 5, which represents the average acidity in the vagina, LR6 displayed a significantly higher growth rate than the ATCC7469 and LGG strains (P-value = 0.029 and 0.029, respectively).

3.7 Microbiome analysis

A rat model was used to assess the impact of *L. rhamnosus* LR6 administration. Rats were orally administered LR6 over a two-week period and fecal and vaginal samples were collected

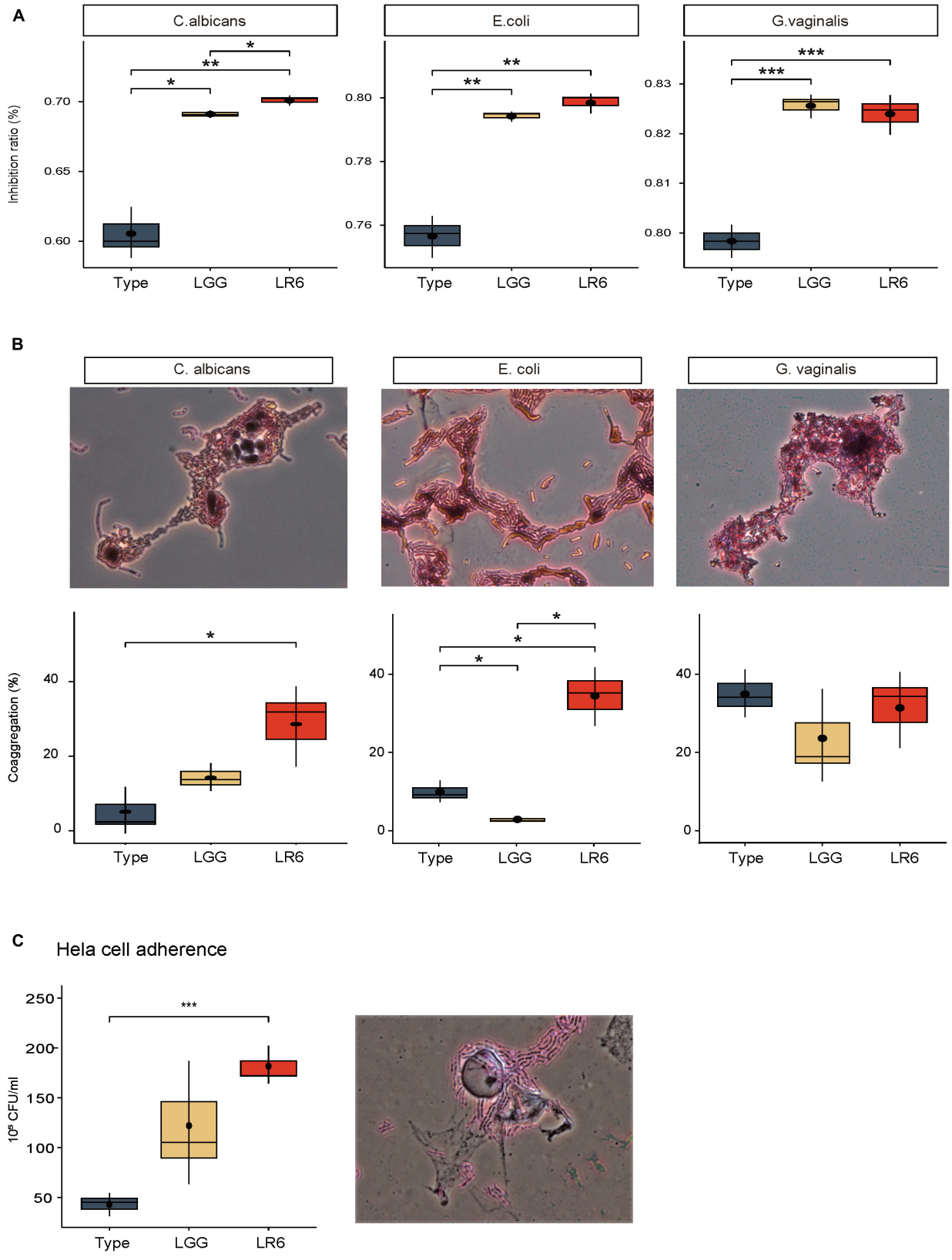


FIGURE 4
Antimicrobial and adhesive activities of *L. rhamnosus* LR6. **(A)** Comparison of antimicrobial activity of *L. rhamnosus strians*, ATCC7469, LGG, and LR6 against vaginal pathogens. **(B)** Evaluation of co-aggregation activity of three *L. rhamnosus* strains with vaginal pathogens 4 h after incubation. **(C)** HeLa cell adhesive activity of three strains of *L. rhamnosus* based on viable cell count method. The co-aggregation activity and HeLa cell adhesive capacity of LR6 were further supported by microscopic observations after Gram staining. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

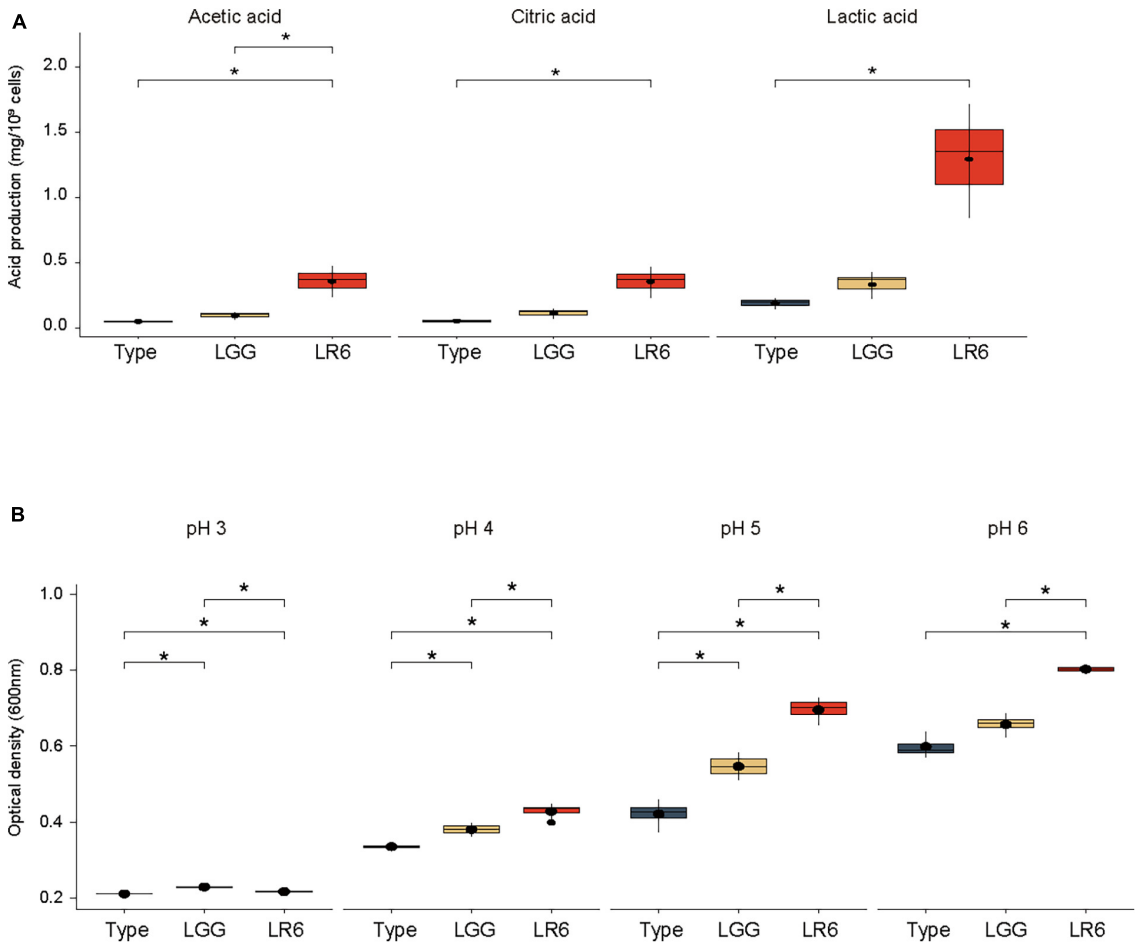


FIGURE 5
Organic acid production and acid tolerance of *L. rhamnosus* LR6. **(A)** Comparison of the concentrations of lactic acid, citric acid, and acetic acid produced by the three strains. **(B)** Comparison of the acidic tolerance under anaerobic conditions (pH ranging from 4 to 6). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

before and after administration. Analysis of the microbiome data revealed distinct shifts in the microbial composition following LR6 administration (**Figure 6**). At the phylum level, both the fecal and vaginal microbiota exhibited an increase in *Firmicutes* compared to the control samples. Notably, compared with that in the control sample, the family *Lactobacillaceae* demonstrated increased abundance in the fecal samples post-administration, indicating the potential colonization or transient presence of LR6 in the gut environment. In vaginal samples, *Enterobacteriaceae* notably decreased compared to that in the control samples, whereas *Enterococcaceae*, *Streptococcaceae*, and *Morganellaceae* increased. The observed changes in the microbiota composition suggest that orally administered *L. rhamnosus* LR6 traversed the gastrointestinal tract and influenced the vaginal microbiome.

4 Discussion

Lactobacillus is considered an essential genus in the vaginal environment that helps maintain the vaginal natural acidic pH, inhibits the growth of potentially harmful microbes, and stabilizes the microbial balance (Chee et al., 2020; Abbe and Mitchell, 2023; Liu et al., 2023). The identification of gene clusters within the genome involved in the synthesis of bacteriocins, as well as the existence of adhesive-associated genes in specific habitats, such as the vaginal tract, is of great importance in the exploration of new vaginal probiotics (Kankainen et al., 2009; Petrova et al., 2021; Wang et al., 2021). Therefore, in this study, the isolation and genomic analysis of *L. rhamnosus* LR6 from the vaginal microbiota of healthy females provided valuable insights into the potential of this strain as a new probiotic for vaginal health. Here, we report a new *L. rhamnosus* strain, LR6, sourced from the vaginas of 20 healthy Korean women, with various probiotic characteristics. Comparative genomic analysis revealed interesting features illustrating the suitability of LR6 for vaginal applications. In the context of vaginal health, the diverse origins of the 45 *L. rhamnosus* strains included in this study highlight the distinctive genomic characteristics of LR6. The prevalence of replication, recombination, and repair functions among cloud genes from diverse sources suggests that these strains adapted dynamically to their respective environments. Phylogenetic analysis classified LR6, along with other vaginal genomes, into four distinct phylogroups reflecting its evolutionary relationship within this

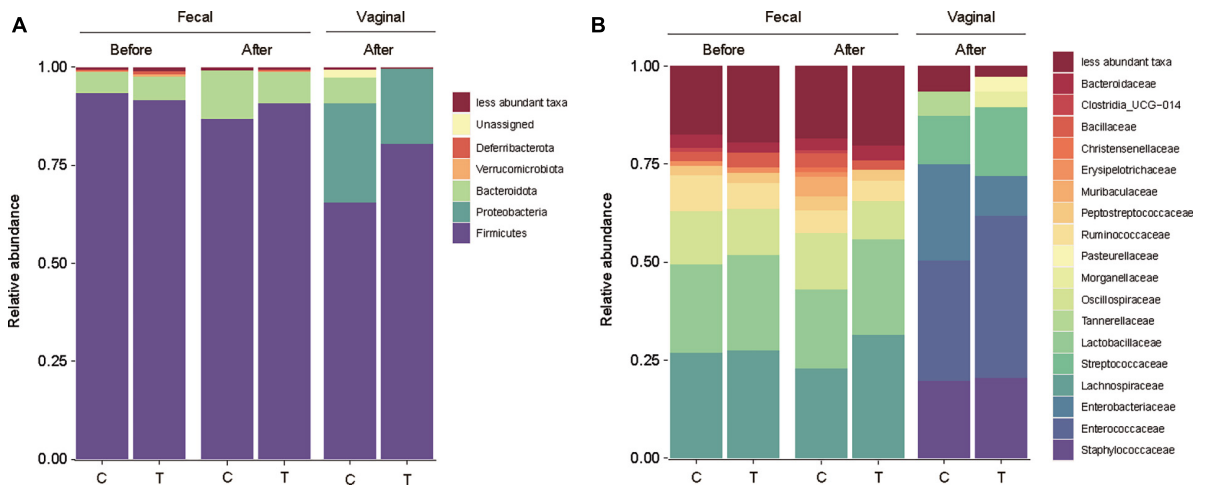


FIGURE 6
Microbial community analysis of fecal and vaginal microbiome of rat. **(A)** Taxonomy bar plot of fecal and vaginal microbiota before and after administration of *L. rhamnosus* LR6 over a two-week at phylum level, and **(B)** family level. Fecal samples were collected on days 0 and 14 and vaginal swabs were collected on day 14 (n = 12).

specific niche (**Figure 2A**). The presence of carbohydrate-utilizing enzymes, as identified by CAZyme profiling, supports the vaginal environment specialization of LR6. Notably, LR6 possessed an abundant number of genes related to GH1, GH13, and α-amylase family, the corresponding enzymes being essential for glycogen depolymerization and lactic acid production (Nunn et al., 2020). Majority of vaginal glycogen is discharged by exfoliated epithelial cells and subsequently broken down by α-amylase, leading to its conversion into lactic acid by the predominant vaginal microbiota, primarily *Lactobacilli* (Nasioudis et al., 2015). This process is a significant source of nutrients and plays a role in maintaining an acidic vaginal environment (Hood Pishchany and Rakoff-Nahoum, 2019).

The genetic makeup of LR6 through mucosal adhesion highlights its potential as a vaginal probiotic. LR6 harbored genes associated with pilus-mediated mucosal adhesion, including the spaFDE-strC2 operon and putative lectin-like protein-coding genes (**Figure 2B**). The presence of MBF including Pfam-MucBP domain repeats and the adhesion modulator MabA was observed as well in the upstream region of the spaFDE-strC2 locus in LR6 (Vélez et al., 2010). Putative lectin-like protein-coding gene, llp, important factors for the adhesion ability of gastrointestinal and vaginal epithelial cells, also observed in the LR6 genome (Petrova et al., 2016). The presence of these genes may improve the ability of LR6 to attach to and colonize vaginal epithelial cells, thereby creating a large biological niche in the vaginal microbiome (Kankainen et al., 2009; Abbe and Mitchell, 2023).

The distribution of bacteriocin gene clusters across the genomes of *L. rhamnosus* gives a glimpse into the antimicrobial potential of LR6 (Turovskiy et al., 2009). The presence of a core peptide, transport/leader cleavage, and immunity/transport/modification genes suggests that this organism is capable of generating class II bacteriocins, which are among the most abundant and diverse secondary metabolites found in lactic acid bacteria (Zhang et al., 2023). This antimicrobial activity is consistent with LR6's inhibitory effects against uropathogenic *E. coli* and two vaginal pathogens, *C. albicans* and *G. vaginalis*, as confirmed by *in vitro* analyses (**Figure 4**). In addition, the ability of LR6 to co-aggregate with vaginal pathogens and its tendency to adhere to HeLa cells indicate its potential to form a barrier against pathogens and prevent colonization (Reuben et al., 2019; Liu et al., 2023).

The vaginal environment is characterized by the dominance of lactic acid-producing *Lactobacillus* species, which form a stable microbiota. Therefore, the ability of vaginal probiotics to produce organic acids is of paramount importance (O'Hanlon et al., 2019). Growth in acidic milieu is a crucial attribute of these probiotic strains. The ability of LR6 to produce lactic acid, citric acid, and acetic acid suggests that it could potentially acidify the vaginal environment coupled with LR6's robust growth under anaerobic conditions, especially at pH 5, to maintain a healthy vaginal pH (**Figure 5**).

In conclusion, *L. rhamnosus* LR6, isolated from the vagina of a healthy woman, is a promising candidate for a new vaginal probiotic. The genomic characteristics of LR6, including its antimicrobial activity, adhesion capabilities, and acidification potential, make it a potential probiotic for vaginal health maintenance. While these findings are promising, comprehensive clinical investigation are essential to validate the efficacy of LR6 and to determine the optimal delivery mechanisms and dosages for use in women's health management. Further investigations, including clinical studies, are necessary to fully explore their benefits and potential applications in promoting vaginal well-being.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1016041.

Ethics statement

The studies involving humans were approved by the Institutional Review Board of the Konkuk University Medical Center (KUMC 2022-04-030). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Institutional Animal Care and Use Committee board in the CellBiotech (IACUC, approval No: CBT-2023-09). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YC: Conceptualization, Data curation, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. SK: Formal analysis, Writing – review and editing. DS: Formal analysis, Writing – review and editing. JL: Resources, Writing – review and editing. MC: Project administration, Writing – review and editing. SL: Formal analysis, Project administration, Resources, Supervision, Writing – review and editing.

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Conflict of interest

YC, SK, DS, MC, and SL employed by Cell Biotech, Co., Ltd.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1308293/full#supplementary-material

References

Abbe, C., and Mitchell, C. M. (2023). Bacterial vaginosis: a review of approaches to treatment and prevention. *Front. Reprod. Health* 5:1100029. doi: 10.3389/frph.2023.1100029

Bradshaw, C. S., Morton, A. N., Hocking, J., Garland, S. M., Morris, M. B., Moss, L. M., et al. (2006). High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J. Infect. Dis.* 193, 1478–1486. doi: 10.1086/503780

Chee, W. J. Y., Chew, S. Y., and Than, L. T. L. (2020). Vaginal microbiota and the potential of *Lactobacillus* derivatives in maintaining vaginal health. *Microb. Cell Fact.* 19:203. doi: 10.1186/s12934-020-01464-4

Ferrés, I., and Iraola, G. (2021). An object-oriented framework for evolutionary pangenome analysis. *Cell Rep. Methods* 1:100085. doi: 10.1016/j.crmeth.2021.100085

Hood Pishchany, M. I., and Rakoff-Nahoum, S. (2019). The fight for glycans in reproductive tract microbial ecology. *Am. J. Obstet. Gynecol.* 221:696. doi: 10.1016/j.ajog.2019.10.064

Kankainen, M., Paulin, L., Tynkkynen, S., Von Ossowski, I., Reunanen, J., Partanen, P., et al. (2009). Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 106, 17193–17198. doi: 10.1073/pnas.0908876106

Letunic, I., and Bork, P. (2021). Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301

Liu, P., Lu, Y., Li, R., and Chen, X. (2023). Use of probiotic lactobacilli in the treatment of vaginal infections: *in vitro* and *in vivo* investigations. *Front. Cell. Infect. Microbiol.* 13:1153894. doi: 10.3389/fcimb.2023.1153894

Martín, R., Chamignon, C., Mhedbi-Hajri, N., Chain, F., Derrien, M., Escribano-Vázquez, U., et al. (2019). The potential probiotic *Lactobacillus rhamnosus* CNCM I-3690 strain protects the intestinal barrier by stimulating both mucus production and cytoprotective response. *Sci. Rep.* 9:5398. doi: 10.1038/s41598-019-41738-5

Nasioudis, D., Beghini, J., Bongiovanni, A. M., Giraldo, P. C., Linhares, I. M., and Witkin, S. S. (2015). α-Amylase in vaginal fluid: association with conditions favorable to dominance of *Lactobacillus*. *Reprod. Sci.* 22, 1393–1398. doi: 10.1177/1933719115581000

Nunn, K. L., Clair, G. C., Adkins, J. N., Engbrecht, K., Fillmore, T., and Forney, L. J. (2020). Amylases in the human vagina. *mSphere* 5:e00943-20. doi: 10.1128/msphere.00943-20

O'Hanlon, D. E., Come, R. A., and Moench, T. R. (2019). Vaginal pH measured *in vivo*: lactobacilli determine pH and lactic acid concentration. *BMC Microbiol.* 19:13. doi: 10.1186/s12866-019-1388-8

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421

Pappas, P. G., Lionakis, M. S., Arendrup, M. C., Ostrosky-Zeichner, L., and Kullberg, B. J. (2018). Invasive candidiasis. *Nat. Rev. Dis. Primers* 4:18026. doi: 10.1038/nrdp.2018.26

Petrova, M. I., Lievens, E., Verhoeven, T. L. A., MacKlaim, J. M., Gloor, G., Schols, D., et al. (2016). The lectin-like protein 1 in *Lactobacillus rhamnosus* GR-1 mediates tissue-specific adherence to vaginal epithelium and inhibits urogenital pathogens. *Sci. Rep.* 6:37437. doi: 10.1038/srep37437

Petrova, M. I., Reid, G., and ter Haar, J. A. (2021). *Lacticaseibacillus rhamnosus* GR-1, a.k.a. *Lactobacillus rhamnosus* GR-1: past and future perspectives. *Trends Microbiol.* 29, 747–761. doi: 10.1016/j.tim.2021.03.010

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, R. U., and Jahid, I. K. (2019). Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiol.* 19:253. doi: 10.1186/s12866-019-1626-0

Schuyler, J. A., Mordechai, E., Adelson, M. E., Sobel, J. D., Gygax, S. E., and Hilbert, D. W. (2016). Identification of intrinsically metronidazole-resistant clades

of *Gardnerella vaginalis*. *Diagn. Microbiol. Infect. Dis.* 84, 1–3. doi: 10.1016/j.diagmicrobio.2015.10.006

Srinivasan, S., Hoffman, N. G., Morgan, M. T., Matsen, F. A., Fiedler, T. L., Hall, R. W., et al. (2012). Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PLoS One* 7:e37818. doi: 10.1371/journal.pone.0037818

Turovskiy, Y., Ludescher, R. D., Aroutcheva, A. A., Faro, S., and Chikindas, M. L. (2009). Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*. *Probiotics Antimicrob. Proteins* 1, 67–74. doi: 10.1007/s12602-008-9003-6

Van Heel, A. J., De Jong, A., Song, C., Viel, J. H., Kok, J., and Kuipers, O. P. (2018). BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. *Nucleic Acids Res.* 46, W278–W281. doi: 10.1093/nar/gky383

Vélez, M. P., Petrova, M. I., Lebeer, S., Verhoeven, T. L. A., Claes, I., Lambrichts, I., et al. (2010). Characterization of MabA, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation. *FEMS Immunol. Med. Microbiol.* 59, 386–398. doi: 10.1111/j.1574-695X.2010.00680.x

Wang, Y., Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., et al. (2021). Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. *Front. Bioeng. Biotechnol.* 9:612285. doi: 10.3389/fbioe.2021.612285

Westerik, N., Kort, R., Sybesma, W., and Reid, G. (2018). *Lactobacillus rhamnosus* probiotic food as a tool for empowerment across the value chain in Africa. *Front. Microbiol.* 9:1501. doi: 10.3389/fmicb.2018.01501

Wu, S., Hugerth, L. W., Schuppe-Koistinen, I., and Du, J. (2022). The right bug in the right place: opportunities for bacterial vaginosis treatment. *NPJ Biofilms Microbiomes* 8:34. doi: 10.1038/s41522-022-00295-y

Zhang, D., Zhang, J., Kalimuthu, S., Liu, J., Song, Z. M., He, B. B., et al. (2023). A systematically biosynthetic investigation of lactic acid bacteria reveals diverse antagonistic bacteriocins that potentially shape the human microbiome. *Microbiome* 11:91. doi: 10.1186/s40168-023-01540-y

Zheng, J., Ge, Q., Yan, Y., Zhang, X., Huang, L., and Yin, Y. (2023). dbCAN3: automated carbohydrate-active enzyme and substrate annotation. *Nucleic Acids Res.* 51, W115–W121. doi: 10.1093/nar/gkad328

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Role of probiotics in managing various human diseases, from oral pathology to cancer and gastrointestinal diseases

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The imbalance of microbial composition and diversity in favor of pathogenic microorganisms combined with a loss of beneficial gut microbiota taxa results from factors such as age, diet, antimicrobial administration for different infections, other underlying medical conditions, etc. Probiotics are known for their capacity to improve health by stimulating the indigenous gut microbiota, enhancing host immunity resistance to infection, helping digestion, and carrying out various other functions. Concurrently, the metabolites produced by these microorganisms, termed postbiotics, which include compounds like bacteriocins, lactic acid, and hydrogen peroxide, contribute to inhibiting a wide range of pathogenic bacteria. This review presents an update on using probiotics in managing and treating various human diseases, including complications that may emerge during or after a COVID-19 infection.

KEYWORDS

microbiome therapeutics, dysbiosis, intestinal barrier, probiotics, gut microbiota

1 Introduction

The gut microbiome represents an intricate ecosystem where microorganisms and their metabolic products engage with host cells, exerting an influence on various bodily functions (Ma et al., 2023). Overall health is closely linked to a "healthy" microbiome, characterized by various bacterial species residing in the gut (Hills et al., 2019; Shanahan et al., 2021). A multitude of environmental variables (e.g., patterns and methods of newborn feeding, delivery mode, dietary patterns, and antibiotic usage) all impact the overall patterns of microbial colonization in the gastrointestinal tract during early life (Beharry et al., 2023; Khan A. et al., 2023; Khan M. T. et al., 2023; Xiao and Zhao, 2023). The disruption of the early colonization process by a "healthy" microbiome has been linked to increased susceptibility to immune-mediated illnesses, such as allergies (Peroni et al., 2020; Parkin et al., 2021). Several factors, including the individual's age, overall health, geographic location, antibiotic treatment dosage and duration, and specific

antibiotics, influence the recovery of the gut microbiota following the antibiotic-induced disruption (FitzGerald et al., 2022). Antibiotic treatment and the resulting resistant bacteria can substantially impact the diversity and makeup of the gut's bacterial microbiota. This disruption can potentially diminish or even eliminate specific microbial species, hence the networking within the bacterial community and with the host (Duan et al., 2022; Patangia et al., 2022). These changes stem from shifts in how intestinal epithelial cells produce mucin, cytokines, and antimicrobial peptides. Reports have pointed out that dietary strategies involving probiotics, prebiotics, omega-3 fatty acids, and the addition of butyrate (Thomas et al., 2021), as well as procedures like fecal microbiota transplantation (Nigam et al., 2022; Quaranta et al., 2022), have shown promise in counteracting the disruptive effects of antibiotic-induced gut dysbiosis and the subsequent harm to the gut barrier (Yadav et al., 2022).

Numerous intestinal bacteria, including *Lactobacillus* (Dempsey and Corr, 2022; Louis et al., 2022; Rastogi and Singh, 2022), *Bifidobacterium* (Sadeghpour Heravi and Hu, 2023), and *Enterococcus* (Im et al., 2023), contribute positively to gut microbiota stability. This imbalance in the microbiome, known as dysbiosis, allows potentially harmful bacteria like *Clostridium perfringens*, *Staphylococcus aureus*, or *Clostridioides difficile* to gain prominence (Ribeiro et al., 2020; Elias et al., 2023) and has also been associated with various health concerns such as obesity, malnutrition, inflammatory bowel disease, neurological disorders, and cancer (Horii et al., 2021).

Beneficial microorganisms and their mutually beneficial relationship with humans are pivotal in maintaining human health (Suman et al., 2022). A deep understanding of this complex interaction has paved the way for innovative personalized healthcare strategies. Probiotics, microorganisms with several health advantages when consumed in adequate quantities, have garnered substantial attention for their potential to prevent and treat a wide range of diseases (Bodke and Jogdand, 2022). Probiotics offer multifaceted benefits to human health, including antimicrobial (Fijan, 2023), anti-inflammatory (Cristofori et al., 2021; Aghamohammad et al., 2022), antioxidant (Rwubuzizi et al., 2023), and immunomodulatory properties (Aghamohammad et al., 2022), alleviating lactose intolerance (Ahn et al., 2023), addressing diarrheal diseases (Kora, 2022), aiding in ulcer treatment (Awasthi et al., 2022), stimulating immunity (Liu G. et al., 2022; Liu Y. et al., 2022), preserving food (Teneva and Denev, 2023), and potentially mitigating colon cancer risk (Singh et al., 2022). Consequently, harnessing the power of probiotics represents a straightforward, cost-effective, adaptable, and inherent approach to achieving improved outcomes in human health (Yadav et al., 2022).

This review presents an update on the utilization of probiotics in managing and treating various human diseases, including complications that may emerge during or after a COVID-19 infection.

2 General characterization of probiotics

Probiotics are microorganisms that engage in a mutually beneficial relationship with the host organism, offering health advantages and performing crucial biological functions when administered in sufficient quantities (Suman et al., 2022). When choosing probiotic strains, specific criteria are employed to ensure compliance with safety and effectiveness standards (Wang et al., 2021). The World Health Organization (WHO) has established criteria for the *in vitro* assessment of probiotics, encompassing safety, effectiveness, functionality, and potential applications in technological and physiological contexts (FAO/WHO, 2002). Probiotic strains chosen for evaluation can be characterized by various vital properties, including their non-pathogenic nature, ability to withstand changes in the human gastrointestinal environment, capacity to adhere to and colonize the intestinal epithelium, antimicrobial properties, genetic and phenotypic stability, and immunomodulatory capabilities (Figure 1; de Melo Pereira et al., 2018; Roe et al., 2022).

Previous research has categorized probiotics into viable and active probiotics and viable inactive probiotics (Silva et al., 2020). The most consumed probiotics in human nutrition belong to genera such as *Lactobacillus*, *Bacillus*, *Bifidobacterium*, and *Saccharomyces*. Lactic acid bacteria (LAB) are considered the main probiotic bacteria that are used as viable cells, including homofermentative lactobacilli, which are represented by three main groups, including *L. acidophilus*, *L. salivarius*, and *L. rhamnosus* (Zielinska and Kolozyn-Krajewska, 2018). Probiotic lactic acid bacteria show significant promise as substitutes for antibiotics, serving as both preventive and curative treatments (Silva et al., 2020; van Zyl et al., 2020; Wang et al., 2022).

In addition, among producers of non-lactic acid are *B. cereus, E. coli Nissle 1917*, *Sporolactobacillus inulinus*, *Propionibacterium freudenreichii*, and *Saccharomyces cerevisiae* (Fijan, 2014). The applications of selected probiotics against pathogenic bacteria and their mechanisms of action are summarized in Figure 1. Notably, *Bacillus* species like *B. coagulans*, *B. subtilis*, *B. clausii*, and *B. licheniformis* have recently gained recognition as viable yet inactive probiotics, finding applications in human nutrition, treatment of intestinal and urinary issues, childhood diarrhea, and managing respiratory infections (Bu et al., 2022; Luise et al., 2022; Aysha Jebin and Suresh, 2023; Bahaddad et al., 2023).

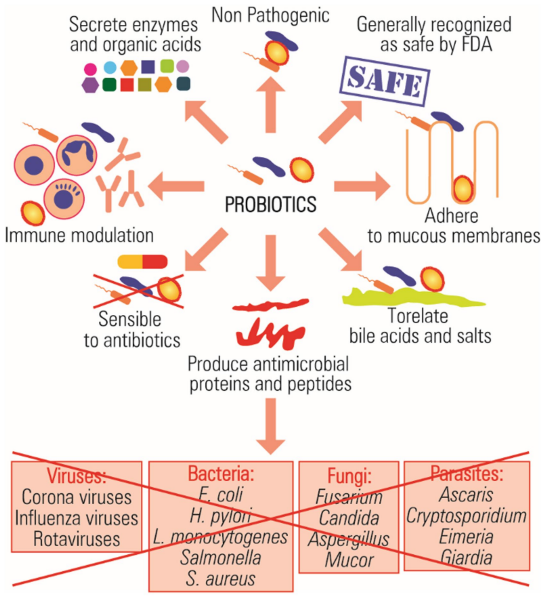


FIGURE 1
Mechanisms of the antimicrobial action of probiotics.

3 Properties and effect mechanisms of probiotics

3.1 Effect mechanisms of probiotics

Probiotics serve a broad spectrum of health-related purposes, which include improving oral and intestinal health, preventing diarrhea, strengthening the immune system, lowering serum cholesterol levels, and exhibiting various beneficial effects such as antimicrobial, anti-biofilm, antioxidant, anti-inflammatory, and anti-diabetic properties. It's important to note that these health-promoting qualities are contingent on the specific probiotic strain in use, and each exerts its effects through distinct mechanisms (Pique et al., 2019). Probiotics have several benefits: (i) reduce vulnerability to infections; (ii) reduce lactose intolerance, relieve allergic episodes and respiratory infections; (iii) reduce serum cholesterol and blood pressure; (iv) prevent the intestine from gastritis and diarrhea; (v) prevent urogenital and vaginal infections and (vi) reduce the chances of colon cancer (Reid et al., 2003).

Some probiotic strains have been found to produce a range of small molecules that can have specific localized effects. These molecules encompass acetylcholine, oxytocin, norepinephrine, dopamine, serotonin, tryptamine, and gamma-aminobutyric acid. Furthermore, research on rats has indicated that probiotics can influence adrenocorticotropic hormone levels and corticosterone levels (Liang et al., 2015).

Before embarking on clinical trials, several *in vitro* tests are available to assess the effectiveness of probiotics. These include methods such as the agar spot test, agar well diffusion test, microdilution assays, antibiofilm assessments, 3D cell cultures, and the utilization of human tissues and animal models (Papadimitriou et al., 2015). Furthermore, probiotics have shown potential in aiding various medical conditions, including but not limited to constipation, diarrhea, polycystic ovary syndrome, ulcerative colitis, stress and anxiety, inflammatory bowel disease, breast cancer, and diabetes (Bodke and Jogdand, 2022). Probiotics also exert antimicrobial effects and modulate the mucosal immune system through several mechanisms summarized in Figure 2.

3.2 Anti-inflammatory properties

Probiotic bacteria have been found to significantly influence the immune system by releasing anti-inflammatory cytokines within the gut. They can potentially impact various types of immune cells, including dendritic cells, monocytes, natural killer (NK) cells, macrophages, lymphocytes, and epithelial cells. A key mechanism of action involves the activation of pattern recognition receptors (PRRs) found on both immune and non-immune cells (Ferlazzo et al., 2011). However, the precise molecular interactions between probiotics and the host organism still need to be fully understood and require further research. Specific strains of *Lactobacillus* sp. have been found to influence cytokine production, while *Bifidobacterium* sp. strains have been associated with promoting immune tolerance (Djaldetti and Bessler, 2017; Azad et al., 2018). Oral administration of probiotic strains like *L. plantarum*, *L. acidophilus*, *B. breve*, and *B. lactis* influence the release of proinflammatory cytokines through toll-like receptor signaling pathways (Yao et al., 2017; Asgari et al., 2018). These varying regulatory activities are associated with the structural characteristics of the probiotic strains, the range of mediators they release, and the different immune pathways they activate simultaneously (Hoffmann et al., 2014).

Bifidobacterium sp. and *Lactobacillus* sp. play significant roles in modulating various aspects of the immune system, including the humoral response, cell-mediated responses, and non-specific immunity. Probiotics such as these can enhance the secretion of immunoglobulin IgA, thus influencing humoral immunity and helping the body defend against invading pathogens. A similar effect was observed when individuals consumed yogurt containing *L. casei* and *L. acidophilus*, resulting in a substantial increase in IgA-producing plasma cells (Cristofori et al., 2021). Moreover, research conducted by Hasan and collaborators demonstrated the ability of heat-killed probiotic *Bacillus* sp. SJ-10 to act as a potent modulator of the innate immune response. This research was conducted in olive bream, underscoring the broad applicability of probiotics in enhancing the innate immune system's capabilities (Hasan et al., 2019).

The commensal microbiota maintains the epithelial environment's balance by stimulating the production of epithelial repair factors and regulating the immune response, safeguarding against epithelial damage. When administered orally, probiotics activate TLR signaling, producing cytokines that activate macrophages and influence intestinal epithelial cells (IEC) and immune cells within the lamina propria. This activation, in turn, stimulates regulatory T cells to release IL-10. Probiotics exhibit immunomodulatory effects that impact humoral immunity, cell-mediated immunity, and the non-specific immune response (Maldonado Galdeano et al., 2019). The regulation of proinflammatory cytokines by probiotics has been shown to decrease inflammation in the gingival area (Ma et al., 2023). Among the commonly used probiotic strains are various *Lactobacillus* and *Bifidobacterium* sp., including *L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. reuteri*, *L. johnsonii*, *L. gasseri*, *B. longum*, *B. bifidum*, and *B. infantis*. Several studies have highlighted the significance of *L. fermentum* and *L. gasseri* in healthy individuals, as they are responsible for inhibiting periodontal pathogens such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Aggregatibacter actinomycetemcomitans*. This inhibition is achieved through various mechanisms, including the production of hydrogen peroxide, antibacterial substances like bacteriocins, and the generation of inorganic acids. By doing so, these probiotic strains contribute to maintaining the dynamic balance of normal microbiota, ultimately restoring homeostasis (Jansen et al., 2021).

3.3 Antimicrobial properties

Probiotics present a promising approach to combat the rise of antibiotic-resistant bacteria (Knipe et al., 2021). Probiotics employ several key antimicrobial mechanisms to achieve this goal, including competitive exclusion, intestinal barrier function improvement by enhancing mucin and tight junction protein expression, antimicrobial molecule secretion, and immune system regulation (Silva et al., 2020).

The antimicrobial activity of probiotics relies significantly on their ability to produce antimicrobial peptides, which play a central role in competitively excluding pathogens. *Lactobacillus* strains, in particular, have demonstrated significant antimicrobial activity against various pathogens, including *Klebsiella* sp., *Clostridium difficile*, *Shigella* sp.,

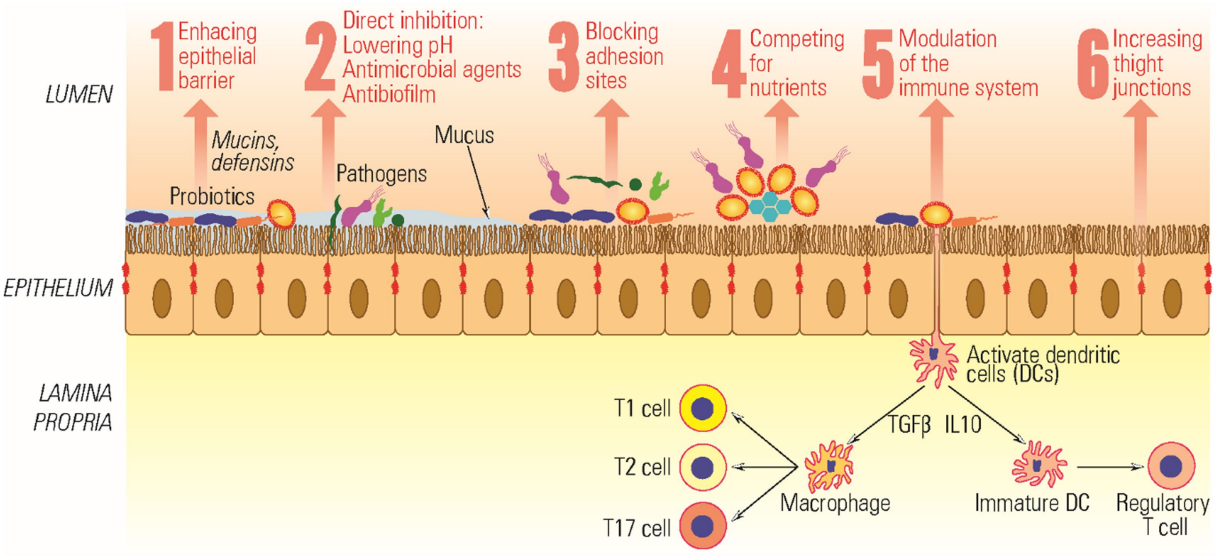


FIGURE 2
Mechanism of action of probiotics.

E. coli, *P. aeruginosa*, *S. mutans*, and *S. aureus* (Prabhurajeshwar and Chandrakanth, 2019). They employ multiple strategies to outcompete and inhibit these pathogenic bacteria, including producing lactic acid, bacteriocin, and hydrogen peroxide, inhibiting the adhesion of pathogenic bacteria to the mucosa and improving the immune response (Plaza-Diaz et al., 2017).

The intestinal barrier serves as a critical line of defense to maintain the homeostasis of the intestine. It accomplishes this by performing various mechanical, chemical, immune, and microbial barrier functions (Vancamelbeke and Vermeire, 2017). Probiotics can improve and reinforce the integrity of the gut barrier through various mechanisms, including upregulating genes and protein expression involved in tight junction signaling. Probiotics can also regulate the apoptosis and proliferation of intestinal epithelial cells, contributing to barrier repair and maintenance (Gou et al., 2022). For instance, *L. acidophilus* can induce rapid and strain-specific enhancement of intestinal epithelial tight junction barrier function. This effect is mediated through TLR complexes, specifically TLR-2/TLR-1 and TLR-2/TLR-6. Strengthening tight junctions helps protect against intestinal inflammation (Al-Sadi et al., 2021). Probiotics can induce mucin expression and promote mucus secretion by goblet cells. Studies have demonstrated that treating mucus-secreting colon epithelial cells with supernatants from a probiotic-rich yogurt mixture can elevate mucin protein expression, including MUC2, a vital constituent of the protective mucus layer. CDX2, a regulator of MUC2 expression, is also influenced by probiotics (Chang et al., 2021).

The primary method by which *Lactobacillus* strains exhibit antimicrobial activity is by releasing substances called bacteriocins (La Storia et al., 2020). Bacteriocins are antimicrobial peptides that combat many bacteria, including Gram-positive and Gram-negative species. Interestingly, the bacteria that produce these bacteriocins have developed specific mechanisms to protect themselves from the action of their antimicrobial peptides (Perez-Ramos et al., 2021). Both *Bifidobacterium* and *Lactobacillus* strains are known to be producers of bacteriocins. These antimicrobial peptides function through various means, which include inhibition of lipid II, a critical component of bacterial cell membranes, prevention of peptidoglycan synthesis, and pore formation. The pore formation process often involves a receptor known as the mannose-phosphotransferase system, as depicted in Figure 3 and Martinez et al. (2013).

3.4 Antioxidant properties

The antioxidant properties of probiotics can be attributed to several actions. Probiotics can chelate metal ions, such as iron, thereby reducing their availability for catalyzing the production of harmful ROS. Certain probiotic strains can synthesize antioxidant metabolites, including vitamins like C and E, glutathione, and various ROS-scavenging enzymes, which help mitigate oxidative stress. Probiotics can stimulate the host's production of antioxidants or enhance dietary antioxidants' absorption, bolstering the body's overall antioxidant capacity. Probiotics can influence oxidative stress and inflammation by modulating signaling pathways, ultimately regulating ROS production. Some probiotic strains can downregulate enzymes responsible for ROS generation, such as NADPH oxidase, leading to decreased ROS levels and reduced oxidative stress. Probiotics also play a role in shaping the composition of the intestinal microbiota, indirectly impacting gut health and potentially contributing to the reduction of oxidative stress (Wang et al., 2017).

Nuclear factor erythroid 2–related factor 2 (Nrf2) is a component belonging to the cap'n'collar transcription factor family and comprises seven NEH domains. In response to OS, Nrf2 plays a critical role in the ubiquitin-dependent signaling pathway (Seminotti et al., 2021). When ROS levels are elevated, Nrf2 disengages from its constant inhibitor, Keap1, migrates to the nucleus, and forms a complex with antioxidant response element (ARE) sequences, thereby initiating the transcription of genes involved in antioxidation, such as NQO1, GST, HMOX1, GCL, and GSH. Substantial research suggests that the

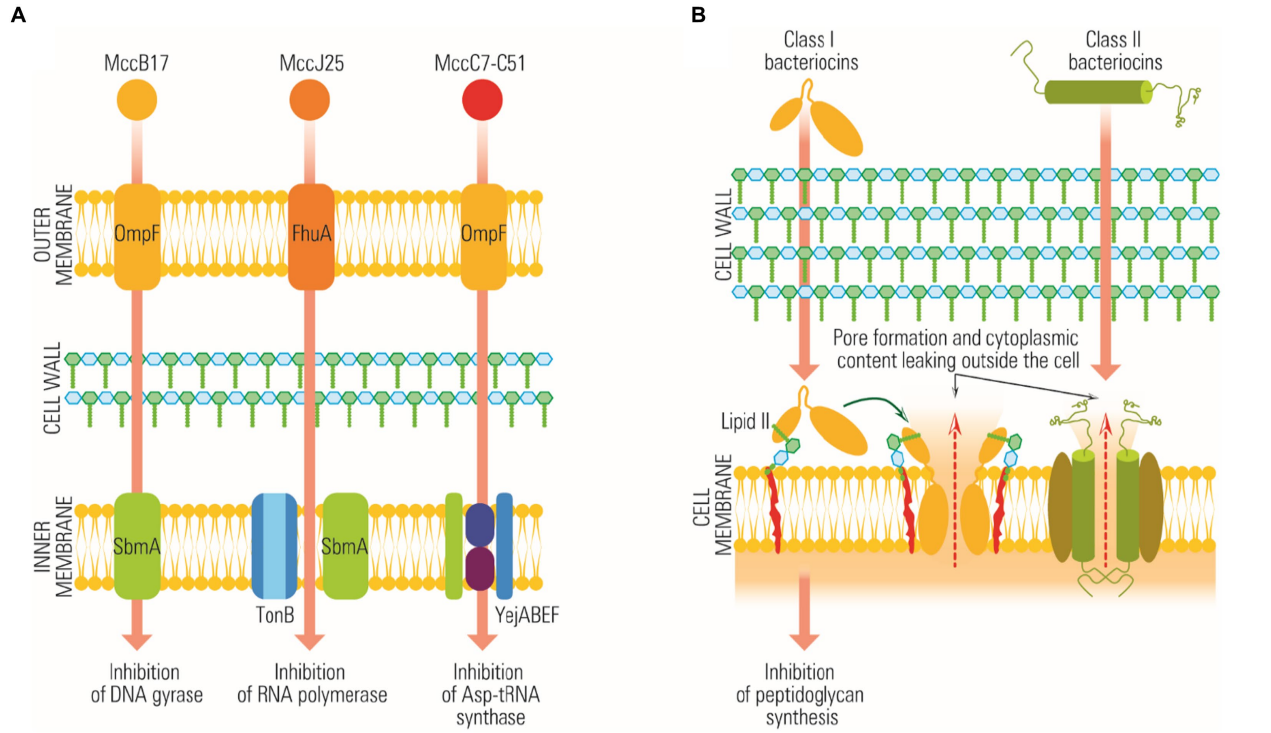


FIGURE 3
Schematically representation of bacteriocins mode of action. **(A)** The action of bacteriocins on Gram-negative targets, without the formation of pores. **(B)** The action of bacteriocins on Gram-positive targets, with the formation of membrane pores.

activation of Nrf2 can inhibit oxidative stress and inflammation, thus potentially averting conditions like UC (Trivedi et al., 2016).

Probiotics are thought to activate the Nrf2 system in the host, representing a crucial mechanism underlying their antioxidant properties. Several *in vitro* studies have illuminated the role of Nrf2 pathway activation in mediating the antioxidant effects of specific probiotic strains such as *B. infantis*, *C. butyricum*, and *L. casei Shirota* in the context of intestinal injury (Ehrlich et al., 2020; Dou et al., 2021). TLRs activation has also been shown to stimulate Nrf2-ARE signaling and heme oxygenase-1 (HO-1) both *in vivo* and *in vitro* (Nadeem et al., 2016). Moreover, several studies have documented that the activation of TLR-Nrf2 signaling by *Lactobacillus* sp. could account for their antioxidant advantages within the gastrointestinal systems of piglets (Yang et al., 2022).

Oxidative stress arises from either the cumulative production of ROS or the inadequate scavenging activity of the antioxidant system, resulting in a disturbance in the body's redox balance (Pizzino et al., 2017). Whether taken alone or alongside food, probiotic consumption has demonstrated the capacity to enhance antioxidant activity, thereby mitigating tissue damage caused by oxidative processes (Wang et al., 2017). Among the various antioxidant activities exhibited by probiotic strains such as *Lactobacillus* sp., *Bifidobacterium* sp., and *Propionibacterium* sp., it has been observed that *P. freudenreichii* displays the highest antioxidant activity (Amaretti et al., 2013). These probiotics release potent antioxidant compounds, including vitamin E, vitamin C, glutathione, beta-carotene, superoxide dismutase (SOD), polysaccharides, prototypical coenzyme I (NADH), and certain unidentified substances, all of which contribute to the promotion of gut health (Hemarajata and Versalovic, 2013). Additionally, probiotics can reduce oxidative stress by inhibiting cytokine production and decreasing levels of interleukin 1 and tumor necrosis factor-alpha while simultaneously increasing glutathione (GSH) levels (Pourrajab et al., 2022).

Various *Lactobacillus* strains, including *L. johnsonii*, *L. reuteri*, *L. brevis* and *L. fermentans* have been observed to activate the NF-κB pathway. In studies conducted with rats, this activation has been associated with reduced OS and inflammation in the intestinal tract (Pan et al., 2016; Wu et al., 2018). Interestingly, genetically engineered *Lactobacillus* strains expressing superoxide dismutase (SOD) have also shown similar positive effects. Additionally, *Bifidobacterium* sp. has been found to downregulate ROS production and inhibit the NF-κB pathway, contributing to the modulation of the intestinal immune system and protecting the intestinal epithelium (Yao et al., 2021). The interaction between SIRT1 and Nrf2/ARE is a critical component of the antioxidant defense system. SIRT1 facilitates the nuclear translocation of Nrf2, consequently elevating the expression of antioxidant proteins and phase II detoxification enzymes. In rats with aging-induced colitis, treatment with *Lactobacillus* C29 led to a decrease in plasma levels of ROS, malondialdehyde (MDA), and C-reactive protein while also increasing SIRT1 expression (Kim et al., 2019). Moreover, the mitigation of high-fat-diet-induced ulcerative colitis (UC) by administering *B. longum* and *L. plantarum* is linked to the activation of SIRT1. Additionally, studies have shown that activated SIRT2 can deacetylate forkhead box proteins (FOXO1a and FOXO3a), thereby augmenting the expression of antioxidant enzymes under the regulation of FoxO (Kitada et al., 2019). The activation of manganese superoxide dismutase (Mn-SOD)/SOD2 by *B. longum* and *L. acidophilus* to reduce cellular ROS levels, mediated by SIRT2, has

been reported (Guo et al., 2017). Additionally, LGG has been shown to prevent H2O2-induced disruption of tight junctions in the human intestinal epithelium, possibly mediated through the ERK1/2 pathway (Zheng et al., 2022). Heat-killed and active *L. brevis* have effectively ameliorated subtotal duodenal and colonic injury caused by dextran sulfate sodium by mitigating oxidative stress and inflammation through a p38-MAPK-mediated pathway (Jiang et al., 2018).

In summary, probiotics exhibit antioxidant properties through several mechanisms, including scavenging free radicals, chelation of metal ions, regulation of antioxidant enzyme expression, and modulation of the gut microbiota. These effects are mediated at the molecular level by the influence of probiotics on various signaling pathways, such as Nrf-2, NF-κB, MAPK, and SIRTs, allowing them to exert their beneficial antioxidant effects.

4 Next-generation and genetically modified probiotics

The rapid evolution of high-throughput sequencing technology and the expanding field of bioinformatics have significantly deepened our understanding of the intricate relationship between the human microbiome (Zhu et al., 2010), particularly in the gut, and a wide range of human diseases. This progress has led to a breakthrough in isolating and identifying numerous gut microorganisms that were previously challenging to cultivate in laboratory settings (Browne et al., 2016; Brown et al., 2022). Many strains with promising health benefits have emerged among these newly discovered microbes. These potential strains are now prime candidates for developing next-generation probiotics (NGPs). They promise to become active biological agents in clinical contexts, offering targeted treatments for specific diseases. This dynamic development underscores the evolving landscape of microbiome research and its potential to revolutionize personalized healthcare approaches (Khan A. et al., 2023; Khan M. T. et al., 2023).

NGPs are specific strains of commensal microbes commonly found within the GIT, influencing the host's natural defenses against gastrointestinal pathogens. NGPs comprise gut bacteria with specific nutritional requirements and oxygen sensitivity, including genera such as *Bacteroides*, *Clostridium*, *Faecalibacterium*, and *Akkermansia*, as well as genetically engineered (GE) strains (Zhang H. et al., 2022). However, working with NGPs presents unique challenges. Their demanding nutritional needs and sensitivity to oxygen make mass production and maintaining their viability during processing and formulation quite challenging. Moreover, NGPs are not universally suitable or safe for all consumers. Unlike conventional probiotics, their application requires careful consideration of target consumers and specific circumstances (O'Toole et al., 2017).

In the context of gut microbiota, *Bacteroides* spp., such as *B. thetaiotamicron*, play a crucial role as essential members, possessing a remarkable ability to metabolize complex polysaccharides, allowing them to interact with intestinal cells. Moreover, they can modulate host gene expression by inducing immunotolerance in dendritic cells (Durant et al., 2020). Another significant player in the gut microbiota is *B. fragilis*, which stands out due to its zwitterionic polysaccharide that enables it to stimulate the host's immune system (Chang et al., 2016). *C. butyricum*, a Gram-positive, spore-forming, butyrate-producing anaerobe commonly found in human and animal intestines, is known for the beneficial effects of butyrate production on the host. However, it's important to note that *C. butyricum* supplementation in neonates can have adverse effects, including the induction of necrotizing enterocolitis and botulism type E (Cassir et al., 2016). *Faecalibacterium prausnitzii*, a non-spore-forming, Gram-negative, butyrate-producing anaerobe, is another key member of the gut microbiota. Its extremely oxygen-sensitive nature has made working within laboratory settings challenging (Lopez-Siles et al., 2017). Consequently, many experiments involving *F. prausnitzii* utilize culture supernatants (SN) rather than live cells to circumvent viability and stability issues. This species is particularly interesting due to its immunomodulatory activities and role in butyrate synthesis, contributing to potential health benefits. Moreover, *F. prausnitzii* has demonstrated the ability to prevent colitis induced by various compounds, including dinitrobenzene sulfonic acid (DNBS), trinitrobenzene sulfonic acid (TNBS), or dextran sulfate sodium (DSS) (Martin et al., 2014). Furthermore, live cells of *F. prausnitzii* have shown promise in reducing the incidence of diarrhea and related mortality in dairy calves and promoting increased body weight (Foditsch et al., 2015).

5 Probiotics as therapeutic tools

5.1 Probiotics in gastrointestinal diseases

5.1.1 Probiotics in diarrhea and constipation

Probiotics treat acute diarrhea as they activate immunological signaling pathways, produce anti-pathogenic factors, and cause the host to secrete them to combat enteric infections. A study conducted by Rashad Hameed and Abdul Sattar Salman aimed to evaluate the effects of 11 isolates of probiotics bacteria (including three *L. plantarum*, one *L. gasseri*, two *L. fermentum*, three *L. acidophilus*, and two *L. garvieae* isolates) against diarrheal causative bacteria. The results showed that probiotic isolates had antibacterial and co-aggregative effects against diarrhea-causative bacteria with an inhibition diameter of 17–49 mm for different *Lactobacillus* spp. and *Lactococcus* spp. isolates (Rashad Hameed and Abdul Sattar Salman, 2023). Higuchi and collaborators led a PROSPERO meta-analysis (CRD 42023405559), including 14 randomized controlled studies to investigate the efficacy of probiotics utilized in Japan in treating acute gastroenteritis in children under 18 years. They evaluated several aspects of data on diarrhea duration, number of hospitalizations, length of hospital stay, and adverse effects. The results showed that diarrhea lasting longer than 48 h and duration of hospitalization were significantly lower in the probiotic group (Higuchi et al., 2023).

A recent meta-analysis delved into the efficacy of probiotics in managing acute diarrhea, revealing notable outcomes in both children and adults. The analysis highlighted a significant 26% reduction in the duration of acute diarrhea in children following probiotic intervention. However, intriguingly, no discernible impact on the risk of hospitalization was observed in this group (Huang et al., 2021). Zheng and collaborators conducted two randomized sub-trials in subjects diagnosed with functional constipation or diarrhea to determine the efficacy of four-week probiotic supplementation on gastrointestinal health. In each sub-trial, 70 eligible adults were randomized to receive a multi-strain probiotic combination or a placebo. The authors assessed several aspects, like gastrointestinal symptoms, defecation

habits, stool characteristics, blood and fecal biochemistry markers, anthropometrics measures, stress-associated responses, and intestinal flora changes. The results showed that 4 weeks of probiotic supplementation reduced overall gastrointestinal symptom scores in participants with functional constipation. For participants with functional diarrhea, probiotics consumption markedly reduced overall gastrointestinal symptom scores and decreased stool frequency thrice weekly (Zheng et al., 2023a).

A meta-analysis involving six randomized controlled trials and 444 geriatric patients revealed that when compared to a placebo, probiotics led to increased stool frequency and a notable impact on ameliorating symptoms associated with constipation (Deng et al., 2023). Recently, Patch and collaborators conducted a double-blinded randomized controlled trial registered in 2021 at the Australian New Zealand Clinical Trials Registry (ACTRN12621001441808p). This study aimed to use a proprietary strain of deactivated *B. subtilis* to treat self-reported functional gastrointestinal disorders in 67 participants. The reports revealed that constipation and diarrhea in the experimental group were improved by 33%/26% compared to placebo (15%/22%). Further analysis revealed that the intervention group's clusters for constipation, indigestion, and dyspepsia were significantly improved compared to the placebo (Patch et al., 2023). Dong and collaborators investigated seven effectiveness indicators and the incidence of adverse reactions in the treatment of constipation (treatment success rate, defecation frequency, frequency of abdominal pain, stool consistency, frequency of defecation pain, frequency of fecal incontinence, and recurrence rate) in nine meta-analyses and systematic reviews. According to the results, this study revealed that the intake of probiotics in children with functional constipation significantly improved treatment success rate and defecation frequency while decreasing the recurrence rate of constipation. The intake of probiotics did not increase the incidence of adverse reactions and demonstrated good safety (Dong et al., 2023).

Another systematic review (PROSPERO ID: CRD42020195869) conducted by Liu and collaborators included seventeen randomized controlled trials involving 1,504 children diagnosed with functional constipation. This study evaluated the efficacy and safety of probiotics and synbiotics in treating childhood constipation and the impact on treatment success, defecation frequency, stool consistency, painful defecation, fecal incontinence, and adverse events in treating childhood functional constipation. Compared to placebo, probiotics significantly improved defecation frequency and fecal incontinence. However, it did not significantly improve treatment success, painful defecation, and abdominal pain (Liu J. et al., 2023).

Radiotherapy and chemotherapy are commonly used therapeutic approaches for cancer patients. However, they often lead to diarrhea, significantly impacting the patient's quality of life and adversely affecting the overall treatment outcomes. However, when administered alongside radiotherapy and chemotherapy, *L. acidophilus* has been shown to reduce the duration of diarrhea in pediatric patients and even inhibit rotavirus infection (Lee et al., 2015). LGG has demonstrated its effectiveness in managing various types of diarrhea, including infectious diarrhea, ulcerative colitis, antibiotic-associated diarrhea, and rotavirus-induced diarrhea. It achieves this by stimulating the production of mucosal immunoglobulin A (IgA) and secretory IgA (sIgA) (Li et al., 2019). The prophylactic and therapeutic oral administration of *B. bifidum G9-1* has ameliorated rotavirus-induced gastroenteritis. It achieves this by producing mucosal protective peptides that effectively manage rotavirus-induced gastroenteritis (Kawahara et al., 2017).

5.1.2 Probiotics in *Helicobacter pylori* infection

Helicobacter pylori, often known as *H. pylori*, is a widely recognized pathogen that infects over 50% of the global population. This is a pathogenic microorganism that can cause health problems since it has several features that make it harmful and virulence genes that are associated with gastrointestinal issues. Additionally, it might potentially lead to immune thrombocytopenic purpura and elevate the risk of cardiovascular disease as acute coronary syndrome and also neurological effects (Tsay and Hsu, 2018). The declining efficacy of current therapeutic regimens in eradicating *H. pylori* poses a substantial challenge for the medical community. Although several therapeutic methods have been utilized, the addition of probiotics as a supplementary therapy has shown encouraging results (Bai et al., 2022).

In the treatment of *H. pylori* infection, the conventional triple therapy includes the use of clarithromycin. However, in areas where there is a high or uncertain level of antibiotic resistance, it is suggested to use either bismuth quadruple therapy or non-bismuth medicines (Malfertheiner et al., 2022). Nevertheless, research on probiotics is expanding as eradication rates continue to decline. Based on our understanding, the use of a *Lactobacillus reuteri* probiotic as an additional medication in managing individuals infected with *H. pylori* has been shown to be advantageous, as demonstrated by prospective, randomized, double-blind, placebo-controlled trials (Emara et al., 2014; Ismail et al., 2022, 2023).

The benefit of probiotics has been assessed in meta-analysis studies, which included 34 eligible randomized controlled trials. These trials mostly focused on triple therapy and showed enhanced eradication rates and decreased occurrence of adverse effects when it was used in combination with different probiotics (Wang et al., 2023a). There are many alternative eradication therapies based on probiotics as adjuvant agents (Penumetcha et al., 2021), such as the combination of multiple antibiotic-resistant lactic acid bacteria preparations and Vonoprazan (Kakiuchi et al., 2020), capsules containing four probiotics (Telaku et al., 2022) and combined mixtures of medicinal plants extracts with different probiotic strains (Hasna et al., 2023).

5.1.3 Probiotics in inflammatory bowel disease

The gastrointestinal tract protects the body from toxic and infectious substances. However, factors like stress, unhealthy diets, dysbiosis, and antibiotic treatment for different pathogens can compromise the intestinal microbiota and barrier function, leading to increased permeability. This can cause diseases like inflammatory bowel disease and irritable bowel syndrome, as well as extra-intestinal conditions like heart disease, obesity, and type 1 diabetes (Aleman et al., 2023). Leaky gut syndrome is a medical disorder characterized by an abnormal increase in the permeability of the human intestine's epithelium, which may allow harmful microorganisms, toxins, or food particles to enter the bloodstream and potentially impact various bodily systems, including the hormonal, immune, nervous, respiratory, and reproductive systems (Obrenovich, 2018; Aleman et al., 2023). The intestinal microbiota plays a crucial role in preserving the integrity and balance of the intestinal epithelium. The multi-strain probiotics are employed to modify the composition of gut microbiota and are recognized for enhancing the integrity of the intestinal barrier,

hence boosting its protective effectiveness (Chaiyasut et al., 2022; Zheng et al., 2023b).

Inflammatory bowel disease (IBD) is a condition with an unknown origin, characterized by an exacerbated immune response in the body against its own gastrointestinal (GI) microflora, primarily affecting the colon and duodenum. IBD is typically classified into two main types: Crohn's disease (CD) and ulcerative colitis (UC). While previous understanding pointed to adaptive immune responses as the main trigger for these conditions, recent research has highlighted the significant role of the innate immune system (So et al., 2023). The innate immune response can disturb the equilibrium between the human gut's beneficial microbiome and commensal microflora. This disruption, known as dysbiosis, can lead to an intensified inflammatory response, thereby impacting the development of inflammatory bowel disease (IBD) (Roy and Dhaneshwar, 2023).

Recent research, encompassing both *in vivo* and *in vitro* studies, has provided substantial evidence that consuming probiotics in sufficient quantities is a potent defense against pathogenic and opportunistic microorganisms. Probiotics can restore the disrupted normal flora of the gastrointestinal tract, which can often occur due to dietary changes, surgical interventions, and antibiotic use in treating intestinal disorders (Das et al., 2022). Yakovenko and collaborators conducted a randomized clinical trial aiming to evaluate the effectiveness of a probiotic drug containing *B. longum* BB-46 and *Enterococcus faecium* ENCfa-68. This study included 62 patients diagnosed with post-infectious irritable bowel syndrome. The results affirmed that the probiotic product enhanced clinical symptoms of the disease, reinstated the natural balance of the intestinal microbiota, and alleviated inflammation in the intestinal mucosa. In most patients receiving probiotics, the remission of the disease was achieved at the end of the course of treatment and persisted even 6 months after its termination (Yakovenko et al., 2022). A clinical trial conceptualized by Bamola and collaborators assessed the impact of *B. clausii* UBBC-07 probiotic strain on gut microbiota and cytokines in IBD patients. The authors concluded that the probiotic strain modulated the gut microbiota and cytokine secretion, decreasing the IBD symptoms (Bamola et al., 2022). More recently, 24 patients with ulcerative colitis were included in a randomized clinical trial and administered probiotics containing nine *Lactobacillus* and five *Bifidobacterium* species. Probiotic therapy in ulcerative colitis patients has significantly improved the quality of life and systemic, social, and emotional scores (Rayyan et al., 2023).

5.2 Probiotics in cardiovascular and cerebrovascular diseases

Probiotics represent a promising avenue in the search for safe and natural alternatives to manage hypercholesterolemia and reduce the risk of cardiovascular disease (Chang et al., 2019). Widely distributed in human and animal body sites, including the intestines, mouth, respiratory tract, and reproductive tract, probiotic strains like *Bacteroides* are gaining attention for their contribution to cardiovascular health improvement (You et al., 2023). He and collaborators isolated 66 *Bacteroides* strains from healthy adult fecal samples and compared their ability to assimilate cholesterol. *In vitro* assessments of bile salt hydrolase activity revealed favorable probiotic characteristics in these strains, including a high survival rate during simulated gastrointestinal digestion, excellent adhesion capabilities, susceptibility to antibiotics, and the absence of hemolysis or virulence genes. Furthermore, the strains exhibited notable bile salt deconjugation activities associated with regulating cholesterol metabolism. The strains also displayed an upregulation of the BT_416 gene, which is linked to cholesterol. This provides valuable insights into a potential molecular mechanism underlying their cholesterol-reducing activity, suggesting a pathway through which these strains may contribute to improving cardiovascular health (He et al., 2023). Ruiz-Tovar and collaborators conducted a prospective non-randomized study to investigate the effect of *L. kefiri* and a hypocaloric diet on cardiovascular risk markers. The addition of *L. kefiri* to a low-calorie diet increased weight loss and further improved the glycemic and lipid profile and also caused a further improvement in obesity-associated dysbiosis, mainly by increasing the muconutritive and regulatory (e.g., *Bifidobacterium* spp., *Akkermansia muciniphila*) microbiome, and decreasing the *Firmicutes*/*Bacteroidetes* ratio (Ruiz-Tovar et al., 2023). In addition, recent studies demonstrated that chronic consumption of probiotics, oats, soymilk, and apples may decrease diastolic blood pressure, triglycerides, total cholesterol, and insulin levels and significantly increase high-density lipoprotein cholesterol, beneficially affecting cardiometabolic health (Naseri et al., 2022; Pavlidou et al., 2022; Hasanpour et al., 2023; Pushpass et al., 2023; Zarezadeh et al., 2023). A cross-sectional study involving a group of 190 schoolchildren from public schools in Brazil aimed to evaluate the fecal abundance of *Bifidobacterium* spp. and their relationship with food consumption and anthropometric characteristics. The results revealed that a high abundance of *Bifidobacterium* spp. is associated with a low prevalence of hyperglycemia and cardiovascular risk markers (Menezes et al., 2023). Two meta-analyses consisting of 16 randomized controlled trials investigating the role of several probiotics (*L. acidophilus*, *B. bifidum*, *L. reuteri*, *L. fermentum*, and *L. rhamnosus*) in treating coronary artery disease and lowering blood pressure, blood lipid, and blood glucose was conducted recently. These studies showed that adding probiotics to conventional medications for coronary artery disease reduced the levels of low-density lipoprotein cholesterol, fasting glucose, and hypersensitive C-reactive protein and increased the levels of high-density lipoprotein cholesterol and nitric oxide (Lei et al., 2023; Liu L. et al., 2023). Similarly, a retrospective cohort study of 4,837 participants aged 65 years or older found that subjects who used probiotics experienced a reduced risk of all-cause mortality by nearly 41% and cardiovascular mortality by 52% (Shen et al., 2023).

An ongoing open-label, randomized, controlled clinical trial (Chinese Clinical Trials Registry ChiCTR2000038797) involving 2,594 adult patients diagnosed with acute myocardial infarction aims to assess the effects of probiotics on in-hospital mortality and the incidence of major adverse cardiovascular events. Patients will receive a bifidobacteria triple viable capsule (*B. longum*, *L. acidophilus*, and *E. faecalis*) twice a day, along with standard treatment, for a maximum of 30 days or standard treatment strategy without the bifidobacterium triple live capsule. This clinical trial will provide evidence for probiotics as a complementary treatment for acute myocardial infarction (Chen et al., 2023).

In the case of stroke, probiotics can ameliorate neurological deficits, decreasing cerebral volume loss and inhibiting neuronal apoptosis by sustaining intestinal barrier function, such as increasing tight junctions, decreasing intestinal injury, and modulating gut

microbiota (Wu and Chiou, 2021; Savigamin et al., 2022). A recent meta-analysis including 26 randomized controlled trials (2,216 patients) was conducted to explore the efficacy and safety of probiotics in stroke patients. The results showed a significantly lower incidence of gastrointestinal complications, a lower incidence of infection, a shorter length of hospital stay, and a lower dysbacteriosis rate in the probiotics group, suggesting that probiotics significantly improve the status of stroke patients (Chen et al., 2022).

5.3 Probiotics in oral diseases

One of the primary benefits of probiotics in the oral cavity is their ability to reduce inflammation. Probiotics play a role in preserving the health of gums and teeth by actively countering harmful microorganisms in the mouth. As a form of natural medicine, probiotics are generally considered safe and are not expected to yield adverse effects. Notably, *L. acidophilus* and *B. lactis* have gained recognition for their effectiveness in combating fungal infections in the oral environment (Lesan et al., 2017).

Oral probiotics should adhere to specific criteria to be effective without causing adverse effects. They should not ferment sugars, as this fermentation can decrease pH and potentially promote dental caries. Additionally, they should be capable of adhering to and colonizing all oral cavity tissues (Saiz et al., 2021). The oral cavity hosts a diverse community of bacteria belonging to several phyla, including *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria, Spirochaetes*, and *Fusobacteria*, as revealed by research (Hernandez et al., 2022). These bacteria are typically normal commensals in healthy individuals, with *Candida* sp. among the most commonly observed organisms (Baumgardner, 2019).

The most common strains being a pillar in the probiotics development are *Lactobacillus* and *Bifidobacterium* sp. Notable strains within the *Lactobacillus* sp. include *L. acidophilus*, *L. johnsonii*, *L. rhamnosus*, *L. casei*, *L. reuteri*, and *L. gasseri*. These probiotic strains are associated with beneficial effects on oral health. They achieve this by engaging with toll-like receptor (TLR) signaling, which contributes to epithelial homeostasis by producing repair factors and immune regulation. This mechanism is crucial for protecting against epithelial injury. Furthermore, these probiotics play a role in regulating the release of inflammatory cytokines such as IL-1β and TNF-α, effectively reducing gingival inflammation (Ma et al., 2023).

5.3.1 Probiotics in the prevention of dental caries progression

Probiotics play a significant role in maintaining and improving overall oral health. One approach involves using *Bifidobacterium* sp., including *B. dentium*, *B. breve*, *B. scardovii*, and *B. longum,* that are particularly effective in preventing the progression of deep dental caries (Abikshyeet et al., 2022). Another noteworthy probiotic, *L. lactis*, has been found to reduce the colonization of various oral bacteria, including *S. oralis*, *Veillonella dispar*, *Actinomyces naeslundii*, and the cariogenic *S. sobrinus* (Guo et al., 2023).

The research on *L. rhamnosus* has yielded promising findings regarding its positive impact on dental health. Consumption of milk containing *L. rhamnosus* has been associated with a beneficial effect in reducing the occurrence of dental caries and inhibiting the colonization of caries-causing streptococcal pathogens (Abikshyeet et al., 2022). Even after discontinuing yogurt consumption, a lasting reduction in caries incidence in children was observed. *L. rhamnosus* was also detected in saliva for an additional 2 weeks, indicating its potential for sustained oral health benefits. Additionally, short-term consumption of cheese containing *L. rhamnosus* significantly reduced caries-associated microbes, with a notable decrease in *S. mutans* (Amargianitakis et al., 2021; Homayouni Rad et al., 2023). Two research groups revealed that, when consumed daily, probiotic yogurt containing *Bifidobacterium* and *Lactobacillus* sp. has effectively reduced resistant streptococci associated with dental caries (Srivastava et al., 2020; Zare Javid et al., 2020). These findings underscore the potential of probiotics, particularly *L. rhamnosus* and *Bifidobacterium* strains, in promoting oral health and preventing dental caries.

Recent research has highlighted the potential of *L. paracasei* and *L. plantarum* in preventing dental caries by regulating the microbiota within dental plaques and inhibiting the *S. mutans* biofilms (Guo et al., 2023; Zeng et al., 2023). Furthermore, a systematic review led by Meng and collaborators, encompassing 17 randomized controlled trials, aimed to assess the clinical effectiveness of probiotics in preventing dental caries in preschool children. They concluded that probiotics can potentially reduce high levels of *S. mutans* in saliva. However, it was noted that probiotics did not significantly impact the levels of *Lactobacillus* sp. in both saliva and dental plaque (Meng et al., 2023). Pallavi and colleagues reported that *Enterobacter cloacae* strains isolated from yogurt exhibit probiotic properties. These strains can inhibit the cariogenic *S. mutans* bacterium by producing organic acids (Pallavi et al., 2023).

5.3.2 Probiotics in the prevention of gingival inflammation

Bifidobacterium strains, including *B. dentium*, *B. breve*, *B. scardovii*, and *B. longum*, have been found to play a crucial role in inhibiting the adhesion of pathogens to oral sites, reducing plaque formation and gingival bleeding (Alkaya et al., 2017). One of the primary mechanisms through which these probiotic strains inhibit the aggregation of oral pathogenic microorganisms on tooth surfaces, thereby hindering the formation of biofilms, involves the disruption of surface adhesive proteins. Furthermore, *L. salivarius* has promising potential in combating subgingival microbiota (Nedzi-Gora et al., 2020).

Recent studies have spotlighted *L. reuteri* due to its possession of the antimicrobial substance reuterin, which has demonstrated efficacy in reducing gingivitis and associated gingival bleeding (Agossa et al., 2022; Garcia et al., 2022). Furthermore, after 4 weeks of probiotic consumption containing *L. reuteri*, it was observed a significant reduction in various oral pathogens, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas intermedia*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (Jansen et al., 2021; Ochoa et al., 2023). Additionally, *L. reuteri* has been found to possess the capability to inhibit pro-inflammatory cytokines through the secretion of bacteriocins, reuterin, and reutericyclin. These compounds are recognized for preventing the growth of various pathogens, including bacteria, viruses, and fungi (Jang et al., 2022).

In a recent randomized clinical trial conducted by Volgenant and colleagues, the potential benefits of probiotic capsules containing one of two oral probiotic strains on gingival bleeding were investigated. Additionally, the authors assessed probiotics' positive effects on oral health, including gingival health, dental plaque accumulation, and

immunological and microbiological factors. Subjects were administered capsules containing *L. plantarum, and L. paracasei*. These findings underscore probiotic capsules' potential to contribute to oral well-being by improving gum health, mitigating inflammation, and modulating the oral microbiome. Notably, the *L. paracasei* strain exhibited a more robust capacity to modulate the oral microbiome than the *L. plantarum* strain (Volgenant et al., 2022).

5.3.3 Probiotics in the prevention of periodontal diseases

Periodontitis is a multifaceted disease with numerous causal factors involving microorganisms and the body's response to them. The main etiology is the presence of biofilm bacteria on tooth surfaces. However, the disease's evolution and severity depend on additional local factors, such as the accumulation of plaque and calculus, genetic influences, environmental factors, the individual's overall health status, and lifestyle choices. Together, these factors collectively shape the development and progression of periodontitis (Sulijaya et al., 2019).

Lactobacillus sp., including *L. fermentum* and *L. gasseri*, can inhibit periodontal pathogens like *P. gingivalis*, *A. actinomycetemcomitans*, and *Prevotella intermedia* through the production of hydrogen peroxide, bacteriocrine antibacterial substances, and inorganic acids (Maitre et al., 2021; Mann et al., 2021). In the case of chronic periodontitis, *L. brevis* has shown promising anti-inflammatory effects due to its ability to significantly reduce key inflammatory markers such as prostaglandin E2 (PGE2) and matrix metalloproteinases (MMP). A recent study revealed that *L. brevis* can help prevent nitric oxide production, thereby reducing bacterial plaque accumulation (Hurtado-Camarena et al., 2023). In addition, regular consumption of *L. salivarius* probiotics, taken three times a day for 8 weeks, has been associated with beneficial outcomes regarding periodontal health. Specifically, it has been linked to improvements in periodontal pocket probing depth and plaque index (Nedzi-Gora et al., 2020).

B. lactis administration in rats with periodontitis has been found to have several beneficial effects. These include a reduction in the levels of IL-1β and the regulation of the expression of TNF-α and IL-6. A recent study assessed the effects of *B. lactis* strain on periodontal parameters, highlighting its immunological and antibacterial properties by examining BD-3, TLR4, and CD4 levels in gingival tissues (Invernici et al., 2020). The composition of organic acids, such as lactic acid, produced by *Bifidobacterium* sp. can disrupt the outer membrane of Gram-negative bacteria. This disruption is known to reduce the adhesion of pathogens like *P. gingivalis* (Argandona Valdez et al., 2021). *Saccharomyces cerevisiae* is a yeast with probiotic properties due to beta-glucans in its cell wall. Beta-glucans can stimulate phagocytic activity and the production of inflammatory cytokines, thereby activating leukocytes (Nguyen et al., 2021). A study conducted by Garcia and collaborators aimed to evaluate the probiotic properties of beta-glucans. They found that this compound increased TGF-β1 concentration in patients with chronic periodontitis. This mechanism contributes to an anti-infective effect and enhances the potential for accelerated periodontal healing (Garcia et al., 2016).

Recent research has sparked interest in several newly discovered *Streptococcus* sp. due to their promising properties, including their ability to colonize oral environments, biocompatibility, and ease of experimental dosage determination. Recently, Deandra and collaborators reported that *S. salivarius* strains possess the capability to inhibit the activity of periodontal pathogens such as *P. gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans* (Deandra et al., 2023). Previous studies have indicated that *S. salivarius* can help maintain immune homeostasis by targeting host cells by inhibiting the release of IL-6 and IL-8 triggered by *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum* (Zhang Y. et al., 2022). In another research, individuals with stage II–III periodontitis who underwent mechanical periodontal treatment were monitored for periodontal outcomes. The study compared chlorhexidine-based toothpaste with toothpaste containing probiotics (including *Bifidobacterium* sp.) and chewing gum containing the same probiotics. After 3 months, an improvement was observed in both the inflammatory and microbial aspects of periodontitis (Butera et al., 2020). Salinas-Azuceno and collaborators conducted a study evaluating the potential of *L. reuteri* as monotherapy to improve periodontal health in a 30-year-old patient with periodontitis. The research indicated that *L. reuteri* exhibited a temporary, targeted antimicrobial effect, reducing the growth of specific harmful oral bacteria associated with periodontitis. During a month of probiotic consumption, the results revealed increased levels of beneficial oral bacteria, including *Actinomyces* sp., *Streptococcus* sp., *Gemella* sp., *Capnocytophaga* sp., and certain purple complex species (Salinas-Azuceno et al., 2022).

5.3.4 Probiotics in halitosis

Halitosis, often called bad breath, can be caused by various factors related to oral and non-oral sources, including poor oral hygiene, periodontal diseases, oral tissue crusts, food impaction, unclean dentures, damaged dental restorations, oral cavity carcinomas, and respiratory and gastrointestinal infections. The key culprits behind halitosis are volatile sulfur compounds (VSCs), which can be categorized into oral and non-oral types depending on their source (Hampelska et al., 2020). Furthermore, an imbalance in the normal commensal microbiota can produce sulfur vapor components such as hydrogen sulfide, dimethyl sulfide, and methyl mercaptan (Yoo et al., 2019). Clinical trials have demonstrated the effectiveness of antimicrobial mouthwashes containing *S. salivarius K12* in reducing VSC-producing bacteria levels, which plays a crucial role in treating halitosis (Hyink et al., 2007). *S. salivarius* has been identified as a producer of salivaricin, a lantibiotic known to inhibit *S. pyogenes*, responsible for throat infections and oral malodor (Barbour et al., 2023). Another approach to combatting halitosis involves oral rinsing with a probiotic suspension of *Weissella cibaria*, which has been found to reduce malodor caused by *F. nucleatum* (Kang et al., 2006). *W. cibaria* produces hydrogen peroxide, which competes with secondary colonizers for attachment sites in the mouth. This competition reduces the reservoir of periodontal pathogens that produce volatile sulfur compounds and subgingival plaque (Kim et al., 2020).

In a randomized, double-blind, placebo-controlled trial, Lee and colleagues investigated the impact of oral probiotic *W. cibaria* tablets on halitosis. Participants took either *W. cibaria* or a placebo daily before bedtime for 8 weeks. The study revealed that for individuals with halitosis, the eight-week intake of this oral probiotic could serve as a beneficial intervention to reduce bad breath and enhance oral health-related quality of life (Lee et al., 2020). Patil and collaborators conducted a recent study to assess the effectiveness of *L. rhamnosus* in

inhibiting bacteria responsible for halitosis. The research found that *L. rhamnosus* exhibited inhibitory effects on halitosis-causing bacteria such as *Tannerella forsythia* and *P. intermedia* after 48 h and on *P. gingivalis* after 72 h (Patil et al., 2023). In another recent randomized, double-blinded study, Han and colleagues aimed to explore the effects of tablets containing *W. cibaria* on halitosis. This study involved 100 adults with halitosis, randomly assigned to either the test group ($n = 50$) or the control group ($n = 50$). The researchers assessed improvements in bad breath, concentrations of VSCs, and the colonization of *W. cibaria*. The study found that the total VSC levels were significantly lower in the probiotics group. However, the difference between hydrogen sulfide and methyl mercaptan was insignificant, supporting the efficacy of *W. cibaria* in improving halitosis (Han et al., 2023).

5.4 Probiotics in the treatment of urogenital infections

Urogenital infections, including yeast vaginitis, bacterial vaginosis (BV), urinary tract infections (UTI), and non-sexually transmitted urogenital infections, constitute common reasons for women to seek gynecological care (Al-Badr and Al-Shaikh, 2013). The composition of normal vaginal commensals in healthy women can vary in premenopausal and postmenopausal stages. In healthy premenopausal women, Lactobacillus species typically predominate. These *Lactobacillus* species include *L. delbrueckii*, *L. brevis*, *L. crispatus*, *L. casei*, *L. jensenii*, *L. fermentum*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *L. rhamnosus*, *L. gasseri*, and *L. vaginalis* (Petrova et al., 2015). Several factors contribute to these variations, including vaginal pH, glycogen levels, hormonal variations (especially estrogen fluctuations), and the menstrual cycle. Together, these factors affect the colonization and attachment of pathogens to vaginal epithelial cells. Elevated estrogen levels in healthy premenopausal women encourage the adherence of lactobacilli while discouraging the colonization of other pathogens. Conversely, in postmenopausal women, estrogen levels decrease, leading to changes in the vaginal microbiota that can contribute to urogenital infections (Witkin, 2015). Bacterial vaginosis (BV), the most prevalent urogenital infection, is primarily attributed to decreased *Lactobacillus* species and subsequent overgrowth of Gram-negative anaerobes (Mestrovic et al., 2021).

Probiotics offer a promising approach to restoring the balance of commensal organisms in the vagina, which can disrupt the growth of pathogenic organisms and inhibit biofilm formation (Barrea et al., 2023). Reid and collaborators initially reported the idea of repopulating the vagina with lactobacilli through oral probiotics (Reid et al., 2001). *Lactobacillus* sp. combat vaginal pathogens through various mechanisms, including the production of antimicrobial agents like bacteriocins (Gaspar et al., 2018) and biosurfactants that alter the surface tension of the environment, preventing pathogen adhesion and further inhibiting their spread in the bladder. Lactobacilli also play a crucial role in maintaining vaginal pH (Ballini et al., 2018). Research has shown that administering the probiotic *Lactobacillus rhamnosus* GR-1 to premenopausal women can increase the expression levels of antimicrobial defenses (Barrea et al., 2023). A randomized, double-blind, placebo-controlled pilot study included 81 premenopausal adult women who experienced recurrent UTIs. The study aimed to evaluate a product containing two Lactobacilli strains and cranberry extract to prevent recurrent UTIs in premenopausal women. The results demonstrated that this product was safe and effective in preventing recurrent UTIs in this group (Koradia et al., 2019). Another study led by Armstrong and collaborators, conducted as part of a phase 2b trial, investigated the impact of the LACTIN-V product based on *L. crispatus* on genital immunology and vaginal microbiota. The findings from this study revealed the role of LACTIN-V in reducing genital inflammation, serving as a biomarker of epithelial integrity (Armstrong et al., 2022).

More recently, Ansari and collaborators investigated the benefits of *Lactobacillus* sp. probiotics in improving vaginal dysbiosis and facilitating the colonization in asymptomatic women. This study included 36 patients who received a combination of *L. acidophilus*, *L. rhamnosus*, and *L. reuteri* and revealed that oral administration of these probiotics improved vaginal dysbiosis in asymptomatic women (Ansari et al., 2023). A recent randomized, double-blind trial (ISRCTN34840624, BioMed Central) conceptualized by Mandar and collaborators investigated the impact of the vaginal probiotic capsules containing *L. crispatus* strains in 182 patients with BV and vulvovaginal candidiasis (VVC). The study highlighted the benefits of vaginal capsules in improving the signs, symptoms, and amount of discharge and itching/irritation in BV and VVC patients (Mandar et al., 2023). Another clinical trial (KCT0005881, Clinical Research Information Service, the Republic of Korea) was conducted to evaluate the effectiveness of a complex of five strains of probiotic candidates in restoring vaginal health in 76 reproductive-aged women. The consequent analyses confirmed the increment of *L. plantarum,* concomitant with inhibiting harmful bacteria such as *Mobiluncus* spp., *Gardnerella vaginalis,* and *Atopobium vaginae.* This clinical trial demonstrated that this probiotic complex can be used for treating BV, as it improves the vaginal microbiota (Park et al., 2023).

Preterm birth (PTB) is a significant global challenge and ranks second leading cause of neonatal mortality (Keelan and Newnham, 2017). Research has highlighted a robust connection between BV and PTB. Consumption of *Lactobacillus* sp. during both early and late pregnancy has been shown to reduce the incidence of PTB-related infections, inflammation, and even conditions like pre-eclampsia (Zheng et al., 2019). A notable characteristic of healthy female reproductive tracts is the predominance of a less diverse community of *Lactobacillus* sp. (Madhivanan et al., 2015). In contrast, a highly diverse vaginal microbiome is associated with BV and serves as a primary risk factor for PTB and the acquisition of pelvic inflammatory disease (Holdcroft et al., 2023).

5.5 Probiotics in cancer prevention and treatment

Probiotics have emerged as promising agents in the fight against cancer, showing their potential in laboratory experiments (*in vitro*) and animal studies (*in vivo*). Various probiotic strains have exhibited their ability to combat different types of cancer. *Lactobacillus* strains (*L. paracasei* SR4, *L. casei* SR1, and *L. casei* SR2) have demonstrated anticancer effects against cervical cancer cells (HeLa) by increasing the expression of apoptotic genes like BAX, BAD, caspase-8, caspase-3, and caspase-9 while reducing the activity of the anti-apoptotic BCl-2 gene (Riaz Rajoka et al., 2018). Studies have revealed that a compound produced by *Enterococcus thailandicus* possesses significant anticancer

properties, particularly against liver cancer cells (HepG2). Yogurt fortified with probiotics has demonstrated the ability to hinder the development of colon tumors induced by 1,2-dimethylhydrazine in laboratory mice (BALB/c mice), suggesting its potential as an anticancer dietary option. An aqueous extract of *Bifidobacterium* sp. has been shown to induce apoptosis in non-small cell lung cancer cells, effectively inhibiting the invasive behavior of cancer cells (Ahn et al., 2020). Various mixtures of probiotics, including *L. casei-01*, combined with dairy beverages, have demonstrated antiproliferative and apoptotic effects on human prostate cell lines (Rosa et al., 2020).

Bacillus Calmette-Guerin (BCG) has long been employed as preventive immunotherapy for recurrent superficial bladder cancer (Guallar-Garrido and Julián, 2020). Additionally, regular lactic acid bacteria consumption is considered a preventative measure against bladder cancer (Andolfi et al., 2020). Probiotics enhance the expression of junctional molecules, thereby preserving the integrity of the intestinal barrier. They also contribute to the production of IgA and short-chain fatty acids, which impede the adherence and proliferation of pathogens (Engevik and Versalovic, 2017).

Moreover, *L. plantarum* releases polysaccharides with recognized antitumor properties. These polysaccharides act by downregulating the expression of MAPK mRNA and upregulating PTEN (Asoudeh-Fard et al., 2017). Research on *L. lactis* strains has revealed their capacity to secrete IL-10 and INF-β, as well as express antioxidants that mitigate the generation of reactive oxygen species (ROS) and reduce colonic damage in animal models (Zhao et al., 2023).

A recent randomized clinical trial conducted by Liu and colleagues confirmed that the perioperative use of probiotic supplements can reduce postoperative infections, improve short-term clinical outcomes, and alleviate common inflammatory symptoms in patients with gastric cancer receiving neoadjuvant chemotherapy (Liu G. et al., 2022; Liu Y. et al., 2022).

The incidence of oral cancer is closely linked to changes in the oral and intestinal microbiota. Variations in the levels of essential vitamins and nutrients can trigger the production of inflammatory cytokines, leading to various pathological conditions (Kany et al., 2019). Certain microbes, including *F. nucleatum*, *P. gingivalis*, and *P. intermedia*, have strong associations with the initiation and progression of oral cancer (Atanasova and Yilmaz, 2014). Among the microbial species extensively studied in this context are *Actinomyces* sp., *Clostridium* sp., *Enterobacteriaceae* sp., *Fusobacterium* sp., *Haemophilus* sp., *Prevotella* sp., *Veillonella* sp., and *Streptococcus* sp. These microorganisms exhibit a robust correlation with precancerous oral lesions and the development of oral cancer (Hu et al., 2016).

Inflammation of the oral mucosa is a frequently encountered condition known as oral mucositis (OM). OM presents with symptoms such as redness (erythema), ulceration, pain, difficulty swallowing (dysphagia), and nutritional deficiencies. This condition is a common consequence in individuals undergoing radiotherapy and chemotherapy. It is primarily attributed to several factors, including the detrimental effects of reactive oxygen species leading to the death of basal stem cells, DNA damage, and inflammatory cytokines synthesis (Lalla et al., 2014).

L. rhamnosus GG (LGG) is a naturally occurring gut commensal bacterium known for its anti-inflammatory properties and has been a pioneer in oncology research (Banna et al., 2017). LGG maintains the equilibrium of the intestinal mucosa by neutralizing harmful pathogens and toxins, effectively preventing breaches in the mucosal barrier through a high-affinity binding system (Okumura and Takeda, 2018). One of its key mechanisms involves regulating the production of IgA (Wang et al., 2017). LGG is also recognized for producing increased levels of geniposide, an anticancer molecule, and its potential as a beneficial adjuvant during cancer treatment. Studies have indicated that LGG, through its probiotic action, can protect epithelial stem cells from radiation-induced damage and reduce their apoptosis. It modulates cyclooxygenase-2 and stimulates the release of prostaglandin E2 (PGE2) (Desai et al., 2018). Furthermore, LGG plays a role in immune system modulation by secreting various cytokines, including IL-6, IL-10, IL-1β, TNF-α, IL-12, and p40. These actions help reduce inflammation, regulate epithelial function, and maintain intestinal mucosa integrity (Andrews et al., 2018). In the context of cancer treatment, *L. brevis CD2* lozenges have been found to reduce the occurrence of oral mucositis in patients undergoing high-dose chemotherapy (Sharma et al., 2017). Additionally, *L. brevis* lozenges have shown benefits in reducing oral ulcers in individuals with recurrent aphthous stomatitis (Abruzzo et al., 2020). These findings underscore the potential of probiotics such as LGG and *L. brevis* in alleviating oral mucosal problems and promoting overall health.

Recently, a meta-analysis conducted by Frey-Furtado and colleagues examined nine articles to evaluate the therapeutic effectiveness of probiotics in managing oral mucositis. Among these studies, four clinical trials reported a decrease in the severity of oral mucositis by using specific strains of bacteria, including *Lactobacillus* (*L. casei* and *L. brevis* CD2) and *B. clausii* UBBC07. Preclinical studies revealed the positive effects of *L. lactis*, *L. reuteri*, and *S. salivarius K12* in reducing the severity of oral mucositis and the size of ulcers (Frey-Furtado et al., 2023). Moreover, two distinct meta-analyses conducted by research teams from Taiwan and China, encompassing a total of 19 randomized clinical trials, investigated the potential of probiotics in preventing oral mucositis induced by cancer therapy and in managing the occurrence of chemotherapy-induced diarrhea and oral mucositis. Both studies revealed the effectiveness of probiotics in preventing and alleviating cancer therapy-induced oral mucositis and addressing adverse reactions associated with chemotherapy (Feng et al., 2022; Liu G. et al., 2022; Liu Y. et al., 2022).

5.6 Probiotics in the treatment of anemia

Numerous studies have highlighted a significant connection between gut microbiota and iron deficiency. Furthermore, research has illuminated the reciprocal relationship between iron deficiency and the composition of gut microbiota, as well as the beneficial impact of probiotic bacteria on enhancing iron absorption (Sandberg et al., 2018; Hadadi et al., 2021).

Folic acid, a water-soluble B vitamin crucial for addressing and managing anemia, is derived from probiotic bacteria, including *L. lactis*, *L. cremoris*, *B. pseudocatenulatum*, *Candida famata*, *B. adolescentis*, *Candida glabrata*, *Candida guilliermondii*, *S. cerevisiae*, *Yarrowia lipolytica*, and *Pichia glucozyma*. These bacteria are harnessed to enhance the intestinal absorption of folic acids. In addition, *P. denitrificans* and *P. shermanii* have found utility in treating vitamin B12 deficiency. Lactic acid-fermented foods are instrumental in increasing iron absorption and are employed in managing anemic patients. They help optimize the pH of the digestive tract and activate the enzyme phytase, which plays a role in nutrient absorption.

Furthermore, a combination of probiotic bacteria has been incorporated into food to treat megaloblastic anemia. These probiotics promote colonic fermentation and counteract the adverse effects of antibiotics (Ohtani et al., 2014).

Numerous studies have shed light on the positive effects of probiotics, particularly *Lactiplantibacillus plantarum 299v*, in addressing iron deficiency anemia and enhancing non-heme iron absorption. A study among Caucasian Europeans revealed that *L. plantarum 299v* had a beneficial impact in preventing iron deficiency anemia and improving the absorption of non-heme iron (Vonderheid et al., 2019). Korcok and collaborators investigated the role of *L. plantarum 299v*, sucrosomal iron, and vitamin C in preventing and treating iron deficiency, administering vitamin C and iron to one group. In contrast, the second group received additional *L. plantarum*. The results showed that the second group had higher iron blood levels due to increased iron absorption, highlighting the additive effect of *L. plantarum 299v* (Korcok et al., 2018). Adiki and colleagues demonstrated that using *L. plantarum 299v* with pear millet enhanced iron absorption. Interestingly, they noted that the increased dose of probiotics did not directly influence hemoglobin and hematocrit levels (Adiki et al., 2019). In individuals with an increased need for iron supplementation, Hoppe and colleagues reported that adding *L. plantarum 299v* to a meal improved iron bioavailability (Hoppe et al., 2017). In a double-blind clinical trial involving patients undergoing hemodialysis, probiotic supplementation containing *L. acidophilus*, *B. bifidum*, *B. lactis*, and *B. longum* led to a significant increase in serum hemoglobin levels compared to the placebo group (Haghighat et al., 2021). A recent randomized placebo-controlled study among athletes found that daily high-protein concentrated pro-yogurt enriched with whey protein isolates significantly increased hemoglobin levels. The study suggested that probiotics in concentrated yogurt, alongside enhanced iron bioavailability through mineral binding, can positively influence iron status and help improve athletic anemia and performance (Gomaa et al., 2022).

5.7 Probiotics in the treatment and prevention of obesity

Obesity is a significant risk factor for various health conditions, including hypertension, coronary heart disease, and type II diabetes. Multiple factors contribute to obesity, including dietary choices, physical activity levels, age, genetics, and developmental stage (Leon Aguilera et al., 2022). In recent times, a novel approach to managing and reducing obesity has emerged through the use of probiotic bacteria. Probiotics have demonstrated their potential to reduce weight gain and combat obesity effectively, regulating food intake and promoting prolonged feelings of satiety, reducing fat deposition in the body, enhancing energy metabolism, and increasing insulin sensitivity (Daniali et al., 2020).

Research into the use of probiotics for managing obesity has yielded several significant findings from various studies. A comprehensive meta-analysis of 105 trials involving 6,826 obese patients concluded that probiotics moderately improved body mass index (BMI) and weight, resulting in a 3–5% reduction. However, there was no statistically significant impact on parameters such as glycated hemoglobin, cholesterol, triglycerides, insulin resistance, or liver function. Notably, in overweight (but not obese) individuals, probiotics, especially strains like bifidobacteria (*B. breve*, *B. longum*), *S. salivarius subsp. thermophilus*, and lactobacilli (*L. acidophilus*, *L. casei*, *L. delbrueckii*), brought about improvements in waist circumference, body fat mass, visceral adipose tissue mass, BMI, and body weight (Koutnikova et al., 2019). In another meta-analysis focusing on women with polycystic ovary syndrome (PCOS), probiotic supplementation was found to have a positive impact. It improved BMI, weight, insulin levels, triglycerides, and low-density lipoprotein (VLDL) cholesterol levels. However, it did not significantly affect dehydroepiandrosterone sulfate levels or total LDL and HDL cholesterol levels (Tabrizi et al., 2022). A comprehensive review encompassing thirty-three randomized clinical trials involving individuals with overweight and obesity revealed that approximately 30% of the trials reported reductions in body weight and BMI, while 50% showed significant reductions in waist circumference and total fat mass (Guedes et al., 2023). In a PROSPERO meta-analysis comprising eleven randomized controlled trials involving morbidly obese patients undergoing bariatric surgery, probiotics demonstrated beneficial effects. The pooled analysis indicated that probiotics could help regulate triglycerides, reduce weight, and influence dietary intake, particularly carbohydrates and fiber. The findings suggested that probiotics might delay the progression of liver function impairment, improve lipid metabolism, and reduce weight and food intake (Wang et al., 2023b).

Moreover, the microbiota administration from a healthy individual to an obese person can alter the composition of the recipient's microbial flora. Recent studies have revealed that probiotics play a role in reducing serum cholesterol levels through mechanisms involving the production of short-chain fatty acids and the conjugation of bile salts (Jia et al., 2023; Momin et al., 2023).

5.8 Probiotics in COVID-19

The global pandemic caused by coronavirus disease 2019 (COVID-19) has tragically impacted countless lives. Despite the widespread adoption of rigorous public health measures such as social distancing, personal care, mask mandates, and lockdowns, the virus continues to proliferate, escalating infections and fatalities worldwide. The primary culprit behind this disease is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which distinguishes itself from previously identified coronaviruses. Consequently, existing antiviral treatments have demonstrated limited effectiveness (Singh and Rao, 2021). In this context, natural products like probiotics and their derivatives are being investigated for their potential contributions to pandemic prevention, treatment, and overall management, extending to post-pandemic periods and other severe infections (Brahma et al., 2022; Banerjee et al., 2023).

Currently, no direct evidence suggests that probiotics can directly inhibit coronaviruses. However, research has shown that certain probiotic strains have inhibitory effects on various viruses, as reviewed by multiple researchers (Olaimat et al., 2020; Tiwari et al., 2020; Singh and Rao, 2021). For example, in an animal study, *L. plantarum* was found to effectively reduce the proliferation of the influenza A (IFV) virus in the lungs in a dose-dependent manner. This probiotic also strengthened the Th1 immune response and increased the production of secretory IgA, contributing to the elimination of IFV from the lungs (Kawashima et al., 2011). This suggests that probiotic consumption

can help reduce viral loads and enhance immune responses, which is particularly important during the pandemic. Bacteriocidins produced by *S. salivarius* 24 SMB have been shown to inhibit the common respiratory pathogen *S. pneumoniae* (Santagati et al., 2012). Additionally, *L. rhamnosus*, alone or in combination with *B. animalis subsp. lactis* Bb-12 has been found to reduce upper respiratory infections in children. It modulates the immune system and reduces nasal colonization with *S. aureus* and *S. pneumoniae* in adults. *S. salivarius* K12 has effectively reduced the incidence of infectious episodes in pharynx-tonsillar infections caused by group A beta-hemolytic streptococci and in children with otitis media. It has also been shown to prevent the recurrence of tonsillitis and pharyngitis (Di Pierro et al., 2016).

While limited data is available, specific probiotic strains such as *L. gasseri* SBT2055, *L. rhamnosus* CRL1505, *B. bifidum*, and *B. subtilis* warrant further exploration for their potential roles in managing COVID-19. Moreover, probiotic bacteria exhibit antioxidant capabilities, which are especially pertinent for COVID-19 management due to the critical role of redox homeostasis in curbing disease progression (Starosila et al., 2017; Singh and Rao, 2021).

Similar to other coronaviruses, SARS-CoV-2 employs the angiotensin-converting enzyme 2 (ACE2) for cellular entry and relies on the transmembrane protease serine 2 precursor (TMPRSS2) for priming and replication within the host (Ahlawat et al., 2020; Hoffmann et al., 2020)(Figure 4). This has suggested that ACE inhibitors could have a role in COVID-19 management (Meng et al., 2020). Notably, certain probiotics, especially lactic acid bacteria, are known to produce bioactive peptides that function as ACE inhibitors (Kim et al., 2021; Bhattacharya et al., 2022). Additionally, the lung-gut axis and its interplay with microbiota, in conjunction with probiotics, have been proposed for investigating COVID-19 management, building on prior work by Ahlawat et al. (2020).

Hence, there is a need to delve into the potential of probiotics and their derivatives, individually and in combinations, which exhibit promising activities that could be harnessed in the future (Singh and Rao, 2021).

Two recent meta-analyses suggest that probiotics may positively impact the overall symptoms experienced by individuals with COVID-19. Specifically, the results showed that probiotics have the potential to restore equilibrium in the intestinal microbiota, decrease the duration of diarrhea, enhance respiratory symptoms such as cough and dyspnea, and reduce the length of hospitalization (Tian et al., 2023; Zhu et al., 2023).

The potential application of microbiome or probiotic therapy holds promise as an emerging therapeutic approach due to its foundation on the multifactorial mechanism of action exhibited by beneficial bacteria in combating respiratory viral diseases (De Boeck et al., 2022). This adjuvant is characterized by its immunomodulatory properties, including the capacity to stimulate interferon production, mitigate excessive immune responses, and inhibit cytokine storms, thereby preventing pathological inflammatory states through immune response modulation (Nayebi et al., 2021). Other studies on different probiotic formulas investigated a throat spray containing specific antiviral lactobacilli. These studies suggest that this formula can decrease nasopharynx viral loads and alleviate acute symptoms (De Boeck et al., 2022). The administration of oral probiotic formula based on *Lactiplantibacillus plantarum* strains and *Pediococcus acidilactici* shows a reduced nasopharyngeal viral load, lung infiltrates, and digestive and non-digestive symptoms duration. The observed effect of the therapy resulted in an elevation of specific IgM and IgG antibodies targeting SARS-CoV2. This finding implies that the intervention may predominantly influence the gut-lung axis through communication with the host's immune system rather than altering the colonic microbiota (Gutierrez-Castrellon et al., 2022). However, studies based on the microbiome analysis confirmed that the abundance of *Lactobacillus rhamnosus* was notably higher in individuals who were administered probiotics with *L. rhamnosus* GG strain compared to those who received a placebo (Tang et al., 2021; Wischmeyer et al., 2022).

Age-related immune response modifications make the elderly substantially more susceptible to COVID-19. Vaccination is crucial in mitigating the severity and fatality rates among older adults. However, it is important to note that age-related deficiencies in immune response might result in diminished levels of protection following vaccination when compared to younger cohorts. Probiotic supplements have been recognized as a supplementary measure to enhance immune function and improve the particular immune responses to vaccines in the aged demographic. The administration of *Loigolactobacillus coryniformis* K8 has shown promising effects, such as increasing the IgA response in correlation with the activation of TGF-β, a key signaling molecule involved in immune regulation and tolerance. Furthermore, the positive immune response induced by K8, as evidenced by the enhanced response to the BNT162b2 mRNA COVID-19 vaccine, suggests that individuals who receive this probiotic may have a more robust immune response if they are exposed to the live virus in the case of infection. This finding underscores the potential benefits of probiotics like K8 in strengthening the body's defenses against viral threats, including COVID-19 (Fernández-Ferreiro et al., 2022).

6 Conclusions and future perspectives

The application of microbial therapy, particularly probiotic bacterial species, has garnered increasing attention in recent years. Researchers have been diligently working to provide conclusive evidence regarding the beneficial effects of probiotic strains on human health and disease outcomes (Tiwari et al., 2020). Probiotic bacteria offer many advantages, including their anti-inflammatory, anti-cancer, antimicrobial, antioxidant, and immunomodulatory properties. These properties enable them to play crucial roles in shaping the physiological and metabolic functions of the host while also combatting pathogenic microbes. The potential contributions of probiotics to the prevention and treatment of conditions such as diabetes, obesity, and cancer have opened up exciting and rapidly growing areas of research (Leong et al., 2018). Incorporating probiotics into one's diet through dairy products and fermented foods represents a straightforward and cost-effective means of enhancing human health. As research advances, there is a growing emphasis on evaluating new strains of human gut microbes and exploring their potential applications in biomedical and clinical research. This ongoing exploration promises to uncover new directions and opportunities for harnessing the benefits of probiotics for human well-being (Nataraj et al., 2020).

Selecting the appropriate probiotic products can be challenging, as their effectiveness hinges on several factors, including the specific

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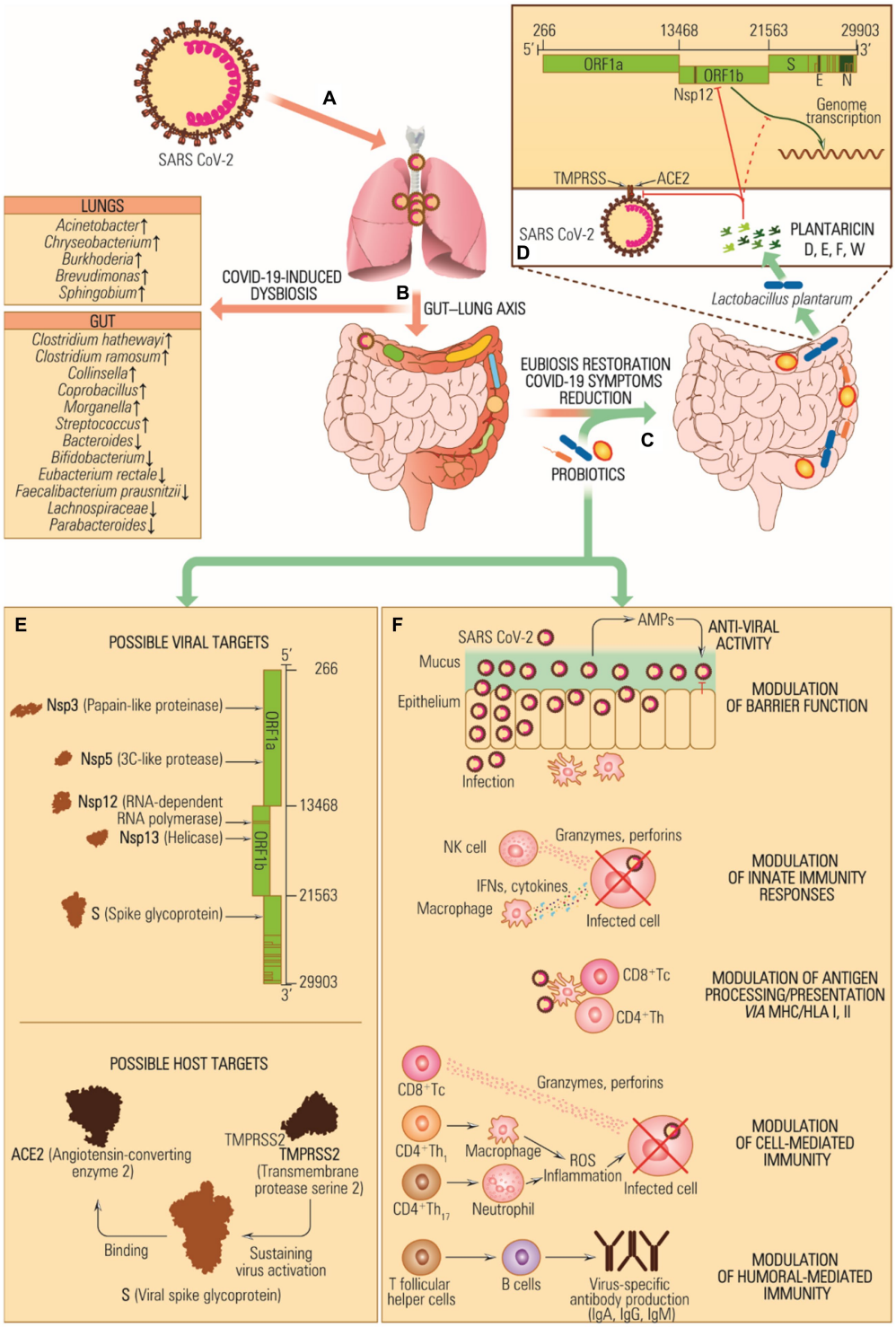


FIGURE 4
Effects of probiotics on SARS-CoV-2 infection. **(A)** The virus enters the body, infecting the respiratory system, including the lungs. **(B)** Via the gut-lung axis, SARS-CoV-2 virus induces dysbiosis in the lungs and colon. **(C)** Dietary supplementation with probiotics is effective in restoring eubiosis and relieving symptoms produced by SARS-CoV-2 infection. **(D)** Example of beneficial effect produced by the probiotic *Lactobacillus plantarum*, whose plantaricins D, E, F, and W inhibit the binding of viral spicular glycoprotein S (S1 subunit) to the cellular ACE2 receptor, they also and binding and inactivate RNA-dependent RNA polymerase (Nsp12), preventing transcription of the viral genome. **(E)** Different compounds secreted by probiotics may target some viral proteins, including Nsp3, Nsp5, Nsp12, Nsp13 and S, or some host cell-expressed proteins, ACE2, which serves as a receptor for viral attachment, and TMPRSS2, which facilitates activation of the viral glycoprotein S, with activation of the virus and its cell binding. **(F)** Probiotics participate in the modulation of barrier function and antiviral activity of epithelia, modulate innate immune response, antigen processing and presentation by antigen-presenting cells via MHC or HLA I and II molecules, and cellular and humoral mediated immunity.

strain, the type of disease or condition being targeted, and the appropriate dosage. Probiotics operate through various mechanisms to combat different pathogens, and what works for one disease may not necessarily be effective for another. For example, while LGG effectively prevents antibiotic-associated pediatric diarrhea, it may not yield the same results for diseases like Crohn's disease, CDI, nosocomial infections, or traveler's diarrhea (Li et al., 2019). Standardizing the required dosage of probiotics is a complex task. Achieving the optimal number of viable probiotic cells for effective gut colonization depends on several factors, including the manufacturing processes, quality control measures, interactions between different bacterial species administered together, and the ability of probiotics to withstand the acidic and bile-rich environment of the digestive system. It's also important to note that, like antibiotics, there is a potential risk of horizontal transfer of AMR-related genes from probiotic strains to other co-infecting pathogens and vice versa. This underscores the need for careful consideration and monitoring when using probiotics, especially when antimicrobial resistance is a concern (Silva et al., 2020).

Despite their potential health benefits, the clinical application of probiotics is facing several challenges that warrant careful consideration and adherence to safety protocols. Probiotics can face viability problems during storage, particularly at room temperature. Ensuring the stability and shelf-life of probiotic products is essential to maintain their effectiveness (Khan A. et al., 2023; Khan M. T. et al., 2023). Probiotics may vary in their ability to colonize the intestinal tract and tolerate the conditions within the gut. There is a concern that probiotics could acquire virulence genes from opportunistic or pathogenic organisms, potentially compromising their safety. Like other bacteria, probiotics can transfer antibiotic-resistance genes within the GIT, raising concerns about the spread of antimicrobial resistance. Some probiotic strains, such as Bacillus species, can produce heat-stable toxins like amylosin, which can potentially induce food poisoning. In light of these challenges, healthcare professionals and researchers must exercise caution when incorporating probiotics into clinical applications (Yadav et al., 2022). It is essential to thoroughly evaluate probiotic strains, their safety profiles, and their specific intended uses. Additionally, adherence to safety protocols and rigorous monitoring can help mitigate potential risks associated with probiotic administration (including storage conditions, gene transfer, and toxin production) while maximizing their potential health benefits (Siciliano et al., 2021).

Developing clinically efficacious probiotics remains challenging and must adhere to various requirements to ensure their safety and effectiveness. Several key considerations in developing and evaluating probiotics include determining the minimum effective dose, including adequate controls, and selecting and combining probiotics relevant to the targeted health outcomes (Brahma et al., 2022). Probiotics hold promise for reducing antibiotic use and addressing various health issues. However, concerns are related to the over-the-counter availability of numerous probiotic combinations with questionable efficacy and the potential for irrational use and undefined dosing. Therefore, researchers and healthcare professionals must exercise caution and conduct rigorous evaluations when considering probiotics for clinical applications (Yadav et al., 2022).

Microbiome therapy aims to establish a healthy native microbial environment in the gut to prevent dysregulation and promote survival. This therapeutic approach leverages the potential of microbes to restore imbalances and enhance the host's survival by influencing various metabolic, nutritional, and physiological pathways, particularly those that affect pathogens. The effectiveness of microbiome therapy has proven challenging under diverse circumstances. Furthermore, research in this field has predominantly relied on rodent models, necessitating a shift toward more human-focused studies. Ensuring the success of microbiome therapy is crucial to the stability and resilience of clinically relevant microbial strains.

In summary, advancing our understanding of these innovative therapeutic strategies is of paramount importance. Until these approaches are well-defined, structured, and readily available in the market, it remains imperative to establish standards for controlling conventional chemical antibiotics, which can significantly disrupt human and animal microbiota in daily consumed foods. Addressing various safety and regulatory concerns is essential for the successful execution of clinical trials involving microbiome therapy. A regulatory framework must be crafted to ensure the biosafety of these therapies, minimizing adverse effects and the inadvertent release of genetically modified microbes into the environment. Additionally, evaluating modified probiotics' long-term safety and efficacy is vital for their sustained therapeutic benefits.

Author contributions

O-AP: Writing – original draft, Writing – review & editing. ICB: Writing – review & editing. A-GN: Writing – review & editing. MC: Writing – review & editing. GAG: Writing – original draft, Writing – review & editing. R-EC: Writing – review & editing. GM: Writing – review & editing. COV: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abikshyeet, P., Mishra, P., Bhuyan, L., Kumar, V., Mahapatra, N., and Adhikary, T. (2022). Probiotics: dawn of a new era in dental caries management. *J. Pharm. Bioallied Sci.* 14, 34–S38. doi: 10.4103/jpbs.jpbs_801_21

Abruzzo, A., Vitali, B., Lombardi, F., Guerrini, L., Cinque, B., Parolin, C., et al. (2020). Mucoadhesive buccal films for local delivery of *Lactobacillus brevis*. *Pharmaceutics* 12:241. doi: 10.3390/pharmaceutics12030241

Adiki, S. K., Perla, C. K., Saha, G., Katakam, P., and Theendra, V. (2019). Enhancement in iron absorption on intake of chemometrically optimized ratio of probiotic strain *Lactobacillus plantarum 299v* with iron supplement pearl millet. *Biol. Trace Elem. Res.* 190, 150–156. doi: 10.1007/s12011-018-1541-5

Aghamohammad, S., Sepehr, A., Miri, S. T., Najafi, S., Pourshafie, M. R., and Rohani, M. (2022). Anti-inflammatory and immunomodulatory effects of Lactobacillus spp. as a preservative and therapeutic agent for IBD control. *Immun Inflamm Dis.* 10:e635. doi: 10.1002/iid3.635

Agossa, K., Dubar, M., Lemaire, G., Blaizot, A., Catteau, C., Bocquet, E., et al. (2022). Effect of *Lactobacillus reuteri* on gingival inflammation and composition of the oral microbiota in patients undergoing treatment with fixed orthodontic appliances: study protocol of a randomized control trial. *Pathogens* 11:112. doi: 10.3390/pathogens11020112

Ahlawat, S., Asha, , and Sharma, K. K. (2020). Immunological co-ordination between gut and lungs in SARS-CoV-2 infection. *Virus Res.* 286:198103. doi: 10.1016/j.virusres.2020.198103

Ahn, S. I., Kim, M. S., Park, D. G., Han, B. K., and Kim, Y. J. (2023). Effects of probiotics administration on lactose intolerance in adulthood: a meta-analysis. *J. Dairy Sci.* 106, 4489–4501. doi: 10.3168/jds.2022-22762

Ahn, J., Kim, H., and Yang, K. M. (2020). An aqueous extract of a *Bifidobacterium* species induces apoptosis and inhibits invasiveness of non-small cell lung cancer cells. *J. Microbiol. Biotechnol.* 30, 885–893. doi: 10.4014/jmb.1912.12054

Al-Badr, A., and Al-Shaikh, G. (2013). Recurrent urinary tract infections management in women: a review. *Sultan Qaboos Univ. Med. J.* 13, 359–367. doi: 10.12816/0003256

Aleman, R. S., Moncada, M., and Aryana, K. J. (2023). Leaky gut and the ingredients that help treat it: a review. *Molecules* 28:619. doi: 10.3390/molecules28020619

Alkaya, B., Laleman, I., Keceli, S., Ozcelik, O., Cenk Haytac, M., and Teughels, W. (2017). Clinical effects of probiotics containing Bacillus species on gingivitis: a pilot randomized controlled trial. *J. Periodontal Res.* 52, 497–504. doi: 10.1111/jre.12415

Al-Sadi, R., Nighot, P., Nighot, M., Haque, M., Rawat, M., and Ma, T. Y. (2021). *Lactobacillus acidophilus* induces a strain-specific and toll-like receptor 2-dependent enhancement of intestinal epithelial tight junction barrier and protection against intestinal inflammation. *Am. J. Pathol.* 191, 872–884. doi: 10.1016/j.ajpath.2021.02.003

Amaretti, A., di Nunzio, M., Pompei, A., Raimondi, S., Rossi, M., and Bordoni, A. (2013). Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. *Appl. Microbiol. Biotechnol.* 97, 809–817. doi: 10.1007/s00253-012-4241-7

Amargianitakis, M., Antoniadou, M., Rahiotis, C., and Varzakas, T. (2021). Probiotics, prebiotics, synbiotics and dental caries. New perspectives, suggestions, and patient coaching approach for a cavity-free mouth. *Appl. Sci.* 11:5472. doi: 10.3390/app11125472

Andolfi, C., Bloodworth, J. C., Papachristos, A., and Sweis, R. F. (2020). The urinary microbiome and bladder cancer: susceptibility and immune responsiveness. *Bladder Cancer* 6, 225–235. doi: 10.3233/BLC-200277

Andrews, C., McLean, M. H., and Durum, S. K. (2018). Cytokine tuning of intestinal epithelial function. *Front. Immunol.* 9:1270. doi: 10.3389/fimmu.2018.01270

Ansari, A., Son, D., Hur, Y. M., Park, S., You, Y. A., Kim, S. M., et al. (2023). *Lactobacillus* probiotics improve vaginal dysbiosis in asymptomatic women. *Nutrients* 15:1862. doi: 10.3390/nu15081862

Argandona Valdez, R. M., Ximenez-Fyvie, L. A., Caiaffa, K. S., Rodrigues Dos Santos, V., Gonzales Cervantes, R. M., Almaguer-Flores, A., et al. (2021). Antagonist effect of probiotic bifidobacteria on biofilms of pathogens associated with periodontal disease. *Microb. Pathog.* 150:104657. doi: 10.1016/j.micpath.2020.104657

Armstrong, E., Hemmerling, A., Miller, S., Burke, K. E., Newmann, S. J., Morris, S. R., et al. (2022). Sustained effect of LACTIN-V (*Lactobacillus crispatus* CTV-05) on genital immunology following standard bacterial vaginosis treatment: results from a randomised, placebo-controlled trial. *Lancet Microbe* 3, e435–e442. doi: 10.1016/S2666-5247(22)00043-X

Asgari, F., Falak, R., Teimourian, S., Pourakbari, B., Ebrahimnezhad, S., and Shekarabi, M. (2018). Effects of oral probiotic feeding on toll-like receptor gene expression of the chicken's cecal tonsil. *Rep Biochem Mol Biol.* 6, 151–157.

Asoudeh-Fard, A., Barzegari, A., Dehnad, A., Bastani, S., Golchin, A., and Omidi, Y. (2017). *Lactobacillus plantarum* induces apoptosis in oral cancer KB cells through upregulation of PTEN and downregulation of MAPK signalling pathways. *Bioimpacts* 7, 193–198. doi: 10.15171/bi.2017.22

Atanasova, K. R., and Yilmaz, O. (2014). Looking in the *Porphyromonas gingivalis* cabinet of curiosities: the microbium, the host and cancer association. *Mol Oral Microbiol* 29, 55–66. doi: 10.1111/omi.12047

Awasthi, A., Corrie, L., Vishwas, S., Gulati, M., Kumar, B., Chellappan, D. K., et al. (2022). Gut dysbiosis and diabetic foot ulcer: role of probiotics. *Pharmaceutics* 14:2543. doi: 10.3390/pharmaceutics14112543

Aysha Jebin, A., and Suresh, A. (2023). Oral microbial shift induced by probiotic Bacillus coagualans along with its clinical perspectives. *J Oral Biol Craniofac Res.* 13, 398–402. doi: 10.1016/j.jobcr.2023.03.013

Azad, M. A. K., Sarker, M., and Wan, D. (2018). Immunomodulatory effects of probiotics on cytokine profiles. *Biomed. Res. Int.* 2018:8063647. doi: 10.1155/2018/8063647

Bahaddad, S. A., Almalki, M. H. K., Alghamdi, O. A., Sohrab, S. S., Yasir, M., Azhar, E. I., et al. (2023). Bacillus species as direct-fed microbial antibiotic alternatives for monogastric production. *Probiotics Antimicrob Proteins* 15, 1–16. doi: 10.1007/s12602-022-09909-5

Bai, X., Zhu, M., He, Y., Wang, T., Tian, D., and Shu, J. (2022). The impacts of probiotics in eradication therapy of *Helicobacter pylori*. *Arch. Microbiol.* 204:692. doi: 10.1007/s00203-022-03314-w

Ballini, A., Santacroce, L., Cantore, S., Bottalico, L., Dipalma, G., Vito, D., et al. (2018). Probiotics improve urogenital health in women. *Open Access Maced J Med Sci.* 6, 1845–1850. doi: 10.3889/oamjms.2018.406

Bamola, V. D., Dubey, D., Samanta, P., Kedia, S., Ahuja, V., Madempudi, R. S., et al. (2022). Role of a probiotic strain in the modulation of gut microbiota and cytokines in inflammatory bowel disease. *Anaerobe* 78:102652. doi: 10.1016/j.anaerobe.2022.102652

Banerjee, A., Somasundaram, I., Das, D., Jain Manoj, S., Banu, H., Mitta Suresh, P., et al. (2023). Functional foods: a promising strategy for restoring gut microbiota diversity impacted by SARS-CoV-2 variants. *Nutrients* 15:2631. doi: 10.3390/nu15112631

Banna, G. L., Torino, F., Marletta, F., Santagati, M., Salemi, R., Cannarozzo, E., et al. (2017). *Lactobacillus rhamnosus* GG: an overview to explore the rationale of its use in cancer. *Front. Pharmacol.* 8:603. doi: 10.3389/fphar.2017.00603

Barbour, A., Smith, L., Oveisi, M., Williams, M., Huang, R. C., Marks, C., et al. (2023). Discovery of phosphorylated lantibiotics with proimmune activity that regulate the oral microbiome. *Proc. Natl. Acad. Sci. U. S. A.* 120:e2219392120. doi: 10.1073/pnas.2219392120

Barrea, L., Verde, L., Auriemma, R. S., Vetrani, C., Cataldi, M., Frias-Toral, E., et al. (2023). Probiotics and prebiotics: any role in menopause-related diseases? *Curr Nutr Rep.* 12, 83–97. doi: 10.1007/s13668-023-00462-3

Baumgardner, D. J. (2019). Oral fungal microbiota: to thrush and beyond. *J Patient Cent Res Rev.* 6, 252–261. doi: 10.17294/2330-0698.1705

Beharry, K. D., Latkowska, M., Valencia, A. M., Allana, A., Soto, J., Cai, C. L., et al. (2023). Factors influencing neonatal gut microbiome and health with a focus on necrotizing enterocolitis. *Microorganisms* 11:2528. doi: 10.3390/microorganisms11102528

Bhattacharya, J., Booy, R., Casadevall, A., Dela Cruz, C., Fedson, D. S., Garcia, J. G. N., et al. (2022). A practical treatment for COVID-19 and the next pandemic. *Pharmacol. Res. Perspect.* 10:e00988. doi: 10.1002/prp2.988

Bodke, H., and Jogdand, S. (2022). Role of probiotics in human health. *Cureus* 14:e31313. doi: 10.7759/cureus.31313

Brahma, S., Naik, A., and Lordan, R. (2022). Probiotics: a gut response to the COVID-19 pandemic but what does the evidence show? *Clin Nutr ESPEN.* 51, 17–27. doi: 10.1016/j.clnesp.2022.08.023

Brown, T. L., Charity, O. J., and Adriaenssens, E. M. (2022). Ecological and functional roles of bacteriophages in contrasting environments: marine, terrestrial and human gut. *Curr. Opin. Microbiol.* 70:102229. doi: 10.1016/j.mib.2022.102229

Browne, H. P., Forster, S. C., Anonye, B. O., Kumar, N., Neville, B. A., Stares, M. D., et al. (2016). Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* 533, 543–546. doi: 10.1038/nature17645

Bu, Y., Liu, Y., Liu, Y., Wang, S., Liu, Q., Hao, H., et al. (2022). Screening and probiotic potential evaluation of bacteriocin-producing *Lactiplantibacillus plantarum* in vitro. *Foods.* 11:1575. doi: 10.3390/foods11111575

Butera, A., Gallo, S., Maiorani, C., Molino, D., Chiesa, A., Preda, C., et al. (2020). Probiotic alternative to chlorhexidine in periodontal therapy: evaluation of clinical and microbiological parameters. *Microorganisms* 9:69. doi: 10.3390/microorganisms9010069

Cassir, N., Benamar, S., and La Scola, B. (2016). *Clostridium butyricum*: from beneficial to a new emerging pathogen. *Clin. Microbiol. Infect.* 22, 37–45. doi: 10.1016/j.cmi.2015.10.014

Chaiyasut, C., Sivamaruthi, B. S., Lailerd, N., Sirilun, S., Khongtan, S., Fukngoen, P., et al. (2022). Probiotics supplementation improves intestinal permeability, obesity index and metabolic biomarkers in elderly Thai subjects: a randomized controlled trial. *Foods* 11:268. doi: 10.3390/foods11030268

Chang, Y. H., Jeong, C. H., Cheng, W. N., Choi, Y., Shin, D. M., Lee, S., et al. (2021). Quality characteristics of yogurts fermented with short-chain fatty acid-producing probiotics and their effects on mucin production and probiotic adhesion onto human colon epithelial cells. *J. Dairy Sci.* 104, 7415–7425. doi: 10.3168/jds.2020-19820

Chang, C. J., Lin, C. S., Martel, J., Ojcius, D. M., Lai, W. F., Lu, C. C., et al. (2016). Modulation of host immune response by *Bacteroides fragilis* polysaccharides: a review of recent observations. *J. Biomed. Lab. Sci.* 28, 1–5.

Chang, C. J., Lin, T. L., Tsai, Y. L., Wu, T. R., Lai, W. F., Lu, C. C., et al. (2019). Next generation probiotics in disease amelioration. *J. Food Drug Anal.* 27, 615–622. doi: 10.1016/j.jfda.2018.12.011

Chen, Y., Chen, R., Wang, X., Zhou, Y., Hong, L., Xiong, N., et al. (2023). Effect of probiotic supplementation on in-hospital mortality in patients with acute myocardial infarction: a study protocol for an open-label, randomized, controlled, superiority clinical trial. *Trials* 24:429. doi: 10.1186/s13063-023-07443-5

Chen, X., Hu, Y., Yuan, X., Yang, J., and Ka, L. I. (2022). Effect of early enteral nutrition combined with probiotics in patients with stroke: a meta-analysis of randomized controlled trials. *Eur. J. Clin. Nutr.* 76, 592–603. doi: 10.1038/s41430-021-00986-3

Cristofori, F., Dargenio, V. N., Dargenio, C., Miniello, V. L., Barone, M., and Francavilla, R. (2021). Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body. *Front. Immunol.* 12:578386. doi: 10.3389/fimmu.2021.578386

Daniali, M., Nikfar, S., and Abdollahi, M. (2020). A brief overview on the use of probiotics to treat overweight and obese patients. *Expert. Rev. Endocrinol. Metab.* 15, 1–4. doi: 10.1080/17446651.2020.1719068

Das, T. K., Pradhan, S., Chakrabarti, S., Mondal, K. C., and Ghosh, K. (2022). Current status of probiotic and related health benefits. *Applied. Food Research* 2:100185. doi: 10.1016/j.afres.2022.100185

De Boeck, I., Cauwenberghs, E., Spacova, I., Gehrmann, T., Eilers, T., Delanghe, L., et al. (2022). Randomized, double-blind, placebo-controlled trial of a throat spray with selected lactobacilli in COVID-19 outpatients. *Microbiol. Spectr.* 10:e0168222. doi: 10.1128/spectrum.01682-22

de Melo Pereira, G. V., de Oliveira Coelho, B., Magalhães Júnior, A. I., Thomaz-Soccol, V., and Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* 36, 2060–2076. doi: 10.1016/j.biotechadv.2018.09.003

Deandra, F. A., Ketherin, K., Rachmasari, R., Sulijaya, B., and Takahashi, N. (2023). Probiotics and metabolites regulate the oral and gut microbiome composition as host modulation agents in periodontitis: a narrative review. *Heliyon* 9:e13475. doi: 10.1016/j.heliyon.2023.e13475

Dempsey, E., and Corr, S. C. (2022). Lactobacillus spp. for gastrointestinal health: current and future perspectives. *Front. Immunol.* 13:840245. doi: 10.3389/fimmu.2022.840245

Deng, X., Shang, X., Zhou, L., Li, X., Guo, K., Xu, M., et al. (2023). Efficacy and safety of probiotics in geriatric patients with constipation: systematic review and meta-analysis. *J. Nutr. Health Aging* 27, 1140–1146. doi: 10.1007/s12603-023-2028-4

Desai, S. J., Prickril, B., and Rasooly, A. (2018). Mechanisms of phytonutrient modulation of cyclooxygenase-2 (cox-2) and inflammation related to cancer. *Nutr. Cancer* 70, 350–375. doi: 10.1080/01635581.2018.1446091

Di Pierro, F., Colombo, M., Giuliani, M. G., Danza, M. L., Basile, I., Bollani, T., et al. (2016). Effect of administration of *Streptococcus salivarius* K12 on the occurrence of streptococcal pharyngo-tonsillitis, scarlet fever and acute otitis media in 3 years old children. *Eur. Rev. Med. Pharmacol. Sci.* 20, 4601–4606.

Djaldetti, M., and Bessler, H. (2017). Probiotic strains modulate cytokine production and the immune interplay between human peripheral blood mononucear cells and colon cancer cells. *FEMS Microbiol. Lett.* 364. doi: 10.1093/femsle/fnx014

Dong, M., Wu, Y., Zhang, M., Chen, P., Zhang, Z., and Wang, S. (2023). Effect of probiotics intake on constipation in children: an umbrella review. *Front. Nutr.* 10:1218909. doi: 10.3389/fnut.2023.1218909

Dou, C., Shang, Z., Qiao, J., Wang, Y., and Li, H. (2021). *Clostridium butyricum* protects IPEC-J2 cells from ETEC K88-induced oxidative damage by activating the Nrf2/ARE signaling pathway. *Oxidative Med. Cell. Longev.* 2021:4464002. doi: 10.1155/2021/4464002

Duan, H., Yu, L., Tian, F., Zhai, Q., Fan, L., and Chen, W. (2022). Antibiotic-induced gut dysbiosis and barrier disruption and the potential protective strategies. *Crit. Rev. Food Sci. Nutr.* 62, 1427–1452. doi: 10.1080/10408398.2020.1843396

Durant, L., Stentz, R., Noble, A., Brooks, J., Gicheva, N., Reddi, D., et al. (2020). *Bacteroides thetaiotaomicron*-derived outer membrane vesicles promote regulatory dendritic cell responses in health but not in inflammatory bowel disease. *Microbiome* 8:88. doi: 10.1186/s40168-020-00868-z

Ehrlich, A. M., Pacheco, A. R., Henrick, B. M., Taft, D., Xu, G., Huda, M. N., et al. (2020). Indole-3-lactic acid associated with Bifidobacterium-dominated microbiota significantly decreases inflammation in intestinal epithelial cells. *BMC Microbiol.* 20:357. doi: 10.1186/s12866-020-02023-y

Elias, A. J., Barna, V., Patoni, C., Demeter, D., Veres, D. S., Bunduc, S., et al. (2023). Probiotic supplementation during antibiotic treatment is unjustified in maintaining the gut microbiome diversity: a systematic review and meta-analysis. *BMC Med.* 21:262. doi: 10.1186/s12916-023-02961-0

Emara, M. H., Mohamed, S. Y., and Abdel-Aziz, H. R. (2014). *Lactobacillus reuteri* in management of *Helicobacter pylori* infection in dyspeptic patients: a double-blind placebo-controlled randomized clinical trial. *Ther. Adv. Gastroenterol.* 7, 4–13. doi: 10.1177/1756283X13503514

Engevik, M. A., and Versalovic, J. (2017). Biochemical features of beneficial microbes: foundations for therapeutic microbiology. *Microbiol Spectr.* 5. doi: 10.1128/microbiolspec.BAD-0012-2016

FAO/WHO (2002). *Guidelines for the Evaluation of Probiotics in Food*. Paris: FAO, 1–11.

Feng, J., Gao, M., Zhao, C., Yang, J., Gao, H., Lu, X., et al. (2022). Oral administration of probiotics reduces chemotherapy-induced diarrhea and oral mucositis: a systematic review and meta-analysis. *Front. Nutr.* 9:823288. doi: 10.3389/fnut.2022.823288

Ferlazzo, G., Rizzello, V., Bonaccorsi, I., Dongarr, M. L., and Fink, L. N. (2011). Role of natural killer and dendritic cell crosstalk in immunomodulation by commensal bacteria probiotics. *J. Biomed. Biotechnol.* 2011:473097. doi: 10.1155/2011/473097

Fernández-Ferreiro, A., Formigo-Couceiro, F. J., Veiga-Gutierrez, R., Maldonado-Lobón, J. A., Hermida-Cao, A. M., Rodriguez, C., et al. (2022). Effects of Loigolactobacillus coryniformis K8 CECT 5711 on the immune response of elderly subjects to COVID-19 vaccination: a randomized controlled trial. *Nutrients* 14:228. doi: 10.3390/nu14010228

Fijan, S. (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. *Int. J. Environ. Res. Public Health* 11, 4745–4767. doi: 10.3390/ijerph110504745

Fijan, S. (2023). Probiotics and their antimicrobial effect. *Microorganisms* 11:528. doi: 10.3390/microorganisms11020528

FitzGerald, J., Patel, S., Eckenberger, J., Guillemard, E., Veiga, P., Schäfer, F., et al. (2022). Improved gut microbiome recovery following drug therapy is linked to abundance and replication of probiotic strains. *Gut Microbes* 14:2094664. doi: 10.1080/19490976.2022.2094664

Foditsch, C., Pereira, R. V., Ganda, E. K., Gomez, M. S., Marques, E. C., Santin, T., et al. (2015). Oral administration of *Faecalibacterium prausnitzii* decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers. *PLoS One* 10:e0145485. doi: 10.1371/journal.pone.0145485

Frey-Furtado, L., Magalhães, I., Azevedo, M. J., and Sampaio-Maia, B. (2023). The role of biotics as a therapeutic strategy for oral mucositis – a systematic review. *Probiotics Antimicrob Proteins* 30. doi: 10.1007/s12602-023-10116-z

Garcia, V. G., Knoll, L. R., Longo, M., Novaes, V. C., Assem, N. Z., Ervolino, E., et al. (2016). Effect of the probiotic *Saccharomyces cerevisiae* on ligature-induced periodontitis in rats. *J. Periodontal Res.* 51, 26–37. doi: 10.1111/jre.12274

Garcia, V. G., Miessi, D. M. J., da Rocha, T. E., Gomes, N. A., Nuernberg, M. A. A., Cardoso, J. M., et al. (2022). The effects of *Lactobacillus reuteri* on the inflammation and periodontal tissue repair in rats: a pilot study. *The Saudi Dental Journal* 34, 516–526. doi: 10.1016/j.sdentj.2022.05.004

Gaspar, C., Donders, G. G., Palmeira-de-Oliveira, R., Queiroz, J. A., Tomaz, C., Martinez-de-Oliveira, J., et al. (2018). Bacteriocin production of the probiotic *Lactobacillus acidophilus* KS400. *AMB Express* 8:153. doi: 10.1186/s13568-018-0679-z

Gomaa, M. A. E., Allam, M. G., Haridi, A. A. I. M., Eliwa, A. M., and Darwish, A. M. G. (2022). High-protein concentrated pro-yogurt (pro-WPI) enriched with whey protein isolate improved athletic anemia and performance in a placebo-controlled study. *Front. Nutr.* 8:788446. doi: 10.3389/fnut.2021.788446

Gou, H. Z., Zhang, Y. L., Ren, L. F., Li, Z. J., and Zhang, L. (2022). How do intestinal probiotics restore the intestinal barrier? *Front. Microbiol.* 13:929346. doi: 10.3389/fmicb.2022.929346

Guallar-Garrido, S., and Julián, E. (2020). Bacillus Calmette-Guérin (BCG) therapy for bladder Cancer: an update. *Immunotargets Ther.* 9, 1–11. doi: 10.2147/ITT.S202006

Guedes, M. R., Pontes, K. S. D. S., Barreto Silva, M. I., Neves, M. F., and Klein, M. R. S. T. (2023). Randomized controlled trials reporting the effects of probiotics in individuals with overweight and obesity: a critical review of the interventions and body adiposity parameters. *Clin. Nutr.* 42, 835–847. doi: 10.1016/j.clnu.2023.03.017

Guo, Q., Li, S., Xie, Y., Zhang, Q., Liu, M., Xu, Z., et al. (2017). The NAD+-dependent deacetylase, *Bifidobacterium longum* Sir2 in response to oxidative stress by deacetylating SigH (O H) and FOXO3a in *Bifidobacterium longum* and HEK293T cell respectively. *Free Radic. Biol. Med.* 108, 929–939. doi: 10.1016/j.freeradbiomed.2017.05.012

Guo, M., Wu, J., Hung, W., Sun, Z., Zhao, W., Lan, H., et al. (2023). *Lactobacillus paracasei* ET-22 suppresses dental caries by regulating microbiota of dental plaques and inhibiting biofilm formation. *Nutrients* 15:3316. doi: 10.3390/nu15153316

Gutierrez-Castrellon, P., Gandara-Martí, T., Abreu Y Abreu, A. T., Nieto-Rufino, C. D., López-Orduña, E., Jiménez-Escobar, I., et al. (2022). Probiotic improves symptomatic and viral clearance in Covid19 outpatients: a randomized, quadruple-blinded, placebo-controlled trial. *Gut Microbes* 14:2018899. doi: 10.1080/19490976.2021.2018899

Hadadi, N., Berweiler, V., Wang, H., and Trajkovski, M. (2021). Intestinal microbiota as a route for micronutrient bioavailability. *Curr Opin Endocr Metab Res.* 20:100285. doi: 10.1016/j.coemr.2021.100285

Haghighat, N., Mohammadshahi, M., Shayanpour, S., Haghighizadeh, M. H., Rahmdel, S., and Rajaei, M. (2021). The effect of synbiotic and probiotic supplementation on mental health parameters in patients undergoing hemodialysis: a double-blind, randomized, placebo-controlled trial. *Indian J Nephrol.* 31, 149–156. doi: 10.4103/ijn.IJN_341_19

Hampelska, K., Jaworska, M. M., Babalska, Z. Ł., and Karpiński, T. M. (2020). The role of oral microbiota in intra-oral halitosis. *J. Clin. Med.* 9:2484. doi: 10.3390/jcm9082484

Han, H. S., Yum, H., Cho, Y. D., and Kim, S. (2023). Improvement of halitosis by probiotic bacterium *Weissella cibaria* CMU: a randomized controlled trial. *Front. Microbiol.* 14:1108762. doi: 10.3389/fmicb.2023.1108762

Hasan, M. T., Jang, W. J., Lee, B. J., Kim, K. W., Hur, S. W., Lim, S. G., et al. (2019). Heat-killed Bacillus sp. SJ-10 probiotic acts as a growth and humoral innate immunity response enhancer in olive flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol.* 88, 424–431. doi: 10.1016/j.fsi.2019.03.018

Hasanpour, A., Babajafari, S., Mazloomi, S. M., and Shams, M. (2023). The effects of soymilk plus probiotics supplementation on cardiovascular risk factors in patients with type 2 diabetes mellitus: a randomized clinical trial. *BMC Endocr. Disord.* 23:36. doi: 10.1186/s12902-023-01290-w

Hasna, B., Hemida, H., Koula, D., Spînu, M., Ungureanu, E., and Assia, B. (2023). In vitro and in vivo study of combined effect of some algerian medicinal plants and probiotics against *Helicobacter pylori*. *Microorganisms* 11:1242. doi: 10.3390/microorganisms11051242

He, Z., Wang, T., Zhang, S., Shi, K., Wang, F., Li, Y., et al. (2023). Evaluation of cholesterol transformation abilities and probiotic properties of *Bacteroides dorei* YGMCC0564. *Front. Microbiol.* 14:1279996. doi: 10.3389/fmicb.2023.1279996

Hemarajata, P., and Versalovic, J. (2013). Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Ther. Adv. Gastroenterol.* 6, 39–51. doi: 10.1177/1756283X12459294

Hernandez, P., Sánchez, M. C., Llama-Palacios, A., Ciudad, M. J., and Collado, L. (2022). Strategies to combat caries by maintaining the integrity of biofilm and homeostasis during the rapid phase of supragingival plaque formation. *Antibiotics (Basel)* 11:880. doi: 10.3390/antibiotics11070880

Higuchi, T., Furuichi, M., Maeda, N., Tsugawa, T., and Ito, K. (2023). Effects of probiotics in children with acute gastroenteritis: a systematic review and meta-analysis focusing on probiotics utilized in Japan. *J. Infect. Chemother.* S1341-321X(23)00277-5 doi: 10.1016/j.jiac.2023.11.005

Hills, R. D. Jr., Pontefract, B. A., Mishcon, H. R., Black, C. A., Sutton, S. C., and Theberge, C. R. (2019). Gut microbiome: profound implications for diet and disease. *Nutrients* 11:1613. doi: 10.3390/nu11071613

Hoffmann, D. E., Fraser, C. M., Palumbo, F., Ravel, J., Rowthorn, V., and Schwartz, J. (2014). Probiotics: achieving a better regulatory fit. *Food Drug Law J.* 69, 237–272, ii.

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cells* 181, 271–280.e8. doi: 10.1016/j.cell.2020.02.052

Holdcroft, A. M., Ireland, D. J., and Payne, M. S. (2023). The vaginal microbiome in health and disease – what role do common intimate hygiene practices play? *Microorganisms* 11:298. doi: 10.3390/microorganisms11020298

Homayouni Rad, A., Pourjafar, H., and Mirzakhani, E. (2023). A comprehensive review of the application of probiotics and postbiotics in oral health. *Front. Cell. Infect. Microbiol.* 13:1120995. doi: 10.3389/fcimb.2023.1120995

Hoppe, M., Önning, G., and Hulthén, L. (2017). Freeze-dried *Lactobacillus plantarum* 299v increases iron absorption in young females-double isotope sequential single-blind studies in menstruating women. *PLoS One* 12:e0189141. doi: 10.1371/journal.pone.0189141

Horii, T., Suzuki, S., Takano, C., Shibuya, H., Ichijima, R., Kusano, C., et al. (2021). Lower impact of vonoprazan-amoxicillin dual therapy on gut microbiota for *Helicobacter pylori* eradication. *J. Gastroenterol. Hepatol.* 36, 3314–3321. doi: 10.1111/jgh.15572

Hu, X., Zhang, Q., Hua, H., and Chen, F. (2016). Changes in the salivary microbiota of oral leukoplakia and oral cancer. *Oral Oncol.* 56, e6–e8. doi: 10.1016/j.oraloncology.2016.03.007

Huang, R., Xing, H. Y., Liu, H. J., Chen, Z. F., and Tang, B. B. (2021). Efficacy of probiotics in the treatment of acute diarrhea in children: a systematic review and meta-analysis of clinical trials. *Transl. Pediatr.* 10, 3248–3260. doi: 10.21037/tp-21-511

Hurtado-Camarena, A., Serafín-Higuera, N., Chávez-Cortez, E. G., and Pitones-Rubio, V. (2023). Lactobacillus probiotics and their impact on periodontal diseases. *EAS J Dent Oral Med* 5, 111–114. doi: 10.36349/easjdom.2023.v05i04.004

Hyink, O., Wescombe, P. A., Upton, M., Ragland, N., Burton, J. P., and Tagg, J. R. (2007). Salivaricin A2 and the novel lantibiotic salivaricin B are encoded at adjacent loci on a 190-kilobase transmissible megaplasmid in the oral probiotic strain *Streptococcus salivarius* K12. *Appl. Environ. Microbiol.* 73, 1107–1113. doi: 10.1128/AEM.02265-06

Im, E. J., Lee, H. H., Kim, M., and Kim, M. K. (2023). Evaluation of enterococcal probiotic usage and review of potential health benefits, safety, and risk of antibiotic-resistant strain emergence. *Antibiotics (Basel)* 12:1327. doi: 10.3390/antibiotics12081327

Invernici, M. M., Furlaneto, F. A. C., Salvador, S. L., Ouwehand, A. C., Salminen, S., Mantziari, A., et al. (2020). *Bifidobacterium animalis* subsp lactis HN019 presents antimicrobial potential against periodontopathogens and modulates the immunological response of oral mucosa in periodontitis patients. *PLoS One* 15:e0238425. doi: 10.1371/journal.pone.0238425

Ismail, N. I., Nawawi, K. N. M., Hsin, D. C. C., Hao, K. W., Mahmood, N. R. K. N., Chearn, G. L. C., et al. (2023). Probiotic containing *Lactobacillus reuteri* DSM 17648 as an adjunct treatment for *Helicobacter pylori* infection: a randomized, double-blind, placebo-controlled trial. *Helicobacter* 28:e13017. doi: 10.1111/hel.13017

Ismail, N. I., Raja Ali, R. A., Wong, Z., Muhammad Nawawi, K. N., Kok, W. H., Mahmood, K. N., et al. (2022). IDDF2022-ABS-0107. The effect of *Lactobacillus reuteri* probiotic as an adjunct treatment for *Helicobacter pylori* infection in adults. *Gut* 71, A9–A10. doi: 10.1136/gutjnl-2022-iddf.10

Jang, A. Y., Rod-In, W., Monmai, C., Sohn, M., Kim, T. R., Jeon, M. G., et al. (2022). Anti-inflammatory potential of *Lactobacillus reuteri* LM1071 via eicosanoid regulation in LPS-stimulated RAW264.7 cells. *J. Appl. Microbiol.* 133, 67–75. doi: 10.1111/jam.15331

Jansen, P. M., Abdelbary, M. M. H., and Conrads, G. (2021). A concerted probiotic activity to inhibit periodontitis-associated bacteria. *PLoS One* 16:e0248308. doi: 10.1371/journal.pone.0248308

Jia, B., Zou, Y., Han, X., Bae, J. W., and Jeon, C. O. (2023). Gut microbiome-mediated mechanisms for reducing cholesterol levels: implications for ameliorating cardiovascular disease. *Trends Microbiol.* 31, 76–91. doi: 10.1016/j.tim.2022.08.003

Jiang, X., Gu, S., Liu, D., Zhao, L., Xia, S., He, X., et al. (2018). Lactobacillus brevis 23017 relieves mercury toxicity in the Colon by modulation of oxidative stress and inflammation through the interplay of MAPK and NF-κB signaling cascades. *Front. Microbiol.* 9:2425. doi: 10.3389/fmicb.2018.02425

Kakiuchi, T., Mizoe, A., Yamamoto, K., Imamura, I., Hashiguchi, K., Kawakubo, H., et al. (2020). Effect of probiotics during vonoprazan-containing triple therapy on gut microbiota in *Helicobacter pylori* infection: a randomized controlled trial. *Helicobacter* 25:e12690. doi: 10.1111/hel.12690

Kang, M. S., Kim, B. G., Chung, J., Lee, H. C., and Oh, J. S. (2006). Inhibitory effect of *Weissella cibaria* isolates on the production of volatile Sulphur compounds. *J. Clin. Periodontol.* 33, 226–232. doi: 10.1111/j.1600-051X.2006.00893.x

Kany, S., Vollrath, J. T., and Relja, B. (2019). Cytokines in inflammatory disease. *Int. J. Mol. Sci.* 20:6008. doi: 10.3390/ijms20236008

Kawahara, T., Makizaki, Y., Oikawa, Y., Tanaka, Y., Maeda, A., Shimakawa, M., et al. (2017). Oral administration of *Bifidobacterium bifidum* G9-1 alleviates rotavirus gastroenteritis through regulation of intestinal homeostasis by inducing mucosal protective factors. *PLoS One* 12:e0173979. doi: 10.1371/journal.pone.0173979

Kawashima, T., Hayashi, K., Kosaka, A., Kawashima, M., Igarashi, T., Tsutsui, H., et al. (2011). *Lactobacillus plantarum* strain YU from fermented foods activates Th1 and protective immune responses. *Int. Immunopharmacol.* 11, 2017–2024. doi: 10.1016/j.intimp.2011.08.013

Keelan, J. A., and Newnham, J. P. (2017). Recent advances in the prevention of preterm birth. *F1000Res* 6. doi: 10.12688/f1000research.11385.1

Khan, M. T., Dwibedi, C., Sundh, D., Pradhan, M., Kraft, J. D., Caesar, R., et al. (2023). Synergy and oxygen adaptation for development of next-generation probiotics. *Nature* 620, 381–385. doi: 10.1038/s41586-023-06378-w

Khan, A., Mi, H., Gao, F., Hu, Q., Gu, X., Ma, F., et al. (2023). Dynamic changes of the gut microbial colonization in preterm infants with different time points after birth. *Front. Microbiol.* 14:1078426. doi: 10.3389/fmicb.2023.1078426

Kim, D., Kang, M., Yeu, J., Lee, M., and Cho, J. (2020). Inhibitory effect of the probiotic bacteria, *Weissella cibaria CMU* on halitosis: a randomized placebo-controlled study. *J. Korean Acad. Oral Health* 44, 246–252. doi: 10.11149/jkaoh.2020.44.4.246

Kim, H. I., Kim, J.-K., Kim, J.-Y., Jang, S.-E., Han, M. J., and Kim, D.-H. (2019). *Lactobacillus plantarum* LC27 and *Bifidobacterium longum* LC67 simultaneously alleviate high-fat diet-induced colitis, endotoxemia, liver steatosis, and obesity in mice. *Nutr. Res.* 67, 78–89. doi: 10.1016/j.nutres.2019.03.008

Kim, E. D., Lee, H. S., Kim, K. T., and Paik, H. D. (2021). Antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities of yogurt supplemented with *Lactiplantibacillus plantarum* NK181 and *Lactobacillus delbrueckii* KU200171 and sensory evaluation. *Foods.* 10:2324. doi: 10.3390/foods10102324

Kitada, M., Ogura, Y., Monno, I., and Koya, D. (2019). Sirtuins and type 2 diabetes: role in inflammation, oxidative stress, and mitochondrial function. *Front. Endocrinol.* 10:187. doi: 10.3389/fendo.2019.00187

Knipe, H., Temperton, B., Lange, A., Bass, D., and Tyler, C. R. (2021). Probiotics and competitive exclusion of pathogens in shrimp aquaculture. *Rev. Aquac.* 13, 324–352. doi: 10.1111/raq.12477

Kora, A. J. (2022). "Chapter 7 – Probiotics in the prevention and treatment of diarrheal disease" in *Probiotics in the Prevention and Management of Human Disease*. eds. M. K. Dwivedi, N. Amaresan, A. Sankaranarayanan and E. H. Kemp (Massachusetts, United States of America: Academic Press), 107–115.

Koradia, P., Kapadia, S., Trivedi, Y., Chanchu, G., and Harper, A. (2019). Probiotic and cranberry supplementation for preventing recurrent uncomplicated urinary tract infections in premenopausal women: a controlled pilot study. *Expert Rev. Anti-Infect. Ther.* 17, 733–740. doi: 10.1080/14787210.2019.1664287

Korcok, D. J., Tršic-Milanovic, N. A., Ivanovic, N. D., and Dordevic, B. I. (2018). Development of probiotic formulation for the treatment of iron deficiency anemia. *Chem. Pharm. Bull.* 66, 347–352. doi: 10.1248/cpb.c17-00634

Koutnikova, H., Genser, B., Monteiro-Sepulveda, M., Faurie, J. M., Rizkalla, S., Schrezenmeir, J., et al. (2019). Impact of bacterial probiotics on obesity, diabetes and non-alcoholic fatty liver disease related variables: a systematic review and meta-analysis of randomised controlled trials. *BMJ Open* 9:e017995. doi: 10.1136/bmjopen-2017-017995

La Storia, A., Di Giuseppe, F. A., Volpe, S., Oliviero, V., Villani, F., and Torrieri, E. (2020). Physical properties and antimicrobial activity of bioactive film based on whey protein and *lactobacillus curvatus* 54M16 producer of bacteriocins. *Food Hydrocoll.* 108:105959. doi: 10.1016/j.foodhyd.2020.105959

Lalla, R. V., Saunders, D. P., and Peterson, D. E. (2014). Chemotherapy or radiation-induced oral mucositis. *Dent. Clin. N. Am.* 58, 341–349. doi: 10.1016/j.cden.2013.12.005

Lee, D. S., Lee, S. A., Kim, M., Nam, S. H., and Kang, M. S. (2020). Reduction of halitosis by a tablet containing *Weissella cibaria* CMU: a randomized, double-blind, placebo-controlled study. *J. Med. Food* 23, 649–657. doi: 10.1089/jmf.2019.4603

Lee, D. K., Park, J. E., Kim, M. J., Seo, J. G., Lee, J. H., and Ha, N. J. (2015). Probiotic bacteria, B. Longum and *L. acidophilus* inhibit infection by rotavirus in vitro and decrease the duration of diarrhea in pediatric patients. *Clin. Res. Hepatol. Gastroenterol.* 39, 237–244. doi: 10.1016/j.clinre.2014.09.006

Lei, Y., Xu, M., Huang, N., and Yuan, Z. (2023). Meta-analysis of the effect of probiotics or synbiotics on the risk factors in patients with coronary artery disease. *Front Cardiovasc Med.* 10:1154888. doi: 10.3389/fcvm.2023.1154888

Leon Aguilera, X. E., Manzano, A., Pirela, D., and Bermúdez, V. (2022). Probiotics and gut microbiota in obesity: myths and realities of a new health revolution. *J Pers Med.* 12:1282. doi: 10.3390/jpm12081282

Leong, K. S. W., Derraik, J. G. B., Hofman, P. L., and Cutfield, W. S. (2018). Antibiotics, gut microbiome and obesity. *Clin. Endocrinol.* 88, 185–200. doi: 10.1111/cen.13495

Lesan, S., Hajifattahi, F., Rahbar, M., and Mohammadi, S. (2017). The effect of probiotic yoghurt on the frequency of salivary Candida. *J. Res. Dent. Maxillofac. Sci.* 2, 1–7. doi: 10.29252/jrdms.2.2.1

Li, Y. T., Xu, H., Ye, J. Z., Wu, W. R., Shi, D., Fang, D. Q., et al. (2019). Efficacy of *Lactobacillus rhamnosus* GG in treatment of acute pediatric diarrhea: a systematic review with meta-analysis. *World J. Gastroenterol.* 25, 4999–5016. doi: 10.3748/wjg.v25.i33.4999

Liang, S., Wang, T., Hu, X., Luo, L., Li, W., Wu, X., et al. (2015). Administration of *Lactobacillus helveticus* NS8 improves behavioral, cognitive, and biochemical aberrations caused by chronic restraint stress. *Neuroscience* 310, 561–577. doi: 10.1016/j.neuroscience.2015.09.033

Liu, G., Cao, S., Liu, X., Li, Z., Tian, Y., Zhang, X., et al. (2022). Effect of perioperative probiotic supplements on postoperative short-term outcomes in gastric cancer patients receiving neoadjuvant chemotherapy: a double-blind, randomized controlled trial. *Nutrition* 96:111574. doi: 10.1016/j.nut.2021.111574

Liu, L., Wang, A., Shi, H., Tao, H., and Nahata, M. C. (2023). Efficacy and safety of probiotics and synbiotics for functional constipation in children: a systematic review and meta-analysis of randomized clinical trials. *Clin. Nutr.* 42, 1817–1826. doi: 10.1016/j.clnu.2023.08.015

Liu, Y., Wang, J., and Wu, C. (2022). Modulation of gut microbiota and immune system by probiotics, pre-biotics, and post-biotics. *Front. Nutr.* 8:634897. doi: 10.3389/fnut.2021.634897

Liu, J., Zhu, R., Song, J., Sohaib, M., Wang, S., Mao, J., et al. (2023). *Limosilactobacillus reuteri* consumption significantly reduces the total cholesterol concentration without affecting other cardiovascular disease risk factors in adults: a systematic review and meta-analysis. *Nutr. Res.* 117, 1–14. doi: 10.1016/j.nutres.2023.06.004

Lopez-Siles, M., Duncan, S. H., Garcia-Gil, L. J., and Martinez-Medina, M. (2017). *Faecalibacterium prausnitzii*: from microbiology to diagnostics and prognostics. *ISME J.* 11, 841–852. doi: 10.1038/ismej.2016.176

Louis, P., Duncan, S. H., Sheridan, P. O., Walker, A. W., and Flint, H. J. (2022). Microbial lactate utilisation and the stability of the gut microbiome. *Gut Microb.* 3:e3. doi: 10.1017/gmb.2022.3

Luise, D., Bosi, P., Raff, L., Amatucci, L., Virdis, S., and Trevisi, P. (2022). *Bacillus* spp. probiotic strains as a potential tool for limiting the use of antibiotics, and improving the growth and health of pigs and chickens. *Front. Microbiol.* 13:801827. doi: 10.3389/fmicb.2022.801827

Ma, T., Shen, X., Shi, X., Sakandar, H. A., Quan, K., Li, Y., et al. (2023). Targeting gut microbiota and metabolism as the major probiotic mechanism – an evidence-based review. *Trends Food Sci. Technol.* 138, 178–198. doi: 10.1016/j.tifs.2023.06.013

Madhivanan, P., Alleyn, H. N., Raphael, E., Krupp, K., Ravi, K., Nebhrajani, R., et al. (2015). Identification of culturable vaginal Lactobacillus species among reproductive age women in Mysore, India. *J. Med. Microbiol.* 64, 636–641. doi: 10.1099/jmm.0.000070

Maitre, Y., Mahalli, R., Micheneau, P., Delpierre, A., Guerin, M., Amador, G., et al. (2021). Pre and probiotics involved in the modulation of Oral bacterial species: new therapeutic leads in mental disorders? *Microorganisms.* 9:1450. doi: 10.3390/microorganisms9071450

Maldonado Galdeano, C., Cazorla, S. I., Lemme Dumit, J. M., Vélez, E., and Perdigón, G. (2019). Beneficial effects of probiotic consumption on the immune system. *Ann. Nutr. Metab.* 74, 115–124. doi: 10.1159/000496426

Malfertheiner, P., Megraud, F., Rokkas, T., Gisbert, J. P., Liou, J. M., Schulz, C., et al. (2022). Management of *Helicobacter pylori* infection: the Maastricht VI/Florence consensus report. *Gut*. gutjnl-2022-327745 doi: 10.1136/gutjnl-2022-327745

Mandar, R., Sõerunurk, G., Štšepetova, J., Smidt, I., Rööp, T., Kõljalg, S., et al. (2023). Impact of *Lactobacillus crispatus*-containing oral and vaginal probiotics on vaginal health: a randomised double-blind placebo controlled clinical trial. *Benef Microbes.* 14, 143–152. doi: 10.3920/BM2022.0091

Mann, S., Park, M. S., Johnston, T. V., Ji, G. E., Hwang, K. T., and Ku, S. (2021). Oral probiotic activities and biosafety of *Lactobacillus gasseri* HHuMIN D. *Microb. Cell Factories* 20:75. doi: 10.1186/s12934-021-01563-w

Martin, R., Chain, F., Miquel, S., Lu, J., Gratadoux, J. J., Sokol, H., et al. (2014). The commensal bacterium *Faecalibacterium prausnitzii* is protective in DNBS-induced chronic moderate and severe colitis models. *Inflamm. Bowel Dis.* 20, 417–430. doi: 10.1097/01.MIB.0000440815.76627.64

Martinez, F. A., Balciunas, E. M., Converti, A., Cotter, P. D., and de Souza Oliveira, R. P. (2013). Bacteriocin production by Bifidobacterium spp. a review. *Biotechnol. Adv.* 31, 482–488. doi: 10.1016/j.biotechadv.2013.01.010

Menezes, C. A., Zanette, D. L., Magalhães, L. B., da Silva, J. T., Lago, R. M. R. S., Gomes, A. N., et al. (2023). Higher Bifidobacterium spp. fecal abundance is associated with a lower prevalence of hyperglycemia and cardiovascular risk markers among schoolchildren from Bahia, Brazil. *PLoS One* 18:e0290813. doi: 10.1371/journal.pone.0290813

Meng, N., Liu, Q., Dong, Q., Gu, J., and Yang, Y. (2023). Effects of probiotics on preventing caries in preschool children: a systematic review and meta-analysis. *J. Clin. Pediatr. Dent.* 47, 85–100. doi: 10.22514/jocpd.2023.014

Meng, J., Xiao, G., Zhang, J., He, X., Ou, M., Bi, J., et al. (2020). Renin-angiotensin system inhibitors improve the clinical outcomes of COVID-19 patients with hypertension. *Emerg. Microbes. Infect.* 9, 757–760. doi: 10.1080/22221751.2020.1746200

Mestrovic, T., Matijašić, M., Perić, M., Čipčić Paljetak, H., Barešić, A., and Verbanac, D. (2021). The role of gut, vaginal, and urinary microbiome in urinary tract infections: from bench to bedside. *Diagnostics* 11:7. doi: 10.3390/diagnostics11010007

Momin, E. S., Khan, A. A., Kashyap, T., Pervaiz, M. A., Akram, A., Mannan, V., et al. (2023). The effects of probiotics on cholesterol levels in patients with metabolic syndrome: a systematic review. *Cureus.* 15:e37567. doi: 10.7759/cureus.37567

Nadeem, A., Siddiqui, N., Al-Harbi, N. O., Al-Harbi, M. M., and Ahmad, S. F. (2016). TLR-7 agonist attenuates airway reactivity and inflammation through Nrf2-mediated antioxidant protection in a murine model of allergic asthma. *Int. J. Biochem. Cell Biol.* 73, 53–62. doi: 10.1016/j.biocel.2016.02.004

Naseri, K., Saadati, S., Yari, Z., Asbaghi, O., Hezaveh, Z. S., Mafi, D., et al. (2022). Beneficial effects of probiotic and synbiotic supplementation on some cardiovascular risk factors among individuals with prediabetes and type 2 diabetes mellitus: a grade-assessed systematic review, meta-analysis, and meta-regression of randomized clinical trials. *Pharmacol. Res.* 182:106288. doi: 10.1016/j.phrs.2022.106288

Nataraj, B. H., Ali, S. A., Behare, P. V., and Yadav, H. (2020). Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. *Microb. Cell Factories* 19:168. doi: 10.1186/s12934-020-01426-w

Nayebi, A., Navashenaq, J. G., Soleimani, D., and Nachvak, S. M. (2021). Probiotic supplementation: a prospective approach in the treatment of COVID-19. *Nutr. Health* 28, 163–175. doi: 10.1177/02601060211049631

Nedzi-Gora, M., WrÓblewska, M., and Gorska, R. (2020). The effect of *Lactobacillus salivarius* SGL03 on clinical and microbiological parameters in periodontal patients. *Pol. J. Microbiol.* 69, 441–451. doi: 10.33073/pjm-2020-047

Nguyen, T., Brody, H., Radaic, A., and Kapila, Y. (2021). Probiotics for periodontal health—current molecular findings. *Periodontol.* 87, 254–267. doi: 10.1111/prd.12382

Nigam, M., Panwar, A. S., and Singh, R. K. (2022). Orchestrating the fecal microbiota transplantation: current technological advancements and potential biomedical application. *Front Med Technol.* 4:961569. doi: 10.3389/fmedt.2022.961569

Obrenovich, M. (2018). Leaky gut, leaky brain? *Microorganisms* 6:107. doi: 10.3390/microorganisms6040107

Ochoa, C., Castro, F., Bulhosa, J. F., Manso, C., Fernandes, J. C. H., and Fernandes, G. V. O. (2023). Influence of the probiotic *L. reuteri* on periodontal clinical parameters after nonsurgical treatment: a systematic review. *Microorganisms.* 11:1449. doi: 10.3390/microorganisms11061449

Ohtani, N., Yoshimoto, S., and Hara, E. (2014). Obesity and cancer: a gut microbial connection. *Cancer Res.* 74, 1885–1889. doi: 10.1158/0008-5472.CAN-13-3501

Okumura, R., and Takeda, K. (2018). Maintenance of intestinal homeostasis by mucosal barriers. *Inflamm Regener.* 38:5. doi: 10.1186/s41232-018-0063-z

Olaimat, A. N., Aolymat, I., Al-Holy, M., Ayyash, M., Ghoush, M. A., Al-Nabulsi, A. A., et al. (2020). The potential application of probiotics and prebiotics for the prevention and treatment of COVID-19. *NPJ Sci. Food* 4:17. doi: 10.1038/s41538-020-00078-9

O'Toole, P. W., Marchesi, J. R., and Hill, C. (2017). Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nat. Microbiol.* 2:17057. doi: 10.1038/nmicrobiol.2017.57

Pallavi, P., Barik, A., Sahoo, N., Rajhans, G., and Raut, S. (2023). Alleviation of dental caries by use of isolated potential probiotic and its characterization. *Biotechnol. Appl. Biochem.* 70, 1518–1529. doi: 10.1002/bab.2471

Pan, N., Wang, K., Qi, Y., Yi, S., and Hu, G. (2016). Effects of *Lactobacillus* on the levels of the NF-N:B and inflammatory mediators in the mouse inflammation model. *Chin. J. Prev. Vet. Med.* 38, 686–689.

Papadimitriou, K., Zoumpopoulou, G., Foligné, B., Alexandraki, V., Kazou, M., Pot, B., et al. (2015). Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. *Front. Microbiol.* 6:58. doi: 10.3389/fmicb.2015.00058

Park, S. H., Lee, E. S., Park, S. T., Jeong, S. Y., Yun, Y., Kim, Y., et al. (2023). Efficacy and safety of MED-01 probiotics on vaginal health: a 12-week, multicenter, randomized, double-blind, placebo-controlled clinical trial. *Nutrients* 15:331. doi: 10.3390/nu15020331

Parkin, K., Christophersen, C. T., Verhasselt, V., Cooper, M. N., and Martino, D. (2021). Risk factors for gut dysbiosis in early life. *Microorganisms* 9:2066. doi: 10.3390/microorganisms9102066

Patangia, D. V., Anthony Ryan, C., Dempsey, E., Paul Ross, R., and Stanton, C. (2022). Impact of antibiotics on the human microbiome and consequences for host health. *MicrobiologyOpen* 11:e1260. doi: 10.1002/mbo3.1260

Patch, C., Pearce, A. J., Cheng, M., Boyapati, R., and Brenna, J. T. (2023). *Bacillus Subtilis* (BG01-4™) improves self-reported symptoms for constipation, indigestion, and dyspepsia: a phase 1/2A randomized controlled trial. *Nutrients* 15:4490. doi: 10.3390/nu15214490

Patil, A. V., Shetty, S. S., and Padhye, A. M. (2023). Comparative evaluation of the inhibitory effect of *Lactobacillus rhamnosus* on halitosis-causing bacteria: an invitro microbiological study. *Cureus* 15:e38568. doi: 10.7759/cureus.38568

Pavlidou, E., Fasoulas, A., Mantzorou, M., and Giaginis, C. (2022). Clinical evidence on the potential beneficial effects of probiotics and prebiotics in cardiovascular disease. *Int. J. Mol. Sci.* 23:15898. doi: 10.3390/ijms232415898

Penumetcha, S. S., Ahluwalia, S., Irfan, R., Khan, S. A., Reddy, S. R., Lopez, M. E. V., et al. (2021). The efficacy of probiotics in the management of *Helicobacter pylori*: a systematic review. *Cureus* 13:e20483. doi: 10.7759/cureus.20483

Perez-Ramos, A., Madi-Moussa, D., Coucheney, F., and Drider, D. (2021). Current knowledge of the mode of action and immunity mechanisms of LAB-bacteriocins. *Microorganisms* 9:2107. doi: 10.3390/microorganisms9102107

Peroni, D. G., Nuzzi, G., Trambusti, I., Di Cicco, M. E., and Comberiati, P. (2020). Microbiome composition and its impact on the development of allergic diseases. *Front. Immunol.* 11:700. doi: 10.3389/fimmu.2020.00700

Petrova, M. I., Lievens, E., Malik, S., Imholz, N., and Lebeer, S. (2015). Lactobacillus species as biomarkers and agents that can promote various aspects of vaginal health. *Front. Physiol.* 6:81. doi: 10.3389/fphys.2015.00081

Pique, N., Berlanga, M., and Miñana-Galbis, D. (2019). Health benefits of heat-killed (tyndallized) probiotics: an overview. *Int. J. Mol. Sci.* 20:2534. doi: 10.3390/ijms20102534

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., et al. (2017). Oxidative stress: harms and benefits for human health. *Oxidative Med. Cell. Longev.* 2017:8416763. doi: 10.1155/2017/8416763

Plaza-Diaz, J., Ruiz-Ojeda, F. J., Vilchez-Padial, L. M., and Gil, A. (2017). Evidence of the anti-inflammatory effects of probiotics and synbiotics in intestinal chronic diseases. *Nutrients* 9:555. doi: 10.3390/nu9060555

Pourrajab, B., Fatahi, S., Sohouli, M. H., Găman, M. A., and Shidfar, F. (2022). The effects of probiotic/synbiotic supplementation compared to placebo on biomarkers of oxidative stress in adults: a systematic review and meta-analysis of randomized controlled trials. *Crit. Rev. Food Sci. Nutr.* 62, 490–507. doi: 10.1080/10408398.2020.1821166

Prabhurajeshwar, C., and Chandrakanth, K. (2019). Evaluation of antimicrobial properties and their substances against pathogenic bacteria in-vitro by probiotic lactobacilli strains isolated from commercial yoghurt. *Clinical Nutrition Experimental* 23, 97–115. doi: 10.1016/j.yclnex.2018.10.001

Pushpass, R. G., Alzoufairi, S., Mancini, A., Quilter, K., Fava, F., Delaiti, S., et al. (2023). Chronic consumption of probiotics, oats, and apples has differential effects on postprandial bile acid profile and cardiometabolic disease risk markers compared with an isocaloric control (cornflakes): a randomized trial. *Am. J. Clin. Nutr.* 117, 252–265. doi: 10.1016/j.ajcnut.2022.10.013

Quaranta, G., Guarnaccia, A., Fancello, G., Agrillo, C., Iannarelli, F., Sanguinetti, M., et al. (2022). Fecal microbiota transplantation and other gut microbiota manipulation strategies. *Microorganisms* 10:2424. doi: 10.3390/microorganisms10122424

Rashad Hameed, S., and Abdul Sattar Salman, J. (2023). Co-aggregative effect of probiotics bacteria against diarrheal causative bacteria. *Arch Razi Inst.* 78, 831–841. doi: 10.22092/ARI.2022.359870.2494

Rastogi, S., and Singh, A. (2022). Gut microbiome and human health: exploring how the probiotic genus *Lactobacillus* modulate immune responses. *Front. Pharmacol.* 13:1042189. doi: 10.3389/fphar.2022.1042189

Rayyan, Y. M., Agraib, L. M., Alkhatib, B., Yamani, M. I., Abu-Sneineh, A. T., and Tayyem, R. F. (2023). Does probiotic supplementation improve quality of life in mild-to-moderately active ulcerative colitis patients in Jordan? A secondary outcome of the randomized, double-blind, placebo-controlled study. *Eur. J. Nutr.* 62, 3069–3077. doi: 10.1007/s00394-023-03207-8

Reid, G., Bruce, A. W., Fraser, N., Heinemann, C., Owen, J., and Henning, B. (2001). Oral probiotics can resolve urogenital infections. *FEMS Immunol. Med. Microbiol.* 30, 49–52. doi: 10.1111/j.1574-695X.2001.tb01549.x

Reid, G., Jass, J., Sebulsky, M. T., and McCormick, J. K. (2003). Potential uses of probiotics in clinical practice. *Clin. Microbiol. Rev.* 16, 658–672. doi: 10.1128/CMR.16.4.658-672.2003

Riaz Rajoka, M. S., Zhao, H., Lu, Y., Lian, Z., Li, N., Hussain, N., et al. (2018). Anticancer potential against cervix cancer (HeLa) cell line of probiotic Lactobacillus casei and *Lactobacillus paracasei* strains isolated from human breast milk. *Food Funct.* 9, 2705–2715. doi: 10.1039/c8fo00547h

Ribeiro, C. F. A., Silveira, G. G. O. S., Cândido, E. S., Cardoso, M. H., Espinola Carvalho, C. M., and Franco, O. L. (2020). Effects of antibiotic treatment on gut microbiota and how to overcome its negative impacts on human health. *ACS Infect. Dis.* 6, 2544–2559. doi: 10.1021/acsinfecdis.0c00036

Roe, A. L., Boyte, M. E., Elkins, C. A., Goldman, V. S., Heimbach, J., Madden, E., et al. (2022). Considerations for determining safety of probiotics: a USP perspective. *Regul. Toxicol. Pharmacol.* 136:105266. doi: 10.1016/j.yrtph.2022.105266

Rosa, L. S., Santos, M. L., Abreu, J. P., Balthazar, C. F., Rocha, R. S., Silva, H. L. A., et al. (2020). Antiproliferative and apoptotic effects of probiotic whey dairy beverages in human prostate cell lines. *Food Res. Int.* 137:109450. doi: 10.1016/j.foodres.2020.109450

Roy, S., and Dhaneshwar, S. (2023). Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: current perspectives. *World J. Gastroenterol.* 29, 2078–2100. doi: 10.3748/wjg.v29.i14.2078

Ruiz-Tovar, J., Llavero, C., and Fernandez-Contreras, M. E. (2023). Effect of *Lactobacillus kefiri*, in conjunction with PENS T6 and a hypocaloric diet, on weight loss, hypertension and laboratory glycemic and lipid profile. *Nutrients* 15:4549. doi: 10.3390/nu15214549

Rwubuzizi, R., Kim, H., Holzapfel, W. H., and Todorov, S. D. (2023). Beneficial, safety, and antioxidant properties of lactic acid bacteria: a next step in their evaluation as potential probiotics. *Heliyon* 9:e15610. doi: 10.1016/j.heliyon.2023.e15610

Sadeghpour Heravi, F., and Hu, H. (2023). *Bifidobacterium*: host-microbiome interaction and mechanism of action in preventing common gut-microbiota-associated complications in preterm infants: a narrative review. *Nutrients* 15:709. doi: 10.3390/nu15030709

Saiz, P., Taveira, N., and Alves, R. (2021). Probiotics in oral health and disease: a systematic review. *Appl. Sci.* 11:8070. doi: 10.3390/app11178070

Salinas-Azuceno, C., Martínez-Hernández, M., Maldonado-Noriega, J. I., Rodríguez-Hernández, A. P., and Ximenez-Fyvie, L. A. (2022). Probiotic monotherapy with *Lactobacillus reuteri* (Prodentis) as a coadjutant to reduce subgingival dysbiosis in a patient with periodontitis. *Int. J. Environ. Res. Public Health* 19:7835. doi: 10.3390/ijerph19137835

Sandberg, A. S., Önning, G., Engström, N., and Scheers, N. (2018). Iron supplements containing *Lactobacillus Plantarum 299v* increase ferric iron and up-regulate the ferric reductase DCYTB in human Caco-2/HT29 MTX co-cultures. *Nutrients* 10:1949. doi: 10.3390/nu10121949

Santagati, M., Scillato, M., Patanè, F., Aiello, C., and Stefani, S. (2012). Bacteriocin-producing oral streptococci and inhibition of respiratory pathogens. *FEMS Immunol. Med. Microbiol.* 65, 23–31. doi: 10.1111/j.1574-695X.2012.00928.x

Savigamin, C., Samuthpongtorn, C., Mahakit, N., Nopsopon, T., Heath, J., and Pongpirul, K. (2022). Probiotic as a potential gut microbiome modifier for stroke treatment: a systematic scoping review of in vitro and in vivo studies. *Nutrients* 14:3661. doi: 10.3390/nu14173661

Seminotti, B., Grings, M., Tucci, P., Leipnitz, G., and Saso, L. (2021). Nuclear factor erythroid-2-related factor 2 signaling in the neuropathophysiology of inherited metabolic disorders. *Front. Cell. Neurosci.* 15:785057. doi: 10.3389/fncel.2021.785057

Shanahan, F., Ghosh, T. S., and O'Toole, P. W. (2021). The healthy microbiome—what is the definition of a healthy gut microbiome? *Gastroenterology* 160, 483–494. doi: 10.1053/j.gastro.2020.09.057

Sharma, A., Tilak, T., Bakhshi, S., Raina, V., Kumar, L., Chaudhary, S., et al. (2017). *Lactobacillus brevis* CD2 lozenges prevent oral mucositis in patients undergoing high dose chemotherapy followed by haematopoietic stem cell transplantation. *ESMO Open.* 1:e000138. doi: 10.1136/esmoopen-2016-000138

Shen, R., Chen, S., Lei, W., Shen, J., Lv, L., and Wei, T. (2023). Nonfood probiotic, prebiotic, and synbiotic use reduces all-cause and cardiovascular mortality risk in older adults: a population-based cohort study. *J. Nutr. Health Aging* 27, 391–397. doi: 10.1007/s12603-023-1921-1

Siciliano, R. A., Reale, A., Mazzeo, M. F., Morandi, S., Silvetti, T., and Brasca, M. (2021). Paraprobiotics: a new perspective for functional foods and nutraceuticals. *Nutrients* 13:1225. doi: 10.3390/nu13041225

Silva, D. R., de Cássia, J. O. S., Nayla de Souza, P., Roque, S. M., da Silva, A. C. B., and Rosalen, P. L. (2020). Probiotics as an alternative antimicrobial therapy: current reality and future directions. *J. Funct. Foods* 73:104080. doi: 10.1016/j.jff.2020.104080

Singh, K., and Rao, A. (2021). Probiotics: a potential immunomodulator in COVID-19 infection management. *Nutr. Res.* 87, 1–12. doi: 10.1016/j.nutres.2020.12.014

Singh, S., Singh, M., and Gaur, S. (2022). Probiotics as multifaceted oral vaccines against colon cancer: a review. *Front. Immunol.* 13:1002674. doi: 10.3389/fimmu.2022.1002674

So, D., Quigley, E. M. M., and Whelan, K. (2023). Probiotics in irritable bowel syndrome and inflammatory bowel disease: review of mechanisms and effectiveness. *Curr. Opin. Gastroenterol.* 39, 103–109. doi: 10.1097/MOG.0000000000000902

Srivastava, N., Ellepola, K., Venkiteswaran, N., Chai, L. Y. A., Ohshima, T., and Seneviratne, C. J. (2020). *Lactobacillus plantarum* 108 inhibits Streptococcus mutans and *Candida albicans* mixed-species biofilm formation. *Antibiotics* 9:478. doi: 10.3390/antibiotics9080478

Starosila, D., Rybalko, S., Varbanetz, L., Ivanskaya, N., and Sorokulova, I. (2017). Anti-influenza activity of a *Bacillus subtilis* probiotic strain. *Antimicrob. Agents Chemother.* 61, e00539–e00617. doi: 10.1128/AAC.00539-17

Sulijaya, B., Takahashi, N., and Yamazaki, K. (2019). Host modulation therapy using anti-inflammatory and antioxidant agents in periodontitis: a review to a clinical translation. *Arch. Oral Biol.* 105, 72–80. doi: 10.1016/j.archoralbio.2019.07.002

Suman, J., Rakshit, A., Ogireddy, S. D., Singh, S., Gupta, C., and Chandrakala, J. (2022). Microbiome as a key player in sustainable agriculture and human health. *Front. Soil Sci.* 2:821589. doi: 10.3389/fsoil.2022.821589

Tabrizi, R., Ostadmohammadi, V., Akbari, M., Lankarani, K. B., Vakili, S., Peymani, P., et al. (2022). The effects of probiotic supplementation on clinical symptom, weight loss, glycemic control, lipid and hormonal profiles, biomarkers of inflammation, and oxidative stress in women with polycystic ovary syndrome: a systematic review and meta-analysis of randomized controlled trials. *Probiotics Antimicrob Proteins.* 14, 1–14. doi: 10.1007/s12602-019-09559-0

Tang, H., Bohannon, L., Lew, M., Jensen, D., Jung, S. H., Zhao, A., et al. (2021). Randomised, double-blind, placebo-controlled trial of probiotics to eliminate COVID-19 transmission in exposed household contacts (PROTECT-EHC): a clinical trial protocol. *BMJ Open* 11:e047069. doi: 10.1136/bmjopen-2020-047069

Telaku, S., Telaku, M., Behluli, E., Isjanovski, V., and Alidema, F. (2022). Impact of Lactobacillus Reuteri and three other probiotics in *Helicobacter pylori* eradication. Macedonian. *J. Med. Sci.* 10, 2215–2219. doi: 10.3889/oamjms.2022.10733

Teneva, D., and Denev, P. (2023). Biologically active compounds from probiotic microorganisms and plant extracts used as biopreservatives. *Microorganisms* 11:1896. doi: 10.3390/microorganisms11081896

Thomas, A., Thomas, A., and Butler-Sanchez, M. (2021). Dietary modification for the restoration of gut microbiome and management of symptoms in irritable bowel syndrome. *Am. J. Lifestyle Med.* 16, 608–621. doi: 10.1177/15598276211012968

Tian, Y., Ran, H., Wen, X., Fu, G., Zhou, X., Liu, R., et al. (2023). Probiotics improve symptoms of patients with COVID-19 through gut-lung axis: a systematic review and meta-analysis. *Front. Nutr.* 10:1179432. doi: 10.3389/fnut.2023.1179432

Tiwari, S. K., Dicks, L. M. T., Popov, I. V., Karaseva, A., Ermakov, A. M., Suvorov, A., et al. (2020). Probiotics at war against viruses: what is missing from the picture? *Front. Microbiol.* 11:1877. doi: 10.3389/fmicb.2020.01877

Trivedi, P., Jena, G., Tikoo, K. B., and Kumar, V. (2016). Melatonin modulated autophagy and Nrf2 signaling pathways in mice with colitis-associated colon carcinogenesis. *Mol. Carcinog.* 55, 255–267. doi: 10.1002/mc.22274

Tsay, F. W., and Hsu, P. I. (2018). *H. pylori* infection and extra-gastroduodenal diseases. *J. Biomed. Sci.* 25:65. doi: 10.1186/s12929-018-0469-6

Van Zyl, W. F., Deane, S. M., and Dicks, L. M. (2020). Molecular insights into probiotic mechanisms of action employed against intestinal pathogenic bacteria. *Gut Microbes* 12:1831339. doi: 10.1080/19490976.2020.1831339

Vancamelbeke, M., and Vermeire, S. (2017). The intestinal barrier: a fundamental role in health and disease. *Expert Rev. Gastroenterol. Hepatol.* 11, 821–834. doi: 10.1080/17474124.2017.1343143

Volgenant, C. M. C., van der Waal, S. V., Brandt, B. W., Buijs, M. J., van der Veen, M. H., Rosema, N. A. M., et al. (2022). The evaluation of the effects of two probiotic strains on the oral ecosystem: a randomized clinical trial. *Front. Oral Health* 3:825017. doi: 10.3389/froh.2022.825017

Vonderheid, S. C., Tussing-Humphreys, L., Park, C., Pauls, H., Hemphill, N. O., Labomascus, B., et al. (2019). A systematic review and meta-analysis on the effects of probiotic species on iron absorption and iron status. *Nutrients* 11:2938. doi: 10.3390/nu11122938

Wang, Z., Li, L., Wang, S., Wei, J., Qu, L., Pan, L., et al. (2022). The role of the gut microbiota and probiotics associated with microbial metabolisms in cancer prevention and therapy. *Front. Pharmacol.* 13:1025860. doi: 10.3389/fphar.2022.1025860

Wang, Y., Wang, X., Cao, X., Zhu, H., and Ma, L. (2023a). Comparative effectiveness of different probiotics supplements for triple *Helicobacter pylori* eradication: a network meta-analysis. *Front. Cell. Infect. Microbiol.* 13:1120789. doi: 10.3389/fcimb.2023.1120789

Wang, Y., Wu, Y., Wang, Y., Xu, H., Mei, X., Yu, D., et al. (2017). Antioxidant properties of probiotic bacteria. *Nutrients* 9:521. doi: 10.3390/nu9050521

Wang, X., Zhang, P., and Zhang, X. (2021). Probiotics regulate gut microbiota: an effective method to improve immunity. *Molecules* 26:6076. doi: 10.3390/molecules26196076

Wang, Y., Zheng, Y., Kuang, L., Yang, K., Xie, J., Liu, X., et al. (2023b). Effects of probiotics in patients with morbid obesity undergoing bariatric surgery: a systematic review and meta-analysis. *Int. J. Obes.* 47, 1029–1042. doi: 10.1038/s41366-023-01375-5

Wischmeyer, P. E., Tang, H., Ren, Y., Bohannon, L., Ramirez, Z. E., Andermann, T. M., et al. (2022). Daily Lactobacillus probiotic versus placebo in COVID-19-exposed household contacts (PROTECT-EHC): a randomized clinical trial. *medRxiv*. doi: 10.1101/2022.01.04.21268275

Witkin, S. S. (2015). The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth. *BJOG* 122, 213–218. doi: 10.1111/1471-0528.13115

Wu, H., and Chiou, J. (2021). Potential benefits of probiotics and prebiotics for coronary heart disease and stroke. *Nutrients* 13:2878. doi: 10.3390/nu13082878

Wu, T., Zhang, Y., Lv, Y., Li, P., Yi, D., Wang, L., et al. (2018). Beneficial impact and molecular mechanism of *Bacillus coagulans* on piglets' intestine. *Int. J. Mol. Sci.* 19:2084. doi: 10.3390/ijms19072084

Xiao, L., and Zhao, F. (2023). Microbial transmission, colonisation and succession: from pregnancy to infancy. *Gut* 72, 772–786. doi: 10.1136/gutjnl-2022-328970

Yadav, M. K., Kumari, I., Singh, B., Sharma, K. K., and Tiwari, S. K. (2022). Probiotics, prebiotics and synbiotics: safe options for next-generation therapeutics. *Appl. Microbiol. Biotechnol.* 106, 505–521. doi: 10.1007/s00253-021-11646-8

Yakovenko, E. P., Strokova, T. V., Ivanov, A. N., Iakovenko, A. V., Gioeva, I. Z., and Aldiyarova, M. A. (2022). The effectiveness of a probiotic containing *Bifidobacterium longum* BB-46 and *Enterococcus faecium* ENCfa-68 in the treatment of post-infectious irritable bowel syndrome. Prospective randomized comparative study. *Ter. Arkh.* 94, 180–187. doi: 10.26442/00403660.2022.02.201368

Yang, X., Pan, X., Jia, Z., Bai, B., Zhi, W., Chen, H., et al. (2022). Oral administration of Lactobacillus brevis 23017 combined with ellagic acid attenuates intestinal inflammatory injury caused by Eimeria infection by activating the Nrf2/HO-1 antioxidant pathway. *Vet. Res.* 53:21. doi: 10.1186/s13567-022-01042-z

Yao, P., Tan, F., Gao, H., Wang, L., Yang, T., and Cheng, Y. (2017). Effects of probiotics on toll-like receptor expression in ulcerative colitis rats induced by 2,4,6-trinitrobenzene sulfonic acid. *Mol. Med. Rep.* 15, 1973–1980. doi: 10.3892/mmr.2017.6226

Yao, S., Zhao, Z., Wang, W., and Liu, X. (2021). *Bifidobacterium longum*: protection against inflammatory bowel disease. *J Immunol Res* 2021:8030297. doi: 10.1155/2021/8030297

Yoo, J. I., Shin, I. S., Jeon, J. G., Yang, Y. M., Kim, J. G., and Lee, D. W. (2019). The effect of probiotics on halitosis: a systematic review and Meta-analysis. *Probiotics Antimicrob Proteins.* 11, 150–157. doi: 10.1007/s12602-017-9351-1

You, H. J., Si, J., Kim, J., Yoon, S., Cha, K. H., Yoon, H. S., et al. (2023). *Bacteroides vulgatus* SNUG 40005 restores *Akkermansia* depletion by metabolite modulation. *Gastroenterology* 164, 103–116. doi: 10.1053/j.gastro.2022.09.040

Zare Javid, A., Amerian, E., Basir, L., Ekrami, A., Haghighizadeh, M. H., and Maghsoumi-Norouzabad, L. (2020). Effects of the consumption of probiotic yogurt containing *Bifidobacterium lactis* Bb12 on the levels of Streptococcus mutans and lactobacilli in saliva of students with initial stages of dental caries: a double-blind randomized controlled trial. *Caries Res.* 54, 68–74. doi: 10.1159/000504164

Zarezadeh, M., Musazadeh, V., Ghalichi, F., Kavyani, Z., Nasernia, R., Parang, M., et al. (2023). Effects of probiotics supplementation on blood pressure: an umbrella meta-analysis of randomized controlled trials. *Nutr. Metab. Cardiovasc. Dis.* 33, 275–286. doi: 10.1016/j.numecd.2022.09.005

Zeng, Y., Fadaak, A., Alomeir, N., Wu, Y., Wu, T. T., Qing, S., et al. (2023). Effect of probiotic *Lactobacillus plantarum* on *Streptococcus mutans* and *Candida albicans* clinical isolates from children with early childhood caries. *Int. J. Mol. Sci.* 24:2991. doi: 10.3390/ijms24032991

Zhang, Y., Ding, Y., and Guo, Q. (2022). Probiotic species in the management of periodontal diseases: an overview. *Front. Cell. Infect. Microbiol.* 12:806463. doi: 10.3389/fcimb.2022.806463

Zhang, H., Duan, Y., Cai, F., Cao, D., Wang, L., Qiao, Z., et al. (2022). Next-generation probiotics: microflora intervention to human diseases. *Biomed. Res. Int.* 2022:5633403. doi: 10.1155/2022/5633403

Zhao, T., Wang, H., Liu, Z., Liu, Y., Ji, D., Li, B., et al. (2023). Recent perspective of *Lactobacillus* in reducing oxidative stress to prevent disease. *Antioxidants (Basel).* 12:769. doi: 10.3390/antiox12030769

Zheng, J., Ahmad, A. A., Yang, Y., Liang, Z., Shen, W., Feng, M., et al. (2022). *Lactobacillus rhamnosus* CY12 enhances intestinal barrier function by regulating tight junction protein expression, oxidative stress, and inflammation response in lipopolysaccharide-induced Caco-2 cells. *IJMS* 23:11162. doi: 10.3390/ijms231911162

Zheng, N., Guo, R., Yao, Y., Jin, M., Cheng, Y., and Ling, Z. (2019). *Lactobacillus iners* is associated with vaginal Dysbiosis in healthy pregnant women: a preliminary study. *Biomed. Res. Int.* 2019:6079734. doi: 10.1155/2019/6079734

Zheng, Y., Xu, L., Zhang, S., Liu, Y., Ni, J., and Xiao, G. (2023a). Effect of a probiotic formula on gastrointestinal health, immune responses and metabolic health in adults

with functional constipation or functional diarrhea. *Front. Nutr.* 10:1196625. doi: 10.3389/fnut.2023.1196625

Zheng, Y., Zhang, Z., Tang, P., Wu, Y., Zhang, A., Li, D., et al. (2023b). Probiotics fortify intestinal barrier function: a systematic review and meta-analysis of randomized trials. *Front. Immun.* 14:1143548. doi: 10.3389/fimmu.2023.1143548

Zhu, J., Pitre, T., Ching, C., Zeraatkar, D., and Gruchy, S. (2023). Safety and efficacy of probiotic supplements as adjunctive therapies in patients with COVID-19: a systematic review and meta-analysis. *PLoS One* 18:e0278356. doi: 10.1371/journal.pone.0278356

Zhu, B., Wang, X., and Li, L. (2010). Human gut microbiome: the second genome of human body. *Protein Cell* 1, 718–725. doi: 10.1007/s13238-010-0093-z

Zielinska, D., and Kolozyn-Krajewska, D. (2018). Food-origin lactic acid bacteria may exhibit probiotic properties: review. *Biomed. Res. Int.* 2018:5063185. doi: 10.1155/2018/5063185

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Whole-genome shotgun sequencing unravels the influence of environmental microbial co-infections on the treatment efficacy for severe pediatric infectious diseases

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Background: The microbiome plays a pivotal role in mediating immune deviation during the development of early-life viral infections. Recurrent infections in children are considered a risk factor for disease development. This study delves into the metagenomics of the microbiome in children suffering from severe infections, seeking to identify potential sources of these infections.

Aims: The aim of this study was to identify the specific microorganisms and factors that significantly influence the treatment duration in patients suffering from severe infections. We sought to understand how these microbial communities and other variables may affect the treatment duration and the use of antibiotics of these patients with severe infections.

Method: Whole-genome shotgun sequencing was conducted on samples collected from children aged 0–14 years with severe infections, admitted to the Pediatrics Department of Xiamen First Hospital. The Kraken2 algorithm was used for taxonomic identification from sequence reads, and linear mixed models were employed to identify significant microorganisms influencing treatment duration. *Colwellia*, *Cryptococcus*, and *Citrobacter* were found to significantly correlate with the duration of clinical treatment. Further analysis using propensity score matching (PSM) and rank-sum test identified clinical indicators significantly associated with the presence of these microorganisms.

Results: Using a linear mixed model after removed the outliers, we identified that the abundance of *Colwellia*, *Cryptococcus*, and *Citrobacter* significantly influences the treatment duration. The presence of these microorganisms is associated with a longer treatment duration for patients. Furthermore, these microorganisms were found to impact various clinical measures. Notably, an increase in hospitalization durations and medication costs was observed in patients with these microorganisms. In patients with *Colwellia*, *Cryptococcus*, and *Citrobacter*, we discover significant differences in platelets levels. We also find that in patients with *Cryptococcus*, white blood cells, hemoglobin, and neutrophils levels are lower.

Conclusion: These findings suggest that *Colwellia, Cryptococcus*, and *Citrobacter*, particularly *Cryptococcus*, could potentially contribute to the

severity of infections observed in this cohort, possibly as co-infections. These microorganisms warrant further investigation into their pathogenic roles and mechanisms of action, as their presence in combination with disease-causing organisms may have a synergistic effect on disease severity. Understanding the interplay between these microorganisms and pathogenic agents could provide valuable insights into the complex nature of severe pediatric infections and guide the development of targeted therapeutic strategies.

KEYWORDS

shotgun sequencing, severe infection, microbiome, pediatric, co-infection

1 Introduction

Children are known to be more susceptible to severe infections, and these infections can have significant physical and economic consequences for patients (Tiru et al., 2015; Sultana et al., 2021). Therefore, accurate identification of pathogenic bacteria is crucial for effective treatment (Brook, 2017). Metagenomic next-generation sequencing (NGS) has emerged as a valuable clinical tool for diagnosing severe infections, particularly those caused by rare pathogens (Wu et al., 2019; Consensus Group of Experts on Application of Metagenomic Next Generation Sequencing in The Pathogen Diagnosis in Clinical Moderate and Severe Infections et al., 2020; Wang et al., 2022). In terms of technological capabilities, shotgun sequencing offers a more powerful tool compared to 16S rRNA sequencing (Bender and Dien, 2018). Its higher resolution and functional profiling capability make it an advantageous approach, particularly as very few studies have applied metagenomic sequencing in pediatric populations.

Shotgun sequencing can simultaneously detect multiple pathogens such as bacteria, fungi, and viruses, thereby broadening the detection spectrum and enhancing the overall pathogen detection rate (Chiu and Miller, 2019). In this study, we aimed to utilize whole-genome shotgun sequencing and taxonomic identification algorithms to identify the microbial species present in samples collected from pediatric patients with severe infections. We hypothesize that the microbiomes of these patients may play a crucial role in influencing disease progression and treatment outcomes.

Therefore, our goals were twofold: (1) we aimed to characterize and analyze the microbiomes present in the infection samples of pediatric patients with severe infections, using shotgun sequencing. This approach allows us to identify and understand the roles and interactions of these microorganisms within the host, which could potentially be contributing to the severity of the infections, and (2) to explore the relationship between microbiomes and clinical characteristic, with a particular focus on identifying microorganisms that may contribute to disease severity through their association with various clinical indicators. This comprehensive analysis of the microbiome composition in different sample types will provide valuable insights into the potential origins of severe infections in children and may contribute to the development of targeted prevention and treatment strategies.

2 Materials and methods

2.1 Patients

123 hospitalized children aged 0–14 years who were admitted to the pediatric department of Xiamen First Hospital with severe infections who were tested for metagenomics next-generation sequencing from 2020 to 2022 were recruited. Children with severe infections exhibit the following characteristics: (1) persistent inflammation (continual presence of fever or abnormal laboratory findings such as procalcitonin (PCT), C-reactive protein (CRP) or sustained white blood cell counts) or disruption of normal activities for more than 1 week (Alkhater, 2009), (2) meeting diagnostic criteria for severe pneumonia (National Health Commission of the People's Republic of China, State Administration of Traditional Chinese Medicine, 2019), (3) concurrent organ failure (Dellinger et al., 2013), and (4) those with extracorporeal metagenomic specimens. The Medical Ethics Committee of Xiamen University approved the study, including all procedures (recruitment of participants and all experimental protocols). Written informed consent was obtained from patients selected from Xiamen First Hospital. A total of 123 patients with severe infections were diagnosed by pathological examination. All participants were Chinese residents of Fujian Province, China.

2.2 Data collection for clinical information

The patient's private information, such as name and medical record number, has been removed from the system. The unique identifier for each patient is a 6-character code composed of letters and numbers. The data under investigation does not contain any personally identifiable information, and all sensitive fields have been anonymized. Clinical information includes visit time, age, sex, treatment time, the cost of medicine, type of antibiotics, diagnosis and laboratory indexes. Laboratory indexes consist in white blood cell count (WBC), neutrophil percentage (NE%), hemoglobin levels (HGB), platelet count (PLT), procalcitonin (PCT), albumin, transaminase, LDH and coagulation function.

2.3 Sample collection

For each patient, multiple samples of the same type were collected at different stages of the disease to monitor the progression and

response to treatment. In addition, samples were also collected from different sites of the same patient, which might indicate possible infection sites. For instance, in cases of suspected pulmonary infection, bronchoalveolar lavage fluid was collected multiple times throughout the course of the disease. In cases where other systemic infections were suspected, a selection of samples from cerebrospinal fluid, urine, intraperitoneal effusion, and blood were collected, specifically from the sites where infection was suspected. All samples were collected according to the standard procedures described above.

(a) As for the bronchoalveolar lavage fluid (BALF), bronchoalveolar lavage was performed on every patient diagnosed with pneumonia. In localized disease, the lavage is most likely perfumed on the segment where the disease is most prominent. In diffuse disease, the right middle lobe or lingula is usually lavage. We want to instill sterilized saline which is prewarming to 37°C by 1 mL/kg aliquots. After performing sufficient suction and recovery, a 5 mL sample was collected for examination. (b) For cerebrospinal fluid, standard lumbar puncture was performed on the patient, and more than 2 mL of cerebrospinal fluid was collected. (c) Regarding urine samples, local digestion of the meatus and adult mucosa were performed with a non-foaming antiseptic solution, such as Dakin's solution. The initial stream of the voided specifics was discarded. At least 5 mL midstream sample was collected and sent to the companies for analysis. (d) Abdominal puncture was performed to collect intraperitoneal effusion, and a minimum volume of 5 mL was obtained for analysis. (e) As for blood samples, the typical procedure involves disinfecting the venous puncture site with 2% chlorhexidine alcohol or alcoholic iodine. Subsequently, a butterfly needle is used to extract 3–5 mL of blood (the specific volume depends on the age of the individual) for testing purposes. All specimens were collected and stored in a −20°C refrigerator. Subsequently, they were collected by the corresponding company and transported under dry ice conditions to ensure proper storage and preservation during transportation.

2.4 Shotgun metagenomic sequencing for each sample

Total genomic DNA and RNA were extracted from five different types of samples, and both DNA and RNA samples were used for shotgun library construction. Subsequently, high-throughput sequencing was performed on the samples using four platforms, including Thermo Fisher Scientific (DA8600), Illumina NextSeq 550, MGI 200/2000, and Q-mNGS™3.0. The sequencing was conducted in paired-end mode with a read length of 150 base pairs (PE150). It is noteworthy that the three batches of samples were obtained from distinct companies. Whole-genome shotgun sequencing was employed to generate paired-end outputs with a read length of 150 base pairs.

2.5 Sequence data pre-processing

The quality control checks on all the raw sequence data by using the FastQC (Andrews, 2010) version 0.11.9 and MultiQC (Ewels, 2016) version 1.12. The raw reads were pre-processed using KneadData (McIver et al., 2018) version 0.10.0 for quality control and host sequence decontamination (based on Trimmomatic version 0.39) (Bolger et al., 2014) and Bowtie2 version 2.4.5 (Langmead and Salzberg, 2012) with the recommended human reference database. In summary, the quality control and criteria of the pre-process procedure: (1) filtering out of the human genome contaminated reads by aligning raw reads to the human reference genome (GRCh37/hg19) (2) removal of adaptor sequences using Trimmomatic (trimming: SLIDINGWINDOW:4:20 MINLEN:25) and (3) ensuring quality scores adhere to phred33 standards. Additionally, (4) Tandem Repeats Finder is not employed to locate and remove reads resembling tandem repeats.

2.6 Taxonomic analysis

Taxonomy assignment were performed using Kraken2 (Wood et al., 2019) version 2.7.7 with default settings. Based on the standard reference database of clade specific marker genes embracing viruses, archaea, bacteria, eukaryotes, human and carriers. In all cases we use the defaults for k-mer length, minimizer length, and minimizer spacing. Results were visualized by MEGAN6 (Beier et al., 2017) and R package phyloseq (McMurdie and Holmes, 2013). In order to reduce the differences in concentration between different samples, the raw data were converted to percentages, and OTUs with a Median Absolute Deviation (MAD) value greater than 0.5 were selected to retain those that had a significant impact on sample differences. The selected OTUs will be used for subsequent multivariate analysis to explore the differences in microbial communities among different samples.

2.7 Statistical analysis

2.7.1 Principal component analysis (PCA)

Due to the diversity of measures and samples included in the study, we expected to observe heterogeneity in effect size estimates. To address potential batch effects between samples, we conducted a principal component analysis and identified outliers based on the first principal component (Leek et al., 2010). Based on the scores of the first principal component, we selected and removed outlier samples in downstream analyses to minimize the impact of batch effects.

2.7.2 Linear mixed models

To account for the within-individual correlations of multiple samples, we used linear mixed-effects models in our statistical analysis. In our statistical analysis, we treated these multiple samples as repeated measures from the same individual and used appropriate statistical methods (mixed-effects models) to account for the within-individual correlations. Linear mixed models (De Boeck et al., 2011) were constructed via R package lme4. In order to assess the significance of differences in clinical variables across samples, the analysis of variance (ANOVA) (McHugh, 2011) with repeated measurements was used. The likelihood ratio tests with Benjamini-Hochberg FDR correction (Benjamini and Hochberg, 1995) were used to screen out non-significant models and non-significant clinical variables. Effect size was estimated using marginal and conditional (pseudo-R2) linear association between standardized variables. Linear mixed models (with random intercepts and slopes) were applied. Firstly, likelihood ratio tests (with Benjamini Hochberg correction)

were applied to the simple models (no condition effects) to assess the significance of the regression coefficient and effect size was estimated by marginal and conditional (pseudo-R2). Then, likelihood ratio tests (with Benjamini-Hochberg correction) were applied to compare simple and extended models to determine whether regression coefficient differ significantly between samples. We tested a series of nested models that included the relevant clinical variables such as age group, gender, and sample type as fixed effects. In these models, the batch was incorporated as a random effect. Based on the proposed empirical importance and practical considerations, individual clinical characteristics were introduced into the model, and the final best-fit model was identified as the one with the lowest Akaike information criterion (AIC) (Liang et al., 2008) value and with statistical significance. After conducting the final screening, we developed a model that included fixed effects such as age group, gender, treatment outcome, and ID specialist antibiotics. The data source was considered as a random effect in the model, taking into account the three different batches from which the data was sourced. In analyzing the taxonomic profiles of the microbial community, we utilized a significant model to compare differences between genera. We performed separate inclusion of each genus in the model as a fixed effect to assess which genus caused the effect on the clinical variable under study. We then selected the microorganisms that were found to have a significant impact on the treatment time, which was the outcome of interest in our study. This study considered potential microorganisms that displayed consistent statistical significance from all analyses above.

2.7.3 Propensity score matching (PSM)

Propensity score matching (PSM) (Hill and Reiter, 2006; Caliendo and Kopeinig, 2008) was performed to match individuals between the presence of pathogens group and the absence of pathogens group based on age group, gender, and sample types in a 1:2 ratio. By using age group, gender, and sample types as covariates for matching, we effectively control for potential confounding factors that could influence the presence of pathogens and treatment outcomes in the dataset. The 1:2 ratio in matching ensures that for each individual in the presence of pathogens group, two individuals with similar age group, gender, and sample type characteristics are selected from the absence of pathogens group, thereby creating a more comparable and representative dataset.

This careful matching process strengthens the validity of our analysis and allows for a more robust assessment of the causal impact of, *Cryptococcus*, and *Citrobacter* on treatment time and various clinical indicators in pediatric infections. By controlling for these important covariates, we can more confidently attribute any observed differences in outcomes to the presence or absence of these three pathogens.

2.7.4 Wilcoxon rank-sum tests

Wilcoxon rank-sum tests were performed to assess the significance of various clinical indicators in relation to the presence or absence of the pathogens. These tests allowed for a robust comparison of clinical metrics between the groups with and without *Cryptococcus*, *Citrobacter*, and *Colwellia*.

The nonparametric rank sum tests were chosen because they do not assume a specific distribution for the data, making them suitable for analyzing clinical indicators that may not follow a normal distribution. By using rank sum tests, we could determine whether there were significant differences in clinical indicators, such as treatment duration, lymphocyte percentage (LY%), neutrophil percentage (NE%), hemoglobin levels (HGB), platelet count (PLT), and white blood cell count (WBC), between the two groups.

By combining the results of propensity score matching (PSM) with Wilcoxon rank-sum test, we were able to gain deeper insights into the potential impact of *Cryptococcus*, *Citrobacter* and *Colwellia* on various clinical outcomes in severe pediatric infections. These statistical analyses strengthen the validity of our findings and support the conclusions drawn from the study.

In the aforementioned statistical analyses, a significance threshold of $p < 0.05$ was utilized to ensure statistical significance across all conducted tests. All statistical analyses were performed using the R environment (version 4.3.0). Bash and R script implementations of the analysis are available on GitHub (https://github.com/iChronostasis/EnvMicrobePediatricTreatmentAnalysis) for academic use.

3 Results

3.1 Patient characteristics and sample analysis in severe infections

123 diagnosed severe infected patients were enrolled in our study, including 69 males and 54 females, and their average age was 3.814 ± 4.15 years. 172 samples were collected for inclusion in this analysis from three batches (Table 1). The 172 samples included in this analysis were collected from 123 patients, with 34 patients contributing multiple samples (Supplementary Table S1). The clinical information of these samples, including gender and age, was

TABLE 1 Demographic of enrolled patients.

Demographic of enrolled patients ($n = 123$)			
Gender	Male	Female	
	69	54	
Age group	4 years old and above	Under 4 years old	
	51	72	
Age	Mean	SD	
	3.814	4.15	
Marrow depression	Yes	No	NA
	23	99	1
Antibiotic treatment	Yes	No	
	121	2	
ID specialist antibiotics	Yes	No	
	78	45	

This table outlines the demographic and clinical characteristics of the 123 patients with severe infections who participated in our study. The data is categorized by sex (69 males and 54 females) and age group (51 patients aged 4 years and above, and 72 patients under 4 years old). The mean age of the patients is 3.814 years with a standard deviation of 4.15 years. In terms of clinical characteristics, 23 patients presented with marrow depression while 99 did not. One patient's marrow depression status was not available. The source of infection was classified as pulmonary (57 patients), intracranial (19 patients), urinary tract (6 patients), and bloodstream (41 patients). The majority of patients (121 out of 123) received antibiotic treatment. The use of ID specialist antibiotics was reported in 78 patients, while 45 patients did not receive these medications.

recorded at the time of sample collection. To account for the potential within-individual correlations, we compared the gender and age group using mixed-effects models that included the individual as a random effect. We did not observe any significant differences in gender or age between the three batches. (a) These three batches' samples number were 106, 33, and 33, respectively (Supplementary Table S2). (b) Blood, bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), urine, and ascites were collected from patients separately, with sample sizes of 74, 53, 36, 4, and 5, respectively (Supplementary Figure S1A). (c) The distribution of treatment time in all samples is shown in Supplementary Figure S1B. (d) Metagenomic results showed a total of 104 samples were positive, 67 were negative and 1 was not analyzed (Supplementary Figure S1C). (e) The culture results of the samples showed that a total of 50 samples were positive, 120 were negative and 2 were not analyzed (Supplementary Figure S1D).

3.2 Comprehensive analysis reveals high microbial diversity in pediatric infection samples

At the kingdom level, WGS identified 312 archaeas, 6,843 bacteria, 660 viruses, and 127 eukaryotes. The abundance information across all samples for each species was calculated and ranked to identify the most abundant species, which is the default parameter implemented in Kraken2, using MEGAN6 to obtain the taxonomic profile in kingdom, phylum and genus. We utilized MEGAN6 to visualize the abundance information of all identified microorganisms at the phylum level in a radial tree chart. This visualization provided a comprehensive view of the microbial composition across all 172 pediatric infection samples, highlighting the relative abundance of different phyla (Supplementary Figure S2). We generated a Taxonomy Rarefaction Plot to assess the microbial diversity in our study. This plot provides valuable insights into the richness and evenness of the microbial taxa across the sampled pediatric infection dataset. As shown in the plot, the rarefaction curve reaches a plateau, indicating that the sampling effort has adequately captured the majority of the microbial diversity present in the samples. This analysis highlights the comprehensive nature of our dataset and strengthens the reliability of our findings (Supplementary Figure S3). After filtering the host sequences of the raw data with quality control and criteria (Method), we identified a total of 7,943 OTUs in this dataset, with an average of 46.18 ($n = 172$) OTUs per sample.

3.3 Batch effects in taxonomic profiles of pediatric infection samples from diverse data sources

Based on the taxonomic profiles from Karken2 and MEGAN6, the principal component analysis was performed on the abundance information of all identified microorganisms in all 172 samples in each phylum (Figure 1A). From the figure of PCA, it can be seen that some samples are far away from most of the samples, and these samples contribute a lot to the first component. In total, six samples contributed abnormally to the first principal component, which means that these samples may show the batch effects compared to the other samples, so these anomalous sample points should be removed and not included in the subsequent analysis (Figure 1B). Upon examining the clinical information of these sample outliers, it was observed that these samples were from cohorts 1, 2, and 3, with the sample types being bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), and blood, respectively. Out of the six patients from whom these samples were sourced, five were under the age of four. All of these patients received antibiotics during their hospitalization and exhibited improved outcomes upon discharge (Supplementary Table S3). However, these six samples are planned to be excluded from further analysis. After excluding these outlier samples, the distribution of sample types and sources of infection in the remaining dataset can be observed in the (Figures 1C,D).

3.4 The existence of *Cryptococcus*, *Citrobacter* and *Colwellia* significantly associates with high costs and prolonged treatment duration in pediatric infections

In Figure 2A, we compared multiple models and selected Model 2, which demonstrated the lowest AIC and BIC values and a significant value of p. The final best-fit model, as mentioned in the methods section, incorporated age group, gender, sample type, and the use of ID specialist antibiotics as fixed effects, while the batch was included as a random effect. The estimates of this optimal model is presented in Supplementary Table S4 and Figure 2B. Utilizing the final best-fit model, we incorporated each genus into the analysis. This allowed us to screen for microorganisms that were both relevant and clinically significant for our variables of interest (Supplementary Figure S4). Our results revealed that *Colwellia*, *Cryptococcus*, and *Citrobacter* all had a statistically significant impact on treatment time according to the linear mixed models (Figure 3A). Moreover, we compared the new models constructed by incorporating each of these three microorganisms. As depicted in Figure 3B, all three microorganisms exert a significant influence on the treatment time, which is our primary concern.

The genus *Colwellia*, which includes some of the most extreme cold-loving and pressure-tolerant species known to date, are heterotrophic and facultatively anaerobic. They thrive in persistently cold marine environments, such as the sea ice of the Arctic and Antarctic. *Colwellia* species have been isolated from organic-rich marine habitats, including sediments or homogenates originating from marine invertebrates like amphipods and squid, or vertebrates like fish (Peoples et al., 2020). However, the microorganisms of *Cryptococcus* and *Citrobacter* spp., are more associated with infections. *Cryptococcus* mainly causes meningoencephalitis, as well as other diseases affecting the central nervous system (CNS) and lungs, and is associated with severe morbidity (Chen et al., 2014; Setianingrum et al., 2019). *Citrobacter* spp. belong to a group of parthenogenic, anaerobic, gram-negative rods in the Enterobacteriaceae family. They are frequently found in water, soil, food, and the intestinal tract of animals and humans. Previously considered low virulence environmental contaminants or colonizing bacteria, they are now known to cause a wide range of infections, including urinary tract, liver, biliary tract, peritoneum, intestine, bone, respiratory tract,

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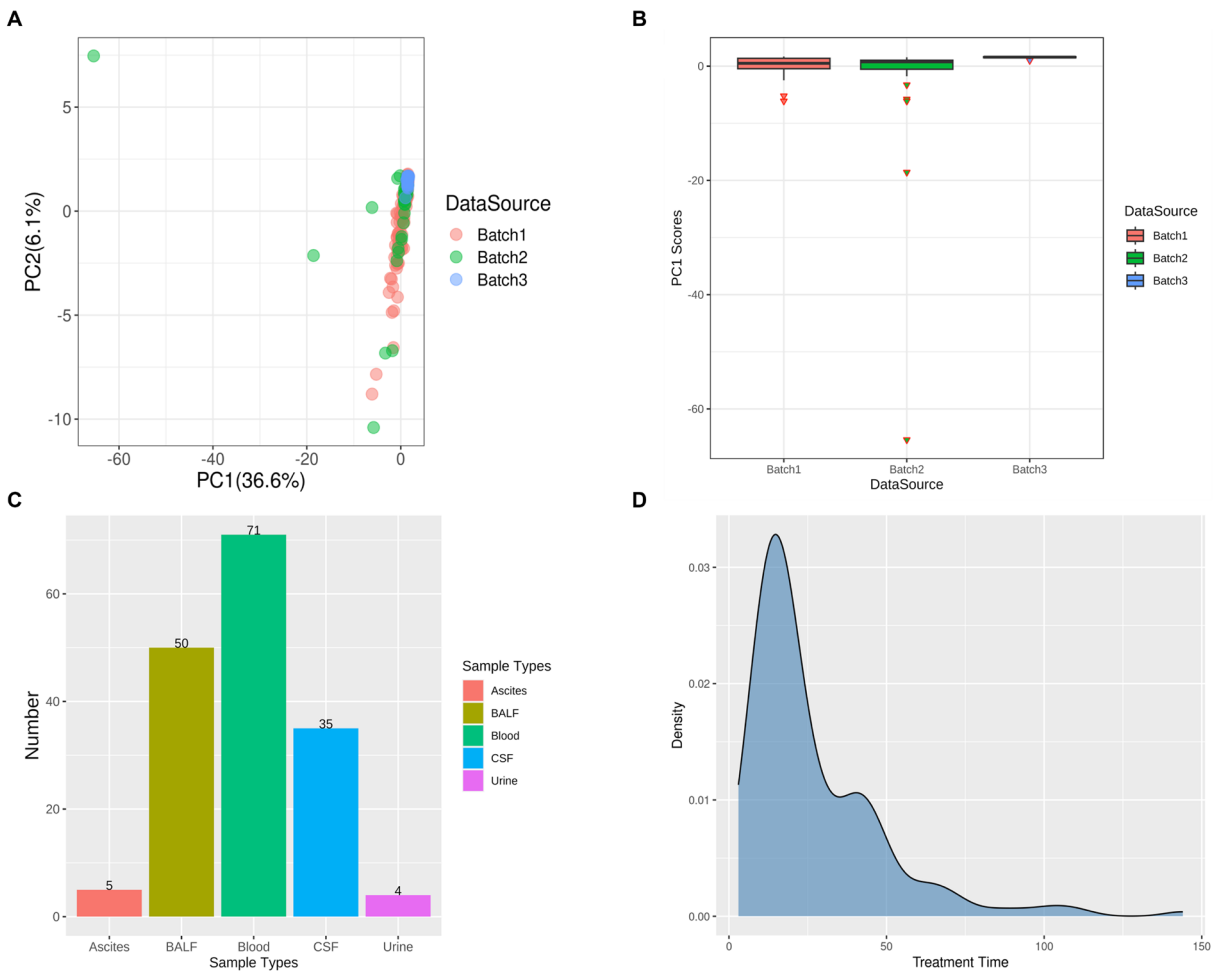


FIGURE 1
Principal Component Analysis (PCA) and Outlier Removal. **(A)** PCA Results Delineated by Data Source: Each batch is uniquely represented by a color scheme, with red, green, and blue corresponding to three distinct batches. **(B)** Identification of Outliers Based on Principal Component 1 (PC1) Scores: The outliers in each batch are represented by inverted triangle symbols, with the batches themselves distinguished by the same color scheme as in **(A)** – red, green, and blue. **(C)** Post-Outlier Removal Distribution of Samples by Sample Type: Different sample types are color-coded for clarity. Red signifies ascites, yellow-green denotes bronchoalveolar lavage fluid (BALF), green represents blood, blue is indicative of cerebrospinal fluid (CSF), and purple corresponds to urine. **(D)** Distribution of Treatment Time in All Samples.

endocardium, wounds, soft tissues, meninges, and blood (Liu et al., 2018; Lee et al., 2019; Liu et al., 2021).

3.5 Significant association between abundance of *Colwellia*, *Cryptococcus*, and *Citrobacter*, and multiple clinical indicators in severe infections

Propensity score matching (PSM) was employed to evaluate the causal impact of three previously identified microorganisms (*Colwellia*, *Cryptococcus*, *Citrobacter*) on outcomes. The presence or absence of these pathogens was transformed into binary variables to indicate their presence or absence. Gender, age group, and sample type were used as covariates to balance the data between the treatment and control groups. Following matching, the Standardized Mean Difference (SMD) in the distribution of covariates between the two groups mostly remained below the threshold of 0.1, indicating high matching quality (Supplementary Figures S5A–C). The similarity in the distribution of covariates between the treatment and control groups is visually demonstrated through jitter plots (Supplementary Figures S5D–F) and histograms of propensity score distribution (Supplementary Figures S5G–I). The matching process helps to mitigate potential confounding factors, ensuring a more accurate assessment of the causal impact of *Cryptococcus*, *Citrobacter* and *Colwellia* on the treatment outcomes and clinical metrics of pediatric infections. Figure 4 is composed of two parts. Figure 4A presents the number of samples, post-propensity score matching (PSM), that are either infected with or free from the three pathogens. Additionally, Figure 4B displays the distribution of samples across different sample types, each categorized based on the presence or absence of the three pathogens.

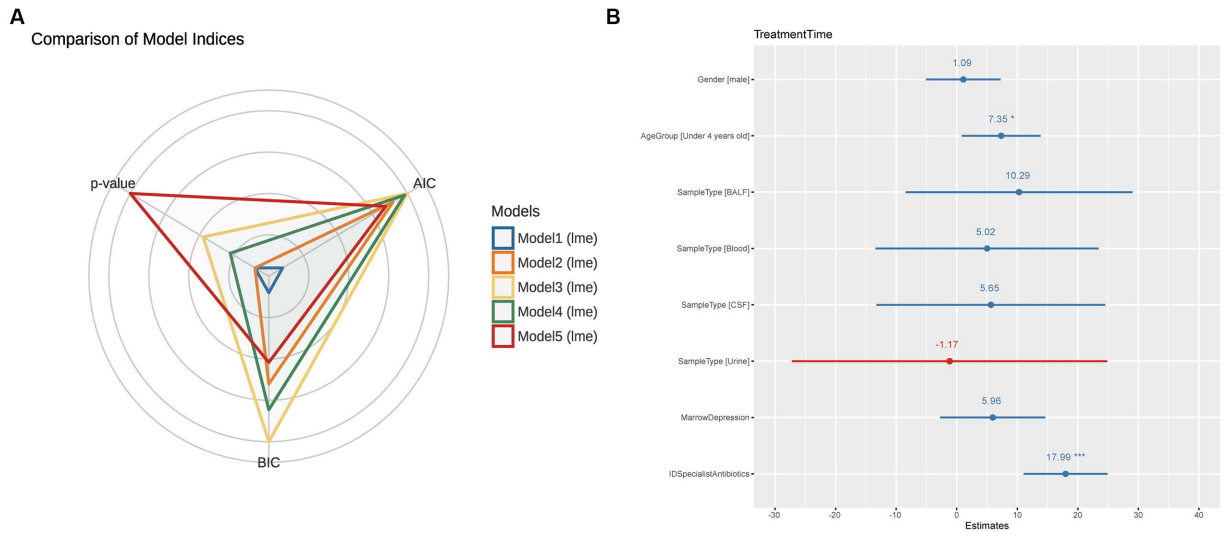


FIGURE 2
Comparison of Statistical Models and Summary of the Optimal Model. **(A)** This figure compares five different statistical models using radar charts, where each axis represents a model and the distance from the center corresponds to the Akaike information criterion (AIC) and Bayesian information criterion (BIC) values. Each model is color-coded as follows: Blue (Model 1): TreatmentTime ~ Gender + AgeGroup + SampleType + MarrowDepression. Orange (Model 2): TreatmentTime ~ Gender + AgeGroup + SampleType + MarrowDepression + IDSpecialistAntibiotics. Yellow (Model 3): TreatmentTime ~ Gender + AgeGroup + MarrowDepression + IDSpecialistAntibiotics + AntibioticTreatment. Green (Model 4): TreatmentTime ~ Gender + AgeGroup + MarrowDepression + IDSpecialistAntibiotics + AntibioticTreatment + MetagenomicResults. Red (Model 5): TreatmentTime ~ Gender + AgeGroup + MarrowDepression + IDSpecialistAntibiotics + AntibioticTreatment + MetagenomicResults + CultureResults. The proximity to the center indicates the model's performance, with a closer distance representing better performance. Among the models, the orange model (Model 2) performed the best, exhibiting the lowest AIC and BIC values and significant *p*-values. **(B)** After selecting Model 2 as the best model, this figure provides a comprehensive summary of the model. As described in the Methods section, Model 2 includes age group, gender, sample type, marrow depression, and ID specialist antibiotic use as fixed effects, with data source considered as a random effect. The summary offers detailed information on coefficients, standard errors, *z*-values, and *p*-values associated with each factor, providing a complete overview of the model's performance and statistical significance.

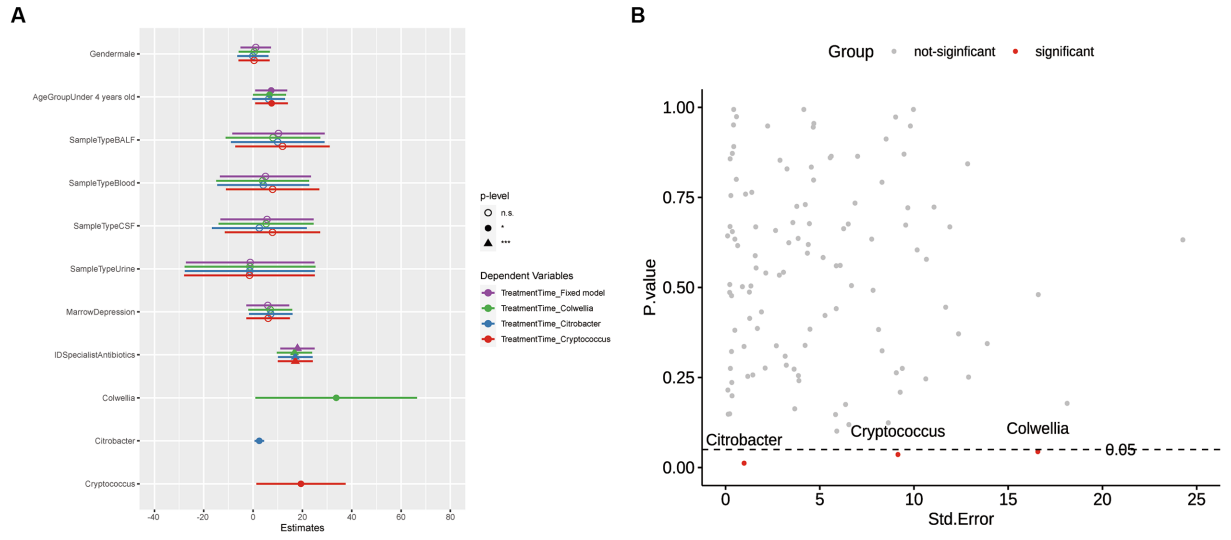


FIGURE 3
Visualization of *p*-values and Model Comparisons. **(A)** Scatterplot of *p*-values versus Standard Errors: This plot visualizes the *p*-values corresponding to each Genus in the model. The color scheme is used to indicate significance, with red denoting significance and gray indicating non-significance. **(B)** Comparison of Models Derived from Significant Microorganisms: The models are color-coded to represent different scenarios. Purple represents a fixed model without the inclusion of microorganisms. Green, blue, and red, respectively, represent models that include Colwellia, Citrobacter, and Cryptococcus. Regarding the *p*-values, the significance levels are annotated as follows: Asterisks denote statistical significance levels (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Additionally, non-significant results ($p \geq 0.05$) are denoted as "n.s." (not statistically significant).

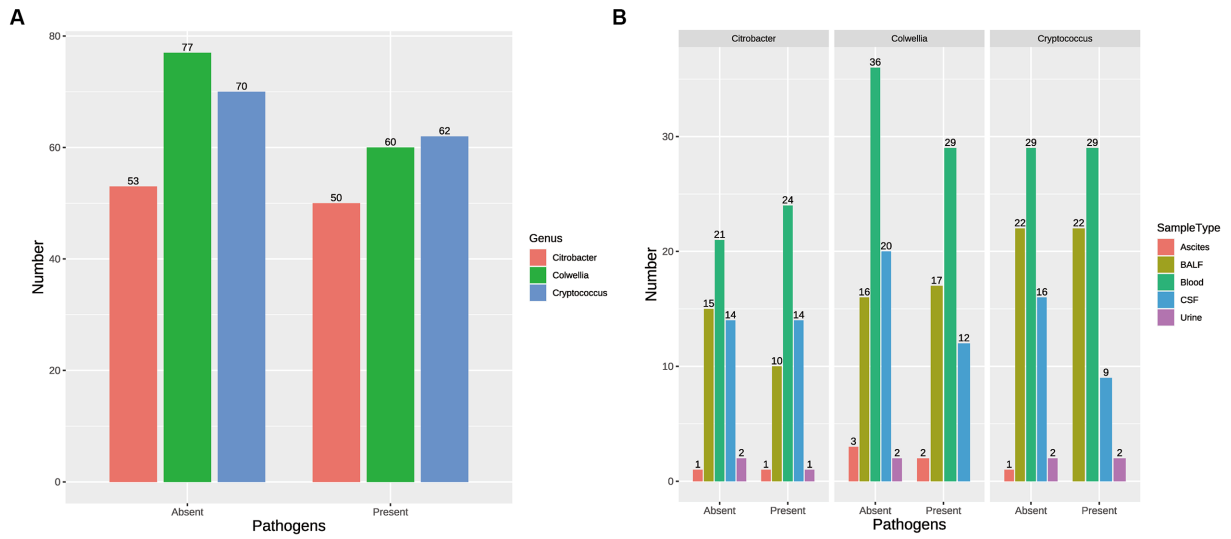


FIGURE 4
Impact of Pathogen Presence on Sample Distribution. **(A)** Number of Samples Post-Propensity Score Matching (PSM): This part of Supplementary Figure S7 presents the number of samples, after propensity score matching, that are either infected with or free from the three pathogens (Colwellia, Cryptococcus, and Citrobacter). The samples are categorized based on the presence or absence of these pathogens. **(B)** Distribution of Samples across Different Sample Types: This part of Supplementary Figure S7 displays the distribution of samples across different sample types, such as blood, bronchoalveolar lavage fluid (BALF), cerebrospinal fluid (CSF), urine, and ascites. The samples in each sample type are further categorized based on the presence or absence of the three pathogens (Colwellia, Cryptococcus, and Citrobacter).

Subsequently, the newly constructed dataset was analyzed to investigate the impact of these pathogens' presence or absence on treatment time and other clinical metrics using the matched dataset. We performed a reanalysis of the matched dataset obtained through propensity score matching (PSM) and explored the associations between the presence or absence of these three pathogens and various clinical indicators using Wilcoxon rank-sum tests.

The reanalyzed results revealed significant differences in the duration of treatment, total cost of hospitalization, and overall hospitalization expenses between the groups with and without these microorganisms (Supplementary Table S5). Additionally, the presence or absence of these microorganisms was found to be associated with lymphocyte percentage (LY%), neutrophil percentage (NE%), hemoglobin levels (HGB), platelet count (PLT), and white blood cell count (WBC).

In our findings, the presence of *Cryptococcus*, *Citrobacter*, and *Colwellia* significantly extended the treatment duration for patients (Figure 5A), resulted in a substantial increase in medication costs (Figure 5B), and caused a noticeable decrease in platelet count (PLT) (Figure 5C). Patients with *Citrobacter* and *Colwellia* also had significantly higher hospitalization costs (Figure 5D). Furthermore, compared to patients without these pathogens, those with *Cryptococcus* showed a significant increase in lymphocyte percentage (LY%), while their neutrophil percentage (NE%), hemoglobin (HGB), and white blood cell count (WBC) significantly decreased (Figures 5E–H).

These findings suggest that the co-occurrence of *Cryptococcus*, *Citrobacter* and *Colwellia* with the pathogenic organism responsible for the severe infection may contribute to prolonged hospital stays and increased treatment costs. Especially, the presence of *Cryptococcus* is significantly correlated with multiple clinical indicators, which may signify a potential impact on the overall health status of patients. This highlights the complexity and multifaceted nature of pediatric infections, emphasizing the importance of considering the presence of these microorganisms in the context of co-infections to better understand and manage the disease course effectively.

4 Discussion

While this study provides valuable insights, it is important to acknowledge its limitations. Firstly, the small sample size and single-center design may limit the generalizability of the findings. Conducting future studies with larger cohorts and multiple centers would be beneficial to validate and extend these results. Additionally, the retrospective nature of the study and the relatively short duration of data collection may introduce biases and restrict the depth of analysis. The identified pathogens in our study are not primary causative agents of the patients' infections; all three of these pathogens are considered background pathogens. Speculations regarding their association with prolonged hospitalization and the use of ID specialist antibiotics include: (a) These three pathogens may potentially participate in influencing the formation of biofilms, thereby affecting the host's defense mechanisms or influencing the drug resistance of colonized bacteria (Van Baarlen et al., 2007; Domínguez-Díaz et al., 2019; Mirzaei et al., 2020). (b) The production of endotoxins or peptides by these three pathogens might lead to the depletion or reduction of cellular ATP levels, thereby enhancing the pathogen's tolerance to antibiotics (Huemer et al., 2020). (c) These three pathogens may release corresponding endotoxins during the host's diseased state, influencing the release of inflammatory factors and sustaining inflammation (Domínguez-Díaz et al., 2019; Rippon et al., 2022). (d) These three pathogens could potentially impact the host's metabolomics, such as carbon or nitrogen sources, thereby enhancing the virulence of pathogenic bacteria (Rohmer et al., 2011). In the future, prospective studies could be conducted to investigate the

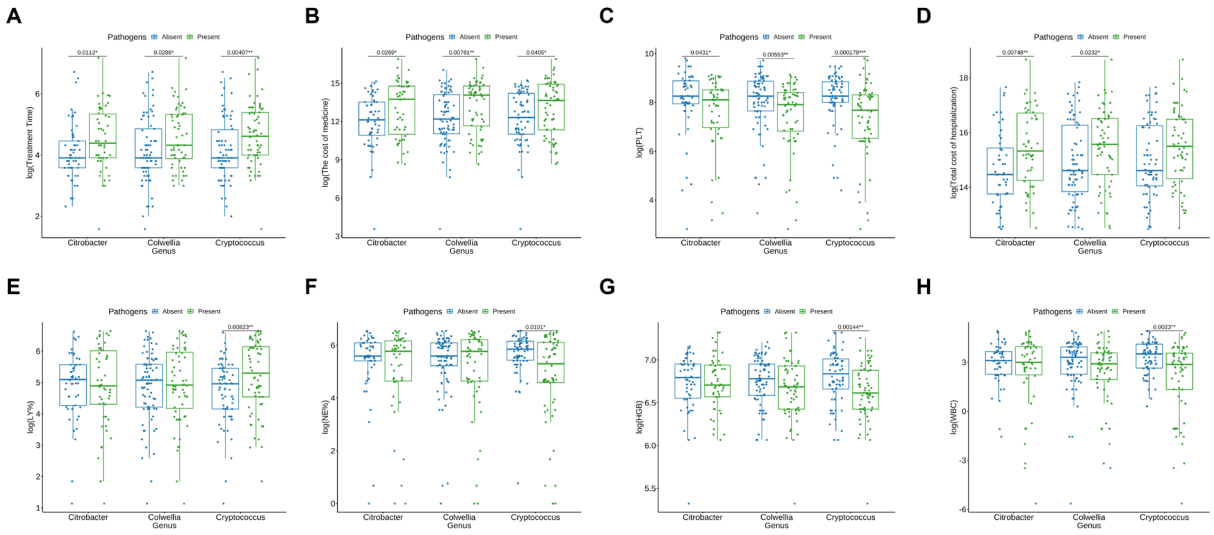


FIGURE 5
Influence of Microorganisms on Clinical Parameters and Costs. **(A)** Influence on Treatment time (days): A significant extension in treatment duration is observed in patients with Cryptococcus, Citrobacter, and Colwellia (represented by green bars) in comparison to those without these microorganisms (blue bars). **(B)** Impact on Medication Costs (RMB): The presence of Cryptococcus, Citrobacter, and Colwellia is associated with a substantial elevation in medication costs (green bars) when compared to patients not harboring these microorganisms (blue bars). **(C)** Effect on Platelet Count (PLT) (x10^9/L): Patients with Cryptococcus, Citrobacter, and Colwellia exhibit a noticeable reduction in platelet count (PLT) (green bars) relative to those without these microorganisms (blue bars). **(D)** Influence on Hospitalization Expenses (RMB): The hospitalization costs are significantly higher for patients with Citrobacter and Colwellia (green bars) compared to those without these microorganisms (blue bars). **(E)** Impact on Lymphocyte Percentage (%): A significant increase in lymphocyte percentage (LY%) is noted in patients with Cryptococcus (green bars) as opposed to patients without this pathogen (blue bars). **(F)** Effect on Neutrophil Percentage (%): Patients with Cryptococcus manifest a significant reduction in neutrophil percentage (NE%) (green bars) in comparison to patients without this pathogen (blue bars). **(G)** Influence on Hemoglobin (HGB) Levels (g/L): A significant decrease in hemoglobin (HGB) levels is observed in patients with Cryptococcus (green bars) relative to patients without this pathogen (blue bars). **(H)** Impact on White Blood Cell Count (WBC) (x10^9/L): Patients with Cryptococcus demonstrate a significant reduction in white blood cell count (WBC) (green bars) compared to patients without this pathogen (blue bars). These findings suggest that the presence of Cryptococcus, Citrobacter, and Colwellia is linked with adverse clinical outcomes, such as extended treatment duration, elevated medication costs, reduced platelet count, and increased hospitalization expenses. The significant alterations in lymphocyte percentage (LY%), neutrophil percentage (NE%), hemoglobin (HGB), and white blood cell count (WBC) further indicate potential impacts on the overall health status of patients harboring these microorganisms.

relevant biological mechanisms associated with severe pediatric infections. By incorporating control and experimental groups, these studies would provide a more comprehensive understanding of the relationships between microorganism co-infections and clinical outcomes. By exploring the biological functions and interactions of these microorganisms, researchers can gain insights into the pathogenesis of severe infections in children. This knowledge can contribute to the development of targeted interventions and improved patient management strategies.

The findings presented here open up several avenues for future research. Firstly, it is crucial to understand the mechanisms underlying the observed associations between microorganism co-infections and clinical outcomes. Investigating the interplay between these microorganisms and the host immune response could shed light on the pathogenesis of severe pediatric infections. Secondly, exploring the impact of different treatment strategies on outcomes in the context of co-infections could guide the development of tailored therapeutic approaches. Moreover, further investigation is warranted to determine the potential role of these microorganisms as prognostic markers or targets for interventions, aiming to improve patient management and outcomes. The results of this study hold significant clinical implications. Identification of *Colwellia*, *Cryptococcus*, and *Citrobacter* in co-infections with pathogenic organisms appears to be associated with prolonged hospital stays and altered clinical indicators in severe pediatric infections. Recognizing the potential impact of these microorganisms could aid healthcare professionals in early risk assessment and appropriate treatment planning for affected patients. By considering the presence of these microorganisms in the clinical decision-making process, medical resources can be better allocated to improve patient care and optimize healthcare resource utilization.

While this study focused on a specific cohort of pediatric patients with severe infections, the results may have broader relevance. However, caution should be exercised when extrapolating the findings to other populations or infection types. Future studies encompassing diverse patient groups and a wide range of infectious diseases will be necessary to confirm the general applicability of these conclusions.

5 Conclusion

Although *Cryptococcus*, *Citrobacter*, and *Colwellia* are recognized as pathogens, they were not identified as the primary causative agents in the analyzed cases. However, the results of this study suggest that the co-infection of these three microorganisms with disease-causing organisms may significantly impact the treatment course in patients with severe pediatric infections. Especially, the presence of *Cryptococcus* was found to be significantly associated with prolonged treatment duration and multiple clinical indicators. These findings

highlight the potential role of *Cryptococcus* in particular, in contributing to longer hospital stays, and altered clinical indicators. It suggests that the presence of these microorganisms in co-infections may contribute to the complexity and severity of pediatric infections, warranting additional medical attention and allocation of resources.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/; PRJEB67633.

Ethics statement

Ethical approval was not required for the study involving human samples in accordance with the local legislation and institutional requirements because [reason ethics approval was not required]. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

CL: Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft. JS: Data curation, Formal analysis, Methodology, Writing – original draft. QC: Data curation, Methodology, Writing – review & editing. DG: Data curation, Investigation, Methodology, Writing – review & editing. QL: Conceptualization, Investigation, Methodology, Project administration, Software, Supervision, Writing – review & editing. YY: Conceptualization, Investigation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1308871/full#supplementary-material

References

Alkhater, S. A. (2009). Approach to the child with recurrent infections. *J Family Commun Med* 16, 77–82. doi: 10.4103/2230-8229.96520

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Beier, S., Tappu, R., and Huson, D. H. (2017). "Functional analysis in metagenomics using MEGAN 6" in *Functional metagenomics: tools and applications*. eds. T. Charles, M. Liles and A. Sessitsch (Cham: Springer).

Bender, J. M., and Dien, B. J. (2018). Metagenomics in pediatrics: using a shotgun approach to diagnose infections. *Curr. Opin. Pediatr.* 30, 125–130. doi: 10.1097/MOP.0000000000000577

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Brook, I. (2017). Microbiology and treatment of brain abscess. *J. Clin. Neurosci.* 38, 8–12. doi: 10.1016/j.jocn.2016.12.035

Caliendo, M., and Kopeinig, S. (2008). Some practical guidance for the implementation of propensity score matching. *J. Econ. Surv.* 22, 31–72. doi: 10.1111/j.1467-6419.2007.00527.x

Chen, S. C.-A., Meyer, W., and Sorrell, T. C. (2014). Cryptococcus gattii Infections. *Clin. Microbiol. Rev.* 27, 980–1024. doi: 10.1128/CMR.00126-13

Chiu, C. Y., and Miller, S. A. (2019). Clinical metagenomics. *Nat. Rev. Genet.* 20, 341–355. doi: 10.1038/s41576-019-0113-7

Consensus Group of Experts on Application of Metagenomic Next Generation Sequencing in The Pathogen Diagnosis in Clinical Moderate and Severe Infections; Professional Committee of Sepsis and Shock Chinese Research Hospital Association; Professional Committee of Microbial Toxins Chinese Society for Microbiology; Professional Committee of Critical Care Medicine Shenzhen Medical Association (2020). Expert consensus for the application of metagenomic next generation sequencing in the pathogen diagnosis in clinical moderate and severe infections (first edition).

Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 32, 531–536. doi: 10.3760/cma.j.cn121430-20200228-00095

De Boeck, P., Bakker, M., Zwitser, R., Nivard, M., Hofman, A., Tuerlinckx, F., et al. (2011). The estimation of item response models with the lmer function from the lme4 package in R. *J. Stat. Softw.* 39, 1–28. doi: 10.18637/jss.v039.i12

Dellinger, R. P., Levy, M. M., Rhodes, A., Annane, D., Gerlach, H., Opal, S. M., et al. (2013). Surviving Sepsis campaign guidelines committee including the pediatric subgroup surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Crit. Care Med.* 41, 580–637. doi: 10.1097/CCM.0b013e31827e83af

Domínguez-Díaz, C., García-Orozco, A., Riera-Leal, A., Padilla-Arellano, J. R., and Fafutis-Morris, M. (2019). Microbiota and its role on viral evasion: is it with us or against us? *Front. Cell Infect. Microbiol.* 9:256. doi: 10.3389/fcimb.2019.00256

Ewels, P., Magnusson, M., Lundin, S., Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*. 1, 3047–8. doi: 10.1093/bioinformatics/btw354

Hill, J., and Reiter, J. P. (2006). Interval estimation for treatment effects using propensity score matching. *Stat. Med.* 25, 2230–2256. doi: 10.1002/sim.2277

Huemer, M., Mairpady Shambat, S., Brugger, S. D., and Zinkernagel, A. S. (2020). Antibiotic resistance and persistence—implications for human health and treatment perspectives. *EMBO Rep.* 21:e51034. doi: 10.15252/embr.202051034

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Lee, R., Choi, S. M., Jo, S. J., Lee, J., Cho, S. Y., Kim, S. H., et al. (2019). Clinical characteristics and antimicrobial susceptibility trends in Citrobacter bacteremia: an 11-year single-center experience. *Infect Chemother* 51, 1–9. doi: 10.3947/ic.2019.51.1.1

Leek, J. T., Scharpf, R. B., Bravo, H. C., Simcha, D., Langmead, B., Johnson, W. E., et al. (2010). Tackling the widespread and critical impact of batch effects in high-throughput data. *Nat. Rev. Genet.* 11, 733–739. doi: 10.1038/nrg2825

Liang, H., Wu, H., and Zou, G. (2008). A note on conditional AIC for linear mixed-effects models. *Biometrika* 95, 773–778. doi: 10.1093/biomet/asn023

Liu, L.-H., Wang, N.-Y., Wu, A. Y.-J., Lin, C.-C., Lee, C.-M., and Liu, C.-P. (2018). *Citrobacter freundii* bacteremia: risk factors of mortality and prevalence of resistance genes. *J. Microbiol. Immunol. Infect.* 51, 565–572. doi: 10.1016/j.jmii.2016.08.016

Liu, L., Zhang, L., Zhou, H., Yuan, M., Hu, D., Wang, Y., et al. (2021). Antimicrobial resistance and molecular characterization of *Citrobacter* spp. Causing extraintestinal infections. *Front. Cell Infect. Microbiol.* 11:737636. doi: 10.3389/fcimb.2021.737636

McHugh, M. L. (2011). Multiple comparison analysis testing in ANOVA. *Biochem Med* 21, 203–209. doi: 10.11613/bm.2011.029

McIver, L. J., Abu-Ali, G., Franzosa, E. A., Schwager, R., Morgan, X. C., Waldron, L., et al (2018). bioBakery: a meta'omic analysis environment. *Bioinformatics* 34, 1235–1237. doi: 10.1093/bioinformatics/btx754

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Mirzaei, R., Mohammadzadeh, R., Sholeh, M., Karampoor, S., Abdi, M., Dogan, E., et al. (2020). The importance of intracellular bacterial biofilm in infectious diseases. *Microb. Pathog.* 147:104393. doi: 10.1016/j.micpath.2020.104393

National Health Commission of the People's Republic of China, State Administration of Traditional Chinese Medicine (2019). Guideline for diagnosis and treatment of community-acquired pneumonia in Children (2019 version). *Chin. J. Clin. Infect. Dis.* 12, 6–13. doi: 10.3760/cma.j.issn.1674-2397.2019.01.002

Peoples, L. M., Kyaw, T. S., Ugalde, J. A., Mullane, K. K., Chastain, R. A., Yayanos, A. A., et al. (2020). Distinctive gene and protein characteristics of extremely piezophilic Colwellia. *BMC Genomics* 21:692. doi: 10.1186/s12864-020-07102-y

Rippon, M. G., Westgate, S., and Rogers, A. A. (2022). Implications of endotoxins in wound healing: a narrative review. *J. Wound Care* 31, 380–392. doi: 10.12968/jowc.2022.31.5.380

Rohmer, L., Hocquet, D., and Miller, S. I. (2011). Are pathogenic bacteria just looking for food? Metabolism and microbial pathogenesis. *Trends Microbiol.* 19, 341–348. doi: 10.1016/j.tim.2011.04.003

Setianingrum, F., Rautemaa-Richardson, R., and Denning, D. W. (2019). Pulmonary cryptococcosis: a review of pathobiology and clinical aspects. *Med. Mycol.* 57, 133–150. doi: 10.1093/mmy/myy086

Sultana, M., Alam, N. H., Ali, N., Faruque, A. S. G., Fuchs, G. J., Gyr, N., et al. (2021). Household economic burden of childhood severe pneumonia in Bangladesh: a cost-of-illness study. *Arch. Dis. Child.* 106, 539–546. doi: 10.1136/archdischild-2020-320834

Tiru, B., DiNino, E. K., Orenstein, A., Mailloux, P. T., Pesaturo, A., Gupta, A., et al. (2015). The economic and humanistic burden of severe Sepsis. *PharmacoEconomics* 33, 925–937. doi: 10.1007/s40273-015-0282-y

Van Baarlen, P., Van Belkum, A., Summerbell, R. C., Crous, P. W., and Thomma, B. P. H. J. (2007). Molecular mechanisms of pathogenicity: how do pathogenic microorganisms develop cross-kingdom host jumps? *FEMS Microbiol. Rev.* 31, 239–277. doi: 10.1111/j.1574-6976.2007.00065.x

Wang, Z. X., Wu, X., Xu, J., Ye, Y. Z., Han, S. Z., Ye, L. J., et al. (2022). Value of metagenomic next-generation sequencing in the etiology diagnosis of bacterial meningitis in children. *Zhonghua Er Ke Za Zhi* 60, 769–773. doi: 10.3760/cma.j.cn112140-20220317-00214

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with kraken 2. *Genome Biol.* 20:257. doi: 10.1186/s13059-019-1891-0

Wu, J., Lu, A. D., Zhang, L. P., Zuo, Y. X., and Jia, Y. P. (2019). Study of clinical outcome and prognosis in pediatric core binding factor-acute myeloid leukemia. *Zhonghua Xue Ye Xue Za Zhi* 40, 52–57. doi: 10.3760/cma.j.issn.0253-2727.2019.01.010

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Urogenital microbiota-driven virulence factor genes associated with recurrent urinary tract infection

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Urinary tract infections (UTIs) are a common health issue affecting individuals worldwide. Recurrent urinary tract infections (rUTI) pose a significant clinical challenge, with limited understanding of the underlying mechanisms. Recent research suggests that the urobiome, the microbial community residing in the urinary tract, may play a crucial role in the development and recurrence of urinary tract infections. However, the specific virulence factor genes (VFGs) driven by urobiome contributing to infection recurrence remain poorly understood. Our study aimed to investigate the relationship between urobiome driven VFGs and recurrent urinary tract infections. By analyzing the VFGs composition of the urinary microbiome in patients with rUTI compared to a control group, we found higher alpha diversity in rUTI patients compared with healthy control. And then, we sought to identify specific VFGs features associated with infection recurrence. Specifically, we observed an increased abundance of certain VGFs in the recurrent infection group. We also associated VFGs and clinical data. We then developed a diagnostic model based on the levels of these VFGs using random forest and support vector machine analysis to distinguish healthy control and rUIT, rUTI relapse and rUTI remission. The diagnostic accuracy of the model was assessed using receiver operating characteristic curve analysis, and the area under the ROC curve were 0.83 and 0.75. These findings provide valuable insights into the complex interplay between the VFGs of urobiome and recurrent urinary tract infections, highlighting potential targets for therapeutic interventions to prevent infection recurrence.

KEYWORDS

recurrent urinary tract infection, urobiome, virulence factor genes, non-invasive diagnostic, metagenomics, urine

Introduction

Urinary tract infections (UTI) pose a significant burden on individuals and healthcare systems worldwide (Jhang and Kuo, 2017; Gaitonde et al., 2019). The UTIs are one of the most common bacterial infections, affecting millions of people each year. While UTIs can occur in both males and females, women are more commonly affected due to anatomical and hormonal factors (Jhang and Kuo, 2017; Gaitonde et al., 2019). Recurrent UTIs (rUTI) are defined as the occurrence of at least two symptomatic infections within a 6 months period or three infections within a year (Malik et al., 2018; Anger et al., 2019). This recurrent nature of UTIs not only

leads to prolonged discomfort for patients but also contributes to antibiotic overuse and the development of antibiotic resistance (Neugent et al., 2022). Unfortunately, the precise mechanisms underlying the rUTI remain poorly understood.

Emerging evidence suggests that the urobiome, comprised of a diverse community of microorganisms inhabiting the urinary tract, may play a critical role in the development and recurrence of urinary tract infections (Wolfe and Brubaker, 2019). The urobiome is not simply a sterile environment but rather a complex ecosystem that can influence the host immune response and disease susceptibility (Karstens et al., 2016; Bučević Popović et al., 2018). Disturbances in the composition and functionality of the urobiome have been associated with UTIs and may contribute to infection recurrence (Neugent et al., 2022). Understanding the underlying factors driving recurrent urinary tract infections is essential for developing effective prevention and treatment strategies. Investigating the relationship between the urobiome and infection recurrence may provide valuable insights into novel therapeutic targets and approaches to manage recurrent urinary tract infections.

In recent years, there has been growing interest in elucidating the precise mechanisms through which microorganisms exert pathogenic effects (Liu et al., 2022; Rudin et al., 2023). One such mechanism involves the presence of virulence factors genes (VFGs), which are encoded by the microbial genome (Colbert et al., 2023). VFGs are molecular components or proteins produced by microorganisms to enhance their capacity to colonize and infect host tissues (Liu et al., 2022). However, effectively utilizing VFGs to assess the virulence characteristics of the microbiome and employing them for disease diagnosis presents certain feasibility challenges.

In this study, we aimed to assess the contribution of VFGs in urobiome to UTI and rUTI. We identified characteristic VFGs associated with UTI and rUTI. These VFGs can provide valuable insights into our understanding of rUTI and aid in the diagnosis of rUTI.

Materials and methods

Shotgun metagenomic sequence collection and quality control

In the Neugent et al. (2022) study, a total of 50 urine samples were collected from patients divided into two groups: 25 with UTI relapse and 25 in remission. Additionally, 25 urine samples were obtained from healthy individuals as controls. All samples in this study were from female participants. The study participants had no underlying issues affecting their urinary tract, immune system, and did not use indwelling or intermittent catheters. To retrieve the required data, we utilized the prefetch v2.10.7 tool from the National Center for Biotechnology Information (NCBI), enabling us to download the necessary datasets for our analysis. Figure 1A outlined the process, providing a visual representation of the entire workflow involving data collection and subsequent processing steps.

To ensure the sequencing data's quality, we utilized Trimmomatic v0.39 (Bolger et al., 2014) to remove adapter sequences and low-quality bases. The parameters used were as follows: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:8:true TRAILING:20 MINLEN:60. Following quality control, an additional processing step was employed to eliminate human genomic sequences. For this purpose, we employed bowtie v2.4.4 (Langmead and Salzberg, 2012) and used the T2T-mY-rCRS genome (Nurk et al., 2022), which can be found at https://github.com/marbl/CHM13. This genome includes hard-masked PARs on chrY replaced with "N" and mitochondrion replaced with rCRS. This approach effectively removed any human genomic contamination.

VFGs annotation

After removing human genomic sequences, we proceeded to align the remaining reads against the virulence factor database (VFDB) (Liu et al., 2022). This database, which can be accessed at http://www.mgc.ac.cn/VFs/main.htm, contains a comprehensive collection of virulence factors. For the alignment process, we utilized bowtie v2.4.4 [13] and performed subsequent analysis using samtools v1.13 (Li et al., 2009). By aligning the reads against the VFDB_setB_nt database (available at http://www.mgc.ac.cn/VFs/download.htm), we were able to identify the number of reads corresponding to VFs in each sample. Subsequently, for any sample N, we calculated the abundance as follows (Equations (1) and (2)), (Ma et al., 2020; Chang et al., 2021):

Step 1: Calculation of the copy number of each gene:

$$b_i = \sqrt{\frac{x_i}{L_i}} \tag{1}$$

Step 2: Calculation of the relative abundance of gene i

$$a_i = \sqrt{\frac{b_i}{\sum_i b_i}} \tag{2}$$

a_i: the relative abundance of gene i, b_i: the copy number of gene i from sample N, L_i: the length of gene i, x_i: the number of mapped reads.

This valuable information will be utilized in subsequent analyses to gain a comprehensive understanding of the involvement of oral microbiome-encoded VFGs in rUTI.

Diagnostic model construction

To develop a diagnostic model for rUTI, we adopted a two-tiered strategy, utilizing both support vector machine (SVM) (Huang et al., 2018) and random forest (RF) classifiers (Rigatti, 2017). Initially, we trained a suite of standard classifiers: random forest, gradient boosting, SVM, and logistic regression. These classifiers were trained with default parameters to establish a baseline performance. Their accuracy was subsequently assessed on test data, with results collated in a dictionary for straightforward comparison. The SVM classifier demonstrated superior accuracy in differentiating between "No UTI History" and the combined "rUTI Relapse" and "rUTI Remission" categories, guiding our choice of SVM for the binary classification task.

SVM for binary classification

We first categorized the data into two groups: "No UTI History" and a combined category of "rUTI Relapse" and "rUTI Remission." This classification was pivotal for distinguishing between individuals without a UTI history and those with rUTI, regardless of their current status. After data preprocessing and partitioning into training and test

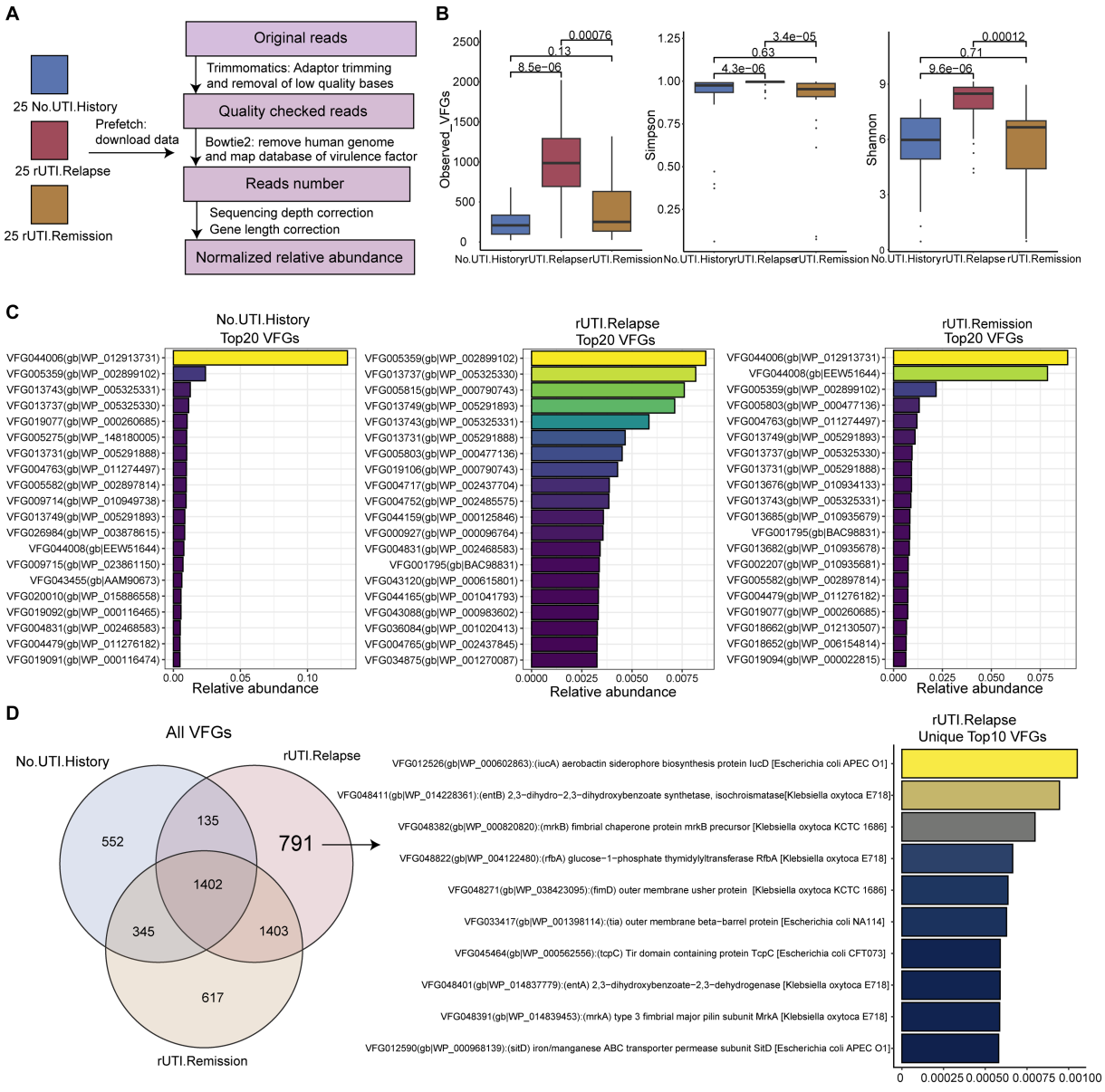


FIGURE 1
Workflow of this study and virulence factor diversity of microbiome. **(A)** Workflow of this study. **(B)** Alpha diversity of VFGs, including the number of observed VFGs, Simpson and Shannon diversity index. **(C)** The top 20 VFGs among three groups. **(D)** The overlap of VFGs among three groups.

sets, we applied the SVM classifier, fine-tuning it with parameters $C = 7$, gamma = 600, and kernel = "rbf." To optimize our SVM model, we executed a grid search to pinpoint the best hyperparameters.

RF for rUTI relapse vs. remission

In the model's second tier, we concentrated on the "rUTI Relapse" and "rUTI Remission" samples, aiming to discern between relapse and remission phases. Employing the RF classifier, we adjusted the model with parameters max_depth = 61 and n_estimators = 230.

Statistical analysis

The statistical analyses in this study were conducted using RStudio. We used the vegan package to calculate alpha diversity measures, such as the Shannon and Simpson indices. The Bray distance was directly computed on the VFG profiles. For principal coordinate analysis (PCoA), we utilized the ade4 package in R (Zapala and Schork, 2006). To assess the significance of group differences, we performed Adonis analysis using the vegan package. To test for differential abundances of VFGs, we employed the Kruskal rank-sum test. The *p*-values were adjusted using the Benjamini–Hochberg (BH) procedure, with a significance threshold set at a *p*-adjust value of <0.05. We used the ggplot2 package to create boxplots and PCoA plots. The pheatmap package was utilized to construct heatmaps visualizing the patterns of VFG abundances. The ggtern was used for the plotting of ternary diagrams (Hamilton and Ferry, 2018). Furthermore, we utilized the pROC package to generate ROC curves, which were employed to evaluate the performance of diagnostic models.

Results

Alpha difference associated with rUTI

According to the workflow depicted in Figure 1A, we obtained the relative abundance of VFGs in all samples. Subsequently, we calculated the alpha diversity measures of the three groups, including observed VFGs, Simpson, and Shannon indices based on the abundance of VFGs. Consistent results were observed, indicating that the alpha diversity of VFGs in the urobiome of patients with rUTI was significantly higher compared to those without a history of UTI (Figure 1B, P-values, observed VFGs: 8.5e−6, Simpson: 4.3e−6, Shannon: 9.6e−6). Furthermore, the alpha diversity was significantly higher in patients with recurrent UTI compared to those with a history of UTI but without recurrence (*p*-values, observed VFGs: 0.00076, Simpson: 3.4e−5, Shannon: 0.00012). There were no notable differences between individuals without a history of UTI and those without recurrence. These results suggest that VFGs may contribute to the recurrence of UTI.

Core and unique VFGs in three group

First, we presented the top 20 abundant VFGs (Figure 1C and Supplementary TableS1) in each group, which we considered as core VFGs. We found that VFGs in urine mainly originated from bacterial genera such as *Gardnerella*, *Streptococcus*, *Corynebacterium*, and *Staphylococcus*. However, patients with rUTI had more virulence factors associated with *Escherichia coli* O157:H7. Furthermore, we identified unique VFGs in each group, including 552, 671, and 791 unique VFGs in the groups without history of UTI, non-recurrent UTI, and rUTI, respectively (Figure 1D). We presented the top 10 features in rUTI, which were distinctly associated with two typical pathogens, *Klebsiella* and *E. coli* O157:H7. This finding once again highlights the presence of unique VFGs in the urine of rUTI patients.

Beta difference among three group

In order to further determine if there are compositional differences in VFGs among the three groups, we conducted PCoA analysis. Based on the Adonis test, we found significant differences in the composition of VFGs from a beta diversity perspective (Figure 2A, $p = 0.001$, $F = 3.2732$). To identify these differential VFGs more explicitly, we represented them using ternary plots with adjusted *p*-values below 0.05 (Figure 2B). We discovered that the majority of differentially abundant VFGs were enriched in women with rUTI, strongly suggesting their potential contribution to UTI recurrence. Additionally, we presented the top 50 VFGs (Figure 2C and Supplementary Table S2), such as VFG043077(gb|WP_000885860). VFG043119(gb|WP_000942326) and VFG043088(gb|WP_000983602). We found that these VFGs are primarily associated with *E. coli* O157:H7 (Supplementary Table S2). It is evident that individuals with a history of UTI and those prone to recurrence exhibit distinct VFG characteristics.

Associations between VFGs and clinical data

To further elucidate potential mechanisms, we investigated the associations between VFGs abundance and clinical data, including age, BMI, and urine pH. We identified six significant correlations using Spearman correlation analysis and canonical correspondence analysis (Figures 3A,B and Supplementary Table S3). One correlation was found with urine pH (Figure 3B and Supplementary Table S3, VFG048953(gb|WP_012967596)), one with age (Figure 3B and Supplementary Table S3, VFG035997(gb|WP_000556543)), and the remaining four with BMI (Figure 3B and Supplementary Table S3, VFG034470(gb|WP_001131106), VFG045826(gb|WP_021526504), VFG048844(gb|WP_014838945) and VFG048845(gb|WP_004122486)).

The rUTI diagnosis model based VFGs

Further investigation is warranted to determine the potential of VFGs profiles in identifying patients with a history of UTI. We developed a machine learning model utilizing SVM to distinguish between females without a UTI history and those with a UTI history. Our findings suggest that the model can achieve an accuracy of 83% (Figure 4A). Moreover, our objective is to create a predictive model that utilizes urobiome information, specifically VFGs, to anticipate UTI recurrence. To accomplish this, we employed a random forest model to evaluate the diagnostic performance of VFGs in detecting rUTI, yielding an accuracy of 75% (Figure 4B). Overall, VFGs at the level of urobiome hold promise as indicators for both UTI history and recurrence. Our combined application of SVM and RF classifiers facilitated the creation of a comprehensive rUTI diagnostic model. The SVM classifier adeptly categorized individuals based on UTI history, while the RF classifier further refined the diagnosis by differentiating relapse and remission states. This dual approach ensures a thorough and precise diagnosis, setting the stage for tailored therapeutic interventions. Future endeavors might incorporate additional features or investigate alternative machine learning algorithms to augment the model's precision.

Discussion

UTI is a frequent and burdensome health issue that affects millions of people worldwide (Gaitonde et al., 2019). While UTIs can generally be treated with antibiotics, some individuals experience rUTI, which present a clinical challenge due to their frequent relapses and limited understanding of the underlying mechanisms. Recent studies have begun to explore the role of the urobiome. The urobiome is believed to contribute to the maintenance of urinary tract health by promoting a balanced microbial environment and resistome (Neugent et al., 2022). However, the annotation of VFGs may provide more direct insights into the microbial contributions to the recurrence of urinary tract infections.

In our study, these VFGs were predominantly associated with two typical pathogens, *Klebsiella* and *E. coli*, further reinforcing their role in rUTI. *E. coli* has been reported to be enriched in rUTI patients and extremely rare in healthy controls and non-recurrent individuals (Magruder et al., 2019; Tornic et al., 2020; Neugent et al., 2022; Worby et al., 2022) and urinary pathogenic *E. coli* is the primary cause of UTI

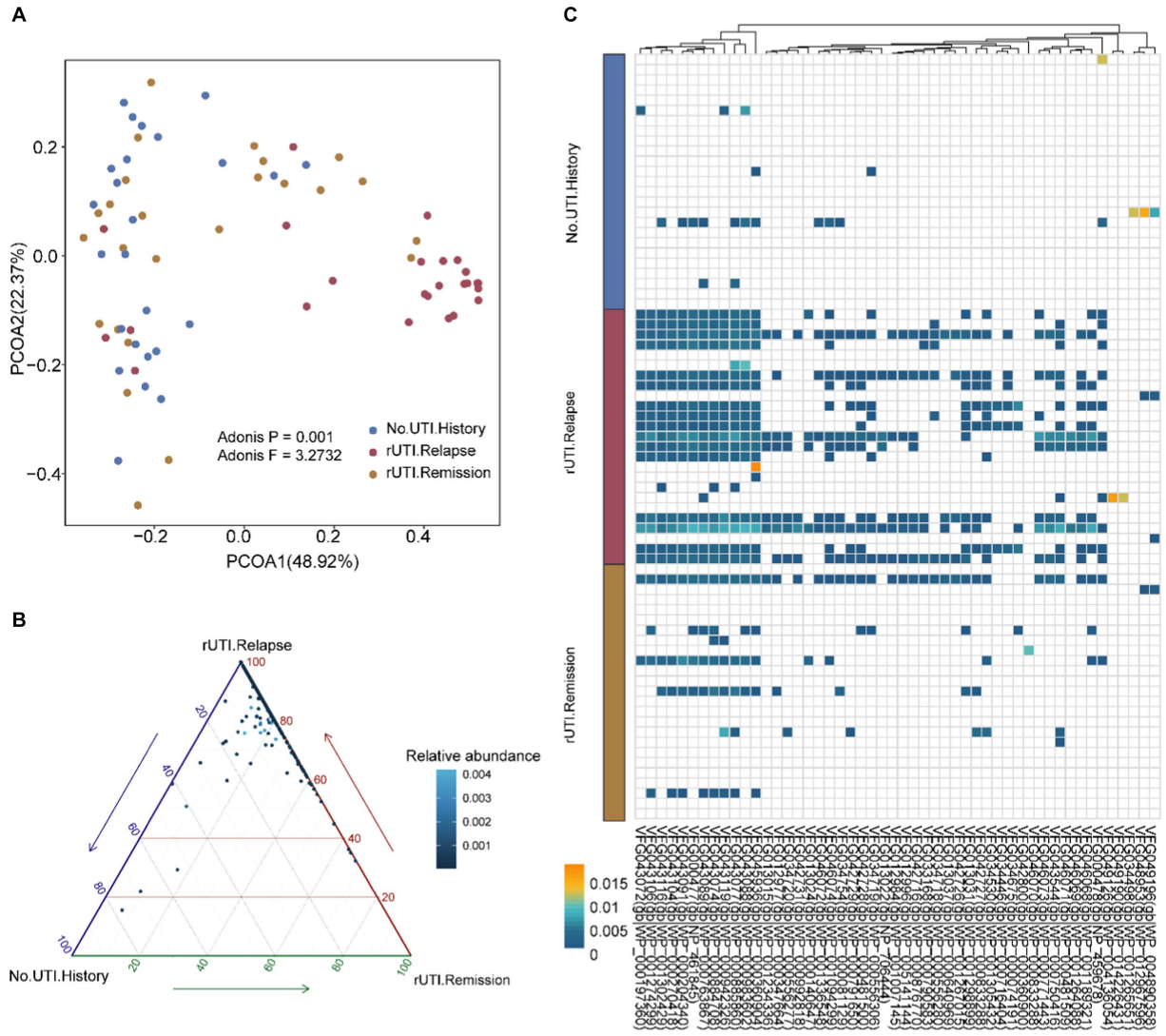


FIGURE 2
Beta diversity and differential VFGs. **(A)** Beta diversity based on bray-cutis distance and differential VFGs profiles. **(B)** Different VFGs with the *p*-values were adjusted using the BH procedure, with a significance threshold set at a p-adjust value of <0.05. **(C)** Top 50 different VFGs were showed using heatmap.

(Lo et al., 2017). which is consistent with our annotation of more *E. coli*-associated VFGs. Previous study revealed that a specific adaptation of *E. coli* to the bladder environment would be predicted to result in decreased fitness in other habitats, such as the gut (Chen et al., 2013). Hence, the virulence factors we are concerned with will provide critical evidence for how *E. coli* colonizes, invades, and acts in the urinary tract. Recent, Naboka et al. (2020) discovered that the Enterobacteria obtained from the urine of female patients experiencing rUTI exhibit a diverse range of VFGs based on qPCR, potentially contributing to the persistence of chronic inflammation in the lower urinary tract. We revealed consistent results using the metagenome.

Although there are many studies predicting urinary tract infections (Heckerling et al., 2007; Gadalla et al., 2019; Sanaee et al., 2020), few have focused on predicting recurrence of urinary tract infections (Cai et al., 2014). To address this, we developed a diagnostic model using machine learning algorithms, namely random forest and support vector machine analysis, based on the levels of the identified VFGs. Our combined application of SVM and RF classifiers facilitated the creation of a comprehensive rUTI diagnostic model, yielding a high accuracy. Our model can help with treatment decisions and enhance clinical outcomes.

Collectively, our findings contribute to the growing body of evidence implicating the role of the urobiome and VFGs in the recurrence of urinary tract infections. By identifying the specific VFGs associated with rUTI, our study provides potential targets for therapeutic interventions aimed at preventing infection recurrence. Future research is warranted to further elucidate the underlying mechanisms by which these VFGs contribute to the pathogenesis of rUTI. In conclusion, our study sheds light on the complex interplay between the VFGs of the urobiome and recurrent urinary tract infections. The identification of specific VFGs associated with rUTI paves the way for the development of personalized treatment strategies and diagnostic approaches, ultimately improving the management of recurrent urinary tract infections.

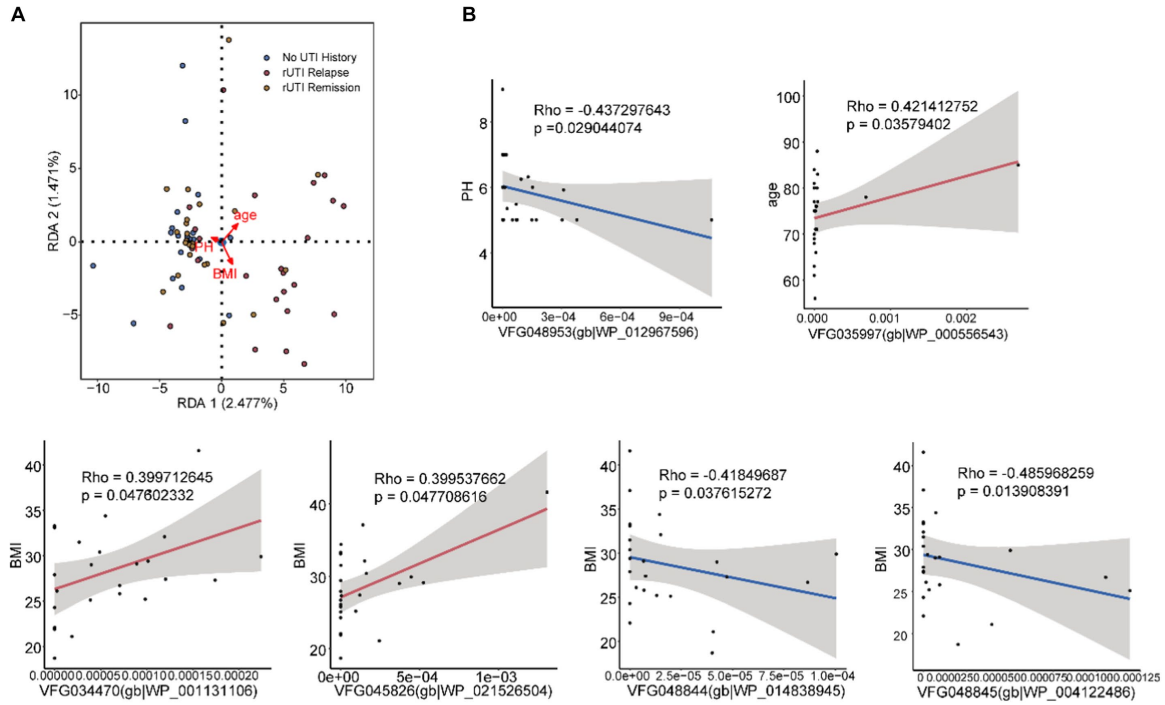


FIGURE 3
The associations between VFGs and clinical data. **(A)** Redundancy analysis. **(B)** Clinical data, urine pH, age, BMI.

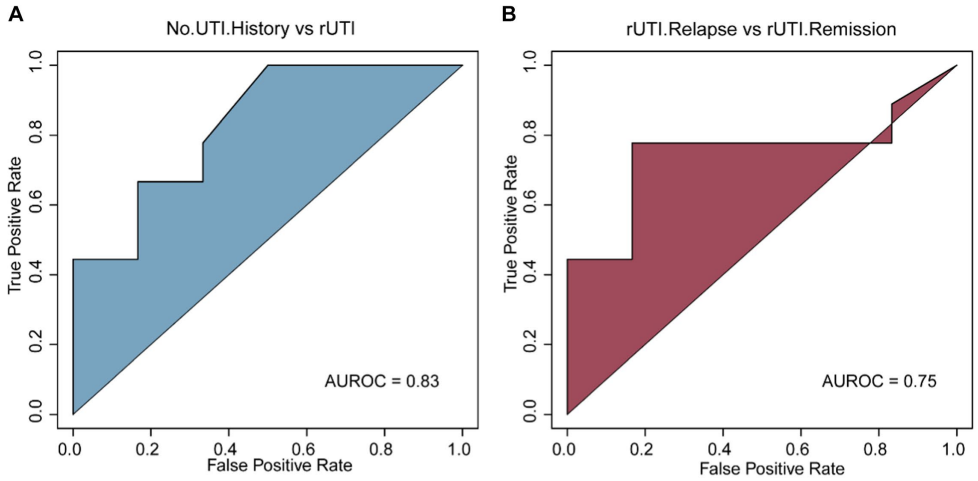


FIGURE 4
Construction of the diagnostic model. **(A)** No UTI history vs. rUTI based on SVM model. **(B)** rUTI relapse vs. rUTI remission based on RF model.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

LJ: Validation, Visualization, Writing – original draft. HW: Software, Writing – original draft. LL: Conceptualization, Writing – original draft. XP: Data curation, Writing – original draft. TL: Methodology, Writing –

original draft. LS: Project administration, Resources, Writing – review & editing. GZ: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Anger, J., Lee, U., Ackerman, A. L., Chou, R., Chughtai, B., Clemens, J. Q., et al. (2019). Recurrent uncomplicated urinary tract infections in women: AUA/CUA/SUFU guideline. *J. Urol.* 202, 282–289. doi: 10.1097/JU.0000000000000296

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bučević Popović, V., Šitum, M., Chow, C. E. T., Chan, L. S., Roje, B., and Terzić, J. (2018). The urinary microbiome associated with bladder cancer. *Sci. Rep.* 8:12157. doi: 10.1038/s41598-018-29054-w

Cai, T., Mazzoli, S., Migno, S., Malossini, G., Lanzafame, P., Mereu, L., et al. (2014). Development and validation of a nomogram predicting recurrence risk in women with symptomatic urinary tract infection. *Int. J. Urol.* 21, 929–934. doi: 10.1111/iju.12453

Chang, H., Mishra, R., Cen, C., Tang, Y., Ma, C., Wasti, S., et al. (2021). Metagenomic analyses expand bacterial and functional profiling biomarkers for colorectal cancer in a Hainan cohort, China. *Curr. Microbiol.* 78, 705–712. doi: 10.1007/s00284-020-02299-3

Chen, S. L., Wu, M., Henderson, J. P., Hooton, T. M., Hibbing, M. E., Hultgren, S. J., et al. (2013). Genomic diversity and fitness of *E. coli* strains recovered from the intestinal and urinary tracts of women with recurrent urinary tract infection. *Sci. Transl. Med.* 5:184ra60. doi: 10.1126/scitranslmed.3005497

Colbert, J. F., Kirsch, J. M., Erzen, C. L., Langouët-Astrié, C. J., Thompson, G. E., McMurtry, S. A., et al. (2023). Aging-associated augmentation of gut microbiome virulence capability drives sepsis severity. *mBio* 14:e0005223. doi: 10.1128/mbio.00052-23

Gadalla, A. A. H., Friberg, I. M., Kift-Morgan, A., Zhang, J., Eberl, M., Topley, N., et al. (2019). Identification of clinical and urine biomarkers for uncomplicated urinary tract infection using machine learning algorithms. *Sci. Rep.* 9:19694. doi: 10.1038/s41598-019-55523-x

Gaitonde, S., Malik, R. D., and Zimmern, P. E. (2019). Financial burden of recurrent urinary tract infections in women: a time-driven activity-based cost analysis. *Urology* 128, 47–54. doi: 10.1016/j.urology.2019.01.031

Hamilton, N. E., and Ferry, M. (2018). ggtern: Ternary diagrams using ggplot2. *J. Stat. Softw.* 87, 1–17. doi: 10.18637/jss.v087.c03

Heckerling, P., Canaris, G., Flach, S., Tape, T., Wigton, R., and Gerber, B. (2007). Predictors of urinary tract infection based on artificial neural networks and genetic algorithms. *Int. J. Med. Inform.* 76, 289–296. doi: 10.1016/j.ijmedinf.2006.01.005

Huang, S., Cai, N., Pacheco, P. P., Narrandes, S., Wang, Y., and Xu, W. (2018). Applications of support vector machine (SVM) learning in cancer genomics. *Cancer Genomics Proteomics* 15, 41–51. doi: 10.21873/cgp.20063

Jhang, J. F., and Kuo, H. C. (2017). Recent advances in recurrent urinary tract infection from pathogenesis and biomarkers to prevention. *Ci Ji Yi Xue Za Zhi* 29, 131–137. doi: 10.4103/tcmj.tcmj_53_17

Karstens, L., Asquith, M., Davin, S., Stauffer, P., Fair, D., Gregory, W. T., et al. (2016). Does the urinary microbiome play a role in urgency urinary incontinence and its severity? *Front. Cell. Infect. Microbiol.* 6:78. doi: 10.3389/fcimb.2016.00078

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352

Liu, B., Zheng, D., Zhou, S., Chen, L., and Yang, J. (2022). VFDB 2022: a general classification scheme for bacterial virulence factors. *Nucleic Acids Res.* 50, D912–D917. doi: 10.1093/nar/gkab1107

Lo, A. W., Moriel, D. G., Phan, M. D., Schulz, B. L., Kidd, T. J., Beatson, S. A., et al. (2017). 'Omic' approaches to study uropathogenic *Escherichia coli* virulence. *Trends Microbiol.* 25, 729–740. doi: 10.1016/j.tim.2017.04.006

Ma, C., Wasti, S., Huang, S., Zhang, Z., Mishra, R., Jiang, S., et al. (2020). The gut microbiome stability is altered by probiotic ingestion and improved by the continuous supplementation of galactooligosaccharide. *Gut Microbes* 12:1785252. doi: 10.1080/19490976.2020.1785252

Magruder, M., Sholi, A. N., Gong, C., Zhang, L., Edusei, E., Huang, J., et al. (2019). Gut uropathogen abundance is a risk factor for development of bacteriuria and urinary tract infection. *Nat. Commun.* 10:5521. doi: 10.1038/s41467-019-13467-w

Malik, R. D., Wu, Y. R., and Zimmern, P. E. (2018). Definition of recurrent urinary tract infections in women: which one to adopt? *Female Pelvic Med. Reconstr. Surg.* 24, 424–429. doi: 10.1097/SPV.0000000000000509

Naboka, Y. L., Mavzyiutov, A. R., Kogan, M. I., Gudima, I. A., Ivanov, S. N., and Naber, K. G. (2020). Does *Escherichia coli* have pathogenic potential at a low level of bacteriuria in recurrent, uncomplicated urinary tract infection? *Int. J. Antimicrob. Agents* 56:105983. doi: 10.1016/j.ijantimicag.2020.105983

Neugent, M. L., Kumar, A., Hulyalkar, N. V., Lutz, K. C., Nguyen, V. H., Fuentes, J. L., et al. (2022). Recurrent urinary tract infection and estrogen shape the taxonomic ecology and function of the postmenopausal urogenital microbiome. *Cell Rep. Med.* 3:100753. doi: 10.1016/j.xcrm.2022.100753

Nurk, S., Koren, S., Rhie, A., Rautiainen, M., Bzikadze, A. V., Mikheenko, A., et al. (2022). The complete sequence of a human genome. *Science* 376, 44–53. doi: 10.1126/science.abj6987

Rigatti, S. J. (2017). Random forest. *J. Insur. Med.* 47, 31–39. doi: 10.17849/insm-47-01-31-39.1

Rudin, L., Bornstein, M. M., and Shyp, V. (2023). Inhibition of biofilm formation and virulence factors of cariogenic oral pathogen *Streptococcus mutans* by natural flavonoid phloretin. *J. Oral Microbiol.* 15:2230711. doi: 10.1080/20002297.2023.2230711

Sanaee, M. S., Pan, K., Lee, T., Koenig, N. A., and Geoffrion, R. (2020). Urinary tract infection after clean-contaminated pelvic surgery: a retrospective cohort study and prediction model. *Int. Urogynecol. J.* 31, 1821–1828. doi: 10.1007/s00192-019-04119-0

Tornic, J., Wöllner, J., Leitner, L., Mehnert, U., Bachmann, L. M., and Kessler, T. M. (2020). The challenge of asymptomatic bacteriuria and symptomatic urinary tract infections in patients with neurogenic lower urinary tract dysfunction. *J. Urol.* 203, 579–584. doi: 10.1097/JU.0000000000000555

Wolfe, A. J., and Brubaker, L. (2019). Urobiome updates: advances in urinary microbiome research. *Nat. Rev. Urol.* 16, 73–74. doi: 10.1038/s41585-018-0127-5

Worby, C. J., Olson, B. S., Dodson, K. W., Earl, A. M., and Hultgren, S. J. (2022). Establishing the role of the gut microbiota in susceptibility to recurrent urinary tract infections. *J. Clin. Invest.* 132:e158497. doi: 10.1172/JCI158497

Zapala, M. A., and Schork, N. J. (2006). Multivariate regression analysis of distance matrices for testing associations between gene expression patterns and related variables. *Proc. Natl. Acad. Sci. USA* 103, 19430–19435. doi: 10.1073/pnas.0609333103

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Longitudinal analysis of microbiome composition in Ghanaians living with HIV-1

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Human immunodeficiency virus (HIV) 1 infection is known to cause gut microbiota dysbiosis. Among the causes is the direct infection of HIV-1 in gut-resident CD4$^+$ T cells, causing a cascade of phenomena resulting in the instability of the gut mucosa. The effect of HIV infection on gut microbiome dysbiosis remains unresolved despite antiretroviral therapy. Here, we show the results of a longitudinal study of microbiome analysis of people living with HIV (PLWH). We contrasted the diversity and composition of the microbiome of patients with HIV at the first and second time points (baseline_case and six months later follow-up_case, respectively) with those of healthy individuals (baseline_control). We found that despite low diversity indices in the follow-up_case, the abundance of some genera was recovered but not completely, similar to baseline_control. Some genera were consistently in high abundance in PLWH. Furthermore, we found that the CD4$^+$ T-cell count and soluble CD14 level were significantly related to high and low diversity indices, respectively. We also found that the abundance of some genera was highly correlated with clinical features, especially with antiretroviral duration. This includes genera known to be correlated with worse HIV-1 progression (*Achromobacter* and *Stenotrophomonas*) and a genus associated with gut protection (*Akkermansia*). The fact that a protector of the gut and genera linked to a worse progression of HIV-1 are both enriched may signify that despite the improvement of clinical features, the gut mucosa remains compromised.

KEYWORDS

HIV, PLWH, Ghana, longitudinal analysis, gut microbiome dysbiois

Background

Human immunodeficiency virus (HIV) is still a worldwide health problem, with 38.4 million living with HIV worldwide. Approximately 1.5 million people acquired HIV, and 650,000 people died from HIV-related causes in 2021 (HIV.gov, 2022). There are still no vaccines available to prevent the disease. Nevertheless, life expectancy and health outcomes in people living with HIV (PLWH) have improved with the availability of combination antiretroviral therapy (ART) that controls HIV replication. HIV incidence has declined 32% since 2010 (HIV.gov, 2022). However, a cure is still not attainable due to the ability of the virus to persist in long-lived cells. These cells cannot be targeted with current ART or HIV-specific immune responses.

Persistence may also be caused by the gut microbiome and its metabolites (Koay et al., 2018; Crakes and Jiang, 2019; Ishizaka et al., 2021). They have been shown to promote inflammation and immune activation in HIV-infected adults. This inflammation and immune activation are correlated with the disruption of gut mucosal integrity. HIV targets gut-associated lymphoid tissues as major sites of viral transmission, replication and seeding, and $CD4^+$ T-cell depletion (Khan et al., 2017). In the gut, altered T-cell homeostasis, particularly of $CD4^+$ Th17 cells, coincides with disruption of the intestinal barrier, where tightly opposed enterocytes lose their adhesion to adjoining cells (Dandekar et al., 2010). When the intestinal barrier is disrupted, microbial products travel from the lumen into systemic circulation, accumulating in the liver and brain tissue and activating the immune system (Estes et al., 2010).

Furthermore, the effect of HIV infection on the dysbiosis of the gut microbiome remains unresolved despite ART therapy (Shacklett and Anton, 2010). HIV replication is effectively suppressed by ART, but HIV in the reservoirs cannot be eradicated and $CD4^+$ T cells cannot be fully restored in the gut or peripheral tissues. The restoration of $CD4^+$ T cells in the gut is delayed compared to those in the peripheral blood (Guadalupe et al., 2003). Initiation of ART fails to reduce chronic immune activation and markers of microbial translocation, such as lipopolysaccharide (LPS) and soluble CD14 (sCD14) (Wallet et al., 2010). The interaction between the host and microbe at the gut mucosal interface is critical in preventing microbial translocation and immunity activation in HIV-infected individuals receiving suppressive ART.

Nevertheless, the elucidation of gut microbial interaction with immune activation is not an easy task, as gut microbiome composition is different all over the world (de Filippo et al., 2010; Brito et al., 2016; He et al., 2018). We previously added to the collective knowledge the results of the microbiome analysis in healthy Ghanaians (Parbie et al., 2021a) and compared it with that in Ghanaian PLWH (Parbie et al., 2021b). Through microbiome analysis on our cohort, we found that our results were mostly in line with previous reports, that is, a significant increase in the abundance of *Proteobacteria* but a decrease in the abundances of *Firmicutes* and *Bacteroidetes* at the phylum level, similar to previous observations in other countries (McHardy et al., 2013; Vujkovic-Cvijin et al., 2013; Monaco et al., 2016). However, some bacteria belonging to *Firmicutes*, namely, *Streptococcus*, *Dorea*, and *Blautia,* are enriched in PLWH (Parbie et al., 2021b). We note that this is different from several other reports, in which the abundance of *Lachnospiraceae*, including *Dorea* and *Blautia*, is decreased in HIV-1-infected individuals (Mutlu et al., 2014; Vázquez-Castellanos et al., 2015). Furthermore, we found a depletion of *Prevotella* in PLWH in Ghana, whereas previous studies on the gut microbiome have described enrichment of *Prevotella* in HIV-1-infected populations (Lozupone et al., 2013; Dillon et al., 2014).

To further complement the data, here, we describe the results of a longitudinal microbiome analysis of PLWH in Ghana.

Materials and methods

Study population

Data on HIV-1-negative (HIV-) and HIV-1-positive (HIV+) status at baseline (baseline_control and baseline_case, respectively) were described in Table 1. We enrolled matching pairs of HIV+ and HIV- individuals. Participants in the study were HIV+ individuals attending the Eastern Regional Hospital, Koforidua (RHK) in Ghana. A community health screening in these communities recruited HIV- individuals. We matched seronegative individuals by age (± 2 years), sex, and community of residence to serve as controls. Only adults above 18 years old were enrolled in this study. Seronegative participants who took antibiotics within four weeks of sample collection were not enrolled. Approximately 6 months after their baseline visit, stool samples were collected from HIV+ individuals, which represent the follow-up_case. $CD4^+$ T-cell count and viral load were not measured on their second visit.

TABLE 1 Clinical and demographic characteristics.

Description	HIV-1-negative ($n = 41$)	HIV-1-positive ($n = 47$)	p-value[1]
Age (years): median (IQR[2])	45 (19–73)	46 (18–75)	0.934
Gender: female number (%)	34 (82.93%)	37 (78.72%)	>0.99
$CD4^+$ T-cell count (cells/μL): median (IQR[2])	ND[3]	434 (31–1,384)	ND[3]
ART duration (months): median (IQR[2]) $n = 37$	ND[3]	54 (6–142)	ND[3]
Viral load (copies/mL): median (IQR[2]) $n = 38$	ND[3]	3,729 (20–200,936)	ND[3]
sCD14 (μg/mL): mean ± SD	0.503 ± 0.443	0.794 ± 0.791	0.238
IFABP (ng/mL): mean ± SD	0.987 ± 0.557	1.409 ± 0.898	0.042
LBP (μg/mL): mean ± SD	4.754 ± 2.14	5.096 ± 2.153	0.252

[1]Statistical comparison between seronegative controls and HIV-1 infected individuals was performed by Wilcoxon rank sum test or Fisher's exact test for gender.
[2]Interquartile range.
[3]Not determined.
RT, antiretroviral therapy; sCD14, soluble CD14; IFABP, intestinal free acid binding protein; LBP, lipoprotein binding protein.

Sample collection

We collected venous blood and stool samples from enrolled participants. The total of 135 samples includes 41 samples from HIV- individuals (baseline_controls), and 94 samples from HIV+ individuals (n = 47) at baseline (baseline_case) and six months later at follow-up time point (follow-up_case). In the 24 h following their collection, all biological samples were transported to the Noguchi Memorial Institute for Medical Research (NMIMR) in Ghana, where they were processed for storage. We prepared plasma and peripheral blood mononuclear cells from venous blood. Stool samples were collected and stored at −80°C until DNA extraction.

Bacterial fraction preparation from fecal samples

One gram of stool was washed with 3 mL of SM-plus buffer (100 mM NaCl, 50 mM Tris–HCl [pH 7.4], 8 mM MgSO4-7H2O, 5 mM CaCl2-2H2O, 0.01% [w/v] gelatin) and centrifuged at 6,000 × g for 5 min. This process was done for additional two times. Pellets were resuspended in 20 mL of SM-plus buffer and filtered through a 100-μm cell strainer (Corning, Tokyo, Japan). One milliliter of the filtered 20 mL of bacterial suspension was used for DNA extraction.

DNA extraction, amplification, and 16S rRNA gene sequencing

DNA was extracted from the fecal sample-derived bacterial fraction as previously described (Kim et al., 2013). Gene libraries for the hypervariable V3-V4 region of 16S rRNA were prepared as previously described (Parbie et al., 2021b) according to the 16S Metagenomic Sequencing Library Preparation guide (Illumina, San Diego, United States; Part # 15044223 Rev. B). Sequencing was performed on the Illumina MiSeq using MiSeq Reagent Kit v3 (600-cycle) with a 20% PhiX (Illumina) spike-in at Noguchi Memorial Institute for Medical Research, Ghana. Sequencing was performed in five batches.

Sequence analyses

Sequences were quality filtered, denoised, and analyzed with Quantitative Insights Into Microbial Ecology (QIIME 2 version 2022.2) (Bolyen et al., 2019). Briefly, raw paired-end reads were imported, demultiplexed, and quality filtered using the q2-demux plugin followed by denoising with DADA2 (Callahan et al., 2016) (via q2-dada2). Taxonomy was assigned to the resulting amplicon sequence variants against the SILVA database (release 138) (Quast et al., 2013) (via q2-feature-classifier). A phylogeny was constructed with the q2-phylogeny plugin. Batch effect was removed with an R package, MMUPHin (Ma et al., 2022) with the adjust_batch() function. Principal coordinate analysis calculated with Curtis-Bray, Unweighted UniFrac, and Weighted UniFrac distances, alpha diversity and microbial composition analyses were conducted with phyloseq (McMurdie and Holmes, 2013). Differentially abundant taxa by HIV-1 status were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) and the Analysis of Composition of Microbiome (ANCOM) (Mandal et al., 2015) approach with default parameters. Metagenomic pathway prediction was performed with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (Douglas et al., 2020) with default parameters. We analyzed only results associated with the MetaCyc database (Caspi et al., 2020).

CD4$^+$ T-cell count and viral load measurement

CD4$^+$ T-cell count was performed with BD FACSCount CD4$^+$ reagents kit following manufacturer's instructions. Viral load was measured with AmpliPrep/ COBAS TaqMan HIV-1 Test V2.0 following manufacturer's instructions.

Analysis of plasma markers for microbial translocation

An enzyme-linked immunosorbent assay (ELISA) was used to measure plasma lipopolysaccharide-binding protein (LBP), soluble CD14 (sCD14), and intestinal fatty acid-binding protein (I-FABP) levels (R&D Systems, Minneapolis, MN, United States).

Statistical analyses

Data were obtained from 2017 to 2019. R 3.6.0 packages were used for statistical analyses. Comparison between categorical variables between groups was performed with the Wilcoxon using an R package, ggpubr with geom_pwc() function (Kassambara, 2023). p values less than 0.05 were considered significant. To test associations involving bacterial taxa showing significant differences in abundance by HIV-1 status and clinical and immunological markers, correlation analysis was performed using the R package Microbiome Multivariable Associations with Linear Models (MaAsLin2) (Mallick et al., 2021) using the following modifiers: analysis_method = "NEGBIN", normalization = "NONE", transform = "NONE", max_significance = 0.5, min_prevalence = 0.5, min_abundance = 0.0001. PICRUSt2 data were visualized with Statistical Analysis of Taxonomic and Functional Profiles (STAMP) (Parks et al., 2014). Significant pathways between two groups (baseline_case vs. baseline_control and baseline_case vs. follow-up_case) were calculated with two-sided Welch's t test and Bonferroni correction with a p value filter of 0.05.

Results

Microbial diversity of some genera rebound in PLWH at the second time point

The total of 135 samples includes 41 samples from HIV- individuals (baseline_control), and 94 samples from HIV+ individuals ($n = 47$) at baseline (baseline_case) and six months later at follow-up time point (follow-up_case). Beta diversity shows that each condition-timepoint has a characteristic expression. Unweighted UniFrac shows that the species mostly overlap at all condition-timepoints. Weighted UniFrac, on the other hand, shows that the abundance of the species in follow-up_case is more similar

to baseline_control than baseline_case (Figure 1A). We measured the alpha diversity of these condition-timepoints. Compared with baseline_control, all indices had lower diversity in baseline_case. Richness and Shannon were statistically insignificant, but two indices, i.e., Fisher and Faith's phylogenetic diversity (PD) (Figure 1B). In the follow-up case, the microbial diversity indices were similar to those in the baseline case. Nevertheless, Faith's PD shows a significant reduction at the second time point (Figure 1B). Compositional plot, which shows the microbial composition for each condition-timepoint, revealed that several genera were up-or downregulated in the follow-up_case compared to the baseline_case, with relative amounts comparable to the baseline_control (Figure 1C). For example, the genera *Subdoligranulum*, *Dorea*, and *Blautia* were less abundant in the follow-up case. On the other hand, the genus *Faecalibacterium* were more abundant in the follow-up case.

We employed the linear discriminant analysis (LDA) effect size (LEfSe) method to identify the taxa characterizing each condition-timepoint (Segata et al., 2011). The phyla *Bacteriodota* and *Euryarchaeota* (archaea) were enriched in healthy individuals (baseline_control), while *Verucomicrobiota* was abundant in PLWH (baseline_case) (Figure 2A). Within the phylum *Firmicutes* (or *Bacillota*), there was abundant diversity. For example, the class *Negativicutes* was enriched in the baseline_control, while *Bacilli* was abundant in the baseline_case. Furthermore, there seems to be an intersection of abundance of baseline_control and follow-up_case, i.e., the phylum *Bacteriodota* and class *Negativicutes* (Figure 2B), which are abundant in both baseline_control and follow-up_case.

The genera *Dorea* and *Blautia* were increased in PLWH at baseline_case (Figure 2C), as well as *Achromobacter* and *Stenotrophomonas*, compared with baseline_control. Some genera in healthy people (baseline_control), such as *Faecalibacterium* and *Phascolarctobacterium* (Figure 2C) were also abundant in the follow-up_case (Figure 2D), corroborating the apparent reversion of the abundance of these genera. Nevertheless, some genera were highly abundant in PLWH regardless of time points, such as *Streptococcus*, *Staphylococcus*, and *Gemella* (Figure 2E). Analysis with ANCOM corroborates the LEfSe results, showing that the abundance in the baseline case and follow-up case was different: the genera *Achromobacter* and *Phascolarctobacterium* were abundant in the baseline case and follow-up case, respectively (Supplementary Figure 1).

The relative abundance of significantly differentially abundant genera of each condition-timepoint are presented in detail in Figure 3. A reversal in the abundance of the *Faecalibacterium* from baseline_case to follow-up_case and a decrease in the abundance of the *Dorea* in PLWH at baseline and follow-up_case was observed. Further, the persistence abundance of *Streptococcus* and *Staphylococcus* in HIV-positive despite the time point.

CD4$^+$ T-Cell count and soluble CD14 are significantly correlated with diversity

The correlation between clinical characteristics and alpha diversity at baseline continued to be examined. We conducted a comparative analysis of alpha diversity, splitting the PLWH population into two groups based on the median values of each of the six parameters, which were CD4$^+$ T-cell count, viral load, ART duration, sCD14, IFABP, and LPS levels. The patients with high CD4$^+$ T-cell counts showed significantly higher alpha diversity than the patients with low counts at baseline (Figure 4A). In contrast, a high sCD14 level showed significantly lower alpha diversity (Figure 4B). Longer ART duration was correlated with lower diversity, although it was not significant in our cohort (Supplementary Figure 2A). Higher IFABP levels were not significantly correlated with lower diversity (Supplementary Figure 2B). Viral load and LPS level did not seem to be correlated with diversity indices (Supplementary Figures 2C,D).

We then sought to link the abundance of microbial genera to the clinical features. Our cohort showed a positive correlation of *Achromobacter* with ART duration, viral load, sCD14, and IFABP and its inverse correlation with CD4$^+$ T-cell count. Likewise, *Stenotrophomonas* followed the same pattern as *Achromobacter*, albeit with an inverse correlation with viral load. *Staphylococcus* showed a positive correlation with sCD14 and IFABP. Other genera did not show a meaningful correlation with the clinical features (Figure 4C). Interestingly, the correlation of ART duration with *Achromobacter* and *Sternotrophomonas* was positive. The abundance of the genus *Akkermansia* was also highly significantly related to this parameter.

Metagenome pathway prediction software predicts a reduction of bacterial pathways activities at follow-up

To evaluate the metabolic pathways, counting the functional genes directly following a shotgun metagenome sequencing is ideal. However, this needs a sequencer with a high sequencing capacity. In a situation where research limitations exist, we may use a prediction software. PICRUSt2 software was developed to predict the functional potential of a bacterial community based on marker gene sequencing profiles (Douglas et al., 2020). Utilizing this software, we compared the functional pathways based on the MetaCyc database (Caspi et al., 2020). Analysis revealed that bacterial pathways predicted a significant increase in the number of functional genes in baseline cases compared to baseline controls, but a decrease in the number of functional genes in follow-up cases compared to baseline cases (Figure 5). Exploring the MetaCyc database revealed the expected taxonomic range to possess the predicted pathways (Supplementary Table 1). While some of the pathways are not specific to bacteria, e.g., NAD biosynthesis II from tryptophan pathway is also found in eukaryotes, some pathways are found to be specific in bacteria, for instance ketogluconate degradation I, 2-nitrobenzoate degradation I, and fatty acid salvage pathways. Furthermore, some upregulated pathways are possessed by groups of bacteria known as pathogenic and opportunistic. Catechol degradation II, L-histidine degradation II, and nicotinate degradation I are pathways possessed by the phylum *Pseudomonadota*, whose members include the families *Neisseriaceae, Campylobacteriaceae, Enterobacteriaceae, Klebsiella, Escherichia, Burkholderiaceae, and Pseudomonadaceae* (Leão et al., 2023).

Leaky gut during the treatment period

Our finding that the abundance of both beneficial and pathogenic bacteria genera is highly correlated with the duration of ART

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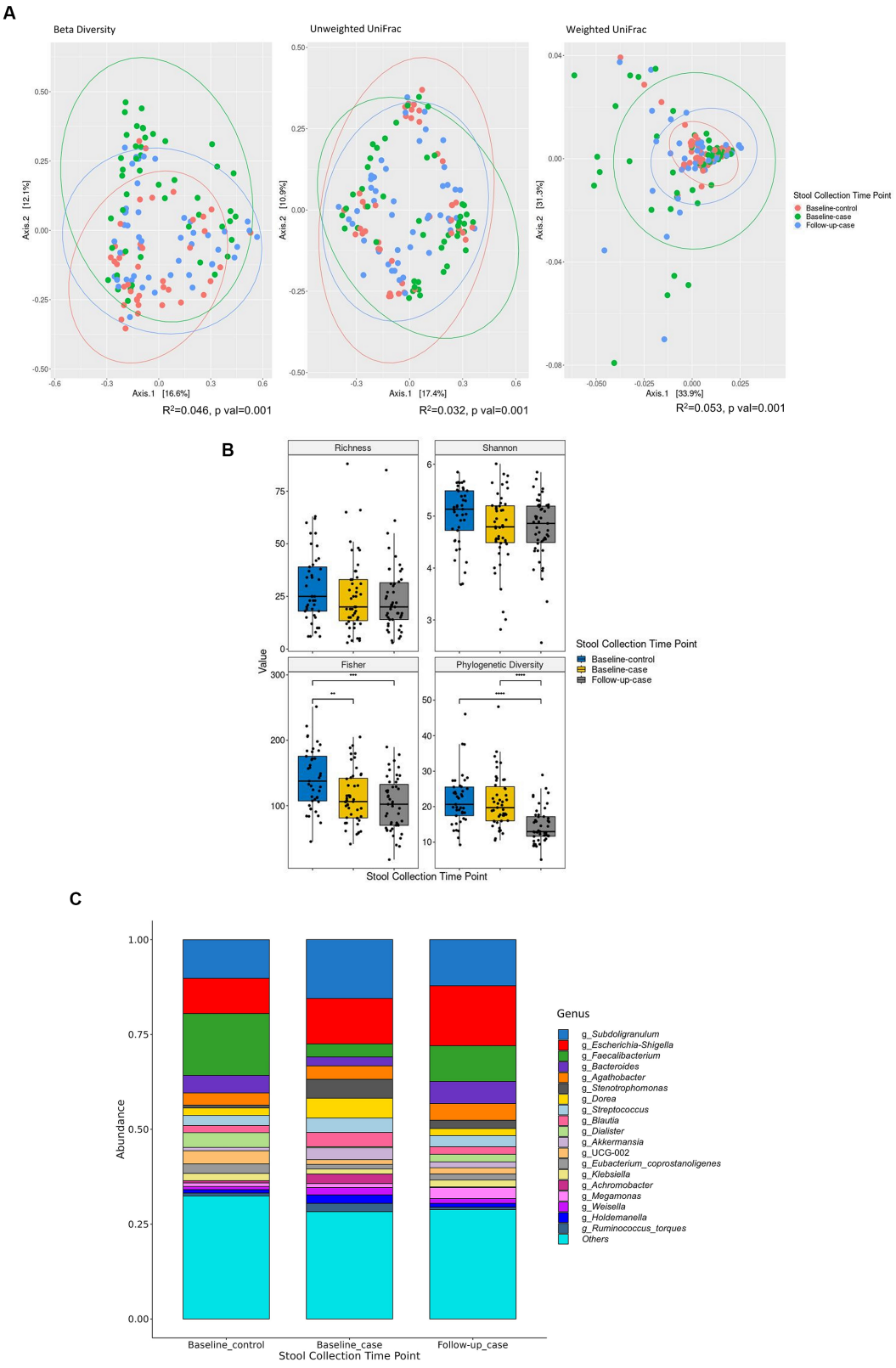


FIGURE 1
Alpha and beta diversity of the cohort. Principal component analysis shows that the microbial abundance of each of the three condition-timepoints can be clustered together. Each dot represents each sample's taxon abundance in two dimensions. Beta diversity is measured using Curtis-Bray, Unweighted UniFrac, and Weighted UniFrac distances **(A)**. **(B)** Shows the diversity indices between condition-timepoint. The relative abundance of the 19 highest expressed genera in each condition-timepoint is shown in **(C)**. Statistical significance is calculated by the Wilcoxon test. $^{**}p <= 0.01$, $^{***}p <= 0.001$, $^{****}p <= 0.0001$.

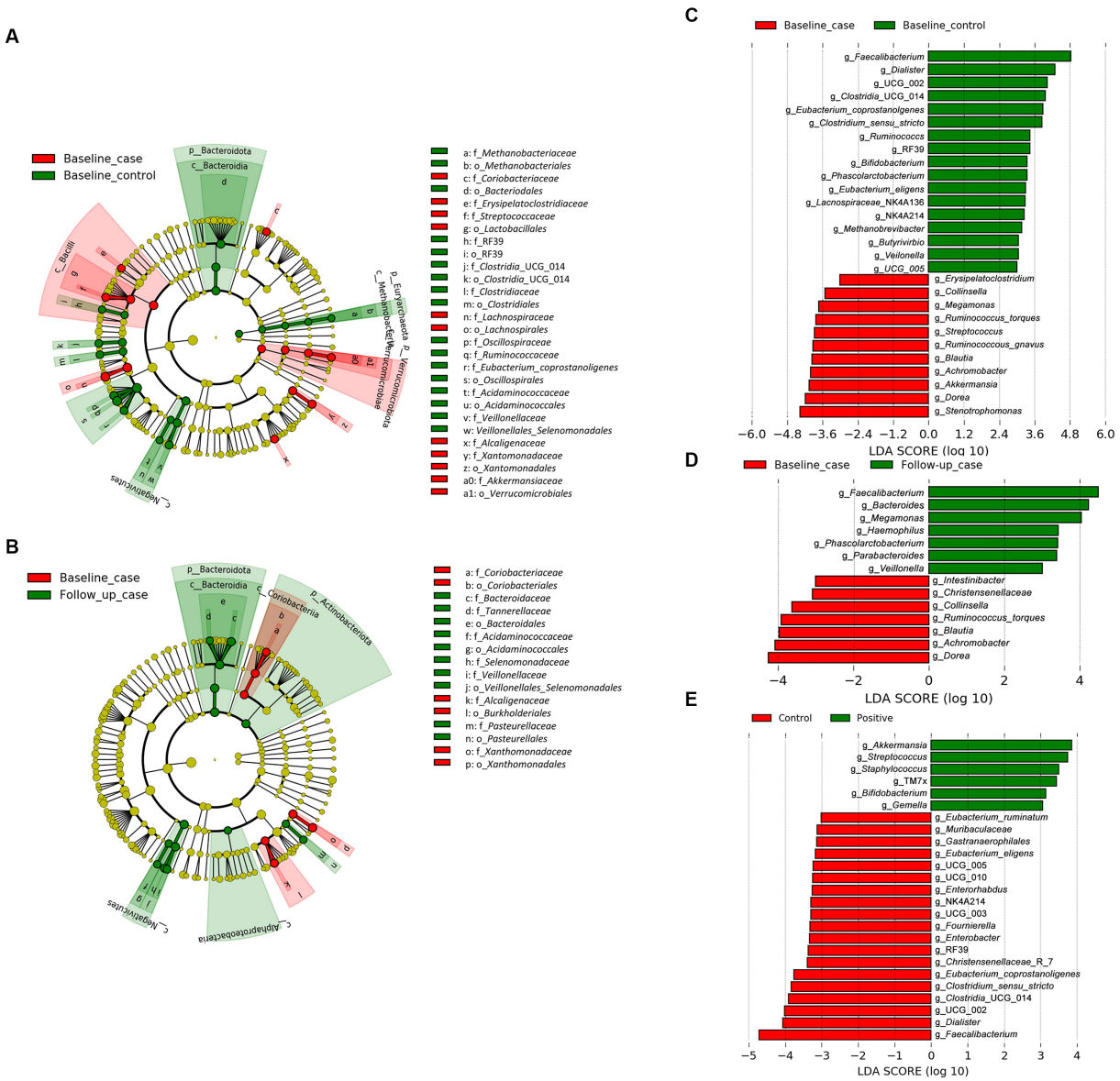


FIGURE 2
The result of linear discriminant analysis (LDA) effect size (LEfSe) analyses. LEfSe analysis between baseline_case and baseline_control and baseline_case and follow-up_case is shown in **(A,B)**, respectively. The difference in genera abundance between baseline_case and baseline_control and baseline_case and follow-up_case is shown in **(C,D)**, respectively. The difference in genera abundance between HIV negative and positive is shown in **(E)**. LDA threshold is 2.5.

motivated us to perform additional longitudinal analysis based on treatment duration. Here, we classified our cohort into ART-naïve, PLWH with ART treatment < 36 months, and PLWH with treatment ≥ 36 months. We found that the correlation between bacterial abundance and clinical features was stronger at treatment ≥36 months than at any other treatment duration (Supplementary Figure 3). $CD4^+$ T-cell count showed that there was no change in cell number in all treatment duration (Figure 6A). Nevertheless, the median $CD4^+$ cell counts of 434 cells/μL was well above 200 cells/μL for all treatment duration. Despite non-significance, viral load was in a decreasing trend with a median value of 3,729 copies/μL (Figure 6B). sCD14 and IFABP showed a higher median value than naïve at early treatment duration but was reduced at late therapy (sCD14, Figure 6C) or no change (LBP, Figure 6D). The median LBP value was of no change (Figure 6E). Nevertheless, sCD14, IFABP, and LBP values showed an increasing trend. Alpha diversity indices were lower in PLWH on treatment at baseline, with more profound significant reduction at late treatment period (Figure 6F). At follow-up, there was no index difference between the treatment conditions. Nevertheless, in comparison to baseline, diversity at follow-up was more significantly reduced in ART-naïve individuals (Figure 6G). LDA LEfSe showed abundance of genus *Faecalibacterium* and class *Negativicutes* on follow up at both <36 and ≥ 36 months ART treatment. The analysis could not identify a taxon with a strong effect size at follow-up in ART-naïve (Figure 7). Collectively these results revealed a leaky gut phenomenon at late treatment period despite relatively controlled disease progression with a mix of beneficial and pathogenic bacterial environment.

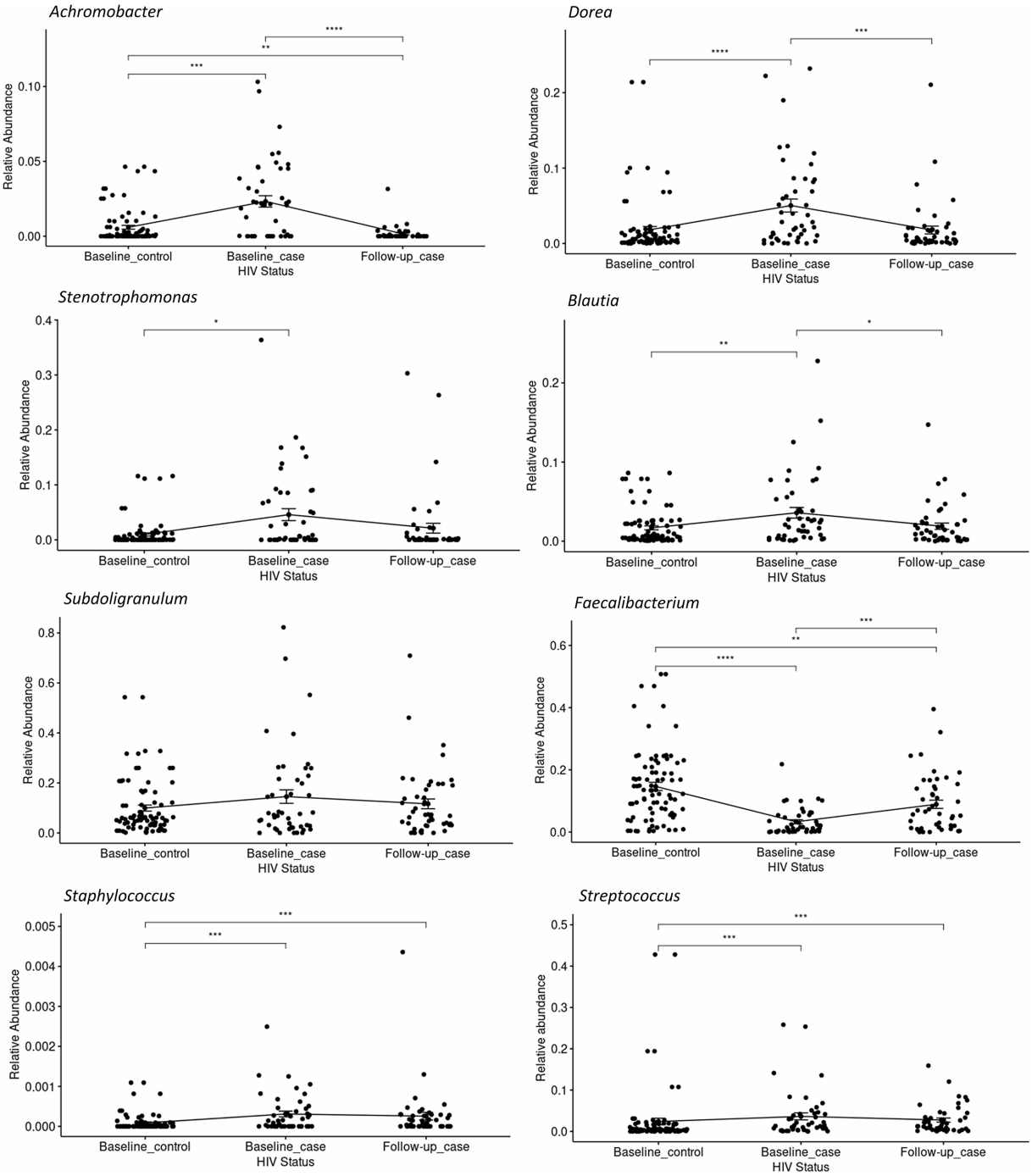


FIGURE 3
The relative abundance of select genera in each condition-timepoint. The genera *Achromobacter*, *Dorea*, *Stenotrophomonas*, *Blautia*, and *Subdoligranulum* shows low abundance in the baseline _control, high abundance in the baseline_case, and low abundance in the follow-up_case, albeit a non-significance in the genus *Subdoligranulum*. In contrast, the genus *Faecalibacterium* has a high abundance in the baseline_control, a reduction in the baseline_case, and a rebound in the follow-up_case. The genera *Staphylococcus* and *Streptococcus*, however, have high abundance in positive cases regardless of time point. Statistical significance is calculated by the Wilcoxon test. $^{**}p <= 0.01$, $^{***}p <= 0.001$, $^{****}p <= 0.0001$.

Discussion

Our previous work confirmed that the gut microbiome composition of Ghanaian PLWH is mostly similar to other reports, with some discrepancies (Parbie et al., 2021a). *Faecalibacterium* as well as *Prevotella* and *Bacteroides* were not included in the top 10 abundant taxa in HIV+ compared to healthy individuals, while the genera *Achromobacter*, *Dorea*, *Blautia*, *Stenotrophomonas*, and *Streptococcus* were abundant, indicating loss of critical commensals in HIV-1-infected adults and proliferation of opportunistic

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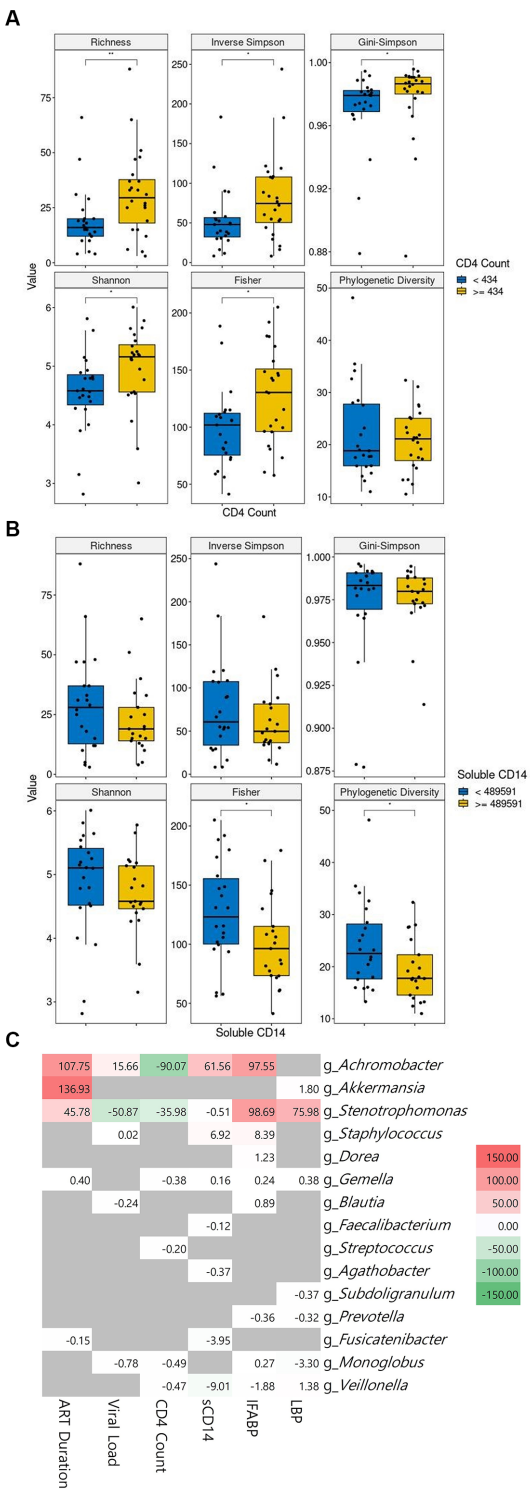


FIGURE 4
The correlation of microbiome abundance with clinical features. The diversity indices between low and high CD4⁺ cell count are shown in **(A)**. The diversity indices between low and high soluble CD14 level are shown in **(B)**. The cutoff value between high and low is the median value. CD4⁺ T-cell count is in cells/μL of blood and soluble CD4 is in pg/mL of blood. The correlation between genera and clinical features is observable in **(C)**. Statistical significance is calculated by the Wilcoxon test. $^{**}p <= 0.01$, $^{***}p <= 0.001$, $^{****}p <= 0.0001$. ART: antiretroviral, sCD14: soluble CD14, IFABP: intestinal fatty acid binding protein, LBP: lipopolysaccharide-binding protein.

pathogens (Parbie et al., 2021b). One discrepancy was that depletion of *Prevotella* in chronic HIV infection was not reported previously (Lozupone et al., 2013; Dillon et al., 2014; Mutlu et al., 2014; Vázquez-Castellanos et al., 2015). In addition, the abundance of *Dorea* and *Blautia* was not reported by the aforementioned authors. The discrepancies observed between this study and others may be explained by differences in inclusion and exclusion criteria, duration of ART treatment, sampling methods and sites, sample sizes, sequencing techniques, and statistical methods (Monaco et al., 2016). For example, the abundance of *Prevotella* is usually linked to MSM community, regardless of HIV status (Kelley et al., 2017; Armstrong et al., 2018; Vujkovic-Cvijin et al., 2020). This is in contrast to our cohort being mostly female.

Lower diversity indices in HIV positive samples have been reported before (Mutlu et al., 2014; Vázquez-Castellanos et al., 2015; Noguera-Julian et al., 2016), while some reports have disagreements (Dillon et al., 2014; Ling et al., 2016). We observed lower Shannon and Fisher indices in our PLWH cohort. These indices are related to low microbial diversity, which lead to the loss of gut integrity and increased microbial migration (Koay et al., 2018). The low microbial diversity was maintained at the second time point in this longitudinal analysis, with Faith's PD index displaying an even lower value than the first time point.

Compositional plot, on the other hand, revealed a different story. At the second time point, the microbial composition showed a partial shift similar to that of healthy Ghanaians. This included abundance rebound of the phylum *Bacteroidota*, class *Negativicutes*, and genus *Faecalibacterium* as well as depletion of genera *Achromobacter*, *Dorea*, and *Blautia*. Some genera, such as *Streptococcus*, *Staphylococcus*, and *Gemella,* did not show this reversal and maintained their high abundance.

The genera *Faecalibacterium*, *Prevotella*, and *Bacteroides* are commensals commonly found in healthy individuals and are correlated with good health (Wexler, 2007; Lopez-Siles et al., 2017; Yeoh et al., 2022). On the other hand, the genera *Achromobacter*, *Streptococcus*, and *Stenotrophomonas* are opportunistic pathogens (Gordon et al., 2002; Calza et al., 2003; Tatro et al., 2014). *Dorea* and *Blautia* from the phylum *Firmicutes* and their correlation to good health are somewhat controversial (Vacca et al., 2020). We hypothesized that the reversal may be contributed by CD4⁺ T cells and sCD14, as these two clinical markers are correlated significantly with microbial diversity. Indeed, a lower CD4⁺ T-cell count in HIV+ patients is associated with gut dysbiosis, a reduced abundance of healthy *Ruminococcaceae* bacteria in the gut, and elevated serum proinflammatory cytokine levels (Guillén et al., 2019; Lu et al., 2021). sCD14, which is a marker of monocyte activation, is indirectly correlated with microbial translocation (Marchetti et al., 2013). Lower diversity in samples with high sCD14 levels may indicate the translocation of gut microbes to the bloodstream. In accordance to this, we found that a higher CD4⁺ T-cell count and lower sCD14 levels were correlated with higher diversity indices in this cohort.

Further, the genera *Achromobacter* and *Sternotrophomonas* were found to be highly correlated with the clinical features. We found that with the improvement of the markers, the abundance of these genera was significantly reduced. Indeed, lower CD4⁺ T-cell percentages, greater CD4⁺ T-cell activation, and higher sCD14 are correlated with the immune response to *Achromobacter xylosoxidans* (Tatro et al., 2014). The repletion of CD4⁺ regulatory T cells in gut-associated

lymphoid tissue may help with the localization of these bacteria in Peyer's patches and mesenteric lymph nodes. *Sternotrophomonas maltophilia* has been shown to induce T-cell exhaustion, which may further increase T-cell apoptosis (Wang et al., 2021). It is likely that the improvement of the immune system limits the vicious cycle caused by the bacteria through an unknown mechanism. We also found that the abundance of one genus, *Akkermansia*, was very significantly correlated with the antiretroviral duration. Considering the role of these bacteria in maintaining the health of the gut mucous (Ouyang et al., 2020), the abundance of *Akkermansia* is a sign that the bacteria are working to preserve the stability of the gut microbiome during disease progression. However, *Achromobacter* and *Stenotrophomonas* were also positively correlated with antiretroviral duration, corroborating the finding that gut mucous integrity is not consistently restored despite the improvement in plasma $CD4^+$ T-cell count and reduction in anti-inflammatory cytokines (Li et al., 2016). One causing factor is $CD4^+$ T-cell depletion in the terminal ileum, including Peyer's patches, which is accompanied by irreversible fibrosis that disrupts normal gut mucosal function (Shacklett and Anton, 2010).

We also predicted the bacterial pathways likely to be enriched using prediction software. Comparison between baseline_control and follow-up_case to baseline_case reveals that samples from baseline_case likely have enrichment of pathways related to bacteria, some even exclusive to the phylum *Pseudomonadota*. The members of this phylum are known to be pathogens of the respiratory tract (*Achromobacter, Klebsiella, Pseudomonas, Sternotrophomonas*), gastrointestinal tract (*Aeromonas, Campylobacter, Escherichia, Helicobacter*), and blood (*Brevundimonas, Neisseria, Serratia, Stenotrophomonas*), among other conditions (*Citrobacter, Enterobacter, Haemophilus, Proteus*) (Leão et al., 2023). This implies that the activity of pathogenic bacteria was high in the baseline case. The activity seemed to be reduced to be similar to control subjects in the follow-up_case, although not completely.

Classifying our cohort into ART-naïve, PLWH with ART treatment <36 months, and ART treatment ≥36 months gives a deeper insight about our cohort. First, we were able to observe that the Ghanaian PLWH might have a relatively good response to therapy. Despite non-significance, $CD4^+$ T-cell count seemed on the increasing trend with a median value above WHO cutoff of 200 cells/μL. Viral load was also on the decreasing trend with a median of 3,729 copies/μL, despite non-significance. Microbial translocation markers were on the increasing trend. This suggests that despite a somewhat controlled viremia and disease progression, leaky gut was occurring. This was corroborated with lower diversity in late treatment period and on follow-up of ART-naïve subjects. LDA LEfSe showed abundance of the genus *Faecalibacterium* and class *Negativicutes* on follow-up at both <36 and ≥36 months ART treatment, showing a mixed bacterial environment. *Negativicutes* is a Gram-negative class belonging to the phylum Firmicutes that is enriched in the gut microbiota of HIV-infected subjects with $CD4^+$ T cell recovery higher than 500 cell/μL. Its abundance was positively correlated with several inflammation markers that mediate an LPS-induced immune response (Baltazar-Díaz et al., 2023).

Taken together, all of these results show that at the second time point, the gut microbial activity partially recovered but not completely, similar to that of healthy subjects. This includes the reduction and the enrichment of some genera correlated to worse and better HIV progression, respectively. This may suggest that the regiment therapy is somewhat effective in our cohort, although not optimal. Additionally, the significant correlation of *Akkermansia*, *Achromobacter*, and *Stenotrophomonas* with antiretroviral duration

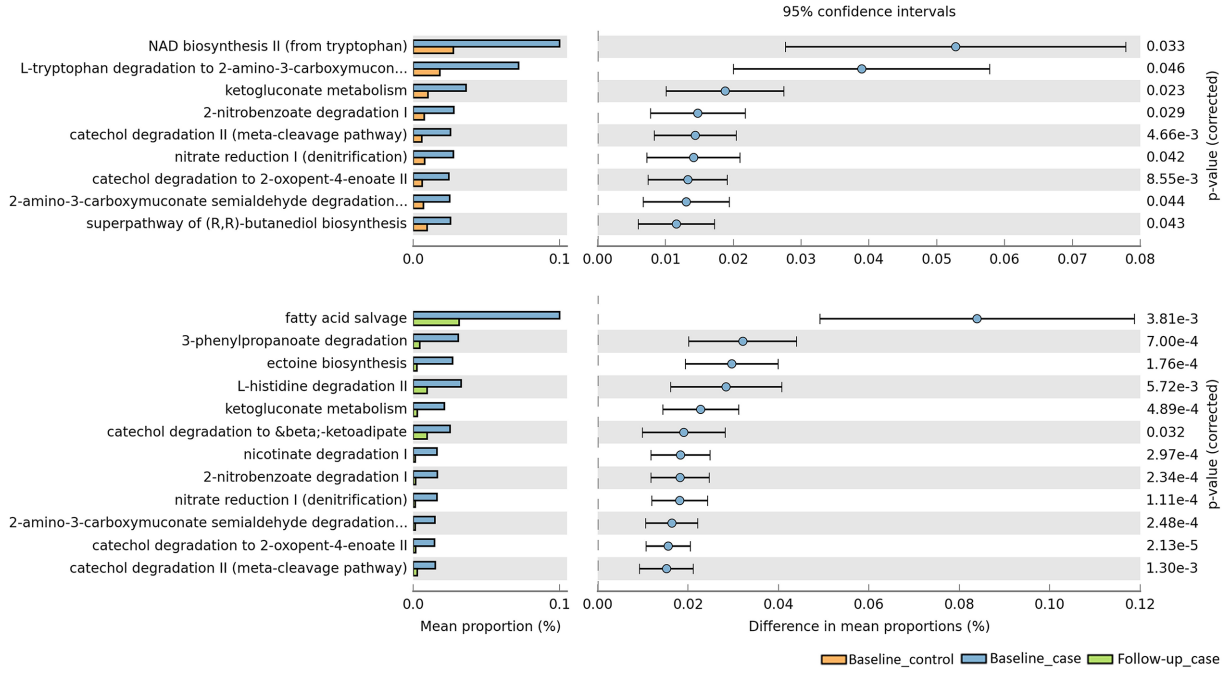


FIGURE 5
Predicted pathways upregulated/downregulated in our cohort. PICRUSt2 predicts that baseline_case samples have pathways that are significantly enriched compared to baseline_control and follow-up_case.

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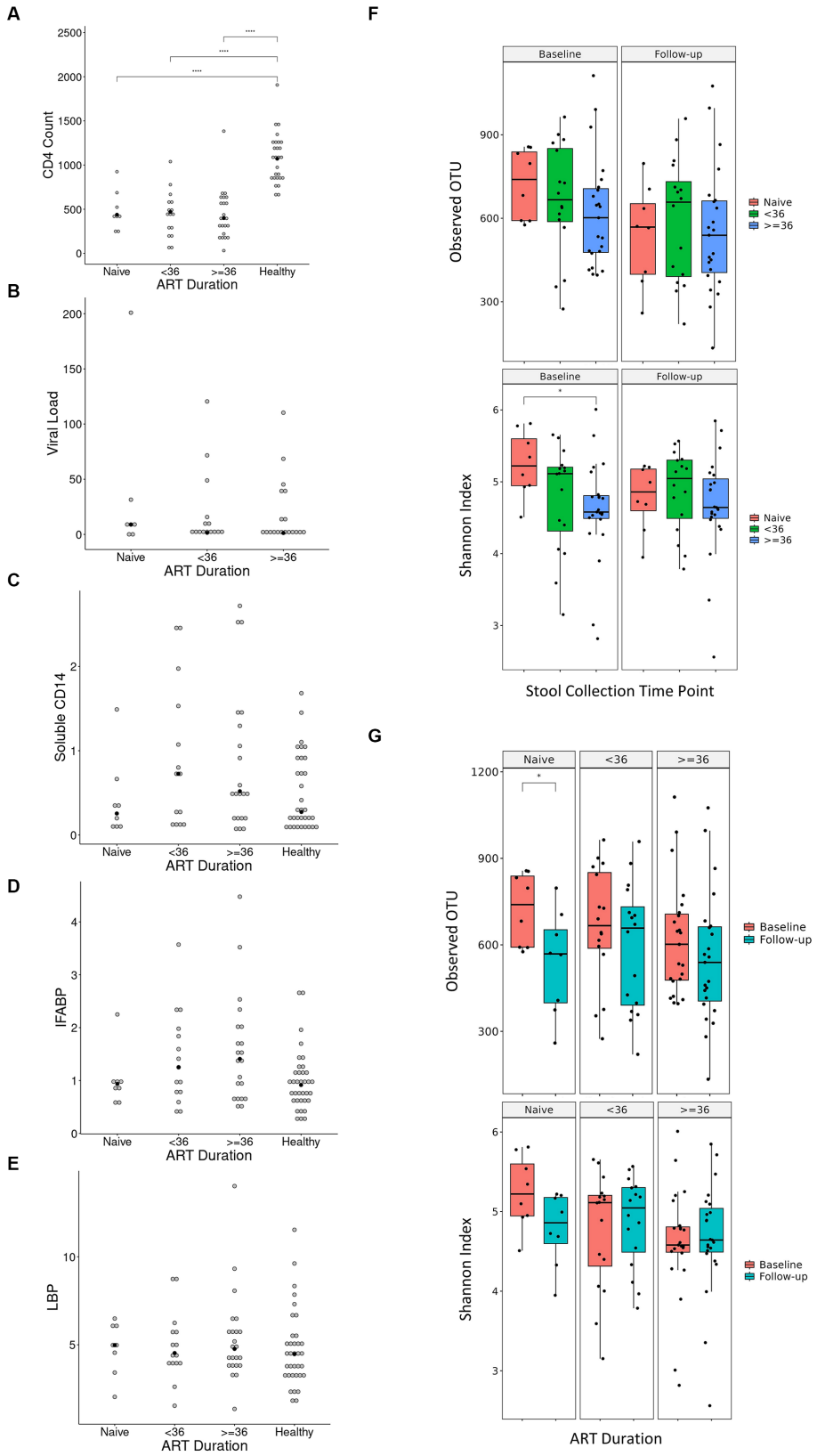


FIGURE 6
Diversity indices and clinical features in regard to treatment duration. Values of CD4⁺ T-cell count, viral load, soluble CD14, IFABP, and LBP in regard to treatment duration is shown in **(A–E)**, respectively. Antiretroviral (ART) duration is in months, CD4⁺ T-cell count is in cells/μL of blood, viral load is in copies/μL of blood, soluble CD14 is in μg/mL of blood, intestinal fatty acid binding protein (IFABP) is in ng/mL of blood, and lipopolysaccharide-binding protein (LBP) is in μg/mL of blood. Each white dot is the value of a sample while the black dots show the median value. Diversity indices in regard to duration treatment is shown in **(F,G)**. Statistical significance is calculated by the Wilcoxon test. $^{**}p <= 0.01$, $^{***}p <= 0.001$, $^{****}p <= 0.0001$.

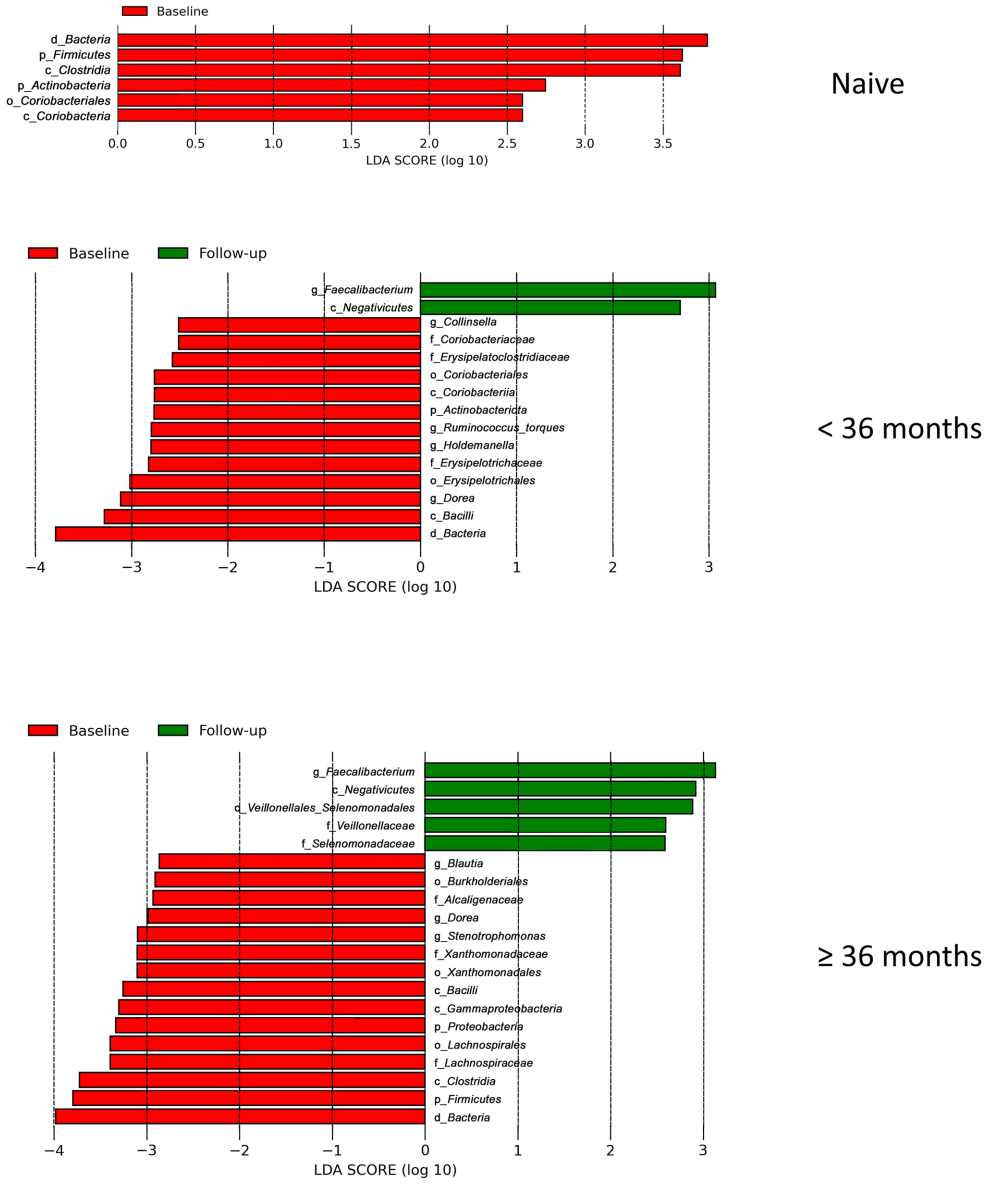


FIGURE 7
Linear discriminant analysis (LDA) effect size (LEfSe) in regard to treatment duration. LEfSe analysis between baseline_case and baseline_control and baseline_case and follow-up_case in regard to treatment duration is shown here. There is no follow-up result in naive due to its effect size is lower than the threshold 2.5.

implies that gut integrity may still be compromised despite the improvement in CD4⁺ T-cell count and sCD14 during the treatment period. However, a longer ART duration does not necessarily mean a better gut microbial environment as we also found an increasing trend of microbial translocation markers at the late treatment period which resulting in a mixed bacterial environment in the gut.

The limitation of this research is that we could only analyze the correlation of clinical features with bacterial composition at one time point (baseline_case) because we could not collect clinical information, such as CD4⁺ T-cell count and viral load from the follow-up_case samples due to logistical problems. The reason was because we could not import the required reagents for the analysis in time. Furthermore, although the viral load range is very large, but the median viral load of 3,729 copies/mL may suggest that this data may only be applicable to PLWH with relatively low viral burden. Our data are also limited to perform additional analyses of bacterial composition in high and low values of the clinical features. Another concern is we could not rule out the time of infection to further classify the PLWH as recently or chronically infected. This limits the understanding that the time to treatment or the antiretroviral itself may have a direct effect in modifying the gut microbiome. At least one study showed that nucleoside reverse-transcriptase inhibitor (NRTI)-based antiretroviral therapy has a more suppressive impact on microbiota composition and diversity in the gut (Imahashi et al., 2021). Lastly, in the publications prior to this analysis, we found a strong correlation of ART treatment with

antibiotic usage (Parbie et al., 2021b). As antibiotics have a direct effect in modifying gut microflora, we could not rule out the possibility of antibiotics reduced the diversity at late treatment period observed here.

In conclusion, we have presented here a longitudinal analysis of the gut microbiome composition in Ghanaian PLWH six months apart. We have shown that at the second time point, gut microbial activity was partially recovered, similar to that of healthy subjects, but not completely. Despite the relatively controlled disease, we found that genera correlated with both worse and better gut microbiomes were enriched and highly correlated with the duration of treatment, likely suggesting a volatile gut environment. This may support the theory that the gut microbiome does not recover despite controlled disease.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ddbj.nig.ac.jp/, DRA010770 https://www.ddbj.nig.ac.jp/, DRA017623.

Ethics statement

The studies involving humans were approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR) and the Ethical Committee of National Institute of Infectious Diseases (NIID). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LR: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. PP: Data curation, Investigation, Methodology, Writing – review & editing. TaM: Data curation, Investigation, Methodology, Supervision, Writing – review & editing. AI: Writing – review & editing. SM: Formal analysis, Writing – review & editing. CA: Data curation, Writing – review & editing. DK: Data curation, Writing – review & editing. EB: Data curation, Writing – review & editing. SO: Data curation, Writing – review & editing. HK: Writing – review & editing. KI: Data curation, Methodology, Supervision, Writing – review & editing. WA: Funding acquisition, Supervision, Writing – review & editing. TeM: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1359402/full#supplementary-material

References

Armstrong, A. J. S., Shaffer, M., Nusbacher, N. M., Griesmer, C., Fiorillo, S., Schneider, J. M., et al. (2018). An exploration of Prevotella-rich microbiomes in HIV and men who have sex with men. *Microbiome* 6, 1–16. doi: 10.1186/s40168-018-0580-7

Baltazar-Díaz, T. A., Amador-Lara, F., Andrade-Villanueva, J. F., González-Hernández, L. A., Cabrera-Silva, R. I., Sánchez-Reyes, K., et al. (2023). Gut bacterial communities in HIV-infected individuals with metabolic syndrome: effects of the therapy with integrase strand transfer inhibitor-based and protease inhibitor-based regimens. *Microorganisms* 11:951, 1–21. doi: 10.3390/microorganisms11040951

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Brito, I. L., Yilmaz, S., Huang, K., Xu, L., Jupiter, S. D., Jenkins, A. P., et al. (2016). Mobile genes in the human microbiome are structured from global to individual scales. *Nature* 535, 435–439. doi: 10.1038/nature18927

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Calza, L., Manfredi, R., and Chiodo, F. (2003). Stenotrophomonas (Xanthomonas) maltophilia as an emerging opportunistic pathogen in association with HIV infection: a 10-year surveillance study. *Infection* 31, 155–161. doi: 10.1007/s15010-003-3113-6

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., et al. (2020). The MetaCyc database of metabolic pathways and enzymes-a 2019 update. *Nucleic Acids Res.* 48, D445–D453. doi: 10.1093/nar/gkz862

Crakes, K. R., and Jiang, G. (2019). Gut microbiome alterations during HIV/SIV infection: implications for HIV cure. *Front. Microbiol.* 10, 1–10. doi: 10.3389/fmicb.2019.01104

Dandekar, S., George, M. D., and Bäumler, A. J. (2010). Th17 cells, HIV and the gut mucosal barrier. *Curr. Opin. HIV AIDS* 5, 173–178. doi: 10.1097/COH.0b013e328335eda3

de Filippo, C., Cavalieri, D., di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S. A.* 107, 14691–14696. doi: 10.1073/pnas.1005963107

Dillon, S. M., Lee, E. J., Kotter, C. V., Austin, G. L., Dong, Z., Hecht, D. K., et al. (2014). An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. *Mucosal Immunol.* 7, 983–994. doi: 10.1038/mi.2013.116

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Estes, J. D., Harris, L. D., Klatt, N. R., Tabb, B., Pittaluga, S., Paiardini, M., et al. (2010). Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. *PLoS Pathog.* 6, 49–50. doi: 10.1371/journal.ppat.1001052

Gordon, S. B., Chaponda, M., Walsh, A. L., Whitty, C. J. M., Gordon, M. A., Machili, C. E., et al. (2002). Pneumococcal disease in HIV-infected Malawian adults: acute mortality and long-term survival. *AIDS* 16, 1409–1417. doi: 10.1097/00002030-200207050-00013

Guadalupe, M., Reay, E., Sankaran, S., Prindiville, T., Flamm, J., McNeil, A., et al. (2003). Severe CD4+ T-cell depletion in gut lymphoid tissue during primary HIV-1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J. Virol.* 77, 11708–11717. doi: 10.1128/JVI.77.21.11708-11717.2003

Guillén, Y., Noguera-Julian, M., Rivera, J., Casadellà, M., Zevin, A. S., Rocafort, M., et al. (2019). Low nadir CD4+ T-cell counts predict gut dysbiosis in HIV-1 infection. *Mucosal Immunol.* 12, 232–246. doi: 10.1038/s41385-018-0083-7

He, Y., Wu, W., Zheng, H. M., Li, P., McDonald, D., Sheng, H. F., et al. (2018). Regional variation limits applications of healthy gut microbiome reference ranges and disease models. *Nat. Med.* 24, 1532–1535. doi: 10.1038/s41591-018-0164-x

HIV.gov. *The global HIV/AIDS epidemic*. (2022). Available at: https://www.hiv.gov/hiv-basics/overview/data-and-trends/global-statistics/. (Accessed: July 16, 2023).

Imahashi, M., Ode, H., Kobayashi, A., Nemoto, M., Matsuda, M., Hashiba, C., et al. (2021). Impact of long term antiretroviral therapy on gut and oral microbiotas in HIV-1-infected patients. *Sci. Rep.* 11, 1–10. doi: 10.1038/s41598-020-80247-8

Ishizaka, A., Koga, M., Mizutani, T., Parbie, P. C., Prawisuda, D., Yusa, N., et al. (2021). Unique gut microbiome in HIV patients on antiretroviral therapy (ART) suggests association with chronic inflammation. *Microbiol. Spectr.* 9, e00708–e00721. doi: 10.1128/Spectrum.00708-21

Kassambara, A. ggpubr: 'ggplot2' Based Publication Ready Plots. (2023).

Kelley, C. F., Kraft, C. S., de Man, T. J. B., Duphare, C., Lee, H. W., Yang, J., et al. (2017). The rectal mucosa and condomless receptive anal intercourse in HIV-negative MSM: implications for HIV transmission and prevention. *Mucosal Immunol.* 10, 996–1007. doi: 10.1038/mi.2016.97

Khan, S., Telwatte, S., Trapecar, M., Yukl, S., and Sanjabi, S. (2017). Differentiating immune cell targets in gut-associated lymphoid tissue for HIV cure. *AIDS Res. Hum. Retrovir.* 33, S-40–S-58. doi: 10.1089/aid.2017.0153

Kim, S. W., Suda, W., Kim, S., Oshima, K., Fukuda, S., Ohno, H., et al. (2013). Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. *DNA Res.* 20, 241–253. doi: 10.1093/dnares/dst006

Koay, W. L. A., Siems, L. V., and Persaud, D. (2018). The microbiome and HIV persistence: implications for viral remission and cure. *Curr. Opin. HIV AIDS* 13, 61–68. doi: 10.1097/COH.0000000000000434

Leão, I., de Carvalho, T. B., Henriques, V., Ferreira, C., Sampaio-Maia, B., and Manaia, C. M. (2023). Pseudomonadota in the oral cavity: a glimpse into the environment-human nexus. *Appl. Microbiol. Biotechnol.* 107, 517–534. doi: 10.1007/s00253-022-12333-y

Li, S. X., Armstrong, A. J. S., Neff, C. P., Shaffer, M., Lozupone, C. A., and Palmer, B. E. (2016). Complexities of gut microbiome dysbiosis in the context of HIV infection and antiretroviral therapy. *Clin. Pharmacol. Ther.* 99, 600–611. doi: 10.1002/cpt.363

Ling, Z., Jin, C., Xie, T., Cheng, Y., Li, L., and Wu, N. (2016). Alterations in the fecal microbiota of patients with HIV-1 infection: an observational study in a Chinese population. *Sci. Rep.* 6:30673. doi: 10.1038/srep30673

Lopez-Siles, M., Duncan, S. H., Garcia-Gil, L. J., and Martinez-Medina, M. (2017). *Faecalibacterium prausnitzii*: from microbiology to diagnostics and prognostics. *ISME J.* 11, 841–852. doi: 10.1038/ismej.2016.176

Lozupone, C. A., Li, M., Campbell, T. B., Flores, S. C., Linderman, D., Gebert, M. J., et al. (2013). Alterations in the gut microbiota associated with HIV-1 infection. *Cell Host Microbe* 14, 329–339. doi: 10.1016/j.chom.2013.08.006

Lu, D., Zhang, J., Wang, Y., Geng, S., Zhang, Z., Xu, Y., et al. (2021). Association between CD4+ T cell counts and gut microbiota and serum cytokines levels in HIV-infected immunological non-responders. *BMC Infect. Dis.* 21, 1–11. doi: 10.1186/s12879-021-06491-z

Ma, S., Shungin, D., Mallick, H., Schirmer, M., Nguyen, L. H., Kolde, R., et al. (2022). Population structure discovery in meta-analyzed microbial communities and inflammatory bowel disease using MMUPHin. *Genome Biol.* 23, 1–31. doi: 10.1186/s13059-022-02753-4

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., et al. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput. Biol.* 17, 1–27. doi: 10.1371/journal.pcbi.1009442

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., Peddada, S. D., et al. (2015). Analysis of composition of microbiomes, a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26, 1–7. doi: 10.3402/mehd.v26.27663

Marchetti, G., Tincati, C., and Silvestri, G. (2013). Microbial translocation in the pathogenesis of HIV infection and AIDS. *Clin. Microbiol. Rev.* 26, 2–18. doi: 10.1128/CMR.00050-12

McHardy, I. H., Li, X., Tong, M., Ruegger, P., Jacobs, J., Borneman, J., et al. (2013). HIV infection is associated with compositional and functional shifts in the rectal mucosal microbiota. *Microbiome* 1, 1–12. doi: 10.1186/2049-2618-1-26

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Monaco, C. L., Gootenberg, D. B., Zhao, G., Handley, S. A., Ghebremichael, M. S., Lim, E. S., et al. (2016). Altered virome and bacterial microbiome in human immunodeficiency virus-associated acquired immunodeficiency syndrome. *Cell Host Microbe* 19, 311–322. doi: 10.1016/j.chom.2016.02.011

Mutlu, E. A., Keshavarzian, A., Losurdo, J., Swanson, G., Siewe, B., Forsyth, C., et al. (2014). A compositional look at the human gastrointestinal microbiome and immune activation parameters in HIV infected subjects. *PLoS Pathog.* 10:e1003829. doi: 10.1371/journal.ppat.1003829

Noguera-Julian, M., Rocafort, M., Guillén, Y., Rivera, J., Casadellà, M., Nowak, P., et al. (2016). Gut microbiota linked to sexual preference and HIV infection. *EBioMedicine* 5, 135–146. doi: 10.1016/j.ebiom.2016.01.032

Ouyang, J., Lin, J., Isnard, S., Fombuena, B., Peng, X., Marette, A., et al. (2020). The bacterium *Akkermansia muciniphila*: a sentinel for gut permeability and its relevance to HIV-related inflammation. *Front. Immunol.* 11, 1–9. doi: 10.3389/fimmu.2020.00645

Parbie, P. K., Mizutani, T., Ishizaka, A., Kawana-Tachikawa, A., Runtuwene, L. R., Seki, S., et al. (2021a). Fecal microbiome composition in healthy adults in Ghana. *Jpn. J. Infect. Dis.* 74, 42–47. doi: 10.7883/yoken.JJID.2020.469

Parbie, P. K., Mizutani, T., Ishizaka, A., Kawana-Tachikawa, A., Runtuwene, L. R., Seki, S., et al. (2021b). Dysbiotic fecal microbiome in HIV-1 infected individuals in Ghana. *Front. Cell. Infect. Microbiol.* 11, 1–13. doi: 10.3389/fcimb.2021.646467

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi: 10.1093/bioinformatics/btu494

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, 590–596. doi: 10.1093/nar/gks1219

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shacklett, B. L., and Anton, P. A. (2010). HIV infection and gut mucosal immune function: updates on pathogenesis with implications for management and intervention. *Curr. Infect. Dis. Rep.* 12, 19–27. doi: 10.1007/s11908-009-0072-9

Tatro, E. T., Purnajo, I., Richman, D. D., Smith, D. M., and Gianella, S. (2014). Antibody response to Achromobacter xylosoxidans during HIV infection is associated with lower CD4 levels and increased lymphocyte activation. *Clin. Vaccine Immunol.* 21, 46–50. doi: 10.1128/CVI.00553-13

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., and de Angelis, M. (2020). The controversial role of human gut Lachnospiraceae. *Microorganisms* 8, 1–25. doi: 10.3390/microorganisms8040573

Vázquez-Castellanos, J. F., Serrano-Villar, S., Latorre, A., Artacho, A., Ferrús, M. L., Madrid, N., et al. (2015). Altered metabolism of gut microbiota contributes to chronic

immune activation in HIV-infected individuals. *Mucosal Immunol.* 8, 760–772. doi: 10.1038/mi.2014.107

Vujkovic-Cvijin, I., Dunham, R. M., Iwai, S., Maher, M. C., and Albright, R. G. (2013). Dysbiosis of the gut microbiota is associated with HIV disease progression and tryptophan catabolism. *Sci. Transl. Med.* 5, 1–7. doi: 10.1126/scitranslmed.3006438

Vujkovic-Cvijin, I., Sortino, O., Verheij, E., Sklar, J., Wit, F. W., Kootstra, N. A., et al. (2020). HIV-associated gut dysbiosis is independent of sexual practice and correlates with noncommunicable diseases. *Nat. Commun.* 11:2448. doi: 10.1038/s41467-020-16222-8

Wallet, M. A., Rodriguez, C. A., Yin, L., Saporta, S., Chinratanapisit, S., Hou, W., et al. (2010). Microbial translocation induces persistent macrophage activation unrelated to HIV-1 levels or T cell activation following therapy. *AIDS* 24, 1281–1290. doi: 10.1097/QAD.0b013e328339e228

Wang, M., Yin, S., Qin, Q., Peng, Y., Hu, Z., Zhu, X., et al. (2021). Stenotrophomonas-maltophilia inhibits host cellular immunity by activating PD-1/PD-L1 signaling pathway to induce T-cell exhaustion. *Mol. Immunol.* 130, 37–48. doi: 10.1016/j.molimm.2020.12.019

Wexler, H. M. (2007). Bacteroides: the good, the bad, and the nitty-gritty. *Clin. Microbiol. Rev.* 20, 593–621. doi: 10.1128/CMR.00008-07

Yeoh, Y. K., Sun, Y., Ip, L. Y. T., Wang, L., Chan, F. K. L., Miao, Y., et al. (2022). Prevotella species in the human gut is primarily comprised of *Prevotella copri*, Prevotella stercorea and related lineages. *Sci. Rep.* 12, 1–9. doi: 10.1038/s41598-022-12721-4

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Hospital antimicrobial stewardship: profiling the oral microbiome after exposure to COVID-19 and antibiotics

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Introduction: During the COVID-19 Delta variant surge, the CLAIRE cross-sectional study sampled saliva from 120 hospitalized patients, 116 of whom had a positive COVID-19 PCR test. Patients received antibiotics upon admission due to possible secondary bacterial infections, with patients at risk of sepsis receiving broad-spectrum antibiotics (BSA).

Methods: The saliva samples were analyzed with shotgun DNA metagenomics and respiratory RNA virome sequencing. Medical records for the period of hospitalization were obtained for all patients. Once hospitalization outcomes were known, patients were classified based on their COVID-19 disease severity and the antibiotics they received.

Results: Our study reveals that BSA regimens differentially impacted the human salivary microbiome and disease progression. 12 patients died and all of them received BSA. Significant associations were found between the composition of the COVID-19 saliva microbiome and BSA use, between SARS-CoV-2 genome coverage and severity of disease. We also found significant associations between the non-bacterial microbiome and severity of disease, with *Candida albicans* detected most frequently in critical patients. For patients who did not receive BSA before saliva sampling, our study suggests *Staphylococcus aureus* as a potential risk factor for sepsis.

Discussion: Our results indicate that the course of the infection may be explained by both monitoring antibiotic treatment and profiling a patient's salivary microbiome, establishing a compelling link between microbiome and the specific antibiotic type and timing of treatment. This approach can aid with emergency room triage and inpatient management but also requires a better understanding of and access to narrow-spectrum agents that target pathogenic bacteria.

KEYWORDS

broad-spectrum antibiotics, COVID-19, sepsis, saliva microbiome, *Candida albicans* (*C. albicans*), *Staphylococcus aureus* (*S. aureus*), hospital antimicrobial stewardship

Introduction

The oral cavity is one of the entry points in the body for SARS-CoV-2. SARS-CoV-2 is known to bind to ACE2 receptors which are highly expressed in the human oral tissue that comprise the gingiva, tongue, and palate (Okui et al., 2021). Studies have shown that dysbiosis of the local airway microbiome induced by SARS-CoV-2 initially occurs in the oral cavity and subsequently impacts distant microbiomes across connected body sites via the oral-lung or oral-gut axis (Bao et al., 2020; Xu et al., 2020; Hazan et al., 2022). A 2021 study found that oropharyngeal microbiota alterations were associated with COVID-19 severity (Ma et al., 2021). As human saliva contains a large amount of human DNA and RNA compared to stool and other specimens (Armstrong et al., 2021), analysis of its microorganisms has received less attention. A recent COVID-19 saliva microbiome study focused on methods to deplete human DNA while preserving the microbiome composition of samples (Armstrong et al., 2021). Moreover, during the COVID-19 pandemic, a preference was to use nasopharyngeal swabs for PCR testing to detect SARS-CoV-2, and most COVID-19 oral microbiome studies have used swabs instead of saliva as a consequence.

A common practice during the COVID-19 outbreak was to treat patients admitted to the hospital for a possible concomitant bacterial pneumonia, at least for a few days until additional test results excluded bacterial pneumonia. In addition, broad-spectrum antibiotics (BSA) were prescribed for patients diagnosed with or at risk of sepsis or septic shock. While many COVID-19 studies have focused on epidemiological associations of SARS-CoV-2 (Rahnavard et al., 2021) and dynamics of microbiome during infection, CLAIRE (CLuster AI pREdiction of disease progression) is the only study to investigate the COVID-19 saliva microbiome in hospitalized patients with a focus on sepsis and BSA (see section "Medical records and outcome analysis"). The SARS-CoV-2 virus was characterized through Illumina's Respiratory Viral Oligo Panel (RVOP) sequencing (Illumina). Our study includes a comparison with 5 healthy subjects from a saliva metagenomic study (Armstrong et al., 2021). We selected saliva as a biospecimen as it is easy to collect at any time compared to stool samples and has not been studied enough in the context of infectious disease and sepsis. We were able to validate our hypothesis that the saliva microbiome can be used as a biomarker for infection disease severity with suggested guidelines on how to incorporate the effect and timing of broad-spectrum antibiotics into a prediction model.

Materials and methods

Patient enrollment

The inclusion criteria for our study were patients at age 18 years and older and admitted to the University of Miami (UM) hospital with a clinically diagnosed COVID-19. COVID-19 infection was determined using one of 3 diagnostics (SARS-CoV-2 NAAT, COVID-19 RT PCR, COVID-19 ACCULA NAAT), all of which can be classified to be at least 95% accurate. Upon obtaining informed consent, participants were asked to provide a one-time saliva sample. The study was approved by the University of Miami IRB ID: 20200724.

Study design and sample collection

Saliva samples were collected after patient enrollment. Instructions to patients were not to eat, drink, smoke, or chew gum for 15 min before giving a sample. The Zymo R1210-E–DNA/RNA Shield Saliva Collection Kit–DX, a 510(k)-Cleared FDA Class II Medical Device, was used to collect the saliva in virus inactivating and preserving DNA/RNA Shield buffer, allowing storage at room temperature for up to 3 weeks. Samples were batched and stored at room temperature and shipped in ice every 2–2.5 weeks to the George Washington University Genomics Core for storage at −20 degrees prior to sequencing. **Supplementary Figure 1** illustrates the study workflow.

Medical records and outcome analysis

Medical records were collected for all patients (**Table 1**). Per hospital COVID-19 management protocol, all patients were started on ceftriaxone and azithromycin when they were admitted to the hospital with clinical suspicion of COVID-19. Patients may have received additional antibiotics if the hospital team deemed it necessary (e.g., doxycycline hyclate used to treat 6 patients). Also, per hospital protocol, patients who met the criteria for sepsis were treated with broad-spectrum sepsis antibiotics (BSAs), specifically vancomycin and one of the following: cefepime, meropenem, or zosyn (piperacillin/tazobactam) (**Table 2**).

Medical records identified 4 patients with a false positive diagnosis of COVID-19 as they had negative COVID-19 PCR tests. One was reclassified and another was added as a false positive. SARS-CoV-2 molecular test results were retrieved from the medical records. For this study, we used the first measurement taken upon admission.

Sepsis was diagnosed with the following elements: 3 main criteria with other sub-criteria. Sepsis is diagnosed if there is (1) clinical suspicion of infection, and (2) either (a) (SBP $<$ 90, MAP $<$ 65, or decrease in SBP $>$ 40 from baseline), or (b) evidence of $>$ 1 organ dysfunction, Creatinine $>$ 2, TBili $>$ 2, PLT $<$ 100, Lactate $>$ 2, IR $>$ 1.5, PTT $>$ 60, and (3) at least 2 of these are present: (a) Temp $<$ 96.8°F (36°C) or $>$ 100.9°F (39.30°C), (b) HR $>$ 90, (c) RR $>$ 20 or PaCO2 $<$ 32, (d) WBC $>$ 12K, or $<$ 4K, or $>$ 10% Bands.

The severity of the COVID-19 disease was categorized using the Adaptive COVID-19 Treatment Trial (ACTT) COVID-19 Stratification Status (**Table 3**) and does not consider viral load as the study sought to investigate whether a one-time saliva sample during admission with ensuing microbiome analysis can predict disease severity. This severity score is based on the trend of the ACTT score, such as improvement or worsening, considering five assessment dates, and considering the treatment outcome (death or recovery). Five assessment dates were considered if the duration of hospitalization covered those dates: Baseline (day of admission), days 5, 15, and 30 after admission, and the day of discharge or expiration. The score was determined for each recruited patient after the outcome of the hospitalization was known.

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TABLE 1 Patients' characteristics.

	Critical (*n* = 25)	Moderate (*n* = 95)		Critical (*n* = 25)	Moderate (*n* = 95)
Age			**BMI**		
Median (range)	60 (34–91)	54 (19–86)	< 18.5 (underweight)	0 (0%)	3 (3.16%)
0–19 (%)	0 (0%)	1 (1.05%)	18.5–24.9 (normal weight)	1 (4.00%)	14 (14.74%)
20–29 (%)	0 (0%)	5 (5.26%)	25–29.9 (overweight)	5 (20.00%)	22 (23.16%)
30–39 (%)	3 (12.00%)	17 (17.89%)	30–34.9 (obesity Class I)	7 (28.00%)	25 (26.32%)
40–49 (%)	3 (12.00%)	15 (15.79%)	35–39.9 (obesity Class II)	7 (28.00%)	19 (20.00%)
50–59 (%)	6 (24.00%)	23 (24.21%)	> 40 (obesity Class III)	4 (16.00%)	11 (11.58%)
60–69 (%)	7 (28.00%)	16 (16.84%)	Unknown	1 (4.00%)	1 (1.05%)
70–79 (%)	2 (8.00%)	14 (14.74%)	**Comorbidities**		
80–100 (%)	4 (16.00%)	4 (4.21%)	Diabetic	12 (48.00%)	28 (29.47%)
Sex			CKD	6 (24.00%)	13 (13.68%)
Female	13 (52.00%)	44 (46.32%)	COPD	5 (20.00%)	14 (14.74%)
Male	12 (48.00%)	51 (53.68%)	Cancer	5 (20.00%)	23 (24.21%)
Ethnicity			HD	14 (56.00%)	29 (30.53%)
Hispanic or Latino	15 (60.00%)	49 (51.58%)	Obesity	7 (28.00%)	29 (30.53%)
African American	1 (4.00%)	1 (1.05%)	**Blood result median (range)**		
White	13 (52.00%)	45 (47.37%)	Leukocyte count (×10^3/μl, 4.00–10.50)	7.83 (3.04–18.63)	6.92 (0.45–15.25)
Other or unknown	1 (4.00%)	3 (3.16%)	Platelets (×10^3/μl, 140–400)	225.96 (113.5–397.5)	231.97 (11–611.75)
Non-Hispanic or Latino	9 (36.00%)	44 (46.32%)	Hemoglobin (g/dL, male = 13.3–16.3, female = 11.1–14.6)	12.59 (8.325–15.55)	11.87 (6.77–15.5)
African American	8 (32.00%)	28 (29.47%)	C-reactive protein (mg/dL, 0–0.5)*	9.88 (0.93–29.5)	6.88 (0.3–31.2)
White	1 (4.00%)	15 (15.79%)	Alanine aminotransferase (U/L, male = 0–41, female = 0–33)	42.02 (13.33–115.33)	53.29 (7.5–323.33)
Other or unknown	0 (0%)	1 (1.05%)	Lactose dehydrogenase (U/L, male = 135–225, female = 135–214)*	456.44 (199.67–720)	385.85 (142–1,578.5)
Ethnicity unknown	1 (4.00%)	2 (2.11%)	D-dimer (ug/ml, < 0.50)	2.06 (0.4–10.8)	2.4 (0.3–18.45)
African American	0 (0%)	2 (2.11%)	NLR (ratio of neutrophils to lymphocyte)*	7.37 (1.04–19.9)	6.65 (0.63–81.74)
Other or unknown	1 (4.00%)	0 (0%)	LMR (ratio of lymphocyte to monocyte)	2.46 (0.75–7.5)	3.1 (0.54–34.5)
			WBC count (×10^3/μl, 4–10.50)	7.83 (18.63–2.04)	6.93 (15.25–0.45)

*Indicates a significant difference between the 2 severity groups.

Extraction and sequencing

The saliva samples were sequenced with two protocols: (1) shotgun DNA metagenomics (SDM) (Illumina), and (2) Illumina Respiratory Viral Oligo Panel (RVOP) sequencing (Illumina). DNA/RNA extraction was performed with the ZymoBIOMICS DNA/RNA Miniprep Kit. Quality control of SDM and RVOP libraries was performed using a Thermo Fisher Qubit 3.0 High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer High Sensitivity DNA kit.

For the SDM, libraries were prepared, along with a ZymoBIOMICS Microbial Community DNA standard, using a Nextera XT DNA Library Prep kit following Illumina's recommended guidelines (Illumina). Libraries were sequenced as paired-end, 2×150 bp, using a NextSeq 500 High-Output kit, with a 1% phi X sequencing control spike-in.

For the RVOP, libraries were prepared using an Illumina RNA Prep with Enrichment kit and enriched using the Respiratory Virus Oligos Panel v2 at 3-plex. Libraries were normalized by mass for three-plex enrichment but were not normalized by viral load as the viral copy number of each sample was unknown (Illumina).

TABLE 2 Broad-spectrum sepsis antibiotics (BSA) treatment categorization.

	Critical (*n* = 25)	Moderate (*n* = 95)	Survived (*n* = 108)	Expired (*n* = 12)
Early broad-spectrum sepsis antibiotics (early BSA)*				
Before sampling	2 (8.00%)	18 (18.95%)	20 (18.52%)	0 (0%)
Same-day of sampling	1 (4.00%)	7 (7.37%)	8 (7.4%)	1 (8.33%)
No early broad-spectrum sepsis antibiotics (no early BSA)*				
After sampling	19 (76.00%)	6 (6.32%)	14 (12.96%)	11 (91.67%)
Never prescribed	3 (12.00%)	64 (67.37%)	67 (62.04%)	0 (0%)

*BSA treatment categorization with two main groups based on the timing of sepsis diagnosis before or after saliva sampling. The "Early BSA" group was split into before and same-day saliva sampling. The "No early BSA" group was split into BSA's "After Sampling" and "Never Prescribed". BSA treatment: vancomycin (glycopeptide), cefepime (cephalosporin), meropenem (carbapenem), zosyn (beta-lactamase inhibitor).

TABLE 3 Classification of patients into two severity groups: critical or moderate.

ACTT code	Description
1	Not hospitalized, no limitations on activities
2	Not hospitalized, limitation on activities, and/or requiring home oxygen
3	Hospitalized, not requiring supplemental oxygen–no longer requires ongoing medical care
4	Hospitalized, not requiring supplemental oxygen–requiring ongoing medical care (COVID-19 related or otherwise)
5	Hospitalized, requiring supplemental oxygen
6	Hospitalized, on non-invasive ventilation or high flow oxygen devices
7	Hospitalized, on invasive mechanical ventilation or ECMO
8	Death during Study
Severity group	**Criteria**
Critical	Expired, or ACTT Status on Day 5 $\geq$ 5, and the patient deteriorated in any of the assessment dates 5, 15, or 30 days after admission
Moderate	Hospitalized but not categorized as critical

Libraries were sequenced as paired-end, 2×75 bp, using a NextSeq 500 Mid-Output kit, with a 1% phi X sequencing control spike-in.

Bioinformatics analyses

Shotgun DNA metagenomics (SDM) reads were analyzed with 3 bioinformatics pipelines, Biobakery (Beghini et al., 2021), Kraken/Bracken (Wood et al., 2019), and Pathoscope (Hong et al., 2014). Biobakery KneadData was used for quality control on the SDM reads. RVOP reads were also analyzed with 3 bioinformatics pipelines: Basespace DRAGEN (Illumina), Kraken (Wood et al., 2019), and Nextflow's nf-core/viralrecon (Ewels et al., 2020).

MetaPhlAn v3.0.7 with database vJan21 (Truong et al., 2015; Nousias and Montesanto, 2021) was run through Biobakery for profiling the composition of bacteria communities from the metagenomic data. Results presented here were run with a confidence parameter of –stat_q 0.1. Kraken and Bracken were run on the same sequence data for metagenomic profiling which includes DNA from viruses, fungi, parasites, and archaea. Results presented here were run with Kraken parameter –confidence 0.1.

HUMAnN2 (Franzosa et al., 2018) was run through Biobakery to profile the abundance of microbial metabolic pathways and other molecular functions from the metagenomics data. Tweedieverse (Mallick et al., 2022) and MaAsLin2 (Mallick et al., 2021) were used for testing statistical associations with microbiome data, to compare community total abundances from MetaPhlAn or HUMAnN2 output with sample metadata. These approaches perform multiple tests with False Discovery Rate Correction using the Benjamini–Hochburg approach. MaAsLin2 uses linear models, and Tweedieverse uses Tweedie models to fit models and test hypotheses for microbial species.

MetagenomeSeq and DESeq2 tools were run within MicrobiomeAnalystR or the online MicrobiomeAnalyst (Chong et al., 2020). The software CARD's Resistance Gene Identifier (RGI) 5.2.1 with the CARD database 3.2.0 (Alcock et al., 2020) was run with –include_wildcard and –aligner bowtie2 settings on the DNA shotgun metagenomic data to predict antimicrobial resistance phenotypes. CARD combines the antibiotic resistance ontology (ARO) with curated antimicrobial resistance (AMR) gene sequences and resistance-conferring mutations to provide an informatics framework for annotation and interpretation of resistomes (Alcock et al., 2020). Nextflow's nf-core/viralrecon was run with default parameters on 83 RVOP samples that had enough viral reads. Basespace DRAGEN and Kraken were run on the RVOP reads to establish SARS-CoV-2 genome coverage.

The healthy subjects (controls) included from Armstrong et al. (2021) were health workers considered in good health and were negative for SARS-CoV-2 infection, based on concurrent qRT-PCR testing. We selected MetaPhlAn results from 5 subjects from the first sampling whose saliva did not undergo human depletion which we merged with our MetaPhlAn data and analyzed with MaAsLin using batch as random effect.

Results

Host clinical characteristics

A total of 120 patients were recruited for the CLAIRE study over a period of 1.5 months to provide a sample of saliva. With the exception of 8 patients whose saliva was sampled after day 3 upon hospital admission, saliva was sampled 3 days or less after admission to verify the hypothesis that an early saliva sample is predictive of disease progression. Four out of the 120 patients had

a false positive COVID-19 test and 116 were confirmed to have COVID-19. Initially, the medical chart identified 4 false positive patients with a negative COVID-19 clinical diagnosis (see "Patient enrollment"). One false negative and one false positive patient were recategorized through virome analysis and clinical chart review, which resulted in 4 "False Positives" (FP) for subsequent analysis. The clinical and demographic characteristics of the patients are depicted in Table 1. The median age for the critical cohort was 60 (range 34–91) and for the moderate cohort 54 (range 19–86). When looking at COVID-19 risk factors other than age, based on past medical history, 43 patients had diabetes type II (12 critical, 28 moderate), 19 patients had chronic kidney disease, 19 patients had chronic obstructive pulmonary disease, 28 patients had or have cancer, and 43 patients had cardiovascular disease. A majority of the patients were Hispanic or Latino, with a 60%/52% breakdown of critical/moderate disease progression.

Correlation tests have been performed to identify collinearity among patient clinical information (metadata). To combine all results, $-\log(p\text{-value})$ of features correlation was calculated with three different tests based on data types (Kruskal–Wallis, Spearman, Chi-Squared, Supplementary Figure 2). A table with p-values for the correlations can be found in Supplementary Table 1. Most significant pairwise correlations are obvious, e.g., race and ethnicity. Of most interest, is the significant correlation between the SARS-CoV-2 genome coverage and severity of disease, discussed in the next section.

Of the 12 patients who died, all of them (100%) received broad-spectrum sepsis antibiotics (BSA) during their hospitalization, resulting in a statistically significant association between sepsis treatment and mortality ($p < 0.0001$, Fisher exact test). 4 BSA subgroups were selected based on the timing of the BSA treatment to understand its effects on the use and timing of saliva collection as a biomarker of disease progression. We therefore stratified the 120 patients into 2 BSA groups, each with 2 subgroups, and into two severity groups, with 95 (79%) subjects classified as being moderate and 25 (21%) subjects classified as being critical (see Table 2 and Table 3). See Supplementary Table 2 for only the 4 FPs' outcomes.

Several clinical features were found to be significantly different between critical and moderate groups, among them C-reactive protein (CRP) values being lower for the moderate group when compared to the critical group (Mann–Whitney U test, $U = 562.5$, $p = 0.030$), which has been confirmed previously using proteomics (Rahnavard et al., 2022). Similarly, we observed significant associations for lactose dehydrogenase (LDH) being lower in the moderate group (Mann–Whitney U test, $U = 470.5$, $p = 0.026$), and the ratio of neutrophils to lymphocyte (NLR) also being lower in the moderate group (Mann–Whitney U test, $U = 741.0$, $p = 0.037$). All three (CRP, LDH, and NLR) are known COVID-19 biomarkers (Gogate et al., 2021).

Disease severity association with SARS-CoV-2 genome coverage and variants

We found a significant association between percentage of SARS-CoV-2 genome coverage and severity of disease (Mann–Whitney U test, $U = 638$, $p = 0.00168$). A total of 8 samples with a low number of total reads for the RVOP sequencing (< 200,000) were removed from consideration because RVOP, which relies on an oligo-capture approach with very short paired 76 bp reads, has been found to be less reliable with low viral loads and low read counts (Lam et al., 2021). 83 samples had enough genome coverage to be categorized as a variant, of those, 61 were categorized as Delta 21J (Nextclade clade) with the most frequent lineage being AY.25.1, a predominant Canada and USA lineage. 1 patient variant was categorized as Mu 21H, a Colombia variant, and 1 patient as Gamma, V3 20J, a Brazilian/Manaus variant (see Supplementary Table 3). The 2 patients with non-Delta variants had moderate disease progression.

Microbiome results

Shotgun metagenomics generated an average of 4 million reads/sample. Quality control with the Biobakery KneadData tool removed an average of 1.5 million low-quality reads/sample, filtering out an average of 2 million human reads/sample using the hg38 human genome as a host reference (Supplementary Table 4). The Biobakery MetaPhlAn tool identified an average of 700,000 bacterial reads/sample and 332 species from which diversity estimates were made (Figure 1). The Kraken/Bracken combination of tools (confidence level 0.1) identified 2264 bacterial species, 123 DNA virus species, and 60 fungi and parasite species from the 120 CLAIRE patient oral samples.

Figures 1A, B show alpha- and beta-diversity at the species level after stratification into 4 groups: moderate with early BSA treatment (M-Yes), Moderate with no early BSA treatment (M-No), Critical with early BSA treatment (C-Yes), Critical with no early BSA treatment (C-No), where "early" was defined as the treatment being initiated before or on the same day as saliva sampling. Alpha-diversity analysis, as shown in Figure 1A, returned a significant result ($p = 2.0337e-5$), as did the beta-diversity analysis ($p = 0.016$), shown in Figure 1B. The taxa abundance plot in Figure 1C shows the abundance of bacteria at the phylum level for the stratified groups for a high-level view of the microbiome composition differences among the groups. When including data from the false positives and 5 healthy subjects (controls) from the study by Armstrong et al. (2021) (see "Materials and methods" section) with the CLAIRE patient data, alpha- and beta-diversity analyses were significant at even lower p-values (Supplementary Figure 3). Beta-diversity for the 4 BSA categories was also significant at the genus level ($p < 0.001$) (Supplementary Figure 4).

Alpha diversity associations with clinical biomarkers are shown in Figure 2. The most significant associations were with age ($p = 0.0298$, Figure 2B), with the 4 BSA categories ($p = 0.0003$, Figure 2C), and the 2 Early BSA groups ($p < 0.0001$, Figure 2D). As expected, BSAs disrupted the bacterial saliva microbiome with a significant reduction in alpha-diversity for the Early BSA group. The difference is slightly more marked at the genus level (p-value = 1.8504e-05) (Supplementary Figure 5).

Healthy subjects and false positives comparison

We compared data from 5 healthy subjects (controls) from the study by Armstrong et al. (2021) (see "Materials and

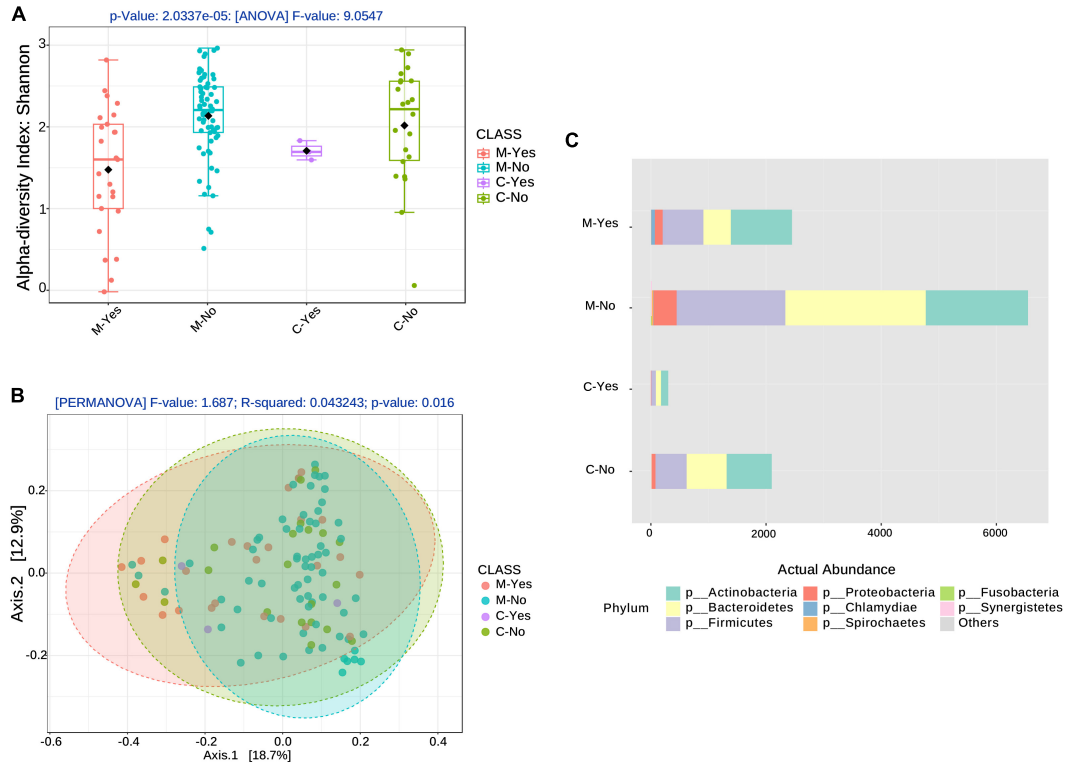


FIGURE 1
Alpha and Beta diversity associated with broad-spectrum sepsis antibiotics (BSA). **(A)** Alpha diversity boxplots for the COVID-19 groups, moderate (M) and critical (C) COVID-19 groups stratified by BSA timing [BSA before or same day as saliva sampling: Yes (Y), No (N)]. **(B)** Beta diversity with COVID-19 groups, as before, stratified by BSA timing. **(C)** Phyla abundance plot.

methods" section) with the CLAIRE patients (Figures 3A–E) and found *Fusobacterium periodonticum* was among the most reduced species in COVID-19 critical patients with respect to controls (Coefficient: -8.45, p = 1.069e-06, FDR = 7.182e-05), see Figure 3C. Other significantly reduced species in the two COVID-19 groups were *Leptotrichia wadei* (Coefficient: −5.19, p = 1.092E-08, FDR = 2.539E-06), *Actinomyces massiliensis* (Coefficient: −3.45, p = 3.666E-06, FDR = 1.607E-04), *Gemella haemolysans* (Coefficient: −6.43, p = 3.097E-06 FDR = 1.487E-04), and *Streptococcus* sp HMSC067H01 (Coefficient: −3.89, p = 3.475E-06 FDR = 1.592E-04) (Supplementary Table 5). Genus level results are provided in Supplementary Table 6.

A significant association distinguishing the bacteria in the saliva of FP patients with that in COVID-19 patients was found. As shown in Supplementary Table 7, *Prevotella shahii* (p = 1.84E-08, FDR = 5.86E-06), *Fusobacterium periodonticum* (p = 8.70E-08, FDR = 1.24E-05) and *Leptotrichia* sp oral taxon_215 (p = 1.17E-07, FDR = 1.24E-05) resulted as the most significantly reduced species in COVID-19 patients with respect to the cluster of FPs.

Disease severity associations with microbiome composition

This section explores disease severity independent of BSA drugs. Our analyses reveal significant differences in the non-bacterial microbiome and its potential as a biomarker of disease progression, presented below, while the bacterial microbiome did not show significant differences. The "No early BSA" section, however, presents significant differences between moderate and critical groups.

Disease severity, DNA viruses, fungi, and parasites

DNA viruses, fungi, and parasites were studied together using the Bracken data as there were fewer species among fungi/parasites: 60, DNA virus: 123, than bacterial species: 2272. Beta diversity of DNA virus, fungi/parasites was significantly different for the moderate and critical groups only at the phylum level (PERMANOVA, $p < 0.041$). DNA virus, fungi/parasites alpha diversity was borderline significant at the family level and lower for the critical group ($p < 0.043$) (Supplementary Figure 6).

When looking at severity of disease, 2 fungi, *Candida dubliniensis* (p = 0.000274, FDR = 0.001369), *Candida albicans* (p = 0.002891, FRD = 0.0096352), were found by all bioinformatics tools to be significantly elevated in the critically ill group. Proton pump inhibitors (PPI) are a risk factor for the identification of *Candida* but medical records show the same percentage of PPI medication, 32%, in both severity groups. *Streptococcus* phage EJ-1 (p = 4.20E-07, FDR = 4.20E-06), *Streptococcus* phage SpSL1 (p = 5.44E-06, FDR = 3.63E-05), *Streptococcus* phage phiARI0131-2 (p = 0.005, FDR = 0.014305), and *Streptococcus* phage PH10 (p = 0.007, FDR = 0.018306), were found to be significantly elevated in the moderate group (see Supplementary Table 8). A random forest classification on the Bracken data finds *Candida albicans*

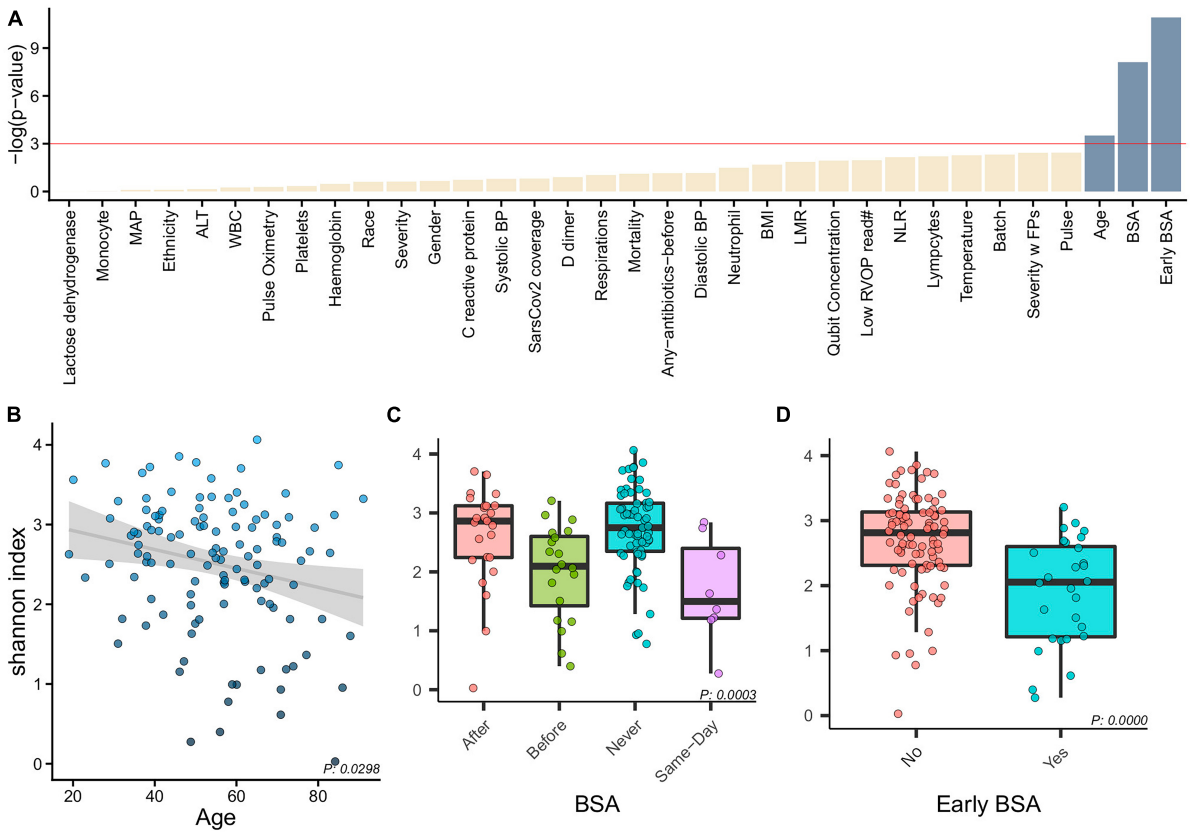


FIGURE 2
Alpha diversity and clinical characteristics. **(A)** Associations between alpha diversity and sample clinical information (*x*-axis) and statistical significance (−log(*p*-value)–*y*-axis) with a horizontal red line showing the −log(0.05) cutoff for significance. With age, BSA and early BSA showing significance, we show alpha diversity against the categorizations of each of these clinical variables, namely, **(B)** Age, **(C)** BSA, and **(D)** early BSA.

as the top feature contributing to classification accuracy (Mean Decrease Accuracy of 0.025) between moderate and critical patients (see Figures 4A, B). The out of bag (OOB) error rate was 16.7% with only 1/95 moderate patients misclassified, and 6/25 critical patients correctly classified.

BSA treatment effects on microbiome composition

BSA and bacteria

We found no significant association between SARS-CoV-2 genome coverage and the timing of BSAs for sepsis treatment (Mann–Whitney U test, $U = 938.5$, p-value = 0.2187) indicating that COVID-19 viral load is not correlated with sepsis. A significant association was found between patients with active cancer and broad-spectrum antibiotic treatment for sepsis early upon admission (Fisher exact test, $p < 0.015$), indicating that immune suppression correlates with sepsis.

MaAsLin finds 72 significant differences between the BSA groups, as shown in Supplementary Table 9. The most significantly reduced bacteria in the Early BSA group was *Prevotella pallens* (Coefficient: −2.834, p = 4.58E-05, FDR = 0.007279), the next significantly reduced species was *Alloprevotella tannerae*, with much higher p-value (Coefficient: −3.131, p = 0.000997, FDR = 0.05283). Multiple bacteria were found to be significantly associated with the 4 BSA categories at the MaAsLin threshold of q-val < 0.25 when analyzing the MetaPhlAn data. *Prevotella pallens* was again significantly reduced in the "Before" group (Coefficient: −3.195, p = 9.30E-05, FDR = 0.044356). The next significant bacteria after *Prevotella pallens* had a much higher p-value and was *Actinomyces* sp oral taxon 1 (p = 0.001892, FDR = 0.23859). The 5 significant genera in common among the results by the three different tools (DESeq2, MetagenomeSeq, MaAsLin) and the two datasets (MetaPhlAn, Bracken) were *Selenomonas*, *Prevotella*, *Gemella*, *Fusobacterium*, and *Haemophilus* (DESeq2 FDR < 0.05).

BSA and DNA viruses, fungi, and parasites

Alpha diversity was not significantly different between the BSA groups when studying fungi and parasite species indicating being less affected by BSAs. Alpha diversity for DNA virus species was significantly lower for the DNA virus species for the Early BSA group (p < 7.43e-5). See Supplementary Figure 7.

BSA and antimicrobial resistance

Both MaAsLin and MetagenomeSeq find tetracycline antibiotic resistance to be significantly lower in the Group with "Early BSA" (MetagenomeSeq, p = 0.0037681, FDR = 0.030898). As the same drug resistance can appear in genes of numerous different bacterial or pathogen species, the analysis focuses on the drug

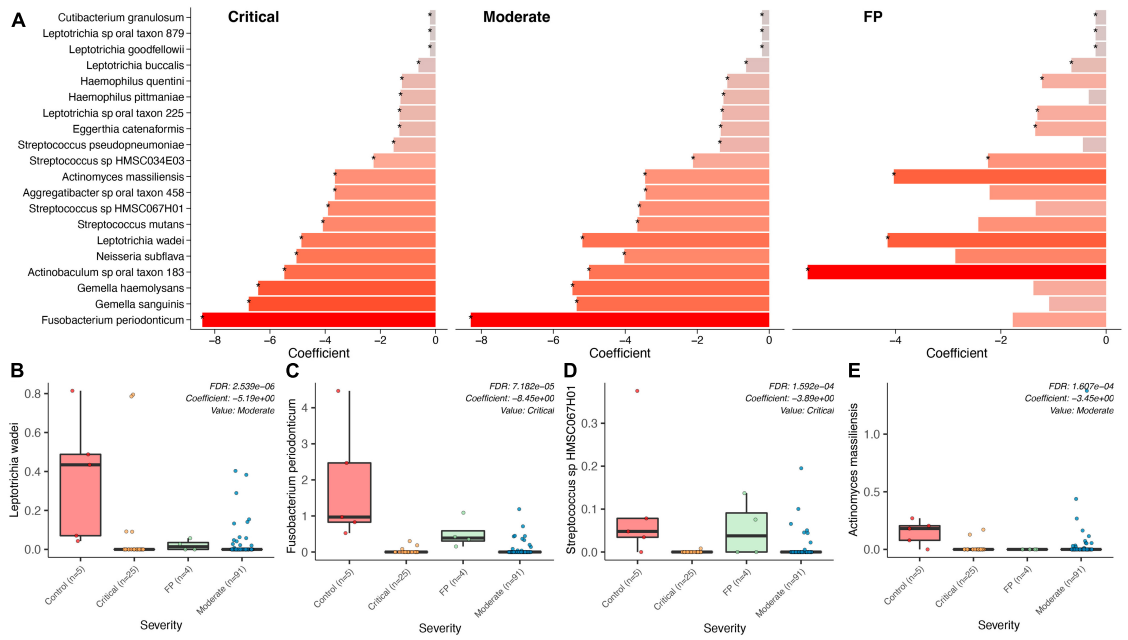


FIGURE 3
Bacterial associations across COVID-19 groups, FP, Controls. **(A)** Top bacteria found in a comparison of the bacterial microbiome composition between the two COVID-19 groups (severe, moderate), the False Positives (FP), and the control group (healthy individuals) using control as reference. **(B–E)** Show specific significance tests for those bacterial species found to be significantly different between controls and our three case groups.

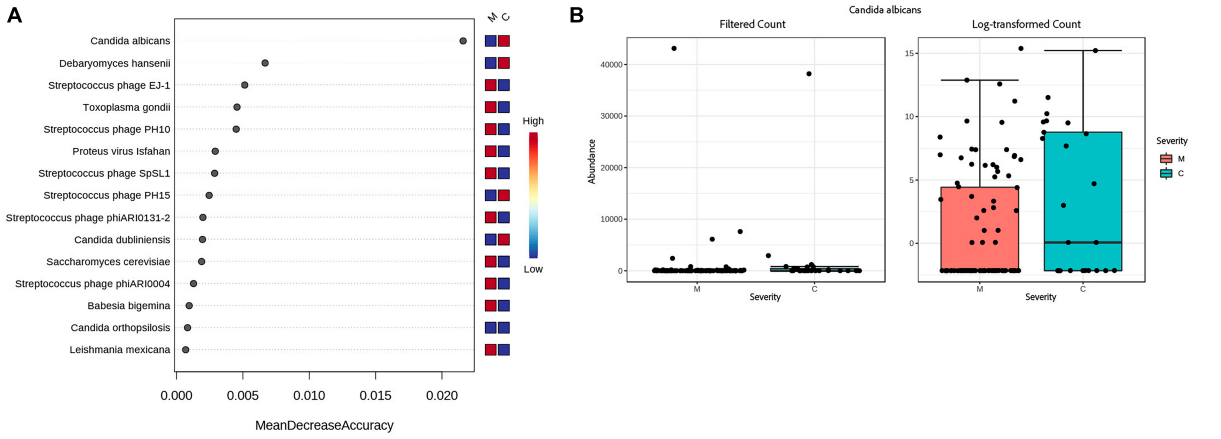


FIGURE 4
Accuracy of predicting disease severity by microbial species. **(A)** Random forest analysis of the decrease in accuracy (*x*-axis) of predicting disease severity with the removal of individual species (*y*-axis). A higher mean decrease accuracy value indicates the importance of that species in predicting severity of disease (moderate vs. critical). **(B)** Because *Candida albicans* was the most important species with respect to disease prediction, we further examined this taxon via a Box and Whisker plot to show individual data points.

classes. When looking at mixed AMR drug classes, MaAsLin and MetagenomeSeq found 2 different mixed drug classes that both contain resistance to fluoroquinolone and were significantly lower in the "Early BSA" group (MetagenomeSeq, p = 1.57E-06, FDR = 0.000101). In both cases, the mixed drug class also contains the tetracycline antibiotic (Supplementary Figure 8). Tetracycline (e.g., doxycycline hyclate) was given to 6 patients after saliva sampling, 4 of whom received BSAs, and neither had high resistance to tetracycline. A frequently used fluoroquinolone agent, ciprofloxacin, was not found in the medical records for the days of hospitalization, but another fluoroquinolone, levofloxacin, was administered to 8 patients, 5 of whom also received BSAs, with 1 having a somewhat elevated resistance to fluoroquinolone but whose disease progression was moderate. Patients P8 and P29 are both patients with elevated numbers of drug resistance bacteria not present in other patients (see dark long red lines in the heatmap in Figure 5). Both were prescribed BSAs after saliva sampling ("After" Group) and developed severe COVID-19 symptoms ("Critical" Group), and patient P8 expired. See the section "No early BSA and antimicrobial resistance".

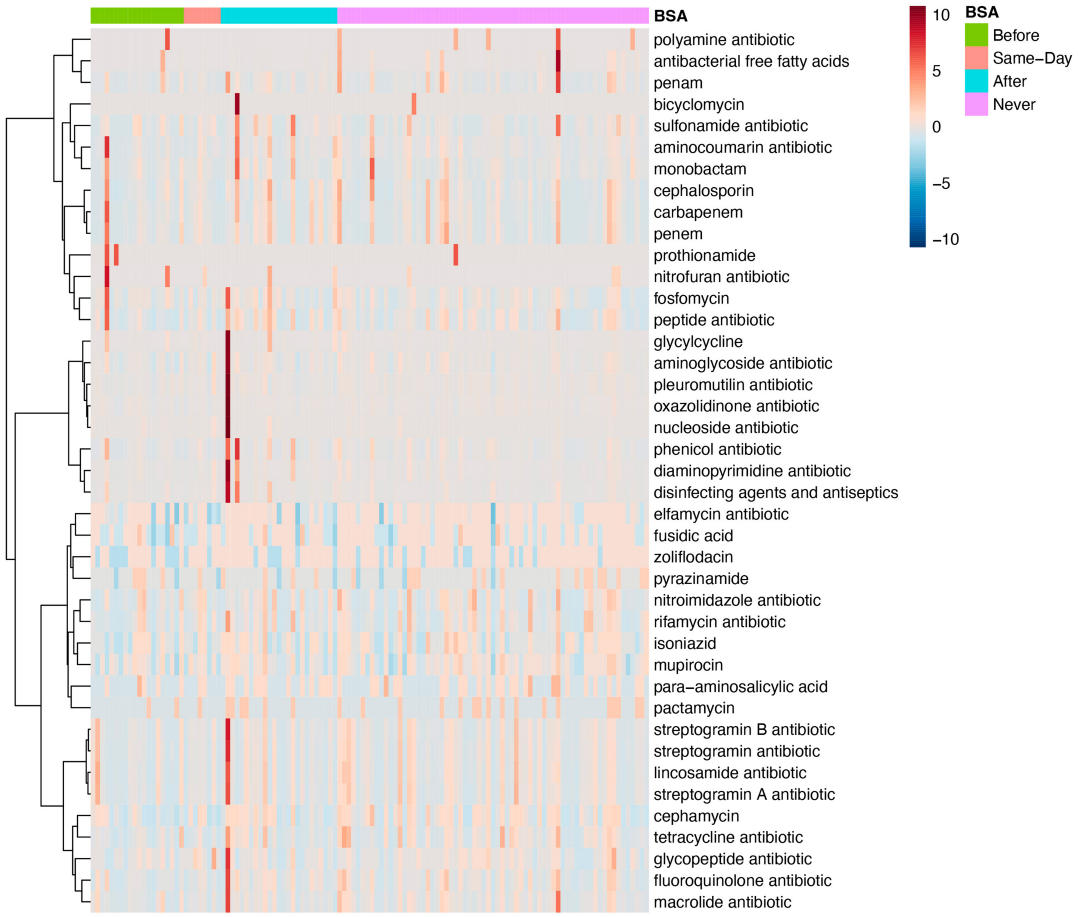


FIGURE 5
Antimicrobial resistance for specific antibiotics by broad-spectrum sepsis antibiotic (BSA) timing. Heatmap of samples with antimicrobial resistance to single drug classes grouped by BSA timing.

BSA and bacterial functional annotation

Bacterial functional annotation data (generated with Humann2) were analyzed by MaAsLin for the BSA groups. Two pathways were significantly elevated in the group that started with BSA early, before saliva sampling (p-value < 0.00015, FDR < 0.123): (1) PWY-702: L-methionine biosynthesis II: *Rothia mucilaginosa*, and (2) PWY-I9: L-cysteine biosynthesis VI (from L-methionine): *Rothia mucilaginosa*. As shown in **Figure 6**, the two pathways were not active in a larger proportion of samples in the No early BSA group (empty area in chart) while a majority of the samples in the Early BSA group showed the two pathways active with a higher number of bacteria associated with the pathways.

Absence of BSA treatment and its effect on microbiome composition and association with severity of disease

Patients who were not treated with BSAs before saliva sampling were studied separately to find patterns of disease progression not affected by these antibiotics (see **Table 2**). Among the "No early BSA" patients ($n = 92$), "After" patients, who developed sepsis after sampling ($n = 25$), were compared to "Never" patients, who never developed sepsis ($n = 67$).

No early BSA, disease severity, and bacteria

Unlike results of analysis including all patients, the "No early BSA" analysis of the Bracken data with MaAsLin finds bacteria that are significantly more elevated in the "Moderate" group: *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, and *Campylobacter concisus* ($p < 0.00043$, FDR < 0.061). MetagenomeSeq finds *Staphylococcus aureus* significantly more elevated in the Critical group ($p = 0.000103$, FDR $= 0.027811$). Medical records show *Staphylococcus aureus* was found in blood culture results of 3 patients. At the genus level, in the MaAsLin analysis, some bacteria are significantly more elevated (*Streptococcus*, *Gemella*, *Lachnoanaerobaculum*) or significantly more reduced (*Bacillus*) in the "Moderate" group (p-value < 0.0015, q-val $= 0.055$).

No early BSA, disease severity, and antimicrobial resistance

When considering only the "No early BSA" patients, MetagenomeSeq finds AMR drug multiclasses significantly elevated in the moderate group and containing drugs that were found to

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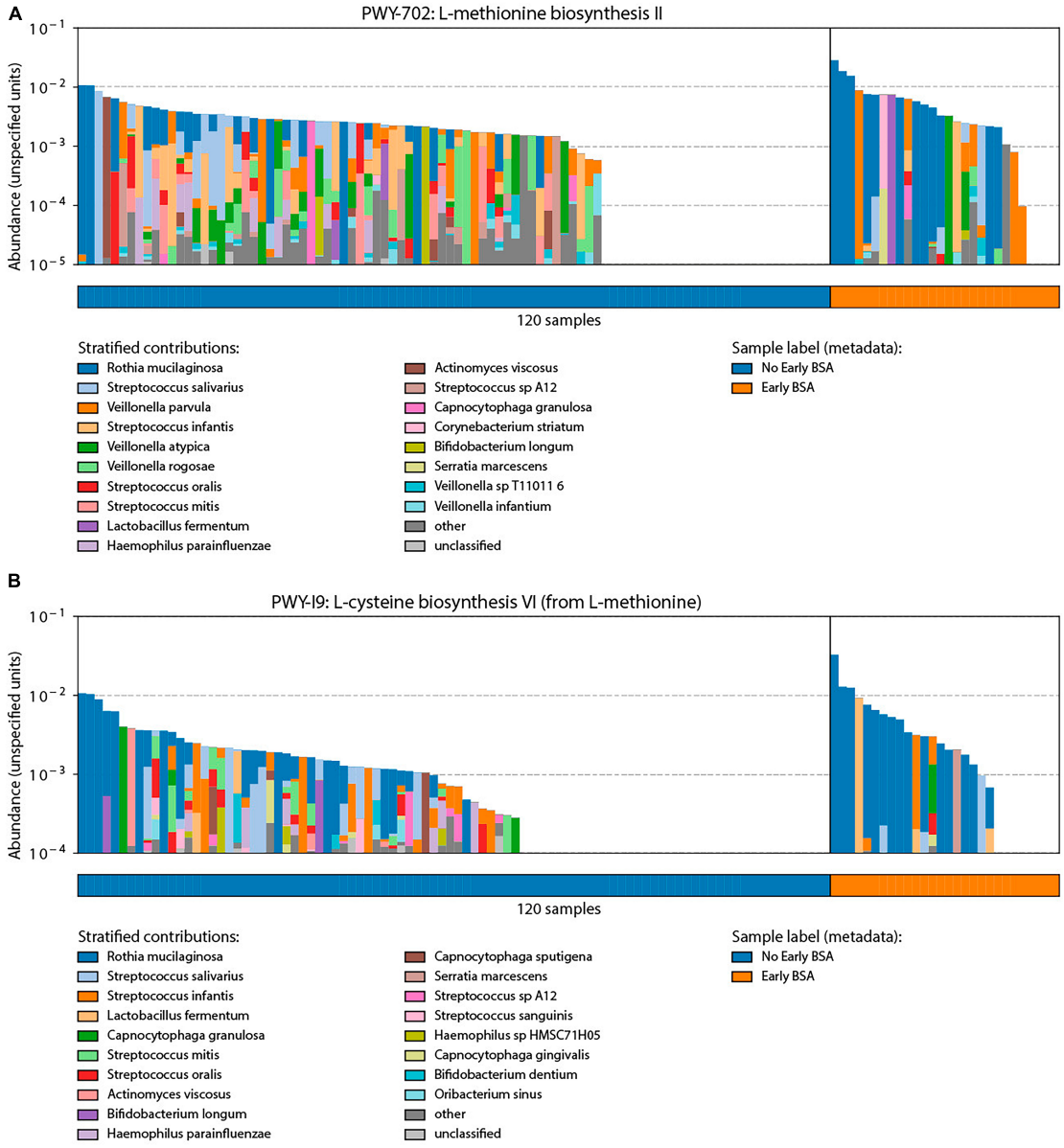


FIGURE 6
Analysis of broad-spectrum sepsis antibiotic (BSA) classification by bacterial functional group. Significant pathways identified through bacterial functional analysis relative to BSA status with *y*-axis length or area of each colored segment showing the relative abundance of a taxonomic group in the given pathway: **(A)** L-methionine biosynthesis II pathway that was preferentially enriched in the Early BSA samples. **(B)** L-cysteine biosynthesis VI pathways were also enriched in the Early BSA samples.

be significant in the earlier analysis of the 2 main BSA groups: "macrolide antibiotic; fluoroquinolone antibiotic; tetracycline antibiotic; phenicol antibiotic; disinfecting agents and antiseptics" (p = 4.33E-16, FDR = 4.29E-14). See "Discussion" section on AMR.

No early BSA, disease severity, DNA virus, fungi and parasites

The results for the "No early BSA" group when studying DNA viruses, fungi, and parasites are comparable to results analyzing all patients, possibly because these components of the microbiome are not as affected by the BSA drugs (Supplementary Table 8).

Discussion and conclusion

In our CLAIRE study, the saliva microbiome was analyzed as a possible biomarker predictive of disease progression in infectious respiratory diseases, specifically COVID-19 and sepsis BSA treatment, to aid in the triage of patients in a hospital setting. We found that bacterial microbiome differences between the groups were mainly due to BSA treatment rather than the COVID-19 infection. Random forest analysis revealed a surprising link between *Candida albicans* and disease severity, suggesting a previously unrecognized role for this fungus as a biomarker for disease progression. Our study found *Staphylococcus aureus* significantly more elevated in the Critical group of patients who

did not receive BSA before saliva sampling. Another relevant finding is the significantly elevated number of *Streptococcus* phage in the moderate group, and while *Streptococcus* phages are one of the least studied bacteriophages, recent publications suggest studying phages as "living antibiotics"(Schooley and Strathdee, 2020; Mehmood Khan et al., 2023).

To date, no studies have completely addressed saliva microbiome profiling, including fungal, parasitic, and viral components in COVID-19 and sepsis treatment and have studied specimens other than saliva, such as oropharynx, nasopharyngeal, oral rinses, throat, or oral swabs (Lebba et al., 2020; Ma et al., 2021; Miller et al., 2021; Nardelli et al., 2021; Soffritti et al., 2021; Li et al., 2023). Our study confirmed the impact of antibiotics in reducing the diversity of the bacterial saliva microbiome compared to the viral, parasite, and fungal saliva microbiome, which contained possible biomarkers of disease progression. Our study reaches similar conclusions to a meta-analysis study by Alshaikh et al. (2022), providing evidence for the need to use narrow-spectrum agents that specifically target pathogenic bacteria supporting better antimicrobial stewardship (AMS) practices using a laboratory-confirmed diagnosis of coinfection instead of the systematic use of broad-spectrum antibiotics. Di Pilato and Giacobbe (2023), noted that "the overall prevalence of bacterial infections in hospitalized COVID-19 patients remains low (4–6%) a striking contrast with the high prevalence of antibiotic use reported by the same population (60–100% in most studies)", recommending AMS interventions that reduce antibiotic resistance (Di Pilato and Giacobbe, 2023). Nandi et al. (2023) found that antibiotic sales were positively associated with COVID-19 cases globally during 2020-2022 and pointed out the ongoing necessity to enhance better antibiotic stewardship practices in the context of COVID-19. Our findings suggest there is a need for research looking into the possible protective or deleterious effect of specific bacteria on the development of an infection and into the effect of timing of BSAs on whether the bacterial microbiome or the non-bacterial microbiome of a saliva sample should be considered as a candidate biomarker of infectious disease severity. Based on Yonkus et al. (2022) findings, nanopore sequencing, which takes 8 h compared to 98 h to return rapid microbial profiling results, has great potential to improve antibiotic stewardship. This method should be accompanied by a prescription of narrow-spectrum antibiotics, once scientific and clinical research align their efforts to develop such agents (Alm and Lahiri, 2020; Datta, 2023).

Associations with severity of disease

Oral COVID-19 microbiome studies have not looked into the effect of antibiotics treatment and have mostly focused on bacteria (Ma et al., 2021; Miller et al., 2021; Nardelli et al., 2021; Soffritti et al., 2021; Li et al., 2023), except for an Ion Gene Studio S5 sequencing study on oral rinse samples that looked at non-bacterial microbiome components (Lebba et al., 2020). Our study expands the focus beyond bacteria in a combined analysis of BSA and sepsis and finds significant associations between DNA viruses, fungi, and parasites and severity of infectious disease (both sepsis and COVID-19). Similar to what was observed in larger series, the majority of patients with COVID-19 (79% or 95 patients) responded to the standard management and were classified as moderate (moderate severity group), while a smaller proportion (21% or 25 patients) became critically ill or died (critical severity group) (see **Table 3**).

DNA viruses, parasites, and fungi

As all patients received antibiotics, the bacterial saliva microbiome did not reveal any statistically significant difference between the moderate and critical severity groups, however, the DNA viral, parasitic, and fungal components of the microbiome, not targeted by the antibiotics, exhibited some significant differences. Specifically, *Candida albicans* emerged as a predictive biomarker of disease severity based on random forest analysis. Immunomodulatory therapy, which became part of the standard therapy for COVID-19, is known to influence the fungal burden in the microbiome. Recent studies have described an increase in deaths from fungal infections during the COVID-19 pandemic (Mina et al., 2022; Gold et al., 2023). A history of proton pump inhibitors (PPI) is a risk factor for the identification of *Candida* (Mojazi Amiri et al., 2012), but PPIs were prescribed proportionally at 32% in both severity groups.

Along with *Candida albicans*, *Candida dubliniensis* was also significantly elevated in the critical group, and several *Streptococcus* phage species were elevated in the moderate group, among them, *Streptococcus* phage EJ-1 and phage PH10, which Soffritti et al. (2021) found increased in COVID-19 patients compared to controls when studying oral rinse samples. *Streptococcus* sp HMSC067H01, an unclassified *Streptococcus* species, was found to be significantly reduced in COVID-19 patients and higher in controls (Soffritti et al., 2021), which could explain the elevated number of *Streptococcus* phage in moderate disease progression. The BSAs used in this study are currently being studied for phage combination therapies (Save et al., 2022; Osman et al., 2023).

No-early BSA group

To investigate whether it is possible to identify biomarkers of disease progression when BSAs were not administered, we excluded patients treated with BSAs before saliva sampling and looked at the "No early BSA" patients (n = 92) (see **Table 2**). At the genus level, *Streptococcus*, *Gemella*, and *Lachnoanaerobaculum* are significantly more elevated and *Bacillus* is significantly more reduced in the "Moderate" group. Interestingly, the results for DNA viruses, parasites, and fungi, when considering only "No early BSA" patients, were comparable to results considering all patients. This provides evidence that unlike bacteria, DNA viruses, parasites, and fungi are not affected by BSAs and can be used as a biomarker of disease progression regardless of whether BSAs were administered.

Sepsis and broad-spectrum sepsis antibiotics

Sepsis

We investigated whether sepsis could be caused by a more severe COVID-19 infection. A Mann–Whitney U test did not support the second hypothesis, with the SARS-CoV-2 genome

coverage between the early or not early BSA groups not being significantly different. This indicates sepsis is associated with other conditions than COVID-19, such as possibly a weaker immune system, which is corroborated by a Fisher exact test finding a significant association between the 2 sepsis groups and patients with active cancer. We saw a significant reduction of *Prevotella pallens* (Pp) in the early BSA group, while Miller et al. (2021) saw a significant enrichment of Pp in COVID-19 patients in their 16S rRNA study but did not consider antibiotics use. In our study, 96% of COVID-19 patients who did not receive BSAs before sampling did not exhibit a critical disease progression and had a statistically significant higher abundance of Pp. It is possible that Pp is enriched in COVID-19 and is also especially susceptible to BSAs. Microorganisms identified from blood culture (sampled on different dates than saliva sampling) in two or more sepsis-treated patients were *Escherichia coli* (5), *Candida albicans* (4), *Staphylococcus aureus* (3), *Enterococcus faecalis* (3), *Pseudomonas aeruginosa* (2) (number of patients shown in parenthesis). In our saliva microbiome analysis, *Candida albicans* and *Staphylococcus aureus* were found to be significantly elevated in critical patients. More research is needed to study the potential of *Staphylococcus aureus* to be predictive of sepsis and potential linkages between the blood microbiome and the saliva microbiome, which would require simultaneous sampling of blood and saliva.

Antimicrobial resistance (AMR)

Significant AMR differences were found between the 2 BSA groups but not between the two COVID-19 disease severity groups. Tetracycline antibiotic resistance (TAR) and fluoroquinolone antibiotic resistance (FAR) were found to be significantly lower in the "Early BSA" group. This could indicate that either BSAs were effective at killing the TAR/FAR bacteria, which is a more likely explanation as they target highly resistant organisms in nosocomial infections, or that the patients with sepsis had fewer resistant bacteria, which is unlikely but supported by two findings. First, the stratification into 4 BSA groups in which the group that developed sepsis after saliva sampling had a lower abundance of TAR bacteria, the group that never developed sepsis had the largest abundance of TAR bacteria (see **Supplementary Figure 8**), and second, an AMR multiclass containing tetracycline and fluoroquinolone resistance was also significantly elevated in the moderate group when looking at the no early BSA subset of patients. Although Tetracycline (e.g., doxycycline hyclate) and fluoroquinolone (e.g., levofloxacin) were given to some patients (less than 10), we could not assess whether the resistance pattern affected disease progression as most also took BSAs, did not show high resistance to those agents, and had a moderate disease progression. In the "No early BSA" group, multiclass AMRs were significantly elevated in the moderate subgroup and contained AMR to drugs mentioned above but also to disinfecting agents and antiseptics. The heightened use of disinfectants during COVID-19 may have led to AMR (van Dijk and Verbrugh, 2022). Two patients with the highest numbers of diverse drug resistance bacteria not present in other patients (see heatmap in **Figure 5**) were in critical condition and one died, but a larger sample size is needed to show AMR affected their disease progression.

Bacterial saliva microbiome diversity and composition in COVID-19 patients

Fusobacterium periodonticum (Fp)

Fusobacterium periodonticum (Fp) was among the most significantly reduced species in the COVID-19 patients as compared to the false positives, in line with a nasopharyngeal microbiome case/control study (Nardelli et al., 2021), with similar trends observed between the salivary and nasopharyngeal microbiome (Kim et al., 2023). Nardelli et al. (2021) reported that Fp can perform surface sialylation and that some sialic acid residues on the cell surface could work as additional S protein of SARS-CoV-2 receptors.

Species richness

Our study noted a significant diminution in bacterial species richness in COVID-19 patients, which was also observed in Lebba et al. (2020), a 16S rRNA microbiome study from naso/oral-pharyngeal swabs (Lebba et al., 2020). In that study, unlike ours, patients did not take antibiotics or probiotics before sampling. We observed that BSA significantly reduced bacterial alpha diversity reinforcing the observations that BSAs have a strong effect on the saliva microbiome (see **Figure 1**). We also found a significant decrease in species diversity with growing age, which is confirmed by other oral microbiome studies (Huang et al., 2020; Willis et al., 2022).

Specific group differences

We found a significant reduction in bacterial abundance in the COVID-19 patients, with the top reduced genera being *Fusobacterium*, *Kingella*, *Actinobaculum*, *Gemella*, *Leptotrichia*, *Cardiobacterium*, *Aggregatibacter*, *Parvimonas*, *Haemophilus*, *Campylobacter*, *Lachnoanaerobaculum*, and *Neisseria*, in that order. Of those, the main bacterial genera in the normal oral cavity include *Fusobacterium*, *Leptotrichia*, and *Neisseria* (Li et al., 2014). Soffritti et al. (2021) found many species of periodontopathogenic bacteria that were significantly increased in COVID-19 oral rinse samples compared to control subjects. In Ma et al. (2021), significantly higher levels of *Veillonella* were found in both COVID-19 and flu patients than in the control group when studying oropharynx swab specimens. Wu et al. (2021) found through 16S rRNA sequencing on throat swab samples that periodontitis-correlated taxa, including species *R. mucilaginosa* (Rm) were elevated in COVID-19 compared to controls. In contrast, Miller et al. (2021) found through 16S rRNA saliva microbiome analysis that Rm was enriched in control patients. In our study, Rm was significantly elevated in patients receiving BSAs the same day of sampling, suggesting that perhaps a more severe early infection, or sepsis, rather than COVID-19, may cause some of these bacteria to become more abundant. Wu et al. (2021) also found that species *H. parainfluenzae* and *N. subflava* were enriched in the oral microbiome of COVID-19 patients, but our study found a significant reduction of those bacteria in COVID-19 and the early BSA groups. Among the three "red

complex" periodontal pathogens (Abdulkareem et al., 2023), only *P. gingivalis* was among the top 33 significantly reduced bacteria in the early BSA groups in our study (**Supplementary Table 8**).

Two of the studies cited above did not mention antibiotic usage, while Wu et al. (2021) reported that 67.9% of patients in their study were treated with antibiotics to prevent potential secondary bacterial co-infections and that they adjusted for it. Antibiotic usage may have had a confounding effect in all those studies, even for the Wu et al. (2021) study after adjustment, and the difference in COVID-19 variants, study population, sequencing method, or sample method has to be considered when comparing results. Our study corroborated some of Wu et al. (2021) findings with *Neisseria*, *Corynebacterium*, and *Aggregatibacter* being significantly lower in the early BSA group and significantly decreased in the COVID-19 group.

Functional pathways

Two L-Methionine pathways were significantly elevated in the early BSA group. A recent hypothesis postulates that L-Methionine may modulate the assembly of SARS-CoV-2 by interfering with the mechanism of RNA polymerase (Benavides, 2022). SARS-CoV-2 could therefore benefit from the enrichment of the two pathways. Some bacteria, such as *Rothia mucilaginosa* (Rm), can synthesize methionine. As pointed out above, different studies found contradicting abundance patterns for Rm (Miller et al., 2021; Wu et al., 2021). In our study, Rm was significantly elevated in patients who started BSA on the same day of sampling. Bacteria involved in the L-Methionine biosynthesis pathway may have contributed to the sepsis symptoms. As most patients in the early BSA group did not develop a critical disease progression, BSAs may have eliminated these bacteria, disrupting the L-Methionine pathways that could no longer be exploited by SARS-CoV-2. The role of Rm may need to be further investigated.

Data availability statement

The data presented in this study is available at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA883997.

Ethics statement

The studies involving humans were approved by the University of Miami, IRB ID: 20200724. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KF: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. CR: Investigation, Methodology, Resources, Validation, Writing – review & editing. AR: Formal analysis, Investigation, Methodology, Software, Visualization, Writing – review & editing. KC: Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing. JC: Conceptualization, Investigation, Methodology, Project administration, Writing – review & editing.

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Conflict of interest

PB and KF were employed by Lifetime Omics.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1346762/full#supplementary-material

References

Abdulkareem, A., Al-Taweel, F., Al-Sharqi, A., Gul, S., Sha, A., and Chapple, I. (2023). Current concepts in the pathogenesis of periodontitis: from symbiosis to dysbiosis. *J. Oral. Microbiol.* 15:2197779. doi: 10.1080/20002297.2023.2197779

Alcock, B., Raphenya, A., Lau, T., Tsang, K., Bouchard, M., Edalatmand, A., et al. (2020). CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 48, D517–D525. doi: 10.1093/nar/gkz935

Alm, R. A., and Lahiri, S. D. (2020). Narrow-spectrum antibacterial agents—benefits and challenges. *Antibiotics* 9:0418. doi: 10.3390/antibiotics9070418

Alshaikh, F., Godman, B., Sindi, O., Seaton, R., and Kurdi, A. (2022). Prevalence of bacterial coinfection and patterns of antibiotics prescribing in patients with COVID-19: a systematic review and meta-analysis. *PLoS One* 17:e0272375. doi: 10.1371/journal.pone.0272375

Armstrong, A., Parmar, V., and Blaser, M. (2021). Assessing saliva microbiome collection and processing methods. *NPJ Biofilms Microbiomes* 7:81. doi: 10.1038/s41522-021-00254-z

Bao, L., Zhang, C., Dong, J., Zhao, L., Li, Y., and Sun, J. (2020). Oral microbiome and SARS-CoV-2: beware of lung co-infection. *Front. Microbiol.* 11:1840. doi: 10.3389/fmicb.2020.01840

Beghini, F., McIver, L., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., et al. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *Elife* 10:e65088. doi: 10.7554/eLife.65088

Benavides, M. (2022). l-Methionine may modulate the assembly of SARS-CoV-2 by interfering with the mechanism of RNA polymerase. *Med. Hypotheses* 161:110798. doi: 10.1016/j.mehy.2022.110798

Chong, J., Liu, P., Zhou, G., and Xia, J. (2020). Using MICROBIOMEANALyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* 15, 799–821. doi: 10.1038/s41596-019-0264-1

Datta, S. (2023). The conundrum of bacteria-specific antibiotics. *J. Antimicrob. Chemother.* 78, 1354–1358. doi: 10.1093/jac/dkad130

Di Pilato, V., and Giacobbe, D. R. (2023). editorial of special issue "the COVID-19 pandemic and bacterial infections: microbiological and clinical aspects". *Microorganisms* 11:1009. doi: 10.3390/microorganisms11041009

Ewels, P., Peltzer, A., Fillinger, S., Patel, H., Alneberg, J., Wilm, A., et al. (2020). The nf-core framework for community-curated bioinformatics pipelines. *Nat. Biotechnol.* 38, 276–278. doi: 10.1038/s41587-020-0439-x

Franzosa, E., McIver, L., Rahnavard, G., Thompson, L., Schirmer, M., Weingart, G., et al. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* 15, 962–968. doi: 10.1038/s41592-018-0176-y

Gogate, N., Lyman, D., Bell, A., Cauley, E., Crandall, K., Joseph, A., et al. (2021). COVID-19 biomarkers and their overlap with comorbidities in a disease biomarker data model. *Brief. Bioinform.* 22:bbab191. doi: 10.1093/bib/bbab191

Gold, J., Ahmad, F., Cisewski, J., Rossen, L., Montero, A., Benedict, K., et al. (2023). Increased deaths from fungal infections during the coronavirus disease 2019 pandemic-national vital statistics system, United States, January 2020-December 2021. *Clin. Infect. Dis.* 76, e255–e262. doi: 10.1093/cid/ciac489

Hazan, S., Stollman, N., Bozkurt, H., Dave, S., Papoutsis, A., Daniels, J., et al. (2022). Lost microbes of COVID-19: Bifidobacterium, Faecalibacterium depletion and decreased microbiome diversity associated with SARS-CoV-2 infection severity. *BMJ Open Gastroenterol.* 9:e000871. doi: 10.1136/bmjgast-2022-000871

Hong, C., Manimaran, S., Shen, Y., Perez-Rogers, J., Byrd, A., Castro-Nallar, E., et al. (2014). PathoScope 2.0: a complete computational framework for strain identification in environmental or clinical sequencing samples. *Microbiome* 2:33. doi: 10.1186/2049-2618-2-33

Huang, S., Haiminen, N., Carrieri, A., Hu, R., Jiang, L., Parida, L., et al. (2020). Human skin, oral, and gut microbiomes predict chronological age. *mSystems* 5:e00630-19. doi: 10.1128/mSystems.00630-19

Illumina Detection and Characterization of Respiratory Viruses, Including SARS-CoV-2, Using Illumina RNA Prep with Enrichment: Illumina. San Diego, CA: Illumina. Available Online at: https://www.illumina.com/content/dam/illumina/gcs/assembled-assets/marketing-literature/illumina-rna-enrichment-coronavirus-app-note-470-2020-005/illumina-rna-enrichment-coronavirus-app-note-470-2020-005.pdf (accessed January 2, 2024).

Illumina. *Illumina Shotgun Metagenomic Sequencing.* Available Online at: :https://www.illumina.com/areas-of-interest/microbiology/microbial-sequencing-methods/shotgun-metagenomic-sequencing.html (accessed January 2, 2024).

Illumina. *Nextera XT DNA Library Prep Kit Reference Guide.* San Diego, CA: Illumina. Available Online at: https://assets.illumina.com/content/illumina-marketing/en/products/by-type/sequencing-kits/library-prep-kits/respiratory-virus-oligo-panel.html

Illumina *BaseSpace Apps.* Available Online at: https://www.illumina.com/products/by-type/informatics-products/basespace-sequence-hub/apps.html (accessed January 2, 2024).

Kim, J., Zhang, A., Rauseo, A., Goss, C., Mudd, P., O'Halloran, J., et al. (2023). The salivary and nasopharyngeal microbiomes are associated with SARS-CoV-2 infection and disease severity. *J. Med. Virol.* 95:e28445. doi: 10.1002/jmv.28445

Lam, C., Gray, K., Gall, M., Sadsad, R., Arnott, A., Johnson-Mackinnon, J., et al. (2021). SARS-CoV-2 genome sequencing methods differ in their abilities to detect variants from low-viral-load samples. *J. Clin. Microbiol.* 59:e0104621. doi: 10.1128/JCM.01046-21

Lebba, V., Zanotta, N., Campisciano, G., Zerbato, V., Di Bella, S., Cason, C., et al. (2020). Profiling of oral microbiota and cytokines in COVID-19 patients. *bioRxiv [Preprint]* doi: 10.1101/2020.12.13.422589

Li, J., Jing, Q., Li, J., Hua, M., Di, L., Song, C., et al. (2023). Assessment of microbiota in the gut and upper respiratory tract associated with SARS-CoV-2 infection. *Microbiome* 11:38. doi: 10.1186/s40168-022-01447-0

Li, Y., He, J., He, Z., Zhou, Y., Yuan, M., Xu, X., et al. (2014). Phylogenetic and functional gene structure shifts of the oral microbiomes in periodontitis patients. *ISME J.* 8, 1879–1891. doi: 10.1038/ismej.2014.28

Ma, S., Zhang, F., Zhou, F., Li, H., Ge, W., Gan, R., et al. (2021). Metagenomic analysis reveals oropharyngeal microbiota alterations in patients with COVID-19. *Signal. Transduct. Target. Ther.* 6:191. doi: 10.1038/s41392-021-00614-3

Mallick, H., Chatterjee, S., Chowdhury, S., Chatterjee, S., Rahnavard, A., and Hicks, S. (2022). Differential expression of single-cell RNA-seq data using Tweedie models. *Stat. Med.* 41, 3492–3510. doi: 10.1002/sim.9430

Mallick, H., Rahnavard, A., McIver, L., Ma, S., Zhang, Y., Nguyen, L., et al. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLoS Comput. Biol.* 17:e1009442. doi: 10.1371/journal.pcbi.1009442

Mehmood Khan, F., Manohar, P., Singh Gondil, V., Mehra, N., Kayode Oyejobi, G., Odiwuor, N., et al. (2023). The applications of animal models in phage therapy: an update. *Hum. Vaccin. Immunother.* 19:2175519. doi: 10.1080/21645515.2023.2175519

Miller, E., Annavajhala, M., Chong, A., Park, H., Nobel, Y., Soroush, A., et al. (2021). Oral microbiome alterations and SARS-CoV-2 saliva viral load in patients with COVID-19. *Microbiol. Spectr.* 9:e0005521. doi: 10.1128/Spectrum.00055-21

Mina, S., Yaakoub, H., Annweiler, C., Dubée, V., and Papon, N. (2022). COVID-19 and fungal infections: a double debacle. *Microbes Infect.* 24:105039. doi: 10.1016/j.micinf.2022.105039

Mojazi Amiri, H., Frandah, W., Colmer-Hamood, J., Raj, R., and Nugent, K. (2012). Risk factors of Candida colonization in the oropharynx of patients admitted to an intensive care unit. *J. Mycol. Med.* 22, 301–307. doi: 10.1016/j.mycmed.2012.08.001

Nandi, A., Pecetta, S., and Bloom, D. E. (2023). Global antibiotic use during the COVID-19 pandemic: analysis of pharmaceutical sales data from 71 countries, 2020-2022. *EClinicalMedicine* 57:101848. doi: 10.1016/j.eclinm.2023.101848

Nardelli, C., Gentile, I., Setaro, M., Di Domenico, C., Pinchera, B., Buonomo, A., et al. (2021). Nasopharyngeal microbiome signature in COVID-19 positive patients: can we definitively get a role to Fusobacterium periodonticum? *Front. Cell Infect. Microbiol.* 11:625581. doi: 10.3389/fcimb.2021.625581

Nousias, O., and Montesanto, F. (2021). Metagenomic profiling of host-associated bacteria from 8 datasets of the red alga Porphyra purpurea with MetaPhlAn3. *Mar. Genomics* 59:100866. doi: 10.1016/j.margen.2021.100866

Okui, T., Matsuda, Y., Karino, M., Hideshima, K., and Kanno, T. (2021). Oral mucosa could be an infectious target of SARS-CoV-2. *Healthcare* 9:1068. doi: 10.3390/healthcare9081068

Osman, A., Kotey, F., Odoom, A., Darkwah, S., Yeboah, R., Dayie, N., et al. (2023). The potential of bacteriophage-antibiotic combination therapy in treating infections with multidrug-resistant bacteria. *Antibiotics* 12:1329. doi: 10.3390/antibiotics12081329

Rahnavard, A., Dawson, T., Clement, R., Stearrett, N., Pérez-Losada, M., and Crandall, K. (2021). Epidemiological associations with genomic variation in SARS-CoV-2. *Sci. Rep.* 11:23023. doi: 10.1038/s41598-021-02548-w

Rahnavard, A., Mann, B., Giri, A., Chatterjee, R., and Crandall, K. (2022). Metabolite, protein, and tissue dysfunction associated with COVID-19 disease severity. *Sci. Rep.* 12:12204. doi: 10.1038/s41598-022-16396-9

Save, J., Que, Y.-A., Entenza, J., and Resch, G. (2022). Subtherapeutic doses of vancomycin synergize with bacteriophages for treatment of experimental methicillin-resistant staphylococcus aureus infective endocarditis. *Viruses* 14:1792. doi: 10.3390/v14081792

Schooley, R. T., and Strathdee, S. (2020). Treat phage like living antibiotics. *Nat. Microbiol.* 5, 391–392. doi: 10.1038/s41564-019-0666-4

Soffritti, I., D'Accolti, M., Fabbri, C., Passaro, A., Manfredini, R., Zuliani, G., et al. (2021). Oral Microbiome Dysbiosis is associated with symptoms severity and local immune/inflammatory response in COVID-19 patients: a cross-sectional study. *Front. Microbiol.* 12:687513. doi: 10.3389/fmicb.2021.687513

Truong, D., Franzosa, E., Tickle, T., Scholz, M., Weingart, G., Pasolli, E., et al. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat. Methods* 12, 902–903. doi: 10.1038/nmeth.3589

van Dijk, H., and Verbrugh, H. (2022). Resisting disinfectants. *Commun. Med.* 2:6. doi: 10.1038/s43856-021-00070-8

Willis, J., Saus, E., Iraola-Guzmán, S., Ksiezopolska, E., Cozzuto, L., Bejarano, L., et al. (2022). Citizen-science reveals changes in the oral microbiome in Spain through age and lifestyle factors. *NPJ Biofilms Microbiomes* 8:38. doi: 10.1038/s41522-022-00279-y

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biol.* 20:257. doi: 10.1186/s13059-019-1891-0

Wu, Y., Cheng, X., Jiang, G., Tang, H., Ming, S., Tang, L., et al. (2021). Altered oral and gut microbiota and its association with SARS-CoV-2 viral load in COVID-19 patients during hospitalization. *NPJ Biofilms Microbiomes* 7:61. doi: 10.1038/s41522-021-00232-5

Xu, J., Li, Y., Gan, F., Du, Y., and Yao, Y. (2020). Salivary glands: potential reservoirs for COVID-19 asymptomatic infection. *J. Dent. Res.* 99:989. doi: 10.1177/0022034520918518

Yonkus, J., Whittle, E., Alva-Ruiz, R., Abdelrahman, A., Horsman, S., Suh, G., et al. (2022). &Answers in hours&;: a prospective clinical study using nanopore sequencing for bile duct cultures. *Surgery* 171, 693–702. doi: 10.1016/j.surg.2021.09.037

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Microbiome markers in HPV-positive and HPV-negative women of reproductive age with ASCUS and SIL determined by V4 region of 16S rRNA gene sequencing

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Introduction: Human papilloma virus (HPV) is the most common sexually transmitted infection worldwide. Cervicovaginal microbiota plays an important role in HPV infection and is associated with the development of squamous intraepithelial lesions (SIL). The natural history of cervical cancer involves reversible changes in the cervical tissue from a normal state, in which no neoplastic changes are detected in the squamous epithelium, to varying states of cellular abnormalities that ultimately lead to cervical cancer. Low-grade SIL (LSIL), like another cytological category - atypical squamous cells of undetermined significance (ASCUS), may progress to high-grade SIL (HSIL) and invasive cervical cancer or may regress to a normal state.

Methods: In this work, we studied cervical canal microbiome in 165 HPV-positive and HPV-negative women of a reproductive age with ASCUS [HPV(+) $n = 29$; HPV(–) $n = 11$], LSIL [HPV(+) $n = 32$; HPV(–) $n = 25$], HSIL [HPV(+) $n = 46$], and the control group with negative for intraepithelial lesion malignancy (NILM) [HPV(–) $n = 22$].

Results and Discussion: HPV16 is the most prevalent HPV type. We have not found any differences between diversity in studied groups, but several genus [like Prevotella (p-value = 0.026), Gardnerella (p-value = 0.003), Fannyhessea (p-value = 0.024)] more often occurred in HSIL group compared by NILM or LSIL regardless of HPV. We have found statistically significant difference in occurrence or proportion of bacterial genus in studied groups. We also identified that increasing of the ratio of *Lactobacillus iners* or age of patient lead to higher chance to HSIL, while increasing of the ratio of *Lactobacillus crispatus* lead to higher chance to LSIL. Patients with a moderate dysbiosis equally often had either of three types of vaginal microbial communities (CST, Community State Type) with the prevalence of *Lactobacillus crispatus* (CST I), *Lactobacillus gasseri* (CST II), and *Lactobacillus iners* (CST III); whereas severe dysbiosis is linked with CST IV involving the microorganisms genera associated with bacterial vaginosis and aerobic vaginitis: *Gardnerella*, *Fannyhessea, Dialister, Sneathia, Anaerococcus, Megasphaera, Prevotella, Finegoldia, Peptoniphilus, Porphyromonas, Parvimonas*, and *Streptococcus*.

KEYWORDS

squamous intraepithelial lesion, 16S rRNA gene sequencing, HPV, cervicovaginal microbiota, SIL prediction

1 Introduction

Studies on the occurrence and mortality in 36 cancer types conducted in 185 countries show that cervical cancer is the 4th cancer among all cancer types in women (Bray et al., 2018). In developing countries, cervical cancer occurrence and mortality occupies the second place (Qingqing et al., 2021).

The natural history of cervical cancer includes reversible changes in the cervical tissue from a normal state with no neoplastic changes, to varying states of cellular abnormalities in the squamous epithelium that ultimately lead to cervical cancer. The Bethesda "Epithelial cell abnormality: Squamous" category encompasses a spectrum of squamous cell lesions starting from the precancerous lesions of low-grade dysplasia associated with transient human papilloma virus (HPV) infection to higher grade lesions. The two-tiered system of low-grade SIL (LSIL) and high-grade SIL (HSIL) matches the HPV carcinogenic potential. LSIL is now recommended to be used as a diagnostic category to describe HPV transient infection-related changes, while HSIL is used to categorize true precancerous lesion. However, depending on qualitative and quantitative factors, some equivocal morphological features may fall under the category "Atypical Squamous Cells" (ASCs), which are subdivided into two categories; "Atypical Squamous Cells of Undetermined Significance" (ASC-US) or "Atypical Squamous Cells-HSIL cannot be excluded" (ASC-H), based on the suspected underlying lesion LSIL versus HSIL, respectively (Alrajjal et al., 2021).

Development of squamous intraepithelial lesions (SIL) and cervical cancer is mostly caused by the persistence of the human papillomavirus (HPV) (Qingqing et al., 2021; Hu et al., 2022; Zuñiga Martinez et al., 2022). According to the pertinent literature, the group of viruses related to high oncogenic risk includes 13 genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66), two of them (16, 18) being associated with 70% of all cases of cervical cancer (Burd, 2003; Cogliano et al., 2005). The World Health Organization (WHO) estimates that HPV infections worldwide are increasing by 9 to 13% each year (Santella et al., 2022).

The microbial communities of the female reproductive system have been extensively studied over the past decade (Al-Nasiry et al., 2020; Andralojc et al., 2021). According to the published data, one of the risk factors promoting persistent HPV infection is the cervicovaginal microbiota disruption (Ritu et al., 2019; Nieves-Ramírez et al., 2021). Studying the cervicovaginal microbiota in the 'Human Microbiome' project revealed that the bacteria colonizing the female genital tract make up 9% of the entire human microbiome (Proctor et al., 2019). In one work, five types of vaginal microbial communities have been outlined (CST – Community State Type) (McClymont et al., 2022; Molina et al., 2022). *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners*, and *Lactobacillus jensenii* prevail in four CST (I, II, III, and V), respectively, while no *Lactobacillus* species is predominant in CST IV ('diverse' group) which is further divided into the following subtypes: CST IV-A comprising the *Anaerococus, Peptoniphilus, Corynebacterium, Prevotella, Finegoldia*, and *Streptococcus* genera; CST IV-B including the *Atopobium, Fannyhessea, Gardnerella, Sneathia, Mobiluncus, Megaspher*agenera.

Some authors consider microbial diversity as well as changes in the qualitative and quantitative composition of the cervicovaginal microbiota to be crucial for triggering the proliferation of the stratified squamous epithelial cells and to accompany HPV persistence. Conversely, the viral infection can alter bacterial composition irrespective of the SIL severity looping a 'vicious circle' in the development and progression of the disease (Kang et al., 2021; Naidoo et al., 2022; Ntuli et al., 2022). Recently, special attention has been paid to the influence of individual microorganisms on SIL development (Norenhag et al., 2020; Gardella et al., 2022; Lin et al., 2022).

In our study, we aimed to estimate the characteristics of the microbiome by NGS sequencing of 16S ribosomal genes in the HPV-positive [HPV(+)] and HPV-negative [HPV(−)] women of a reproductive age with cervical pathology.

2 Methods

2.1 Clinical material

From January 2022 to May 2022, 165 women of reproductive age who sought consultation at the Department of Outpatient Clinical Research Development of Kulakov National Medical Research Center for Obstetrics, Gynecology, and Perinatology for diagnosis and treatment of cervical pathology. The patients visited the department because of a wide list of reasons, for example, pregnancy management, absence of menstruation, examination and treatment of female infertility and etc. The primary diagnosis was established based on the results of liquid-based cytology. The cytological material was obtained in accordance with the laws and regulations of the Russian Federation. Air-dried cervical epithelial swabs were prepared by routine Papanicolaou staining. The specimens were collected from patients undergoing cytological and histological examination Departments of Kulakov National Medical Research Center for Obstetrics, Gynecology, and Perinatology (Moscow, Russia). The samples were classified according to the Bethesda system: normal cytology (negative for intraepithelial lesion or malignancy; NILM), low- and high-grade squamous intraepithelial lesions (LSILs and HSILs), or atypical squamous cells of undetermined significance (ASCUS). The final diagnosis was confirmed by histological examination carried out for 76 (46%) women. If the diagnosis had not been verified by histology, patients were placed under dynamic monitoring for cervix condition due to the lack of indications for treatment, and their diagnosis was interpreted by cytological methods. The criteria for inclusion in the study were as follows:

1. The age of women ranging from 19 to 45 years old;
2. The cytological conclusion: ASCUS, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous

intraepithelial lesion (HSIL), and negative for intraepithelial lesion or malignancy (NILM);
3. The provided written informed consent for enrollment in the study.

The criteria for exclusion were as follows:

1. Pregnancy;
2. Lactation;
3. Treatment with antibiotics in the previous 14 days;
4. Neuropsychiatric diseases;
5. Acute inflammatory diseases;
6. Renal, liver, or lung dysfunction at the decompensation stage.

This study conformed to the principles of the Declaration of Helsinki. The involved human participants were reviewed and approved by the Local Ethics Committee at the FSBI "National Medical Research Center For Obstetrics, Gynecology And Perinatology Named After Academician V.I.Kulakov" Ministry of Health of the Russian Federation (protocol no. 3 of 26 March 2020).

2.2 HPV testing

HPV testing was performed by qPCR using the reagent kit for detecting, genotyping, and identifying of the HPV viral load by PCR "HPVquant-21" (DNA technology, Russia) and involved 21 HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 44(55), 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82).

2.3 Collecting the biomaterial for metagenomic study

The swab was collected into the 1.5 tubes containing 300 microliters of 20 mM Tris–HCl buffer (pH = 7.2).

2.4 DNA isolation and library preparation for sequencing

DNA was isolated from the biomaterial using the DNeasy Blood and Tissue kit (Qiagen, United States) following the manufacturer's instructions. Quality control of the isolated prokaryotic DNA was performed by qPCR using the primers recognizing the V4 DNA region encoding 16S rRNA (515F and 806R) (Parada et al., 2016).

The libraries were prepared for sequencing in two steps. At the first step, the V4 region of 16S rRNA was amplified using the primers to the prokaryotic V4 DNA region encoding 16S rRNA (515F and 806R) containing technical sequences for the MGI adapters. At the second step, amplification was performed using primers containing unique barcodes and primer technical sequences. The concentrations of prepared libraries were measured by Qubit Flex (Life Techonologies, United States) using dsDNA HS Assay Kit (Life Technologies, United States) following the manufacturer's protocol. The quality of the prepared libraries was assessed using Bioanalyzer 2,100 with the High Sensitivity DNA kit (Agilent Technologies) according to the manufacturer's instructions. Next, the libraries were circularized and sequenced in the paired-end mode using the DNBSEQG-400 platform with the DNBSEQ-G400RS High-throughput Sequencing Set PE150 kit according to manufacturer's protocol (MGI Tech). FastQ files were generated using the zebracallV2 software by the manufacturer (MGI Tech).

2.5 Sequencing data processing

The obtained overlapping paired reads were merged into unified nucleotide sequences and grouped based on the sequence identity and possible polymerase errors using Qiime2 v.2022.8. Each sequence group was assigned with the taxonomic class (family and genus) using the RDP classificator. Furthermore, each sequence group was aligned using blast v2.13.0 with default settings against the 16S rRNA database followed by determining the species in a read group. Sequence groups with the content of <0.01% in the sample were excluded from the analysis.

2.6 Statistical analysis

For statistical analysis, we used compositional data without any transformation to avoid complexity with clinical interpretation. The differences in age between the groups were assessed using One-way ANOVA, followed by multiple pairwise comparisons using the *t*-test with Bonferroni correction. The statistical significance of differences in the ratio of categorical variables (including the qualitative assessment of microorganisms) between the groups was assessed using the Chi-square test, provided that there were no cells in the contingency table with the values below 5. The exact Fisher's test was used if at least one cell in the contingency table contained a value of <5. Only those microorganisms with the content of >1% in at least two patients were subjected to further qualitative estimation (the frequency of occurrence of a microorganism in the sample). The microorganisms with the content >5% for at least two patients were subjected to further quantitative estimation. The statistical significance of differences upon quantitative assessment between groups was evaluated using the Kruskal–Wallis test. Alpha diversity was assessed using the Shannon and the Simpson index with using amount of reads per taxon. Beta diversity between all groups was assessed by adonis algorithm (Bray-Curtis distance) from "vegan" package (R language). Statistical significance was assessed using the Kruskal–Wallis test for all groups, followed by pairwise comparisons using the Mann–Whitney test with Bonferroni correction. The differences were considered statistically significant at $p < 0.05$. The charts were created using the R programming language (ver 4.2.0). Logistic regression was created using the *'glm'* and *'step'* functions from the *'stats'* package in the R programming language (ver 4.2.0).

3 Results

3.1 Demographic and clinical indicators did not revealed any significant differences

The study included 165 female participants with the age ranging from 19 to 45 years (mean age: 31 ± 6.3 years). The patients were stratified into 4 groups (Table 1).

The mean ages of the patients from the ASCUS, HSIL, LSIL, and NILM groups were 30.7 ± 6.2, 33 ± 5.8, 30 ± 6.3, and 30.4 ± 5.8 years (Figure 1). There were no statistically significant differences between the groups ($p = 0.093$).

The median ages of sexual initiation in the ASCUS, LSIL, HSIL, and NILM groups were 18 years (IQR (Q25–Q75) is 16–18 years), 18 years (16–18 years), 17 years (16–18 years), and 18 years (18–19 years), respectively (Figure 2). We detected statistically significant differences in the age of sexual initiation between the LSIL and NILM groups (adjusted p-value = 0.018) and between the HSIL and NILM groups (adjusted p-value = 0.03). On average, patients from the HSIL and LSIL groups have a lower age of sexual initiation (1 year less) compared to the control group.

Statistical analysis of the patients based on anamnestic and clinical parameters (somatic morbidity, pregnancies, childbirths, contraception methods, surgical interventions, gynecological diseases, and past infectious diseases) did not reveal any significant differences between the groups (the data are shown in Supplementary Table S1).

TABLE 1 The numbers of patients in groups.

Cytological/ Histological conclusion	Total number	Number HPV(+)	Number HPV(–)
NILM (control group)	22	0	22
ASCUS	40	29	11
LSIL	57	32	25
HSIL	46	46	0

3.2 HPV16 type and overall amount of infected patient was higher in HSIL group

The number of patients with at least one HPV type was higher in the HSIL group (100%) than in the LSIL (72.5%) and ASCUS (56.1%) groups (adjusted p-value = 0.0086). The most common HPV type in the LSIL, HSIL, NILM was HPV16. After applying the correction for multiple comparisons, only HPV16 exhibited statistically significant differences (adjusted p-value = 0.013) (Tables 2, 3; Figure 3). The Spearman's correlation coefficient between the age and the Lactobacillus representation across all studied groups was 0.095 being not statistically significant (p-value = 0.22), similarly to the correlation coefficient between the age and HPV carriage (rho = −0.059, p-value = 0.46). Estimating the correlation between the Lactobacillus representation among the studied groups did not provide any statistically significant results (Table 3). The groups under scrutiny showed the reverse correlation between the age and Lactobacillus representation in the sample. In particular, the correlation coefficient raised from -0.28 in the HSIL group to 0.38 in the control NILM group (Figure 4, Table 3). Estimating the linear correlation between the HPV carriage and age in the HSIL and NILM groups was impossible since they comprised only one HPV carriage group. The ASCUS group showed the negative statistically significant correlation (p-value = 0.047) between the age and HPV carriage (Table 3).

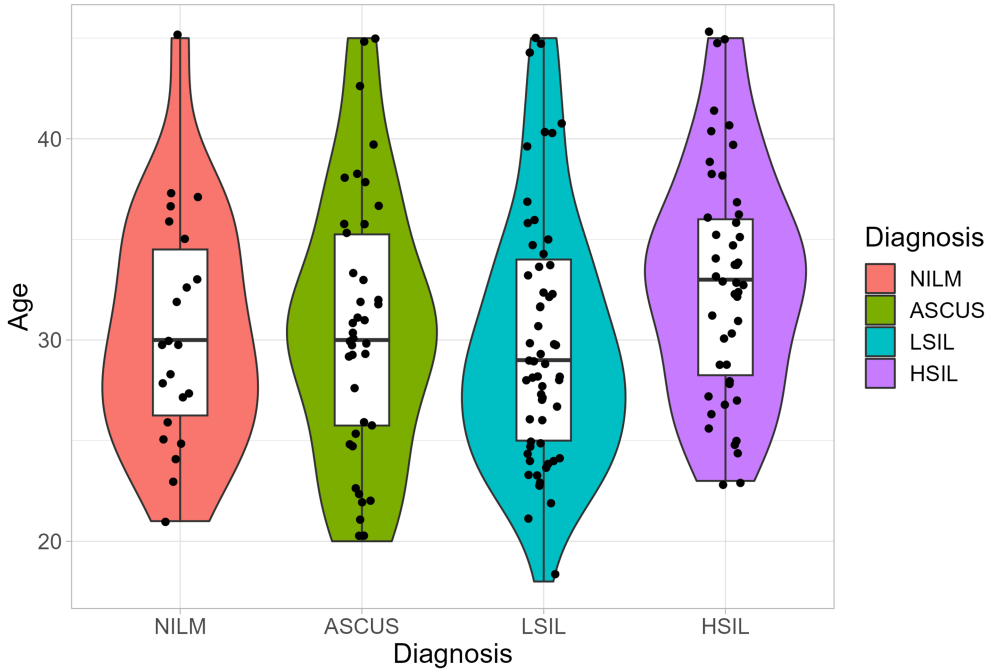


FIGURE 1
The age distribution in the studied groups. Boxplot shows standard sample metrics (median, Q25–Q75 and Q25 – 1.5 * IQR; Q75 + 1.5 * IQR by whiskers). No statistically significant difference were found between groups.

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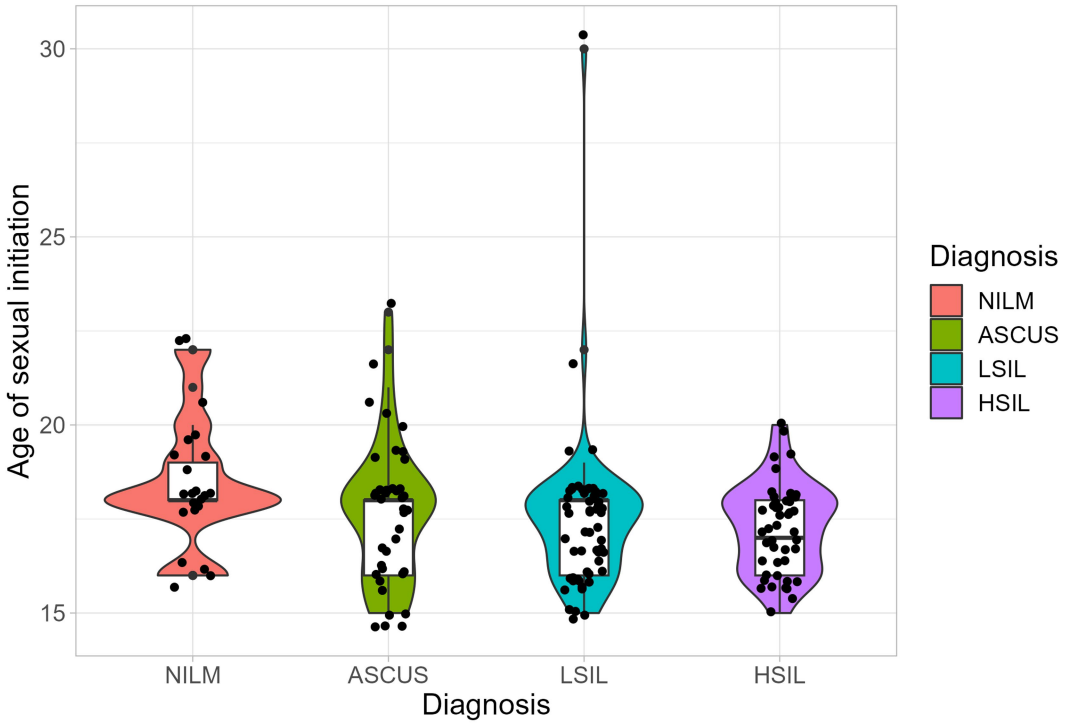


FIGURE 2
The distribution of the age of sexual initiation. Boxplot shows standard sample metrics (median, Q25–Q75 and Q25 – 1.5 * IQR; Q75 + 1.5 * IQR by whiskers). No statistically significant difference were found between groups.

TABLE 2 The ratio of HPV types across the groups.

HPV type	ASCUS (*n*=40)	LSIL (*n*=57)	HSIL (*n*=46)
11	1	0	1
16	**10**	**16**	**30**
18	3	1	3
31	4	4	10
33	2	2	5
35	0	0	4
39	1	0	1
44	1	2	1
45	0	1	2
51	4	2	3
52	3	1	2
53	2	1	1
56	4	4	2
58	1	1	2
59	4	1	0
6	2	4	2
66	2	4	1
68	3	2	2
73	1	1	0
82	0	1	0
At least one type	**29**	**32**	**46**

Statistically significant differences (adjusted p-value < 0.05) in the ratio of HPV types across the groups are highlighted in bold.

TABLE 3 The Spearman's correlation coefficient and statistical significance between the age and *Lactobacillus* representation.

	The age and the *Lactobacillus* percentage	The age and HPV carriage
Group	*r* (*p*-value)	*r* (*p*-value)
NILM	0.38 (0.078)	–
ASCUS	−0.017 (0.92)	−0.32 (**0.047**)
LSIL	−0.14 (0.29)	−0.23 (0.08)
HSIL	−0.28 (0.059)	–

Rho, the Spearman's correlation coefficient. Statistically significant correlation (r) in bold.

3.3 Evaluation of the alpha and beta microorganism diversity

Statistically significant differences in the alpha diversity between the groups were not detected (Table 4). The control group (NILM) displayed the lowest diversity level. Analyzing all patients as well as only HPV(+) patients revealed the greatest level of diversity in the HSIL group. In the HPV(−) patients, the highest diversity level was observed for the ASCUS group.

The Beta diversity analysis did not revealed any statistically significant difference between groups (p-value = 0.15). Pairwise comparison taking into account HPV status also did not show difference (Supplementary Table S8). Pairwise comparison without HPV revealed only one statistically significant difference (p-value = 0.036) between HSIL and LSIL group, but after applying multiple comparisons correction it was gone (p-value = 0.22).

10.3389/fmicb.2024.1334502

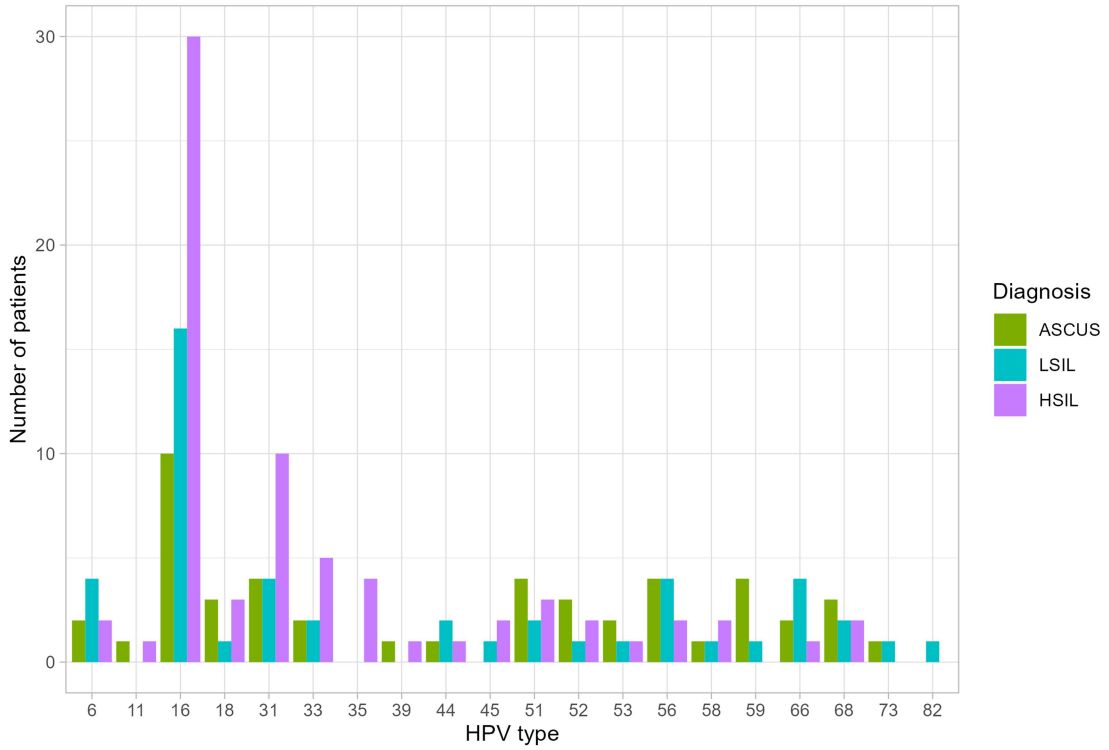


FIGURE 3
The distribution of HPV types among the studied groups. Statistically significant difference were identified for HPV 16 type in HSIL group and at least one HPV type was higher in the HSIL group.

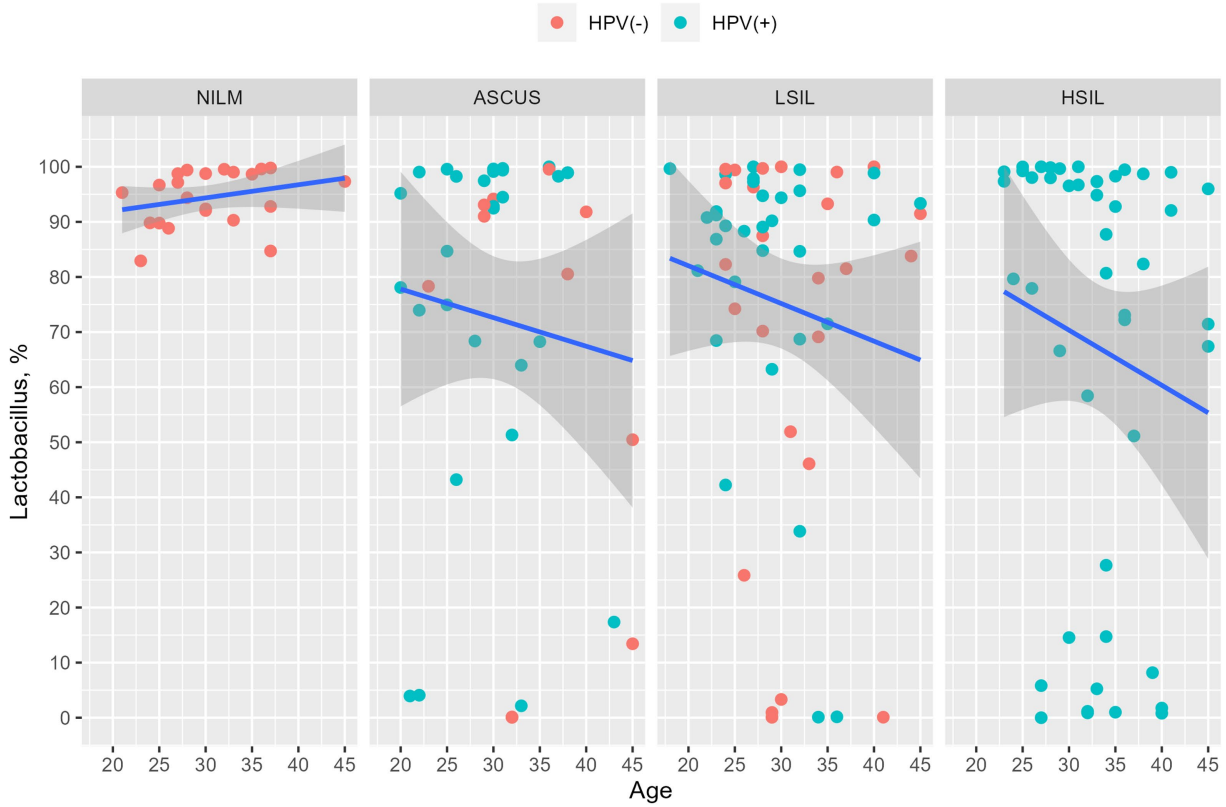


FIGURE 4
Dependency of the *Lactobacillus* genus representation from age. The HPV-negative and HPV-positive patients are indicated in red and blue, respectively. It was observed that the percentage of *Lactobacillus* is decreased in all groups except NILM. The changes were not statistically significant.

TABLE 4 Estimation of the microorganism diversity in the analyzed groups.

Diversity index	NILM	ASCUS	LSIL	HSIL	*p*-value
Shannon	0.41 (0.26–0.54)	0.582 (0.24–1.11)	0.56 (0.33–1.04)	0.81 (0.25–1.25)	0.101
Simpson	0.15 (0.09–0.27)	0.31 (0.11–0.58)	0.28 (0.16–0.52)	0.41 (0.11–0.62)	0.115
		HPV(−)			
Shannon	0.41 (0.26–0.54)	0.82 (0.37–1.54)	0.68 (0.27–1.21)	–	0.064
Simpson	0.15 (0.09–0.27)	0.35 (0.14–0.69)	0.30 (0.11–0.58)	–	0.093
		HPV(+)			
Shannon	0.41 (0.26–0.54)	0.57 (0.2–1.04)	0.52 (0.37–1.00)	0.81 (0.25–1.25)	0.104
Simpson	0.15 (0.09–0.27)	0.27 (0.07–0.53)	0.27 (0.17–0.49)	0.41 (0.11–0.62)	0.108

Data are shown as a median with the interquartile range (Q25–Q75).

TABLE 5 The qualitative and quantitative characteristics of the *Lactobacillus* species content in the HPV(+) patients and control group.

Lactobacillus species	Data	NILM (*n* = 22)	ASCUS HPV(+) (*n* = 29)	LSIL HPV(+) (*n* = 32)	HSIL HPV(+) (*n* = 46)	*p*-value
L. crispatus	*n* (%)	16 (72.7)	20 (69)	26 (81.2)	33 (71.7)	0.71
	Median (Q25–Q75)	0.36% (0.01–87.1)	7.04% (0–92)	26.5% (0.2–88.4)	0.56% (0–27.7)	0.28
L. jensenii	*n* (%)	9 (41)	12 (41.4)	19 (59.4)	21 (45.7)	0.44
	Median (Q25–Q75)	0% (0–5.3)	0% (0–2)	0.54% (0–5.4)	0% (0–4.1)	0.67
L. gasseri	*n* (%)	4 (18.2)	6 (20.7)	9 (28.1)	11 (23.9)	0.85
	Median (Q25–Q75)	0% (0–0)	0% (0–0)	0% (0–0)	0% (0–0.13)	0.90
L. iners	*n* (%)	15 (68.2)	14 (48.3)	20 (62.5)	25 (54.4)	0.47
	Median (Q25–Q75)	18.51% (0–82.8)	0% (0–39.4)	0.25% (0–45.4)	0.29% (0–72.2)	0.34
L. delbrueckii	*n* (%)	1 (4.5)	–	–	1 (2.2)	0.97
	Median (Q25–Q75)	4% (0–0)	–	–	0.01% (0–0)	0.97

Data are presented as a number of patients in a group that have a certain *Lactobacillus* species (the percentage from the total number of patients per group is given in the parentheses). *p*-value, (Pearson's Chi-squared test or Exact Fisher's test). The quantitative metrics are presented as a median with an interquartile range. *p*-value, Kruskal–Wallis test.

TABLE 6 The qualitative and quantitative characteristics of the content of various *Lactobacillus* species in the HPV(−) patients and the control group.

Lactobacillus species	Data	NILM (*n* = 22)	ASCUS HPV(−) (*n* = 11)	LSIL HPV(−) (*n* = 25)	*p*-value
L. crispatus	*n* (%)	16 (72.7)	7 (63.6)	17 (68)	0.87
	Median (Q25–Q75)	0.36% (0.01–87.1)	0.31% (0–3.2)	7.99% (0–87.5)	0.51
L. jensenii	*n* (%)	9 (41)	3 (27.3)	11 (44)	0.72
	Median (Q25–Q75)	0% (0–5.3)	0% (0–0.01)	0% (0–3)	0.51
L. gasseri	*n* (%)	4 (18.2)	4 (36.4)	7 (28)	0.49
	Median (Q25–Q75)	0% (0–0)	0% (0–4.3)	0% (0–0.04)	0.54
L. iners	*n* (%)	15 (68.2)	8 (72.7)	12 (48)	0.28
	Median (Q25–Q75)	18.5% (0–82.8)	11% (0.03–83)	0% (0–10.5)	0.07
L. delbrueckii	*n* (%)	1 (4.5)	–	1 (4)	0.98
	Median (Q25–Q75)	0% (0–0)	–	0% (0–0)	0.97

Data are presented as a number of patients in a group that have a certain *Lactobacillus* species (the percentage from the total number of patients per group is given in the parentheses). *p*-value, (Pearson's Chi-squared test or Exact Fisher's test). The quantitative metrics are presented as a median with an interquartile range. *p*-value, Kruskal–Wallis test.

3.4 The qualitative and quantitative estimations of the *Lactobacillus* species did not reveal any significant difference

The qualitative and quantitative estimations of the *Lactobacillus* content in the HPV(+) or HPV(−) patients, and the control group are shown in Tables 5, 6. There was no statistically significant difference in the *Lactobacillus* content between the groups ($p > 0.05$). Below is the list of the species sorted based on their content: *Lactobacillus crispatus, L. iners, L. jensenii, L. gasseri, L. delbrueckii*. It is worth noting that among the *Lactobacillaceae* family, we also detected the species of *Limosilactobacillus* genus (including *L. antri*, *L. coleohominis*,

L. reuteri, *L. mucosae*) in the HPV(+) patients with ASCUS (2 cases, 5%), LSIL (4 cases, 12.5%), HSIL (3 cases, 6.52%), and in the control group (2 cases, 9.09%). No statistically significant difference was observed between studied groups by genus.

3.5 The qualitative and quantitative estimations of the content of microorganisms (except *Lactobacillus* species) in the HPV(+) and HPV(−) patients

In this work, the cervical canal of the women of reproductive age comprised more than 100 various bacterial genera, 44 being the most common (not <1% of reads in two or more samples). Estimating the frequencies of detecting taxons across all the groups studied revealed statistically significant differences in the occurrence frequencies of *Fannyhessea* and *Gardnerella* among the ASCUS, HSIL, LSIL, and NILM groups (Table 7). Pairwise group comparisons showed the differences in the occurrence frequencies between the control group and the groups under scrutiny (ASCUS, HSIL, LSIL). For the *Prevotella* genus, significant differences were found only between the HSIL and NILM groups. In all detected statistically significant differences, these microorganism genera were more common in the groups under investigation (ASCUS, HSIL, LSIL) compared to the NILM group. Other comparisons did not provide any statistically significant results (Supplementary Table S2).

The qualitative estimation of all groups of HPV(−) patients revealed statistically significant differences for 4 microorganism genera: *Prevotella*, *Gardnerella*, *Porphyromonas*, *Dialister* (Table 8). Pairwise comparisons demonstrated the significant differences only between the ASUS and control groups for all 4 genera. They occurred significantly more frequently in the ASCUS group compared to the control group. Other comparisons did not provide any statistically significant results (Supplementary Table S3).

The quantitative estimation of the microorganism content across all studied groups revealed statistically significant differences in case of the *Ureaplasma* genus (Table 9). On average, *Ureaplasma* is more

TABLE 7 The qualitative estimation of the microorganism occurrence frequencies in the HPV(+) patients.

Genera	NILM ($n = 22$)	ASCUS ($n = 29$)	LSIL ($n = 32$)	HSIL ($n = 46$)	Difference between all groups	ASCUS versus NILM	LSIL versus NILM	HSIL versus NILM
					p-value	*p*-value	*p*-value	*p*-value
Fannyhessea	0 (0%)	5 (17.2%)	2 (6.3%)	10 (21.7%)	**0.036**	**0.062**	0.508	**0.024**
Prevotella	0 (0%)	4 (13.8%)	2 (6.6%)	9 (19.6%)	0.0764	0.12	0.508	**0.026**
Gardnerella	1 (4.6%)	9 (31%)	12 (37.5%)	18 (39.1%)	**0.0132**	**0.03**	**0.008**	**0.003**

p-value, (Pearson's Chi-squared test or Exact Fisher's test). Statistically significant differences are highlighted in bold.

TABLE 8 The qualitative estimation of the microorganism occurrence frequencies in the HPV(−) patients.

Genera	NILM ($n = 22$)	ASCUS ($n = 11$)	LSIL ($n = 25$)	Difference between all groups	ASCUS versus NILM	ASCUS versus LSIL	LSIL versus NILM
				p-value	*p*-value	*p*-value	*p*-value
Prevotella	0 (0%)	3 (27.27%)	4 (16%)	**0.046**	**0.03**	0.65	0.11
Gardnerella	1 (4.55%)	6 (54.55%)	6 (24%)	**0.005**	**0.003**	0.16	0.10
Porphyromonas	0 (0%)	3 (27.27%)	1 (4%)	**0.019**	**0.03**	0.07	1
Dialister	0 (0%)	3 (27.27%)	4 (16%)	**0.046**	**0.03**	0.65	0.11

p-value, (Pearson's Chi-squared test or Exact Fisher's test). Statistically significant differences are highlighted in bold.

TABLE 9 The quantitative estimation of the microorganism representation in the HPV(+) patients.

Genera	NILM ($n = 22$)	ASCUS ($n = 29$)	LSIL ($n = 32$)	HSIL ($n = 46$)	Difference between all groups	ASCUS versus NILM	HSIL versus LSIL	HSIL versus NILM	LSIL versus NILM
					p-value				
Gardnerella	0 (0–0.6)	0 (0–19.2)	0.05 (0–14)	0 (0–23)	0.27	0.15	0.91	0.11	**0.04**
Porphyromonas	0 (0–0)	0 (0–0.01)	0 (0–0)	0 (0–0.5)	0.20	0.12	0.33	**0.04**	0.14
Staphylococcus	0.05 (0–0.2)	0.02 (0–0.2)	0 (0–0.2)	0 (0–0.2)	0.22	0.44	0.81	**0.043**	0.10
Streptococcus	0 (0–0.3)	0.06 (0–6.3)	0 (0–2.9)	0 (0–1.2)	0.07	**0.012**	0.87	0.20	0.311
Parvimonas	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0.4)	0.06	0.21	**0.035**	0.08	-
Ureaplasma	0 (0–2.1)	0 (0–3.6)	0.02 (0–6.4)	0 (0–2.8)	**0.02**	0.58	**0.004**	0.72	**0.039**

Data are presented as a median fraction of a microorganism in a group with 10 and 90% quantiles. *p*-value, Kruskal–Wallis test. Statistically significant differences are highlighted in bold.

common in the LSIL group than in the other groups. Pairwise comparisons confirmed the statistically significant differences of *Ureaplasma* occurrence between the HSIL and LSIL as well as LSIL and NILM groups. Furthermore, the *Parvimonas* species was shown to occur more frequently in the HSIL group compared to the LSIL group. Significant differences were observed also for the *Parvimonas*, *Streptococcus*, *Staphylococcus*, *Porphyromonas*, *Gardnerella* genera. On average, the representation percentages of *Streptococcus*, *Staphylococcus*, *Porphyromonas*, *Gardnerella* genera were higher in the ASCUS, HSIL, and LSIL groups compared to the NILM group. Other comparisons did not provide any statistically significant results (Supplementary Table S4).

The quantitative estimation of the microorganism representation among all studied groups in HPV(−) patients revealed statistically significant differences in the mean fraction for 4 microorganism genera *Gardnerella*, *Porphyromonas*, *Dialister*, *Campylobacter* (Table 10). Pairwise comparisons showed most differences in the mean microorganism fractions between the ASCUS and NILM groups. On average, the fractions of six microorganism genera (*Gardnerella*, *Porphyromonas*, *Dialister*, *Campylobacter, Prevotella*, *Fusobacterium*) and two family (*Ruminococcaceae, Porphyromonadaceae*) were higher fraction in the ASCUS group than in the NILM group. Other comparisons did not provide any statistically significant results (Supplementary Table S5).

The microorganisms detected in the ASCUS HPV(+) group (along with the microorganisms listed in Table 11) included *F. vaginae* (with median of 0 and 10% quantile – 90% quantile of 0–8.3) as well as small fractions (90% quantile not exceeding 3%) of *Prevotella* spp., *M. organophilum, Anaerococcus* spp., *F. magna*, *Peptoniphilus* spp., *R. syzygii*, and *R. pickettii*.

In the LSIL HPV(+), *U. urealyticum* and *U. parvum* occurred more frequently and had a higher mean content compared to the other HPV(+) groups. The microorganisms detected in the LSIL group (apart from the microorganisms listed in Table 9): *R. syzygii and R. pickettii* (0%, 0–3.5) as well as small fractions (90% quantile not exceeding 3%) of *F. magna, M. organophilum, Sphingomonas* spp., *Acinetobacter* spp., *Streptococcus* spp., *R. syzygii*, and *R. pickettii*.

In the HSIL HPV(+) groups, the fractions of *F. vaginae*, *G. vaginalis*, *Prevotella* spp. were 0% (0–15.52), 0% (0–23), 0% (0–10.3), respectively. They had the highest occurrence frequencies (Table 7) among all HPV(+) groups. The microbiome of the cervical canal in the HSIL HPV(+) group comprised the following microorganisms (along with the microorganisms listed in Table 9): *Prevotella* spp. (0%, 0–10.3) and *S. sanguinegens* (0%, 0–6.35) as well as small fractions (90% quantile not exceeding 3%) of *Streptococcus* spp., *Anaerococcus* spp., *F. magna, D. micraerophilus, Parvimonas* spp., *Peptoniphilus* spp., *R. syzygii, R. pickettii, U. urealyticum*, *U. parvum, Gemella* spp., *M. massiliensis, and Sphingomonas* spp.

Along with the microorganisms listed in Table 10, the microorganisms detected in the LSIL HPV(−) group included *Bifidobacterium* (0, 0–7.79); *U. urealyticum* and *U. parvum* (0, 0–6.26). They also comprised small fractions (90% quantile not exceeding 3%) of *Sphingomonas* spp., *Peptoniphilus* spp., *F. magna*.

Comparing the quantitative contents of anaerobic microorganisms (*Anaerococcus* spp. - (2.3%, 0–0.1), *D. micraerophilus* (1.8%, 0–1.5), *S. sanguinegens* (6.6%, 0–0.03), *G. vaginalis* (8.3%, 0.2–8.7), *Prevotella* (5.6%, 0–8.7), *Porphyromonas* (1.6%, 0–1.3)) showed that their average percentage was the highest in the ASCUS HPV(−) group compared to the other groups (Table 11).

TABLE 11 Major representatives (species) of the genera detected in this study.

Genus	Major representatives (species)	Abbreviation
Fannyhessea	*Fannyhessea vaginae*	*F. vaginae*
Gardnerella	*Gardnerella vaginalis*	*G. vaginalis*
Ralstonia	*Ralstonia syzygii* *Ralstonia pickettii*	*R. syzygii* *R. pickettii*
Ureaplasma	*Ureaplasma urealyticum* *Ureaplasma parvum*	*U. urealyticum* *U. parvum*
Methylobacterium	*Methylobacterium organophilum*	*M. organophilum*
Finegoldia	*Finegoldia magna*	*F. magna*
Sneathia	*Sneathia sanguinegens*	*S. sanguinegens*
Dialister	*Dialister micraerophilus*	*D. micraerophilus*
Megasphaera	*Megasphaera massiliensis*	*M. massiliensis*

TABLE 10 The quantitative estimation of the microorganism representation in the HPV(−) patients.

Genera	NILM (n = 22)	ASCUS (n = 11)	LSIL (n = 25)	Difference between all groups	ASCUS versus LSIL	ASCUS versus NILM	LSIL versus NILM
				p-value			
Prevotella	0 (0–0)	0 (0–21.2)	0 (0–2)	0.1	0.32	**0.036**	0.13
Gardnerella	0 (0–0.6)	6.27 (0–14.9)	0 (0–22)	**0.02**	0.06	**0.004**	0.55
Porphyromonas	0 (0–0)	0 (0–5.79)	0 (0–0)	**0.01**	**0.035**	**0.012**	0.35
Dialister	0 (0–0)	0 (0–7.63)	0 (0–2)	**0.044**	0.54	**0.016**	**0.03**
Campylobacter	0 (0–0)	0 (0–1.01)	0 (0–0)	**0.007**	0.05	**0.003**	0.18
Ruminococcaceae	0 (0–0)	0 (0–2.69)	0 (0–0)	0.075	0.14	**0.04**	0.35
Fusobacterium	0 (0–0)	0 (0–0.7)	0 (0–0.06)	0.15	0.55	**0.04**	0.1
Porphyromonadaceae	0 (0–0)	0 (0–0.08)	0 (0–0)	0.08	0.16	**0.04**	0.35

Data are presented as a median with 10 and 90% quantiles. p-value, Kruskal–Wallis test. Statistically significant differences are highlighted in bold.

TABLE 12 The coefficients (standard error) of the logistic regression models including different type of predictors.

	Age only model	Model with *L. crispatus*	Model with *L. iners*
Age	−0.08* (0.03)	−0.08* (0.035)	−0.1** (0.037)
Lactobacillus crispatus	–	0.012* (0.005)	–
Lactobacillus iners	–	–	−0.01* (0.006)
Constant	2.78* (1.1)	2.4* (1.12)	3.64 ** (1.23)
Observation	103	103	103
AIC	139.5	136.11	137.13
Dependent variable	SIL status (LSIL or HSIL)		

*$p < 0.05$.; **$p < 0.01$.

3.6 Influence of age and proportion of *Lactobacillus iners* and *Lactobacillus crispatus* on SIL status

The logistic regression based on the age and age of sexual initiation as interacting independent variables and on the SIL status (HSIL/LSIL) as a dependent variable revealed no significant difference for either feature. The HPV status was not included in the model since the HSIL group comprised no HPV(−) patients. The stepwise selection of the best model based on the AIC parameter revealed the statistically significant relationship between the age and an elevated probability of being sorted into the HSIL group rather than LSIL (Table 12; age only model).

Adding the data on *L. jensenii, L. gasseri* as well as the most represented dominants from CST IV such as *Prevotella*, *Streptococcus*, *Gardnerella*, *Sneathia*, *Fannyhessea* did not exert significant effects on the coefficients for the other features included in the model. The statistical significance remained unaltered, while the absolute value changed only within 2 decimal places. The coefficient for the feature indicating the representation of the taxon based on an absolute value did not exceed 0.05 and was not statistically significant.

Creating the model that includes the values of representation of *L. crispatus, L. iners* did not affect the features previously included in the model as well (Table 12, model with *L. crispatus* and *L. iners*, respectively). The coefficients for these features were statistically significant (p-value < 0.05) yet opposite in sign.

In particular, the positive coefficient for *L. crispatus* shows that an elevated percentage leads to an increase of the probability of detecting LSIL rather than HSIL. At the same time, elevated content of *L. iners* results in a higher probability of detecting HSIL rather than LSIL given the equal age of a patient.

3.7 The predominant *Lactobacillus* species patients with normocenosis, moderate and severe dysbiosis (anaerobic, aerobic, and mixed)

In this study, we detected all previously isolated CST I-V including IV-A and IV-B (Figure 5). However, in two patients (one was from the NILM group, the other one - from the LSIL group), *L. delbrueckii* prevailed with the contents of 90 and 74%, respectively. CST was termed CST Ldelb in these patients.

Among all groups under investigation, the predominant microorganisms were CST I and II, with *L. crispatus* and *L. iners* prevailing in the cervical canal. The highest fraction of the CST IV group which correlates with pathologic microflora state was observed in the HSIL group (Figure 5).

In the HSIL group, the most severe dysbiosis was found in 24% of patients being the most common dysbiosis among all studied groups. In the patients with the established normocenosis, *L. delbrueckii* was absent in all the groups (ASCUS, LSIL, HSIL).

The microorganisms most frequently prevailing in the NILM, ASCUS, LSIL, HSIL groups were *L. crispatus*, *L. iners*, and *L. gasseri* (Figures 6, 7; Table 13). The highest *L. gasseri* content was observed in the HSIL patients. The *L. jensenii* fraction was relatively small. The fraction of the samples containing *L. crispatus* was the smallest in the HSIL group. Furthermore, in normocenosis, *L. gasseri* content was the smallest in all groups.

In each microbiota state (normocenosis, moderate dysbiosis, pronounced dysbiosis), HSIL samples exhibit a certain decrease of the representation of *L. crispatus* with the predominance of other species (Table 13; Supplementary Table S6).

4 Discussion

Among women with cervical dysfunction confirmed by cytological and/or histological examination, cervical HPV colonization was observed in 100, 72.5, and 56.1% for HSIL, ASCUS, and LSIL, respectively. The most common HPV type was HPV16 which occurred twice as often in the patients with severe cervical dysfunction (HSIL, 58.8%) compared to LSIL (28%) and ASCUS (25%) patients. Alpha and beta diversity did not show any statistically significant difference between studied group. We suggest that it may be due to lack of influence of HPV to cervical canal microbiota. Nevertheless, some CST (for example CST IV or CST III) could be more vulnerable to entry and spread of HPV. In healthy women of a reproductive age, equally often prevailing types of Lactobacillus are *L. crispatus* and *L. iners*. For example, *L. crispatus* turned out to be the predominant *Lactobacillus* species in women with normocenosis for the ASCUS and LSIL groups, while *L. iners* prevailed in the HSIL group. There are contradictions in the works of other authors concerning *L. iners*: some studies show that it accompanies HPV infection and SIL (Wang et al., 2019; Sasivimolrattana et al., 2022; Xu et al., 2022), whereas other studies demonstrate no association of the kind (McKee et al., 2020; Gomez Cherey et al., 2023; Ivanov et al., 2023). *L. crispatus* is considered to protect from SIL progressing in HPV (Cheng et al., 2020), since it produces a lot of the D-isomer of lactic acid which increases the viscosity of the vaginal secrete and elevates its ability to sequester virions, in contrast to *L. iners* which produces only the L-isomer (Kalia et al., 2020; Nicolò et al., 2023). Moreover, *L. iners* produces inerolysin that creates pores in the vaginal epithelium potentially promoting HPV infection (Xu et al., 2022; Sharifian et al., 2023). This theory agrees to our results. All taxons with statistically significant difference are usually found in cervical microbiota, but they have higher occurrence and ratio in HSIL group compared with NILM and LSIL groups. The logistic regression allowed for individually evaluating the impact of the age and the role

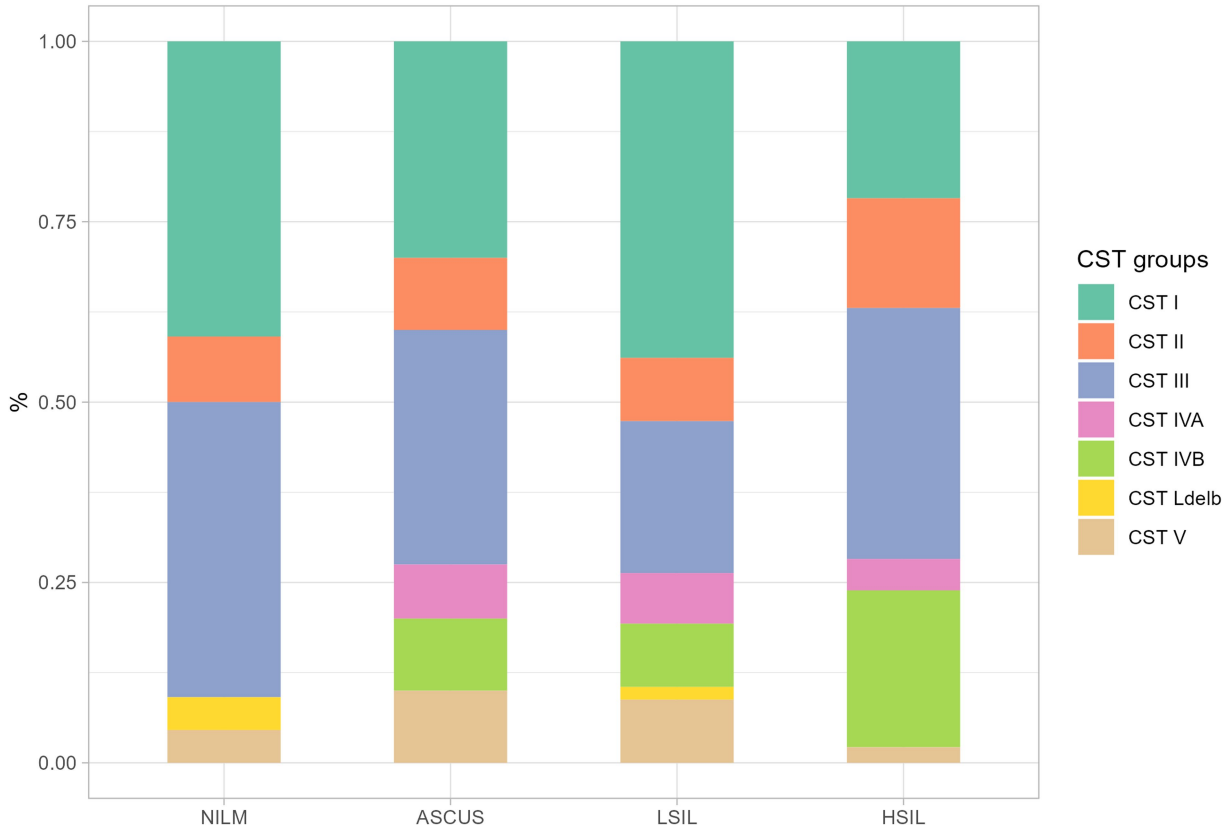


FIGURE 5
The ratio of CST fraction in the groups under scrutiny. The plot shows the proportion of samples from different. All CST groups was determined by predominant *Lactobacillus* (CST I – CST III, CST V, CST Ldelb) or other non-*Lactobacillus* (CST IVA, CST IVB) genus.

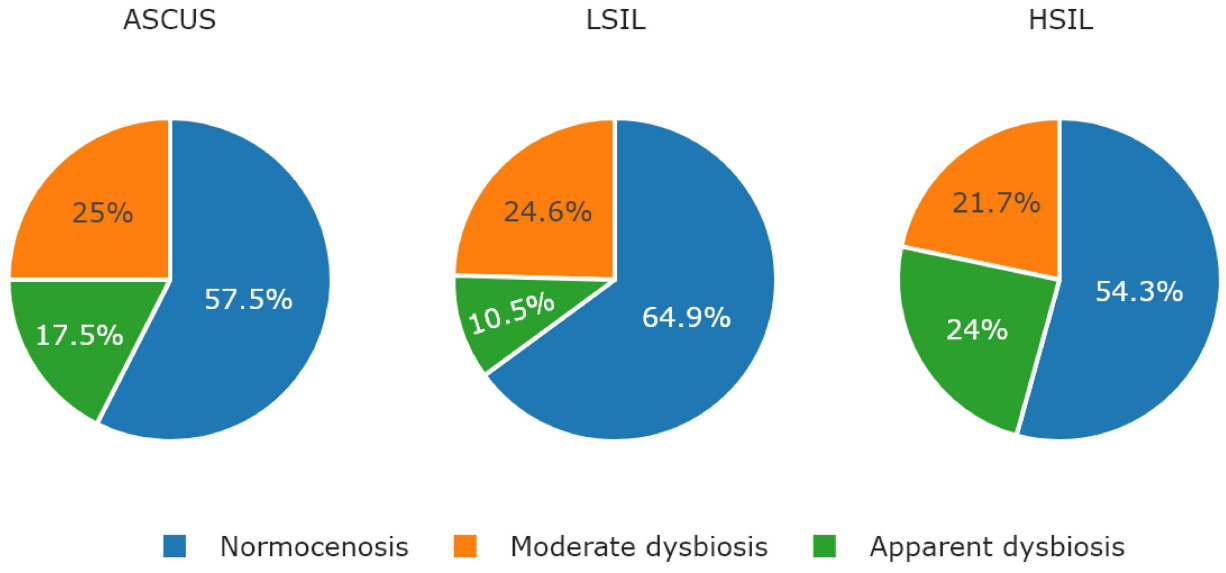


FIGURE 6
The ratio of microbiological cenosises were observed in the studied groups. The type of cenosis was determined by the percentage of *Lactobacillus* genus in the sample. Normocenosis – more than or equal 80%; moderate dysbiosis – from 20 to 80%; apparent dysbiosis – <20%.

of certain microorganisms in HSIL and LSIL development. The older was the patient as well as the higher was *L. iners* fraction in the sample from the cervical canal, the higher was the probability of detecting HSIL rather than LSIL. Conversely, the younger age and high *L. crispatus* content increased the chance of being sorted into the LSIL group. In our work, the ASCUS patients with moderate dysbiosis more

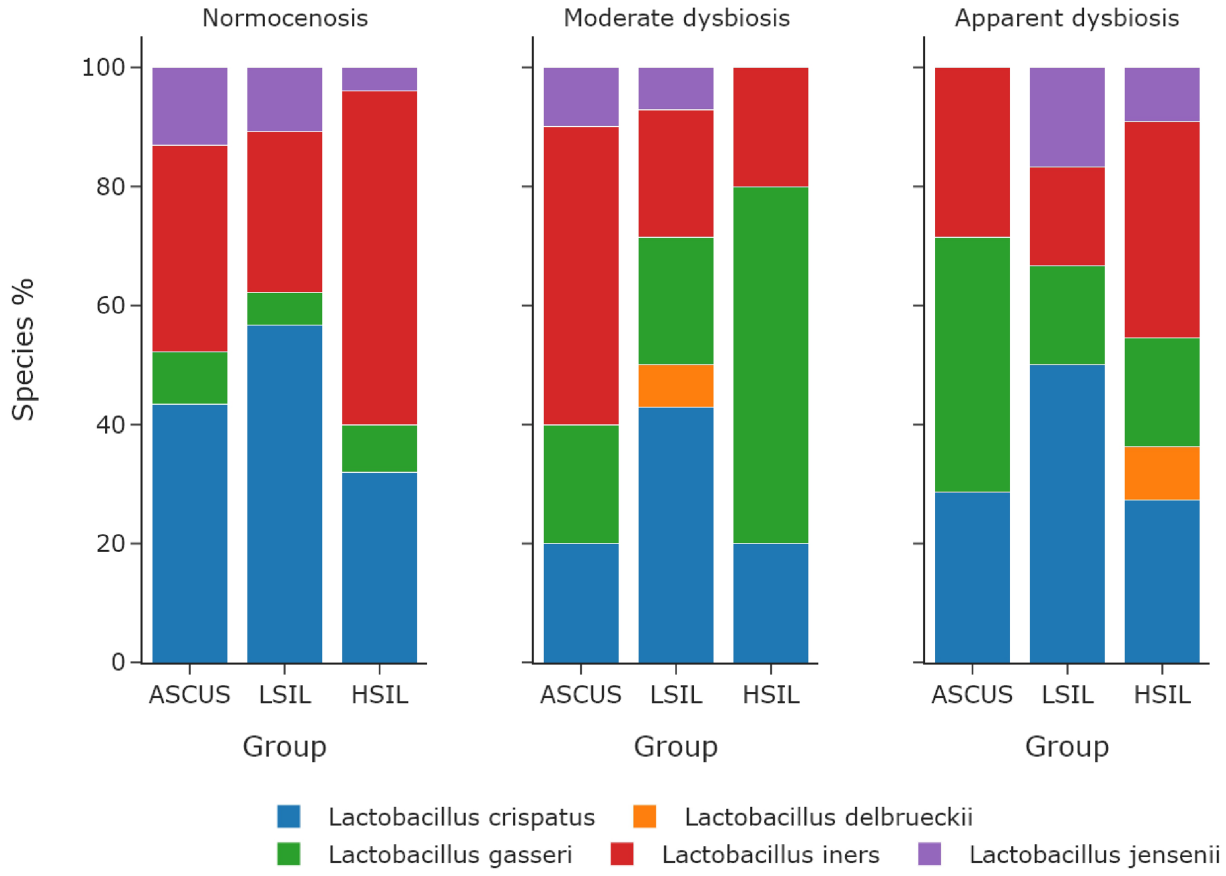


FIGURE 7
The dependence of the fraction of predominant *Lactobacillus* species on microbiome cenosises. The type of cenosis was determined by the percentage of *Lactobacillus* genus in the sample. Normocenosis – more than or equal 80%; moderate dysbiosis – from 20 to 80%; apparent dysbiosis – <20%.

TABLE 13 The dependence of predominant *Lactobacillus* species on cenosis in a sample.

Group	State	*n* (% of total amount patients)	Predominant *Lactobacillus* species	*n* of the predominant species (% of total amount cenosis)
NILM (*n* = 22)	Normocenosis	22 (100)	*L. iners/L. crispatus*	9 (40.9)/9 (40.9)
ASCUS (*n* = 40)	Normocenosis	23 (57.5)	*L. crispatus*	10 (43.5)
	Moderate dysbiosis	10 (25)	*L. iners*	5 (50)
	Apparent dysbiosis	7 (17.5)	*L. gasseri*	3 (42.9)
LSIL (*n* = 57)	Normocenosis	37 (65)	*L. crispatus*	21 (56.8)
	Moderate dysbiosis	14 (24.6)	*L. crispatus*	6 (42.9)
	Apparent dysbiosis	6 (10.5)	*L. crispatus*	3 (50)
HSIL (*n* = 46)	Normocenosis	25 (54.3)	*L. iners*	14 (56)
	Moderate dysbiosis	10 (21.7)	*L. gasseri*	6 (60)
	Apparent dysbiosis	11 (24)	*L. iners*	4 (36.4)

often contained *L. iners*, whereas LSIL patients contained *L. crispatus*, and HSIL patients had *L. gasseri*. Severe dysbiosis correlated with *L. gasseri*, *L. crispatus*, and with both *L. crispatus and L. iners* in the ASCUS, LSIL, and HSIL groups, respectively. *L. gasseri* was the least common *Lactobacillus* in the women of reproductive age. *L. delbrueckii* was the predominant *Lactobacillus* being present in 1.8% of the enrolled women, although it did not belong to any type of vaginal communities. In the ASCUS group, LSIL, and HSIL dysbiosis were observed in 42.5, 35, and 45.6% of patients, respectively. Anaerobic dysbiosis or bacterial vaginosis was the most prevalent in all groups. In bacterial vaginosis, the following anaerobic microorganisms prevailed: *G. vaginalis, F. vaginae, D. micraerophilus, S. sanguinegens,*

Anaerococcus spp., *M. massiliensis, Prevotella* spp., *F. magna, Peptoniphilus* spp., *Porphyromonas* spp., *and Parvimonas* spp. Aerobic vaginitis was more rare, and in all cases under scrutiny, the predominant microorganism to cause it was *Streptococcus* spp. The bacteria associated with bacterial vaginitis produce carcinogenic nitrosamines, which trigger the release of cytokines such as interleukine-1b involved in SIL development. During HPV infection, the cytokines weaken the immune system, while carcinogenic nitrosamines facilitate DNA damage (Muzny et al., 2020; Tidbury et al., 2021). The predominance of certain groups of microorganisms such as *Sneathia, Prevotella, Megasphaera, Dialister, Gardnerella, Streptococcus*, and *Fannyhessea* is associated with SIL development (Mitra et al., 2020; Dong et al., 2022) which is in line with our data. However, it is believed that the predominance of anaerobic microorganisms is associated with LSIL (Lin et al., 2021), whereas aerobic vaginitis is associated with HSIL (Plisko et al., 2021). Colonization of the cervical canal and vagina by *U. urealyticum* and *U. parvum* lead to SIL development (Condic et al., 2023) which is supported by our results, since *Ureaplasma* spp. prevailed both qualitatively and quantitatively in the LSIL HPV(+) patients. Other works suggest that microbial diversity is elevated upon HPV infection (Chen et al., 2020; Usyk et al., 2020) which, however, was not observed in this work (Figure 7).

The qualitative and quantitative comparisons of microbiomes between the groups allowed us to detect statistically significant differences for several genera: *Prevotella, Gardnerella, Porphyromonas, Dialister, Staphylococcus, Ureaplasma*. Estimating the possible relationship of various *Lactobacillus* species with dysbiotic impairments of the microbiome and HPV-associated cervical diseases did not reveal any statistical significant difference in the *Lactobacillus* species composition and its relationship with HPV-associated cervical disorders. Of note, that *L. iners, L. gasseri, L. crispatus* were often detected upon dysbiosis, whereas *L. gasseri* was the least common in all groups. Evaluating dysbiotic impairments of the microbiome in HPV(+) patients based on 16S sequencing demonstrated that dysbiosis was most common in the patients with ASCUS (42.5%), LSIL (35%), and HSIL (45.6%). Bacterial vaginosis was associated with *G. vaginalis, F. vaginae, D. micraerophilus, S. sanguinegens, Anaerococcus* spp., *M. massiliensis, Prevotella* spp., *F. magna, Peptoniphilus* spp., *Porphyromonas* spp., and *Parvimonas* spp., while aerobic vaginitis was related to *Streptococcus* spp. Detecting HPV along with the impaired microbiome, such as bacterial vaginosis and aerobic vaginitis, may allow for identifying the groups of women with the high risk of SIL development and progression.

Data availability statement

The data presented in the study are deposited in the NCBI BioProject repository, accession number PRJNA1041987.

Ethics statement

The studies involving humans were approved by Local Ethics Committee at the FSBI "National Medical Research Center for Obstetrics, Gynecology and Perinatology Named After Academician V.I.Kulakov" Ministry of Health of the Russian Federation (protocol no. 3 of 26 March 2020). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AP: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. VC: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AK: Conceptualization, Investigation, Methodology, Software, Writing – original draft. AAn: Conceptualization, Investigation, Methodology, Writing – original draft. ZR: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – original draft. AAs: Conceptualization, Investigation, Writing – original draft. DK: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. DR: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. GB: Conceptualization, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1334502/full#supplementary-material

References

Al-Nasiry, S., Ambrosino, E., Schlaepfer, M., Morré, S. A., Wieten, L., Voncken, J. W., et al. (2020). The interplay between reproductive tract microbiota and immunological system in human reproduction. *Front. Immunol.* 11, 1–20. doi: 10.3389/fimmu.2020.00378

Alrajjal, A., Pansare, V., Choudhury, M. S. R., Khan, M. Y. A., and Shidham, V. B. (2021). Squamous intraepithelial lesions (SIL: LSIL, HSIL, ASCUS, ASC-H, LSIL-H) of uterine cervix and Bethesda system. *Cytojournal* 18:16. doi: 10.25259/Cytojournal_24_2021

Andralojc, K. M., Molina, M. A., Qiu, M., Spruijtenburg, B., Rasing, M., Pater, B., et al. (2021). Novel high-resolution targeted sequencing of the cervicovaginal microbiome. *BMC Biol.* 19, 267–218. doi: 10.1186/s12915-021-01204-z

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., and Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* 68, 394–424. doi: 10.3322/caac.21492

Burd, E. M. (2003). Human papillomavirus and cervical cancer. *Clin. Microbiol. Rev.* 16, 1–17. doi: 10.1128/CMR.16.1.1-17.2003

Chen, Y., Qiu, X., Wang, W., Li, D., Wu, A., Hong, Z., et al. (2020). Human papillomavirus infection and cervical intraepithelial neoplasia progression are associated with increased vaginal microbiome diversity in a Chinese cohort. *BMC Infect. Dis.* 20, 629–612. doi: 10.1186/s12879-020-05324-9

Cheng, L., Norenhag, J., Hu, Y. O. O., Brusselaers, N., Fransson, E., Ährlund-Richter, A., et al. (2020). Vaginal microbiota and human papillomavirus infection among young Swedish women. *NPJ Biofilms Microbiomes* 6, 39–10. doi: 10.1038/s41522-020-00146-8

Cogliano, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., et al. (2005). Carcinogenicity of human papillomaviruses. *Lancet Oncol.* 6:204. doi: 10.1016/s1470-2045(05)70086-3

Condic, M., Neidhöfer, C., Ralser, D. J., Wetzig, N., Thiele, R., Sieber, M., et al. (2023). Analysis of the cervical microbiome in women from the German national cervical cancer screening program. *J. Cancer Res. Clin. Oncol.* 149, 6489–6500. doi: 10.1007/s00432-023-04599-0

Dong, B., Huang, Y., Cai, H., Chen, Y., Li, Y., Zou, H., et al. (2022). Prevotella as the hub of the cervicovaginal microbiota affects the occurrence of persistent human papillomavirus infection and cervical lesions in women of childbearing age via host NF-κB/C-myc. *J. Med. Virol.* 94, 5519–5534. doi: 10.1002/jmv.28001

Gardella, B., Pasquali, M. F., La Verde, M., Cianci, S., Torella, M., and Dominoni, M. (2022). The complex interplay between vaginal microbiota, HPV infection, and immunological microenvironment in cervical intraepithelial neoplasia: a literature review. *Int. J. Mol. Sci.* 23:7174. doi: 10.3390/ijms23137174

Gomez Cherey, J. F., Payalef, S. N., Fleider, L., Reyes, A. P., Maldonado, V. A., Losada, M. O., et al. (2023). Microbiota unbalance in relation to high-risk human papillomavirus cervical infection. *Int. J. Gynecol. Cancer* 33, 482–488. doi: 10.1136/ijgc-2022-003760

Hu, C., Liu, T., Han, C., Xuan, Y., Jiang, D., Sun, Y., et al. (2022). HPV E6/E7 promotes aerobic glycolysis in cervical cancer by regulating IGF2BP2 to stabilize m6A-MYC expression. *Int. J. Biol. Sci.* 18, 507–521. doi: 10.7150/ijbs.67770

Ivanov, M. K., Brenner, E. V., Hodkevich, A. A., Dzyubenko, V. V., Krasilnikov, S. E., Mansurova, A. S., et al. (2023). Cervicovaginal-microbiome analysis by 16S sequencing and real-time PCR in patients from Novosibirsk (Russia) with cervical lesions and several years after Cancer treatment. *Diagnostics* 13:140. doi: 10.3390/diagnostics13010140

Kalia, N., Singh, J., and Kaur, M. (2020). Microbiota in vaginal health and pathogenesis of recurrent vulvovaginal infections: a critical review. *Ann. Clin. Microbiol. Antimicrob.* 19:5. doi: 10.1186/s12941-020-0347-4

Kang, G.-U., Jung, D.-R., Lee, Y. H., Jeon, S. Y., Han, H. S., Chong, G. O., et al. (2021). Potential association between vaginal microbiota and cervical carcinogenesis in Korean women: a cohort study. *Microorganisms* 9:294. doi: 10.3390/microorganisms9020294

Lin, W., Zhang, Q., Chen, Y., Chen, L., Dong, B., and Sun, P. (2021). The prevalence of human papillomavirus and bacterial vaginosis among young women in China: a cross-sectional study. *BMC Womens Health* 21, 409–410. doi: 10.1186/s12905-021-01504-0

Lin, S., Zhang, B., Lin, Y., Lin, Y., and Zuo, X. (2022). Dysbiosis of cervical and vaginal microbiota associated with cervical intraepithelial neoplasia. *Front. Cell. Infect. Microbiol.* 12:767693. doi: 10.3389/fcimb.2022.767693

McClymont, E., Albert, A. Y., Wang, C., Dos Santos, S. J., Coutlée, F., Lee, M., et al. (2022). Vaginal microbiota associated with oncogenic HPV in a cohort of HPV-vaccinated women living with HIV. *Int. J. STD AIDS* 33, 847–855. doi: 10.1177/09564624221109686

McKee, K. S., Carter, K. A., Bassis, C., Young, V. B., Reed, B., Harper, D. M., et al. (2020). The vaginal microbiota, high-risk human papillomavirus infection, and cervical cytology: results from a population-based study. *Gynecol. pelvic Med.* 3:18. doi: 10.21037/gpm-20-10

Mitra, A., MacIntyre, D. A., Ntritsos, G., Smith, A., Tsilidis, K. K., Marchesi, J. R., et al. (2020). The vaginal microbiota associates with the regression of untreated cervical intraepithelial neoplasia 2 lesions. *Nat. Commun.* 11:1999. doi: 10.1038/s41467-020-15856-y

Molina, M. A., Andralojc, K. M., Huynen, M. A., Leenders, W. P. J., and Melchers, W. J. G. (2022). In-depth insights into cervicovaginal microbial communities and hrHPV infections using high-resolution microbiome profiling. *npj Biofilms Microbiomes* 8:8. doi: 10.1038/s41522-022-00336-6

Muzny, C. A., Łaniewski, P., Schwebke, J. R., and Herbst-Kralovetz, M. M. (2020). Host-vaginal microbiota interactions in the pathogenesis of bacterial vaginosis. *Curr. Opin. Infect. Dis.* 33, 59–65. doi: 10.1097/QCO.0000000000000620

Naidoo, K., Abbai, N., Tinarwo, P., and Sebitloane, M. (2022). BV associated bacteria specifically BVAB 1 and BVAB 3 as biomarkers for HPV risk and progression of cervical neoplasia. *Infect. Dis. Obstet. Gynecol.* 2022, 1–11. doi: 10.1155/2022/9562937

Nicolò, S., Antonelli, A., Tanturli, M., Baccani, I., Bonaiuto, C., Castronovo, G., et al. (2023). Bacterial species from vaginal microbiota differently affect the production of the E6 and E7 Oncoproteins and of p53 and p-Rb Oncosuppressors in HPV16-infected cells. *Int. J. Mol. Sci.* 24:7173. doi: 10.3390/ijms24087173

Nieves-Ramírez, M. E., Partida-Rodríguez, O., Moran, P., Serrano-Vázquez, A., Pérez-Juárez, H., Pérez-Rodríguez, M. E., et al. (2021). Cervical squamous intraepithelial lesions are associated with differences in the vaginal microbiota of Mexican women. *Microbiol. Spectr.* 9:e0014321. doi: 10.1128/Spectrum.00143-21

Norenhag, J., Du, J., Olovsson, M., Verstraelen, H., Engstrand, L., and Brusselaers, N. (2020). The vaginal microbiota, human papillomavirus and cervical dysplasia: a systematic review and network meta-analysis. *BJOG An Int. J. Obstet. Gynaecol.* 127, 171–180. doi: 10.1111/1471-0528.15854

Ntuli, L., Mtshali, A., Mzobe, G., Liebenberg, L. J. P., and Ngcapu, S. (2022). Role of immunity and vaginal microbiome in clearance and persistence of human papillomavirus infection. *Front. Cell. Infect. Microbiol.* 12, 1–10. doi: 10.3389/fcimb.2022.927131

Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18, 1403–1414. doi: 10.1111/1462-2920.13023

Plisko, O., Zodzika, J., Jermakova, I., Pcolkina, K., Prusakevica, A., Liepniece-Karele, I., et al. (2021). Aerobic vaginitis—underestimated risk factor for cervical intraepithelial neoplasia. *Diagnostics* 11, 1–9. doi: 10.3390/diagnostics11010097

Proctor, L. M., Creasy, H. H., Fettweis, J. M., Lloyd-Price, J., Mahurkar, A., Zhou, W., et al. (2019). The integrative human microbiome project. *Nature* 569, 641–648. doi: 10.1038/s41586-019-1238-8

Qingqing, B., Jie, Z., Songben, Q., Juan, C., Lei, Z., and Mu, X. (2021). Cervicovaginal microbiota dysbiosis correlates with HPV persistent infection. *Microb. Pathog.* 152:104617. doi: 10.1016/j.micpath.2020.104617

Ritu, W., Enqi, W., Zheng, S., Wang, J., Ling, Y., and Wang, Y. (2019). Evaluation of the associations between cervical microbiota and HPV infection, clearance, and persistence in Cytologically Normal women. *Cancer Prev Res (Phila)* 12, 43–56. doi: 10.1158/1940-6207.CAPR-18-0233

Santella, B., Schettino, M. T., Franci, G., De Franciscis, P., Colacurci, N., Schiattarella, A., et al. (2022). Microbiota and HPV: the role of viral infection on vaginal microbiota. *J. Med. Virol.* 94, 4478–4484. doi: 10.1002/jmv.27837

Sasivimolrattana, T., Chantratita, W., Sensorn, I., Chaiwongkot, A., Oranratanaphan, S., Bhattarakosol, P., et al. (2022). Cervical microbiome in women infected with HPV16 and high-risk HPVs. *Int. J. Environ. Res. Public Health* 19:14716. doi: 10.3390/ijerph192214716

Sharifian, K., Shoja, Z., and Jalilvand, S. (2023). The interplay between human papillomavirus and vaginal microbiota in cervical cancer development. *Virol. J.* 20, 73–79. doi: 10.1186/s12985-023-02037-8

Tidbury, F. D., Langhart, A., Weidlinger, S., and Stute, P. (2021). Non-antibiotic treatment of bacterial vaginosis—a systematic review. *Arch. Gynecol. Obstet.* 303, 37–45. doi: 10.1007/s00404-020-05821-x

Usyk, M., Zolnik, C. P., Castle, P. E., Porras, C., Herrero, R., Gradissimo, A., et al. (2020). Cervicovaginal microbiome and natural history of HPV in a longitudinal study. *PLoS Pathog.* 16, e1008376–e1008320. doi: 10.1371/journal.ppat.1008376

Wang, H., Ma, Y., Li, R., Chen, X., Wan, L., and Zhao, W. (2019). Associations of cervicovaginal lactobacilli with high-risk human papillomavirus infection, cervical intraepithelial neoplasia, and cancer: a systematic review and meta-analysis. *J. Infect. Dis.* 220, 1243–1254. doi: 10.1093/infdis/jiz325

Xu, X., Zhang, Y., Yu, L., Shi, X., Min, M., Xiong, L., et al. (2022). A cross-sectional analysis about bacterial vaginosis, high-risk human papillomavirus infection, and cervical intraepithelial neoplasia in Chinese women. *Sci. Rep.* 12, 6609–6612. doi: 10.1038/s41598-022-10532-1

Zuñiga Martinez, M. L., López Mendoza, C. M., Tenorio Salazar, J., García Carrancá, A. M., Cerbón Cervantes, M. A., and Alcántara-Quintana, L. E. (2022). Establishment, authenticity, and characterization of cervical cancer cell lines. *Mol. Cell. Oncol.* 9:2078628. doi: 10.1080/23723556.2022.2078628

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Multi-omics analyses reveal interactions between the skin microbiota and skin metabolites in atopic dermatitis

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Introduction: Atopic dermatitis (AD) is one of the most common inflammatory skin diseases. Skin microecological imbalance is an important factor in the pathogenesis of AD, but the underlying mechanism of its interaction with humans remains unclear.

Methods: 16S rRNA gene sequencing was conducted to reveal the skin microbiota dynamics. Changes in skin metabolites were tracked by LC–MS metabolomics. We then explored the potential mechanism of interaction by analyzing the correlation between skin bacterial communities and metabolites in corresponding skin-associated samples.

Results: Samples from 18 AD patients and 18 healthy volunteers (HVs) were subjected to 16S rRNA gene sequencing and LC–MS metabolomics. AD patients had dysbiosis of the skin bacterial community with decreased species richness and evenness. The relative abundance of the genus *Staphylococcus* increased significantly in AD, while the abundances of the genera *Propionibacterium* and *Brevundimonas* decreased significantly. The relative abundance of the genera *Staphylococcus* in healthy females was significantly higher than those in healthy males, while it showed no difference in AD patients with or without lesions. The effects of AD status, sex and the presence or absence of rashes on the number of differentially abundant metabolites *per capita* were successively reduced. Multiple metabolites involved in purine metabolism and phenylalanine metabolism pathways (such as xanthosine/xanthine and L-phenylalanine/trans-cinnamate) were increased in AD patients. These trends were much more obvious between female AD patients and female HVs. Spearman correlation analysis revealed that the genus *Staphylococcus* was positively correlated with various compounds involved in phenylalanine metabolism and purine metabolic pathways. The genera *Brevundimonas* and *Lactobacillus* were negatively correlated with various compounds involved in purine metabolism, phenylalanine metabolism and sphingolipid signaling pathways.

Discussion: We suggest that purine metabolism and phenylalanine metabolism pathway disorders may play a certain role in the pathogenic mechanism of

Staphylococcus aureus in AD. We also found that females are more likely to be colonized by the genus *Staphylococcus* than males. Differentially abundant metabolites involved in purine metabolism and phenylalanine metabolism pathways were more obvious in female. However, we should notice that the metabolites we detected do not necessarily derived from microbes, they may also origin from the host.

KEYWORDS

atopic dermatitis, skin microbiome, skin metabolome, correlation analysis, purine metabolism, phenylalanine metabolism

1 Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases and clinically manifests as an eczema-like rash with pruritus (Ständer, 2021; Zhang et al., 2023). The prevalence of AD is 15–20% among children and up to 10% among adults, making AD the 15th most common nonfatal disease and the skin disease with the highest disease burden in disability-adjusted life years (Ständer, 2021; Schuler et al., 2023). In addition, AD may be associated with an increasing prevalence of psychosocial disorders, such as depression, anxiety, sleep disorders and suicidal ideation (Kage et al., 2020). Epidermal barrier dysfunction, skin microecological imbalance and immune disorders, which are dominated by type 2 inflammation, contribute to the outbreak of AD (Langan et al., 2020).

The skin microbiota has a complex interaction with the host (Chen et al., 2018). In recent years, with the application of high-throughput DNA sequencing technologies based on two principal methods (16S rRNA gene sequencing and metagenomics sequencing), the study of the AD skin microbiome has shown great progress (Byrd et al., 2018; Ghosh et al., 2018). Skin microbiota disturbances characterized by increased *Staphylococcus aureus* colonization and decreased microbiota diversity occurred in both lesion and nonlesion sites of AD patients (Koh et al., 2022). *S. aureus* may destroy the skin barrier and induce an inflammatory response (Langan et al., 2020). The local Th2 immune response further diminishes barrier function and facilitates the growth of *Staphylococcus,* especially *S. aureus* (Langan et al., 2020; Schuler et al., 2023). However, the composition of the skin microbiome is complex, and the specific mechanism of its interaction with the host remains unclear.

Metabolomics is a technique that reflects the phenotypic outcome of biological activities and elucidates the relationship between metabolite changes and physiological/pathological changes (Zhang et al., 2023). Skin cells and glandular secretions, skin resident microbiota, and external environmental factors (such as cosmetics or pollution) can affect the composition of skin metabolites (Afghani et al., 2022). The metabolomic profile of 15 AD patients and 17 controls was studied through skin punch biopsies, which revealed that a total of 77 metabolites differed significantly between the lesional skin of atopic dermatitis, nonlesional skin of atopic dermatitis and skin of controls (Ilves et al., 2021). Until now, much of the metabolomics research on AD skin has focused on the proportion of changes in the lipid composition of the skin due to its role in the structural integrity of the skin barrier (Emmert et al., 2021; Zhang et al., 2023).

Theoretically, the skin microbiome and the skin would constantly interact with surrounding skin metabolites. The identification of AD skin metabolites may help to determine the changes in host status and the influence of the skin microbiome on the local skin. Several previous studies have explored the interactions between the skin microbiota and skin metabolites in AD. AD skin has an increase in free fatty acids (FFA), and this effect appears to be *S. aureus*-dependent (Emmert et al., 2021). Saturated shorter-chain FFA are negatively correlated with *Staphylococci* (Emmert et al., 2021), and long-chain FFA are decreased in *S. aureus*-colonized AD skin (Li et al., 2017). Shorter fatty acids can more easily traverse the skin to acidify it, and *S. aureus* does not grow well in acidic healthy skin pH conditions (Afghani et al., 2022). However, lipids are not the only metabolites present in the skin, and an increasing number of studies are starting to turn from lipids to other molecules in AD (Afghani et al., 2022). Herein, we performed 16S rRNA gene sequencing of the skin microbiome and untargeted metabolomics analysis of AD patients' skin samples, and searched for metabolites closely related to the skin microbiome of AD patients through association analysis. We also explored the effects of sex and rash status on the results through subgroup analysis. We established a correlation map of the skin microbiome and skin metabolome in AD patients and healthy people. These results highlight the role of the skin microbiome in regulating overall metabolism and provide new insights into the pathological mechanisms of AD.

2 Materials and methods

2.1 Study population

AD patients and healthy volunteers (HVs) were recruited from Guangdong Provincial Hospital of Traditional Chinese Medicine from October 2019 to May 2021. The inclusion criteria were as follows: over 18 years of age; patients meeting the Williams AD diagnostic criteria (Williams et al., 1994), with or without rash in both elbow fossa; and volunteers with no skin disease. The exclusion criteria were as follows: patients who received systemic immunosuppressive agents, biological agents, antibiotics, antifungals and glucocorticoid drugs, or ultraviolet rays and other systematic treatment in the previous 1 month; participants with severe kidney or liver damage, mental illness, or other serious organ disease; and participants who had used topical glucocorticoid, anti-biological ointment, skin care product or lotion on the elbow fossa in the previous week. All clinical information and

samples were obtained with informed consent. This study was conducted with the approval of the institutional review board of the Guangdong Provincial Hospital of Traditional Chinese Medicine and in accordance with the Declaration of Helsinki (World Medical Association, 2013).

2.2 Sample collection

A sterile double-sided polyester swab was soaked with 0.9% NaCl and forcefully rubbed back and forth 50 times at the elbow fossa within a range of approximately 4 cm × 4 cm (regardless of whether there were rashes at the elbow fossa). The head of each swab was then placed in a sterile liquid-holding tube, fully oscillated, and cut aseptically from the handle before closing the tube cap. The same procedure was used to sample the skin of the elbow fossa again, and the samples were stored in another sterile storage tube. The two samples were refrigerated at −80°C within 2 h until 16S rRNA high-throughput sequencing and metabolomics were performed. The workflow of this study is shown in Figure 1.

2.3 Microbiome DNA extraction and 16S sequencing

The DNA of the microbial community was extracted by a MagPure Stool DNA KF kit B (Magen, China) following the manufacturer's instructions. The quality of all extracted DNA was assessed with a Qubit® dsDNA BR Assay kit (Invitrogen, United States) and agarose gel electrophoresis. Variable region V4 of the bacterial 16S rRNA gene was amplified with the common PCR primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′- GGACTACH VGGGTWTCTAAT-3′). Both forward and reverse primers were tagged with Illumina adapter, pad, and linker sequences.

The PCR products were purified using Agencourt AMPure XP beads. The validated libraries were used for sequencing on the Illumina HiSeq 2,500 platform (BGI, Shenzhen, China) following Illumina's standard pipelines and generating 2 × 250 bp paired-end reads.

2.4 16S amplicon sequencing data analysis

After Illumina sequencing, barcode and primer sequences were removed. Specific tags were generated by FLASH software (version 1.2.11) according to the overlap information of the reads (Magoč and Salzberg, 2011). Tags were clustered into operational taxonomic units (OTUs) with a 97% threshold by USEARCH (v7.0.1090), where the unique OTU representative sequences can be obtained (Edgar, 2010). Chimeras were filtered by UCHIME (v4.2.40) (Edgar et al., 2011). OTU representative sequences were aligned against the database for taxonomic annotation by RDP classifier (v2.2) software (sequence identity was set to 0.6) (Wang et al., 2007). Alpha diversity and beta diversity analyses were conducted by mothur (v1.31.2) and QIIME (v1.80), respectively (Schloss et al., 2009; Caporaso et al., 2010).

2.5 Liquid chromatography–mass spectrometry metabolomic data collection

Samples (100 μL) were placed into an EP tube and extracted with 400 μL extract solution (methanol:acetonitrile:water 2:2:1), vortexed

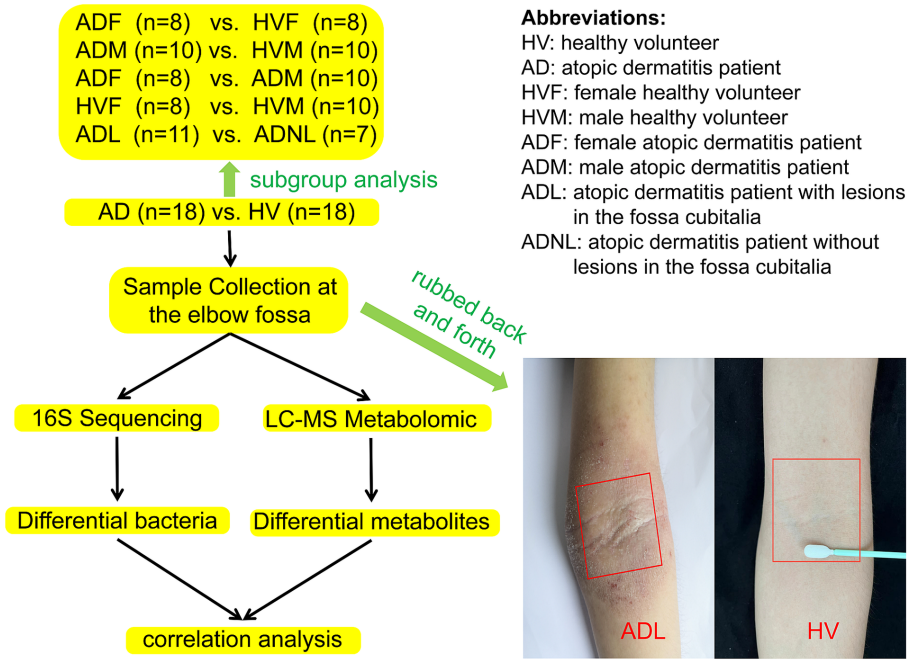


FIGURE 1
Analysis workflow.

for 1 min, sonicated for 10 min, and incubated for 1 h at −20°C. Samples were centrifuged for 15 min at 25000 rpm at 4°C, and the supernatant was then transferred for vacuum freeze drying. The metabolites were resuspended in 200 μL of 10% methanol and sonicated for 10 min at 4°C. After centrifuging for 15 min at 25000 rpm, the supernatants were transferred to a new glass vial for further analysis. The QC samples were then mixed from each sample.

The samples were analyzed on a Waters 2D UPLC (Waters, United States) coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific, United States) with a heated electrospray ionization (HESI) source and controlled by the Xcalibur 2.3 software program (Thermo Fisher Scientific, Waltham, MA, United States), according to previously reported methods with minor modifications (Chen et al., 2021). Briefly, the mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in positive mode and 10 mM ammonium formate (A) and acetonitrile (B) in negative mode. The column temperature was maintained at 45°C. The flow rate was 0.35 mL/min, and the injection volume was 5 μL. The mass spectrometric settings for positive/negative ionization modes were as follows: spray voltage, 3.8/−3.2 kV; sheath gas flow rate, 40 arbitrary units (arb); aux gas flow rate, 10 arb; aux gas heater temperature, 350°C; capillary temperature, 320°C.

2.6 LC–MS metabolomic data analyses

LC–MS/MS data processing was performed using Compound Discoverer 3.1 (Thermo Fisher Scientific, United States) software, including peak extraction, peak alignment, and compound identification. Data preprocessing, statistical analysis, metabolite classification annotations and functional annotations were performed using the metabolomics R package metaX (BGI, Shenzhen, China) and the metabolome bioinformatic analysis pipeline (Wen et al., 2017). The multivariate raw data are dimensionally reduced by principal component analysis (PCA) to analyze the groupings, trends (intra- and intergroup similarities and differences) and outliers of the observed variables in the data set (whether there is an abnormal sample). Partial least squares method-discriminant analysis (PLS-DA), the variable importance in projection (VIP) values of the first two principal components of the model, combined with the variability analysis, the fold change and Student's *t* test were used to screen for differentially abundant metabolites.

2.7 Statistical analysis

Statistical analysis was performed in the R platform (v3.5.1). The differences in alpha diversity indexes were determined by Student's *t* test. The beta diversity difference between the two groups was analyzed by analysis of similarity (ANOSIM). Differences in the relative abundance of genera between the two groups were evaluated with the Wilcoxon rank sum test. False-discovery rate (FDR) values were estimated using the Benjamini-Hochberg method to control for multiple testing. Linear discriminant analysis coupled with effect size (LEfSe) was applied to identify microorganisms that can be used to discriminate AD patients from HVs (Segata et al., 2011). A *p* value threshold cutoff at 0.05 was considered. Spearman correlation was carried out to determine the relationship between the skin microbiota and metabolites.

3 Results

3.1 Study population characteristics

After quality control, samples from 18 AD patients and 18 HVs were successfully subjected to 16S rRNA gene sequencing and LC–MS metabolomic analyses. Subgroup analysis was conducted according to whether there were rashes in the sampling site of the elbow fossa and by sex. The characteristics of the participants in each group are shown in Figure 1 and Supplementary Table S1.

3.2 Altered skin microbiota composition in AD patients

In the present study, a total of 2,073,232 effective 16S rRNA gene sequencing reads were obtained from the skin samples of 18 AD patients and 18 HV, with an average of 57589.8 reads per sample (ranging from 38,199 to 64,529). A total of 3,097 OTUs were obtained according to 97% similarity. After taxonomic assignment against the Greengenes (v201305) database, they were annotated at different phylogenetic levels (Supplementary Table S2). According to the rarefaction curve (Figure 2A), the current sequencing depth and samples were sufficient for taxa identification.

Alpha diversity represents the species richness and evenness within the microbiota, while beta diversity can reflect the shared diversity within the microbiota at different ecological distances (Yang et al., 2022). The alpha diversity indexes, including the ACE index, Shannon index and Chao index, of the skin of AD patients were less than those of the HV group ($p < 0.05$) (Figure 2B; Supplementary Figures S1A,B), indicating decreased richness and evenness of the skin microbiome in AD patients. In addition, the ACE index and Chao index of female atopic dermatitis patients (ADFs) were less than those of female healthy volunteers (HVFs) ($p < 0.05$) (Supplementary Figures S1C,D). The score plot of principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances showed differences in the composition and structure of the bacterial community between the AD group and HV group ($p < 0.05$) (Figures 2C,D). There were significant differences in beta diversity analyzed by weighted UniFrac PCoA of all subgroups (Supplementary Figures S2A–E).

There was considerable variation in the relative abundance of 569 differentially abundant bacteria at the genus level (Figure 2E). Compared with HVs, AD patients showed a significantly greater relative abundance of the genus *Staphylococcus* (44.1% vs. 10.6%) and lower levels of the genera *Propionibacterium* (0.9% vs. 5.9%) and *Brevundimonas* (0.6% vs. 2.9%) ($p < 0.05$) (Figure 2F). The relative abundances of the genera *Staphylococcus, Acinetobacter, Lactobacillus* and *Streptococcus* in HVFs were significantly higher than those in male healthy volunteers (HVMs) ($p < 0.05$) (Supplementary Figure S4A). Microbiome compositions at the genus level in other subgroups are shown in Supplementary Figures S3A–E, 4B–E.

To further examine the alterations associated with AD, we conducted LEfSe analysis. The main differences were the increase in the abundance of *Bacillales* (class *Bacilli* and order *Bacillales*) in AD patients and the reduction in *Caulobacterales* (class *Alphaproteobacteria* and order *Caulobacterales*), *Rhodobacterales* (class *Alphaproteobacteria* and order *Rhodobacterales*) and *Xanthomonadales* (class *Gammaproteobacteria* and order

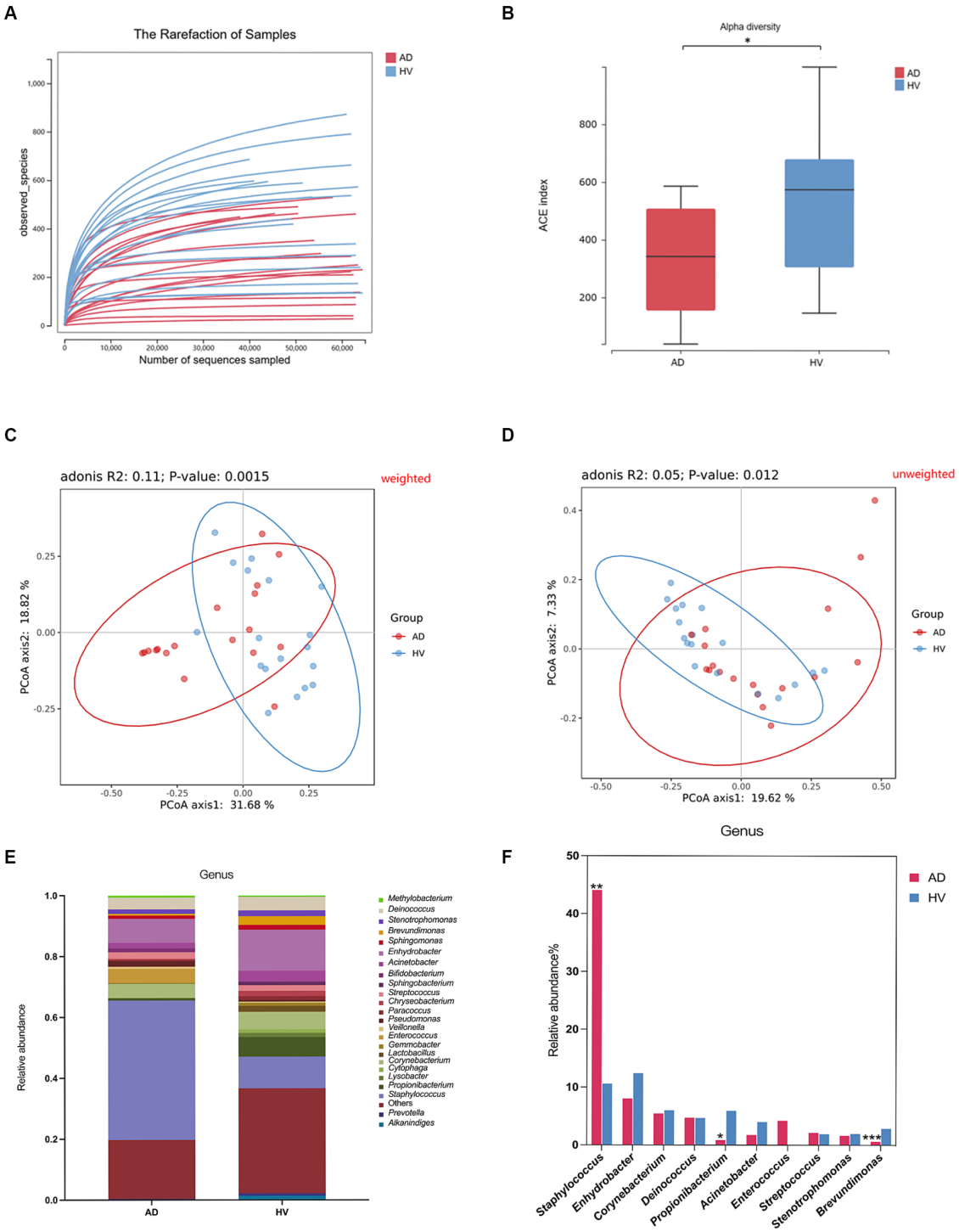


FIGURE 2
Skin microbiome diversity and structure comparison between the AD group and HV group. **(A)** Rarefaction curve of all samples. **(B)** Alpha diversity analyzed by the ACE index. **(C)** Weighted Unifrac PCoA plot. **(D)** Unweighted Unifrac PCoA plot. **(E)** Comparison of bacteria at the genus level. **(F)** Top 10 genera in relative abundance. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Xanthomonadales) (Figure 3A). Some differences were also observed at a lower taxonomic level. AD patients showed an increase in *Staphylococcaceae* (genus *Staphylococcus*). In contrast, AD patients exhibited a loss of *Propionibacterium*, *Brevundimonas*, *Lactobacillus* and *Lysobacter* at the genus level (Figure 3B). Taken together, these data indicate alterations in the commensal skin microbiome composition in AD patients, suggesting dysregulation of the microbial community.

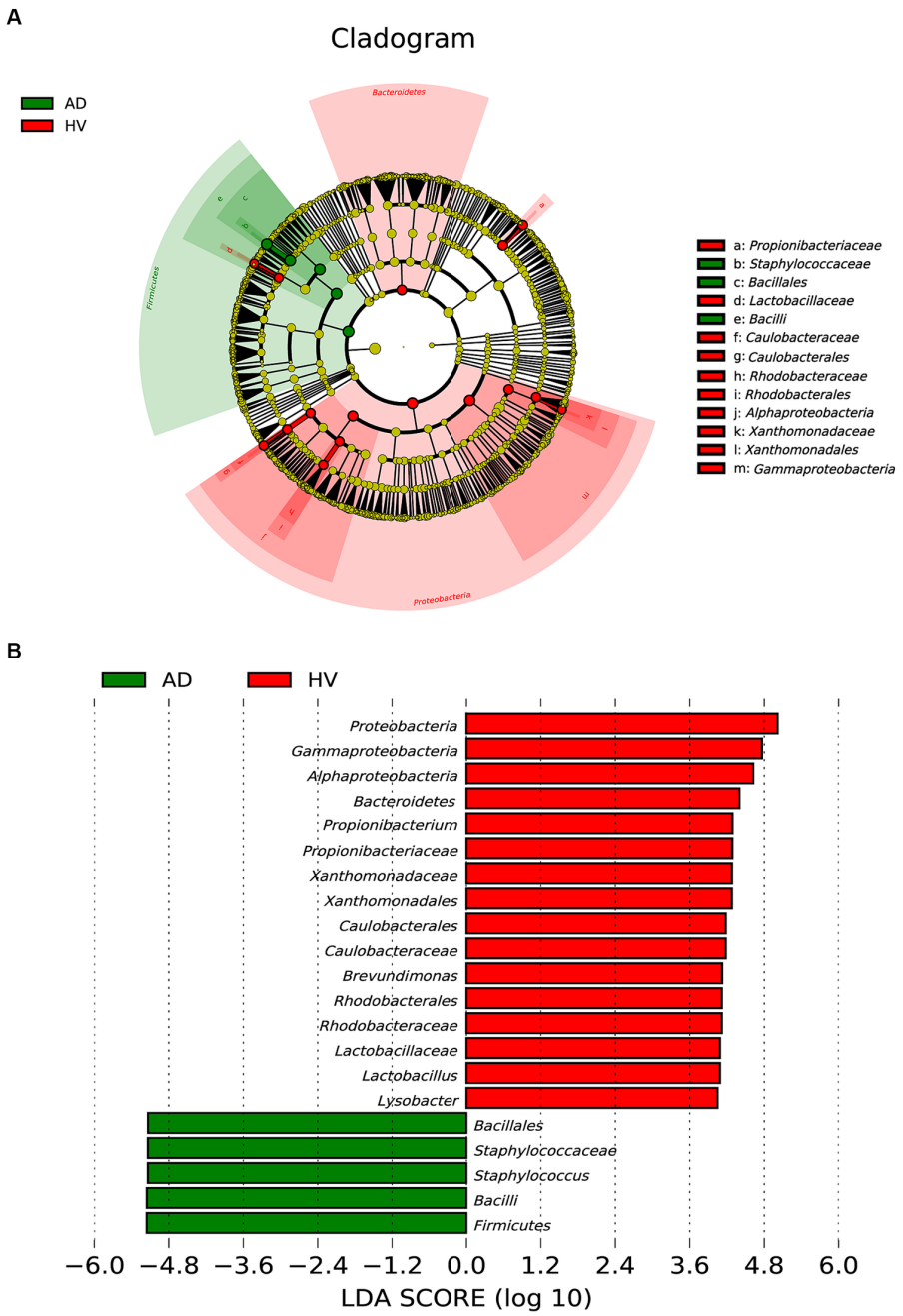


FIGURE 3
Linear discriminant analysis (LDA) effect size of the AD group and HV group. **(A)** Cladogram of LEfSe of the skin microbiome from 16S rRNA gene sequencing results. **(B)** Histogram of the LDA scores for differentially abundant microbes in AD patients and healthy controls (LDA > 4.0).

3.3 Skin metabolic profiling of AD patients

According to the information available for matching (including MS1 molecular weight, MS2 fragment spectra, column retention time and whether there are reference standards), the credibility levels of the identified substances are annotated to divide them into different credibility levels (Supplementary Table S3). In general, a total of 23,700 features and 3,867 metabolites (level 1–5) were identified (Supplementary Table S4). All metabolites were classified into several classes, including compounds with biological roles (benzene and derivatives, amino acids, peptides, organic acids, carbohydrates, etc.), lipids (polyketides, fatty acyls, etc.), phytochemical compounds (terpenoids, alkaloids, flavonoids, etc.) and others. KEGG pathway analysis was conducted and showed that metabolism (including amino acid metabolism and lipid metabolism) was the main function of the metabolites.

PCA, a multivariate technique, was used to determine whether samples from different groups could be segregated based on their metabolic profiles. The PCA results showed that the distribution of samples in the HV group was more concentrated than that in the AD

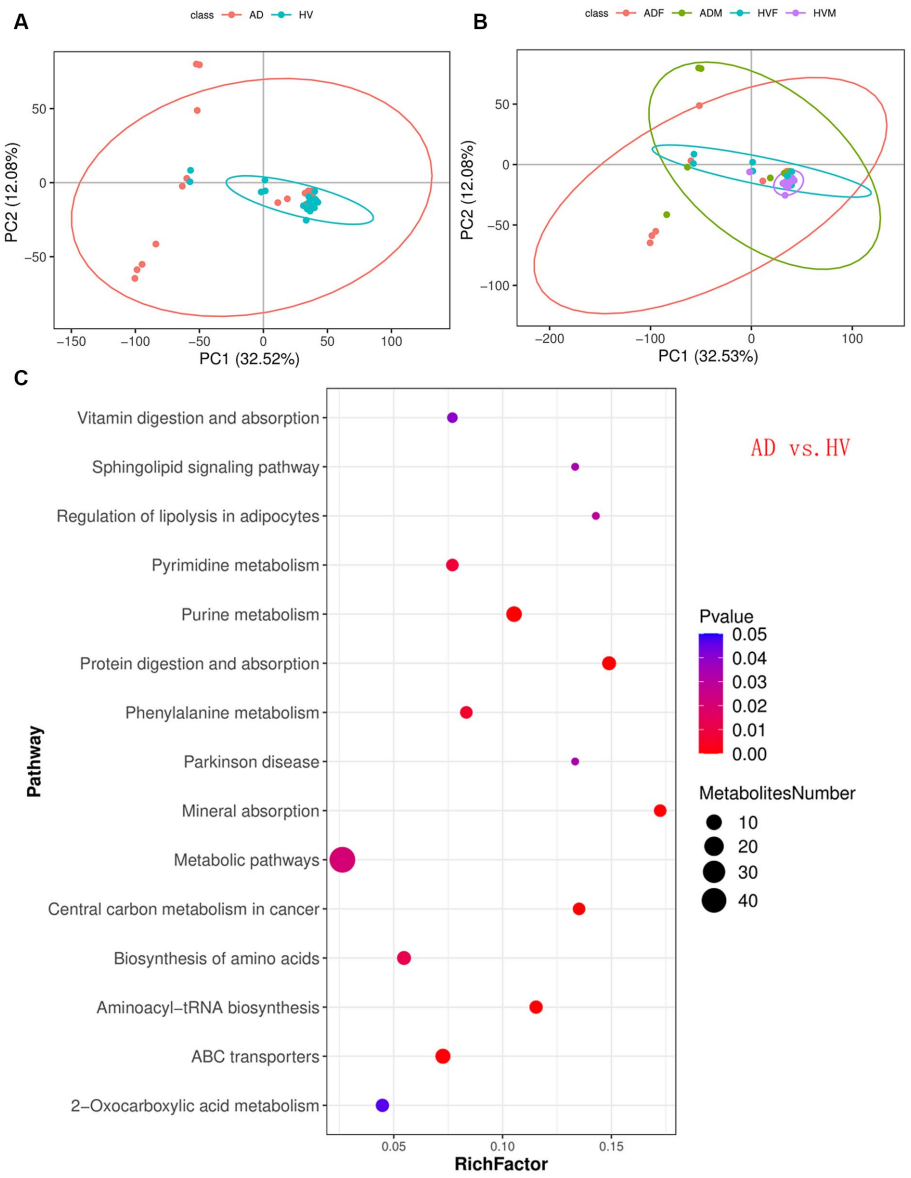


FIGURE 4
Skin metabolomic profiling. **(A)** PCA revealed that the distribution of samples in the HV group was more concentrated than that in the AD group. **(B)** PCA revealed that the distributions of samples in the ADM group and HVM group were more concentrated than those in the ADF group and HVF group, respectively. **(C)** Bubble plot of the metabolic pathway enrichment analysis results of the AD group and HV group.

group (Figure 4A). Similarly, the distributions of samples in the male atopic dermatitis patient (ADM) group and HVM group were more concentrated than those in the ADF group and HVF group, respectively (Figure 4B).

A total of 773 differentially abundant metabolites (VIP $\geq$ 1; fold-change $\geq$1.2 or $\leq$0.83; $p < 0.05$) between the AD group and HV group, at the credibility level of level 1 to level 4, were obtained (Supplementary Table S5). Similarly, differentially abundant metabolites in other subgroups were calculated, and the number of differentially abundant metabolites in each subgroup is shown in Table 1. Compared with the total number, the *per capita* number was more appropriate to reflect the effect of different influencing factors. We found that the number of differentially abundant metabolites *per capita* could be divided into three levels. Under the same gender, the number of differentially abundant metabolites *per capita* between AD patients and HVs was the largest, at more than 20. In the same state of illness or health, the number of differentially abundant metabolites *per capita* between different genders was in the middle, approximately 10. The number of differentially abundant metabolites *per capita* in AD patients with or without rashes was the lowest, approximately 5. Thus, we inferred that the state of AD is the most important factor that causes significant changes in skin metabolites. The gender factor ranked second. The presence or absence of rashes in AD has relatively little influence on skin metabolites.

KEGG pathway analysis of the differentially abundant metabolites between the AD group and HV group was conducted to uncover the metabolic pathway alterations (Figure 4C). The dot size of metabolic pathways was the largest, indicating the largest amounts of

TABLE 1 The number of differentially abundant metabolites in each group.

Group	Up	Down	Total number	*Per capita* number
AD vs. HV	334	439	773	21.5
ADF vs. HVF	213	161	374	23.4
ADM vs. HVM	189	300	489	24.5
ADF vs. ADM	93	65	158	8.8
HVF vs. HVM	66	128	194	10.8
ADL vs. ADNL	49	32	81	4.5

Differentially abundant metabolite screening conditions: (1) VIP of the first two principal components of the PLS-DA model ≥ 1; (2) fold-change ≥ 1.2 or ≤ 0.83; (3) $p < 0.05$.

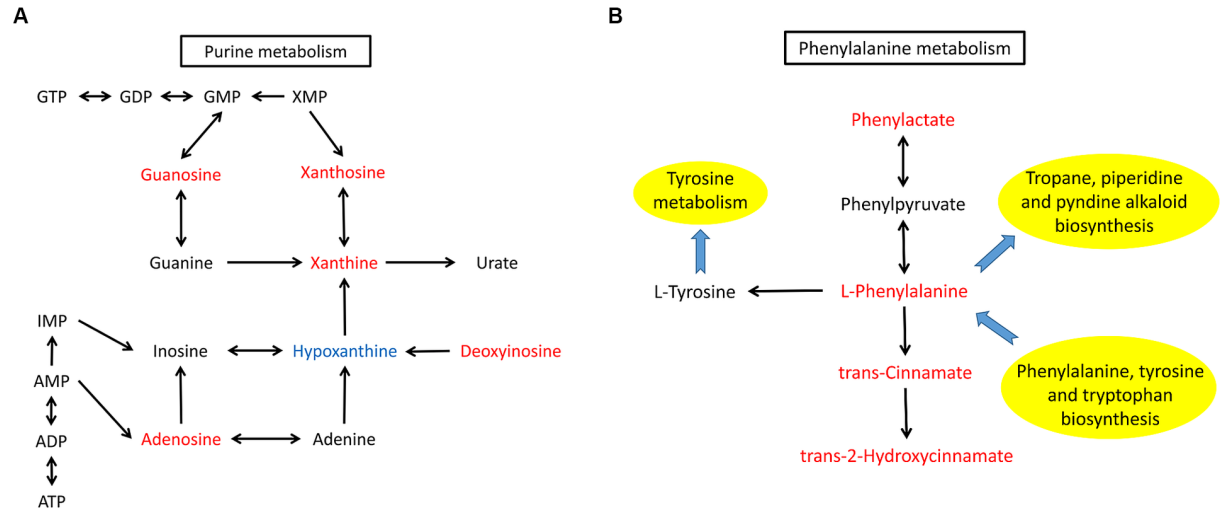


FIGURE 5
Altered metabolites and metabolic pathways observed in AD patients. **(A)** The purine metabolism pathway. **(B)** The phenylalanine metabolism pathway. Differentially abundant metabolites are shaded in red or blue. Red represents elevation compared with the HV group, and blue indicates a decrease compared with the HV group. The black arrow represents molecular interaction or relation. The blue arrow indicates the link to/from another KEGG pathway map.

differentially abundant metabolites annotated to metabolic pathways. Furthermore, the differentially abundant metabolites of each enriched pathway were analyzed, and it was found that there were upstream and downstream cascades in the purine metabolism and phenylalanine metabolism pathways (Figures 5A,B). The content of xanthosine and xanthine in the skin of AD patients was 54 times and 38 times that of HV, and the content of L-phenylalanine and trans-cinnamate was approximately 4 times and 5 times that of HV, respectively, suggesting that the metabolic pathways related to xanthosine/xanthine and L-phenylalanine/trans-cinnamate are upregulated in AD. In addition, sphingosine levels were also significantly elevated in AD patients, approximately 9 times higher than in HV.

Similarly, KEGG pathway analysis of other subgroups was conducted (Supplementary Figures S5A–E). We found significant enrichment of differentially abundant metabolites in purine metabolism and phenylalanine metabolism pathways between ADF and HVF (Supplementary Figure S5A). The content of xanthosine and xanthine in ADF is 99 times and 75 times that of HVF, and the content of L-phenylalanine and trans-cinnamate is approximately 7 times and 9 times that of HVF, respectively. However, the comparison between ADM and HVM showed only differentially abundant metabolite enrichment in the purine metabolism pathway (Supplementary Figure S5B). The xanthosine and xanthine contents of ADM were 30 times and 7 times that of HVM, respectively. In addition, the differentially abundant metabolites of purine metabolism and phenylalanine metabolism pathways were also enriched between ADF and ADM (Supplementary Figure S5C). However, there was no significant difference in the above two pathways between HVF and HVM (Supplementary Figure S5D). Through the above subgroup analysis, it can be inferred that the skin metabolic phenotype of AD patients differed by sex, and the differences in the purine metabolism and phenylalanine metabolism pathways between AD patients and HV were more obvious in females.

3.4 Correlation analysis between the skin microbiota and skin metabolism

In total, 569 differentially abundant bacteria at the genus level and 81 differentially abundant metabolites at credibility levels of 1 to 3 between the AD group and HV group were obtained. Spearman correlation analysis was performed between the above differentially abundant bacteria and differentially abundant metabolites (Supplementary Table S6). Furthermore, the correlation between five

TABLE 2 The correlation between specific bacteria and differentially abundant metabolites.

Compound	*Staphylococcus*	*Brevundimonas*	*Propionibacterium*	*Lactobacillus*	*Lysobacter*
L-phenylalanine[a]	***0.610***	***−0.508***	−0.325	***−0.578***	−0.221
Trans-cinnamate[a]	***0.642***	***−0.541***	***−0.330***	***−0.578***	−0.266
Tran-2-hydroxycinnamate[a]	***0.570***	***−0.505***	−0.293	***−0.564***	−0.259
Phenylactate[a]	***0.591***	***−0.368***	−0.195	***−0.442***	−0.426
Xanthine[b]	***0.523***	***−0.436***	***−0.418***	−0.282	−0.096
Xanthosine[b]	***0.772***	***−0.549***	***−0.372***	***−0.467***	−0.244
Hypoxanthine[b]	−0.321	***0.421***	***0.416***	0.217	−0.033
Adenosine[b]	***0.448***	***−0.509***	0.027	***−0.354***	−0.278
Guanosine[b]	***0.430***	−0.295	−0.126	***−0.408***	***−0.338***
Deoxyinosine[b]	***0.437***	***−0.535***	−0.131	***−0.468***	−0.282
Sphingosine[c]	0.257	***−0.395***	−0.243	***−0.381***	−0.191
$r \leq -0.6$ / $-0.6 < r \leq -0.4$	$-0.4 < r \leq -0.2$	$-0.2 < r \leq 0$	$0 < r \leq 0.2$	$0.2 < r \leq 0.4$ / $0.4 < r \leq 0.6$	$r > 0.6$

Bold and italic values indicate nominal significant associations ($p < 0.05$).

[a]These metabolites are involved in phenylalanine metabolism.

[b]These metabolites are involved in purine metabolism.

[c]These metabolites are involved in sphingolipid signaling pathway.

important kinds of differentially abundant bacteria revealed by LEfSe analysis and the key differentially abundant metabolites in purine metabolism, phenylalanine metabolism and sphingolipid signaling pathways are shown in Table 2. We found that the genus *Staphylococcus* was positively correlated with various compounds in phenylalanine metabolism and purine metabolic pathways. The genera *Brevundimonas* and *Lactobacillus* were negatively correlated with various compounds in purine metabolism, phenylalanine metabolism and sphingolipid signaling pathways.

4 Discussion

Skin microecological imbalance plays an important role in the pathogenesis of AD (Langan et al., 2020). The relationship between human and skin microbiota is bidirectional. Skin microbiota can affect host gene expression, and different skin characteristics can also form microbiota with specific preferences (Fyhrquist et al., 2019). There are also complex interactions between different skin bacteria. Previous studies have suggested that *S. aureus* is often dominant in the skin of AD patients, which can promote the onset of AD and limit the growth of other bacteria, which may have regulatory or protective effects (such as *Staphylococcus epidermidis* and *Corynebacterium*) (Fyhrquist et al., 2019). In contrast, some strains of *Staphylococcus epidermidis, Roseomonas* and *Propionibacterium* can inhibit the growth of *S. aureus* and are potential probiotics for the treatment of AD (Koh et al., 2022). Our study is based on 16S high-throughput sequencing technology, and the results are difficult to accurately annotate to the species level, nor can they be used to detect viruses or eukaryotic communities (Ghosh et al., 2018). Our study showed that the alpha diversity of the skin microbiota decreased in AD patients. The relative abundance of the genus *Staphylococcus* significantly increased, while the relative abundances of the genera *Propionibacterium*, *Brevundimonas*, *Lactobacillus* and *Lysobacter* significantly decreased, suggesting that the genus *Staphylococcus* affected the growth of other skin symbiotic flora and destroyed the skin microecological balance. Thus, it is reasonable to infer that *S. aureus* should account for most of the detected genus *Staphylococcus* and play an important role in the pathogenesis of AD.

In addition to disease factors, the skin bacterial structure is also affected by other confounding factors. It has been reported that the number of bacteria at the genus level does not change from birth to 1 year of age, but the relative abundance of microbiota fluctuates, with the relative abundances of the genera *Staphylococcus* and *Streptococcus* decreasing as infants grow, while the relative abundances of other genera increase (Capone et al., 2011). This trend was maintained through childhood (2–12 years old), with more diversity of skin microbiota on the forearms of children than adults (Shi et al., 2016). The composition of skin microbiota in adolescents was stable, similar to that of adults (Shi et al., 2016). The skin microbiome structure in different sites of AD was also different (Bjerre et al., 2021). Therefore, the subjects included in this study were all adults, and the sampling sites were all elbows, which can better reduce the interference of age and site factors on the research results. To investigate the effect of sex and local rash factors on the results, we performed a subgroup analysis. We revealed that there were significant differences in the composition and structure of the bacterial community between different sexes and lesion states.

Due to the important role of skin microbiota in the pathogenesis of AD, restoring skin microbial homeostasis is becoming a novel therapeutic strategy for treating AD (Hrestak et al., 2022; Koh et al., 2022). Application of prebiotics and probiotics, whether by gastrointestinal approach or external use, may help to increase the diversity of the skin microbiota and have potential therapeutic effect of AD (Hrestak et al., 2022). Coagulase-negative *Staphylococcus* (CoNS) strains with antimicrobial activity were common on the normal population but rare on AD subjects, and application of antimicrobial CoNS strains to human subjects with AD may decreased colonization by *S. aureus* (Nakatsuji et al., 2017). Topical microbiome transplantation with *Roseomonas mucosa* for AD patients was associated with significant decreases in measures of disease severity and *S. aureus* burden (Myles et al., 2018). A Living symbiotic bacteria-involved skin dressing was developed for microbiome-based

biotherapy toward AD, which may recover skin barrier functions and alleviate AD-associated inflammation responses (Liu et al., 2023).

Samples for metabolomics analyses can be serum, urine, sweat, skin, etc. (Afghani et al., 2022). Both host gene expression products and substances produced by skin microorganisms influence skin metabolites (Afghani et al., 2022). Skin samples can be collected by invasive or noninvasive methods; the former is by skin biopsy, and the latter is by tape strips (Afghani et al., 2022). It has been reported that the swabbing method is comparable to the tape stripping method for collecting viable skin bacteria without losing fidelity to the composition of the skin microbiome (Ogai et al., 2018). However, to our knowledge, there have been no previous studies in AD metabolomics applying the swabbing method for sampling. In this study, a polyester swab was used to collect samples from skin, and large amounts of metabolites were successfully obtained. This noninvasive method for skin sample collection was proven to be safe, convenient and efficient.

Diet, age, sex, sampling method and other factors may affect the metabolites detected (Afghani et al., 2022). We evaluated the influence of disease, sex and rashes on the metabolomics analyses. The PCA results revealed that metabolites of healthy people have better consistency than those of AD patients, and males have better consistency than females. The number of differentially abundant metabolites *per capita* suggested that the effects of the state of AD, sex and the presence or absence of rashes on metabolomics analyses were successively reduced.

The differentially abundant metabolites between AD patients and HVs were enriched in the purine metabolism and phenylalanine metabolism pathways. The contents of xanthosine/xanthine and L-phenylalanine/trans-cinnamate are significantly elevated in AD, especially among females. However, we should notice that the metabolites we detected do not necessarily derived from microbes, they may also origin from the host. Xanthine is the substrate of xanthine oxidase and xanthine dehydrogenase, and it is also the intermediate product of the process from hypoxanthine to uric acid (Kulikowska et al., 2004). Xanthine is involved in a variety of intracellular metabolic pathways, such as purine nucleotide catabolism (Kulikowska et al., 2004). Exogenous guanosine and xanthosine, which are fluxed through the GTP branch of purine biosynthesis, were shown to significantly reduce methicillin-resistant *S. aureus* (MRSA) β-lactam resistance (Nolan et al., 2023). Further study demonstrated that exposure of MRSA to guanosine and xanthosine can significantly reduce the levels of the cyclic dinucleotide c-di-AMP, which is needed for β-lactam resistance (Nolan et al., 2023).

Phenylalanine is involved in a variety of metabolic activities in the body, and tyrosine, phenylactate and trans-cinnamate can be produced under the action of different enzymes (Matthews, 2007; Teufel et al., 2010; Otto et al., 2019). Bacteria in the environment can participate in the metabolism of phenylalanine and phenylacetate (Teufel et al., 2010). *Pseudomonas taiwanensis* has been used to catalyze trans-cinnamate formation from phenylalanine (Otto et al., 2019). The functional predictions based on metagenomic analysis in previous study revealed that AD skin samples exhibited enrichment in phenylalanine tyrosine and tryptophan biosynthesis (Chng et al., 2016). In addition, human lack the ability to synthesize essential amino acids such as phenylalanine, and they acquire essential amino acids from their diet or perhaps their associated microbial communities (Shrode et al., 2022; McCann and Rawls, 2023). Essential amino acid may act as chemical mediators of host–microbe interaction (McCann and Rawls, 2023). Thus, it was reasonable to infer that the significant proportion of increasing phenylalanine in AD we detected by skin metabolomics was probably derived from dominant microbes in AD. As far as we know, there are no relevant studies on purine metabolism and phenylalanine metabolism pathways in the pathogenesis of AD, but there is evidence that xanthosine, phenylalanine and trans-cinnamate participate in bacterial metabolic activities (Otto et al., 2019; Nolan et al., 2023). The important role of *S. aureus* in the pathogenesis of AD has been generally confirmed, and this study suggested that xanthosine/xanthine and L-phenylalanine/trans-cinnamate were positively correlated with *Staphylococcus*. Therefore, the purine metabolism and phenylalanine metabolism pathways may play a certain role in the pathogenic mechanism of *S. aureus* in AD, which is worthy of further exploration.

Epidermal barrier dysfunction is also a major characteristic of AD (Langan et al., 2020). Ceramide plays an important role in maintaining skin barrier function, and sphingosine is an important component of ceramide (Toncic et al., 2020). Ceramides can be divided into several subtypes, and they are dysregulated in AD patients (Ghosh et al., 2018). Previous studies have observed a decrease in long-chain ceramides and an increase in short-chain ceramides in AD patients (Afghani et al., 2022). It was reported that the colonization of *Staphylococcus* was positively correlated with the epidermal ceramide subspecies AS, ADS, NS and NDS in AD (Emmert et al., 2021). Our study showed that the amount of sphingosine in AD patients was approximately 10 times that in healthy controls ($p < 0.05$), which was consistent with previous studies (Toncic et al., 2020). However, we did not identify ceramide substances in this study, which may be due to the wide variety of ceramides and lack of a specialized ceramide comparison database.

There are some limitations in our study. First, the correlation between the skin microbiota and skin metabolites is only statistically significant, and it was difficult to determine whether the differential metabolites between the two groups detected in skin samples came from the host or microorganisms. Second, our study only detected the metabolites in skin samples without untargeted metabolomics analysis on serum samples, which can better reflect the metabolic situation of the body. It is necessary to collect both skin samples and blood samples in future research. In addition, relatively fewer subjects were included in the subgroup analysis, which inevitably increased the random error. Thus, the specific mechanism of interaction between skin microbiota, human skin and skin metabolites still needs to be further studied.

In summary, AD patients had dysbiosis of the skin microbiome with the features of decreased species richness and evenness. The relative abundance of the genus *Staphylococcus* increased significantly in AD, while the relative abundances of the genera *Propionibacterium*, *Brevundimonas*, *Lactobacillus* and *Lysobacter* were significantly decreased. Multiple metabolites related to purine metabolism and phenylalanine metabolism pathways (such as xanthosine/xanthine and L-phenylalanine/trans-cinnamate) were upregulated in AD patients, and they were positively correlated with the genus *Staphylococcus,* which suggested that purine metabolism and phenylalanine metabolism pathways may play a certain role in the pathogenic mechanism of *S. aureus* in AD. We also found that different sexes had certain effects on skin microbiota and metabolite composition. Females are more likely to be colonized by the genus *Staphylococcus* than males, and the differentially abundant metabolites

involved in purine metabolism and phenylalanine metabolism pathways were more obvious in female.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Guangdong Provincial Hospital of Chinese Medicine (BE2019-165-01). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KH: Software, Writing – original draft, Visualization, Funding acquisition, Formal analysis. FL: Writing – original draft, Investigation, Data curation. YLiu: Writing – original draft, Investigation, Data curation. BL: Data curation, Writing – original draft, Investigation. PQ: Writing – original draft, Data curation. LY: Writing – original draft, Formal analysis. SH: Writing – original draft, Formal analysis. WL: Writing – review & editing, Project administration, Funding acquisition, Formal analysis. XM: Writing – original draft, Funding acquisition, Data curation. LD: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. YLin: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1349674/full#supplementary-material

References

Afghani, J., Traidl-Hoffmann, C., Schmitt-Kopplin, P., Reiger, M., and Mueller, C. (2022). An overview of the latest metabolomics studies on atopic eczema with new directions for study. *Int. J. Mol. Sci.* 23:8791. doi: 10.3390/ijms23158791

Bjerre, R. D., Holm, J. B., Palleja, A., Sølberg, J., Skov, L., and Johansen, J. D. (2021). Skin dysbiosis in the microbiome in atopic dermatitis is site-specific and involves bacteria, fungus and virus. *BMC Microbiol.* 21:256. doi: 10.1186/s12866-021-02302-2

Byrd, A. L., Belkaid, Y., and Segre, J. A. (2018). The human skin microbiome. *Nat. Rev. Microbiol.* 16, 143–155. doi: 10.1038/nrmicro.2017.157

Capone, K. A., Dowd, S. E., Stamatas, G. N., and Nikolovski, J. (2011). Diversity of the human skin microbiome early in life. *J. Invest. Dermatol.* 131, 2026–2032. doi: 10.1038/jid.2011.168

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chen, Y. E., Fischbach, M. A., and Belkaid, Y. (2018). Skin microbiota-host interactions. *Nature* 553, 427–436. doi: 10.1038/nature25177

Chen, D., He, J., Li, J., Zou, Q., Si, J., Guo, Y., et al. (2021). Microbiome and metabolome analyses reveal novel interplay between the skin microbiota and plasma metabolites in psoriasis. *Front. Microbiol.* 12:643449. doi: 10.3389/fmicb.2021.643449

Chng, K. R., Tay, A. S. L., Li, C., Ng, A. H. Q., Wang, J., Suri, B. K., et al. (2016). Whole metagenome profiling reveals skin microbiome-dependent susceptibility to atopic dermatitis flare. *Nat. Microbiol.* 1:16106. doi: 10.1038/nmicrobiol.2016.106

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Emmert, H., Baurecht, H., Thielking, F., Stölzl, D., Rodriguez, E., Harder, I., et al. (2021). Stratum corneum lipidomics analysis reveals altered ceramide profile in atopic dermatitis patients across body sites with correlated changes in skin microbiome. *Exp. Dermatol.* 30, 1398–1408. doi: 10.1111/exd.14185

Fyhrquist, N., Muirhead, G., Prast-Nielsen, S., Jeanmougin, M., Olah, P., Skoog, T., et al. (2019). Microbe-host interplay in atopic dermatitis and psoriasis. *Nat. Commun.* 10:4703. doi: 10.1038/s41467-019-12253-y

Ghosh, D., Bernstein, J. A., Khurana Hershey, G. K., Rothenberg, M. E., and Mersha, T. B. (2018). Leveraging multilayered "omics" data for atopic dermatitis: a road map to precision medicine. *Front. Immunol.* 9:2727. doi: 10.3389/fimmu.2018.02727

Hrestak, D., Matijašić, M., Paljetak, H. Č., Drvar, D. L., Hadžavdić, S. L., and Perić, M. (2022). Skin microbiota in atopic dermatitis. *Int. J. Mol. Sci.* 23:3503. doi: 10.3390/ijms23073503

Ilves, L., Ottas, A., Kaldvee, B., Abram, K., Soomets, U., Zilmer, M., et al. (2021). Metabolomic analysis of skin biopsies from patients with atopic dermatitis reveals hallmarks of inflammation, disrupted barrier function and oxidative stress. *Acta Derm. Venereol.* 101:adv00407. doi: 10.2340/00015555-3766

Kage, P., Simon, J. C., and Treudler, R. (2020). Atopic dermatitis and psychosocial comorbidities. *J. Dtsch. Dermatol. Ges.* 18, 93–102. doi: 10.1111/ddg.14029

Koh, L. F., Ong, R. Y., and Common, J. E. (2022). Skin microbiome of atopic dermatitis. *Allergol. Int.* 71, 31–39. doi: 10.1016/j.alit.2021.11.001

Kulikowska, E., Kierdaszuk, B., and Shugar, D. (2004). Xanthine, xanthosine and its nucleotides: solution structures of neutral and ionic forms, and relevance to substrate properties in various enzyme systems and metabolic pathways. *Acta Biochim. Pol.* 51, 493–531. doi: 10.18388/abp.2004_3587

Langan, S. M., Irvine, A. D., and Weidinger, S. (2020). Atopic dermatitis. *Lancet* 396, 345–360. doi: 10.1016/s0140-6736(20)31286-1

Li, S., Villarreal, M., Stewart, S., Choi, J., Ganguli-Indra, G., Babineau, D. C., et al. (2017). Altered composition of epidermal lipids correlates with *Staphylococcus aureus* colonization status in atopic dermatitis. *Br. J. Dermatol.* 177, e125–e127. doi: 10.1111/bjd.15409

Liu, X., Qin, Y., Dong, L., Han, Z., Liu, T., Tang, Y., et al. (2023). Living symbiotic bacteria-involved skin dressing to combat indigenous pathogens for microbiome-based biotherapy toward atopic dermatitis. *Bioact. Mater.* 21, 253–266. doi: 10.1016/j.bioactmat.2022.08.019

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Matthews, D. E. (2007). An overview of phenylalanine and tyrosine kinetics in humans. *J. Nutr.* 137:1549S-1555S; discussion 1573S-1575S. doi: 10.1093/jn/137.6.1549S

McCann, J. R., and Rawls, J. F. (2023). Essential amino acid metabolites as chemical mediators of host-microbe interaction in the gut. *Ann. Rev. Microbiol.* 77, 479–497. doi: 10.1146/annurev-micro-032421-111819

Myles, I. A., Earland, N. J., Anderson, E. D., Moore, I. N., Kieh, M. D., Williams, K. W., et al. (2018). First-in-human topical microbiome transplantation with *Roseomonas mucosa* for atopic dermatitis. *JCI Insight* 3:e120608. doi: 10.1172/jci.insight.120608

Nakatsuji, T., Chen, T. H., Narala, S., Chun, K. A., Two, A. M., Yun, T., et al. (2017). Antimicrobials from human skin commensal bacteria protect against *Staphylococcus aureus* and are deficient in atopic dermatitis. *Sci. Transl. Med.* 9:eaah4680. doi: 10.1126/scitranslmed.aah4680

Nolan, A. C., Zeden, M. S., Kviatkovski, I., Campbell, C., Urwin, L., Corrigan, R. M., et al. (2023). Purine nucleosides interfere with c-di-AMP levels and act as adjuvants to re-sensitize MRSA to β-lactam antibiotics. *MBio* 14:e0247822. doi: 10.1128/mbio.02478-22

Ogai, K., Nagase, S., Mukai, K., Iuchi, T., Mori, Y., Matsue, M., et al. (2018). A comparison of techniques for collecting skin microbiome samples: swabbing versus tape-stripping. *Front. Microbiol.* 9:2362. doi: 10.3389/fmicb.2018.02362

Otto, M., Wynands, B., Lenzen, C., Filbig, M., Blank, L. M., and Wierckx, N. (2019). Rational engineering of phenylalanine accumulation in *Pseudomonas taiwanensis* to enable high-yield production of trans-cinnamate. *Front. Bioeng. Biotechnol.* 7:312. doi: 10.3389/fbioe.2019.00312

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/aem.01541-09

Schuler, C. F., Billi, A. C., Maverakis, E., Tsoi, L. C., and Gudjonsson, J. E. (2023). Novel insights into atopic dermatitis. *J. Allergy Clin. Immunol.* 151, 1145–1154. doi: 10.1016/j.jaci.2022.10.023

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shi, B., Bangayan, N. J., Curd, E., Taylor, P. A., Gallo, R. L., Leung, D. Y. M., et al. (2016). The skin microbiome is different in pediatric versus adult atopic dermatitis. *J. Allergy Clin. Immunol.* 138, 1233–1236. doi: 10.1016/j.jaci.2016.04.053

Shrode, R. L., Cady, N., Jensen, S. N., Borcherding, N., and Mangalam, A. K. (2022). Isoflavone consumption reduces inflammation through modulation of phenylalanine and lipid metabolism. *Metabolomics* 18:84. doi: 10.1007/s11306-022-01944-1

Ständer, S. (2021). Atopic dermatitis. *N. Engl. J. Med.* 384, 1136–1143. doi: 10.1056/NEJMra2023911

Teufel, R., Mascaraque, V., Ismail, W., Voss, M., Perera, J., Eisenreich, W., et al. (2010). Bacterial phenylalanine and phenylacetate catabolic pathway revealed. *Proc. Natl. Acad. Sci. USA* 107, 14390–14395. doi: 10.1073/pnas.1005399107

Toncic, R. J., Jakasa, I., Hadzavdic, S. L., Goorden, S. M., Vlugt, K. J. G., Stet, F. S., et al. (2020). Altered levels of sphingosine, sphinganine and their ceramides in atopic dermatitis are related to skin barrier function, disease severity and local cytokine milieu. *Int. J. Mol. Sci.* 21:1958. doi: 10.3390/ijms21061958

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/aem.00062-07

Wen, B., Mei, Z., Zeng, C., and Liu, S. (2017). metaX: a flexible and comprehensive software for processing metabolomics data. *BMC Bioinform.* 18:183. doi: 10.1186/s12859-017-1579-y

Williams, H. C., Burney, P. G., Hay, R. J., Archer, C. B., Shipley, M. J., Hunter, J. J., et al. (1994). The U.K. working Party's diagnostic criteria for atopic dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br. J. Dermatol.* 131, 383–396. doi: 10.1111/j.1365-2133.1994.tb08530.x

World Medical Association (2013). World medical association declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA* 310, 2191–2194. doi: 10.1001/jama.2013.281053

Yang, L., Xiang, Z., Zou, J., Zhang, Y., Ni, Y., and Yang, J. (2022). Comprehensive analysis of the relationships between the gut microbiota and fecal metabolome in individuals with primary Sjogren's syndrome by 16S rRNA sequencing and LC-MS-based metabolomics. *Front. Immunol.* 13:874021. doi: 10.3389/fimmu.2022.874021

Zhang, L., Zeng, Y., and Sun, J. (2023). Progress of metabolomics in atopic dermatitis: a systematic review. *J. Dtsch. Dermatol. Ges.* 21, 229–236. doi: 10.1111/ddg.14960

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Integrated 16S rRNA sequencing and metagenomics insights into microbial dysbiosis and distinct virulence factors in inflammatory bowel disease

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Introduction: The escalation of urbanization correlates with rising rates of inflammatory bowel disease (IBD), necessitating research into new etiological factors. This study aims to elucidate the gut microbiota profiles in IBD patients and compare them with healthy controls in a western city of China.

Methods: We conducted a multicenter case-control study from the end of 2020, using 16S rRNA gene sequencing ($n = 36$) and metagenomic sequencing ($n = 12$) to analyze the gut microbiota of newly diagnosed IBD patients, including those with Crohn's disease (CD) and ulcerative colitis (UC).

Results: Our results demonstrated a significant enrichment of the phylum Proteobacteria, particularly the genus *Escherichia-Shigella*, in CD patients. Conversely, the genus *Enterococcus* was markedly increased in UC patients. The core gut microbiota, such as the *Christensenellaceae R-7* group, *Fusicatenibacter*, and *Holdemanella*, were primarily identified in healthy subjects. Additionally, significant interactions between the microbiome and virulence factors were observed.

Discussion: The findings suggest that oxidative stress may play a pivotal role in the pathology of IBD. This study contributes to the growing dialogue about the impact of gut microbiota on the development of IBD and its variations across different geographies, highlighting potential avenues for further research.

KEYWORDS

16S rRNA, metagenome, inflammatory bowel disease, Crohn's disease, ulcerative colitis, case-control study

1 Introduction

Inflammatory bowel disease (IBD) represents a group of chronic inflammatory disorders of the gastrointestinal tract, primarily encompassing Crohn's disease (CD) and ulcerative colitis (UC). IBD is a major public health burden due to its substantially increased prevalence in many regions (GBD 2017 Inflammatory Bowel Disease Collaborators, 2020). Traditionally, the incidence and prevalence of IBD were lower in Asian populations compared to western populations (Kaplan and Windsor, 2021). However, the latest data revealed a significant shift, particularly in newly industrialized countries where occurrences are influenced by environmental pressures such as nutrition and lifestyle behaviors, with China and the USA currently reporting the highest numbers

of IBD cases worldwide (Park and Cheon, 2021; Wang et al., 2023). Due to the speed of industrialization and a fast-growing aging population, the burden of IBD in China has steadily increased and is expected to increase further by 2030 (Shao et al., 2022; Ma et al., 2023). Although the prevalence and incidence in the Western region of China lag behind those in the Eastern region (Yang et al., 2022), the implications of rapid urbanization should not be ignored.

Although the cause of IBD remains unknown, emerging evidence increasingly highlights the critical role of the microbiome in IBD patients. A cohort study and Mendelian randomization analyses have further confirmed the causal relationship between individual taxa and CD (Raygoza Garay et al., 2023; Li et al., 2024). The advent of high-throughput sequencing technologies, particularly 16S rRNA gene and metagenome sequencing, have revolutionized our comprehension of the intricate microbial communities in the human gut and their contributions to the onset and progression of IBD (Basha et al., 2023; Ning et al., 2023). Gut microbiota's integral connection to human physiology and diet, alongside the interplay between geographical origin and disease, adds complexity to assessing disease-associated alterations in the microbial populations (Rehman et al., 2016; He et al., 2022; Zhang and Gérard, 2022). Given the fact that the Western region exhibits a multi-ethnic composition and distinct dietary patterns characterized by animal husbandry lifestyles (Zhang et al., 2023), the heterogeneity of IBD geographical distribution makes it intriguing to delineate the microbial factor associated with IBD conditions.

The human gastrointestinal tract is a sophisticated and ever-evolving ecosystem, teeming with a diverse consortium of microorganisms, including bacteria and viruses. The intricate trans-kingdom interplay among gut viruses, bacteria, and the mammalian host plays a critical role in influencing both health and disease, and the gut virome is highly heterogeneous across populations (Cao et al., 2022). Recent studies have highlighted the role of the gut virome in the pathophysiology of IBD and its correlation with therapeutic success, indicating that these viruses might serve as novel targets for treatment (Jansen et al., 2023; Massimino et al., 2023). Furthermore, the expansive realm of metagenomics has redirected our focus toward the broader implications of the interactions in this complex community.

Given the limited research on IBD in western China and its distinct dietary patterns, this study aims to utilize 16S rRNA gene and metagenome sequencing to investigate the gut microbiota profiles of IBD patients. The objective of this study is to explore the relationship between microbial community networks and IBD, thereby deepening our understanding of these intricate interactions across diverse geographical areas.

2 Materials and methods

2.1 Participants and samples

A total of 36 participants were recruited from two hospitals (Qinghai Provincial Traditional Chinese Medicine Hospital and Qinghai Provincial People's Hospital) at Xining in Qinghai Province, China, from 2020. The *de novo* diagnosis of 18 IBD patients was matched by age and gender with 18 healthy individuals (CTL). The CTL group was further differentiated into distinct controls for cases in CD (CD_CTL) and UC (UC_CTL), enabling a more accurate comparative analysis. Patients with CD (n = 4) and UC (n = 14) were further classified into subtypes using the Montreal classification system, and disease severity was meticulously assessed (Table 1). Demographic characteristics were collected by questionnaire, and biochemical examinations were conducted using the collected fecal samples. A minimum of 2 g of the central part of the fecal sample was collected with a spoon in the gut collection cup; then, the cup was numbered and kept frozen in the laboratory at −80°C within 2 h of collection of the sample. This project was approved by the ethics committee of the Qinghai Provincial Traditional Chinese Medicine Hospital.

TABLE 1 The detail of *de novo* diagnosis of IBD patients.

Categories	Variables	Levels	CD (n = 4)	UC (n = 14)
Disease subtype	Subtype A (the age of onset)	A1	0 (0%)	
		A2	1 (25%)	
		A3	3 (75%)	
	Subtype B/P (disease behavior)	B1	2 (50%)	
		B2	0 (0%)	
		B3	2 (50%)	
	Subtype L (disease location)	L1	1 (25%)	
		L2	0 (0%)	
		L3	3 (75%)	
	Subtype E (disease behavior)	E1		0 (0)
		E2		4 (28.57%)
		E3		10 (71.43%)
Severity grading	CD Activity Index (Mean ± SD)		124.30 ± 39.65	
	UC Mayo Score (Mean ± SD)			7.14 ± 2.28

2.2 Microbiota 16s rRNA sequencing

Total genomic DNA was extracted from the gut microbiome samples, and the 16S V4-V5 region was amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Then, the products were purified and quantified to create a library and sequenced by the Illumina MiSeq PE300 platform (Illumina, San Diego, USA). The raw amplicon sequence variants (ASVs) dataset was acquired by the Qiime2 pipeline (version 2020.2), employing the recommended parameters. The taxonomy of ASVs was classified with the Q2-feature-classifier's classify-sklearn tool, setting the confidence threshold at 0.8. We then conducted taxonomy-based filtering

to exclude ASVs identified as mitochondria, chloroplasts, or archaea. Additionally, we removed ASVs that were present in <10% of the samples or had a relative abundance below 0.0001%. The rarefaction curve analysis confirmed that the sequencing depth for each sample met the anticipated targets (Supplementary Figure 1A).

2.3 Metagenomic sequencing and data analysis

Metagenomic sequencing was performed on samples randomly selected from 16s rRNA sequencing samples with six IBD (three UC and three CD) patients and their six matched healthy counterparts (CTL). DNA was extracted, and libraries were constructed; then paired-end sequencing was executed using an Illumina NovaSeq/Hiseq X Ten platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. Adapter sequences and low-quality reads (shorter than 50 bp, with a quality score below 20, or containing ambiguous bases denoted as "N") were filtered out using fastp (Chen et al., 2018). The remaining high-quality reads were then mapped to the human genome using the Burrows-Wheeler Alignment (BWA) tool l (Li and Durbin, 2009), with any human-aligned reads, and their mate discarded. The MEGAHIT was conducted to assemble the metagenomic data, and representative sequences of the non-redundant gene catalog were aligned to the NR database with an e-value cutoff of 1e-5 using Diamond for taxonomic annotations. A cluster of orthologous groups of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the Virulence Factor Database (VFDB; Kanehisa and Goto, 2000; Liu et al., 2022) were also used for functional annotation and further function assignment.

2.4 Alpha and beta diversity analysis

Alpha-diversity, represented by the Chao, Sob, and Shannon indexes, was estimated based on the ASV profiles from 16S rRNA sequences and at the species level from metagenomic profiles. The richness of the microbiomes was evaluated using the Sobs and Chao indexes, while the diversity was assessed by the Shannon index. Beta-diversity was visualized through non-metric multidimensional scaling (NMDS), employing a Bray-Curtis dissimilarity matrix estimated from the square-root-transformed and Wisconsin double-standardized ASV table. The beta diversity distance value was calculated using the Bray-Curtis dissimilarity matrix from the metagenomic profile.

2.5 Statistical analysis of demographic and microbiome data

Demographic information was expressed as the mean ± standard deviation, unless otherwise indicated, and analyzed using the paired *t*-test or chi-square test for statistical significance. Considering that microbial data were sparse with a non-normal distribution, relative statistics were performed with a non-parametric test, such as the Wilcoxon test. The tableone package was conducted to construct "Table 1" (https://github.com/kaz-yos/tableone). All statistical analyses were conducted using the "stats" package in R. For identifying taxa with the most pronounced differences in abundance and function across groups, we performed linear discriminant analysis effect size (LEfSe) analysis. The co-occurrence network calculated by networkX vividly illustrates the symbiotic relationships between the genus and samples, thereby facilitating an enhanced understanding of the distribution patterns of dominant species across various samples, and is visualized in Cytoscape (v 3.10). The comprehensive Spearman correlation analysis was performed between the microbiome at the genus level (top 50) and various virulence factors (top 50) in metagenome sequencing. We established a correlation coefficient threshold of 0.5, and only associations with a p-value of <0.05 were considered statistically significant and were retained for further visualization in Gephi (v 0.1). The visualization of the results was facilitated by the "ggplot2" package (Wickham, 2016) and the "microeco" package (Liu et al., 2021) in R, enabling a comprehensive and detailed graphical representation of our findings.

3 Results

3.1 Characteristics of participants

We enrolled 36 participants, 24 men and 12 women, aged 46.17 ± 11.48 years, and most of them were of Han ethnicity. IBD patients, including 14 UC and 4 CD patients, were paired with healthy volunteers of the same age and gender. The comparison results revealed no differences between patients and healthy participants (Table 2), indicating that these demographic characteristics were comparable across groups.

3.2 Decreased richness and diversity in IBD patients

Significant differences in the gut microbiota composition and diversity were observed between patients with IBD and healthy individuals (Supplementary Figure 1B). These variations were characterized using 16S rRNA sequencing to analyze microbial composition and diversity, complemented by metagenomic approaches for an extensive microbial overview. Notably, bacteria emerged as the most abundant microorganisms in the participants, with a marked enrichment of viruses in CD (40%) compared to UC (3%) patients (Figure 1A). Furthermore, 16S rRNA sequencing highlighted substantial alterations in the composition, richness, and diversity of the gut microbiota among IBD patients. At the phylum level, the dominant bacteria included *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, and *Bacteroidetes* (Figure 1B). At the genus level, the alterations were equally striking, and *Escherichia-Shigella* was predominant in both CD and UC patients, accounting for 59 and 50% of the microbiota, respectively. Furthermore, *Bifidobacterium* and *Streptococcus* were identified as the sub-dominant bacterial genera in CD (21%) and UC (23%) patients, respectively (Supplementary Figure 1C). The richness and

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TABLE 2 Demographic characteristics of participants ($n = 36$).

	UC_CTL ($n = 14$)	UC ($n = 14$)	*p*	CD_CTL ($n = 4$)	CD ($n = 4$)	*p*
Age [mean (SD)]	46.43 (12.70)	45.64 (12.62)	0.9	48.50 (9.85)	44.75 (6.24)	0.54
Gender (%)						
Men	8 (57.1)	8 (57.1)		4 (100.0)	4 (100.0)	
Women	6 (42.9)	6 (42.9)		0 (0.0)	0 (0.0)	
Ethnic group (%)			0.4			0.15
Han	8 (57.1)	10 (71.4)		1 (25.0)	0 (0.0)	
Tibetans	5 (35.7)	2 (14.3)		2 (50.0)	1 (25.0)	
Others	1 (7.1)	2 (14.3)		1(25.0)	3 (75.0)	
Education (%)			0.7			0.29
<9 years of schooling	6 (42.9)	5 (35.7)		4 (100.0)	3 (75.0)	
≥9 years of schooling	8 (57.1)	9 (64.3)		0 (0.0)	1 (25.0)	
Marital (%)			0.6			1
Unmarried/widowed/divorced/separated	3 (21.4)	2 (14.3)		0 (0.0)	0 (0.0)	
Married	11 (84.6)	12 (85.7)		4 (100.0)	4 (100.0)	
Occupational labor intensity (%)			0.3			0.31
Light	9 (69.2)	9 (64.3)		1 (25.0)	0 (0.0)	
Moderate	5 (30.8)	3 (21.4)		2 (50.0)	1 (25.0)	
Heavy	0 (0.0)	2 (14.3)		1 (25.0)	3 (75.0)	
BMI [mean (SD)]	23.30 (3.70)	22.88 (3.11)	0.8	25.70 (3.82)	21.52 (3.27)	0.15

diversity of the bacterial community at the ASV level, as measured by the Sobs and Chao indices, were significantly reduced in both UC and CD patients (Figure 1C). Moreover, the Shannon index, which was expanded at the species level in the metagenomic data (Figure 1D), corroborated with the 16S rRNA findings, indicating a reduction in alpha diversity within the IBD patients.

3.3 Changes in beta diversity and the differentially dominant bacteria

To investigate the difference between IBD patients and healthy participants, the NMDS analysis was conducted to explore beta diversity. The differences of beta diversity in gut microbiota were identified at the ASV level (Figure 2A), with IBD samples primarily distributed in the left region, showing some overlaps between CD and UC patients, whereas healthy participants were located in the right region. The NMDS analysis of the metagenome profile (stress = 0.115) revealed a similar relationship between the sampling sites, indicating that these regions contained distinct bacterial community structures (Figure 2B). To figure out the difference among the dominant bacteria, analyses at both phylum and genus levels were conducted. The phylum Proteobacteria showed a significant increase in IBD patients (Supplementary Figure 1D), while further analysis indicated that this enrichment was specific to CD patients (Figure 2C). In CD patients, at the genus level, *Escherichia-Shigella* significantly increased, while *Faecalibacterium*, *Agathobacter*, and *Subdoligranulum* significantly decreased (Figure 2D).

3.4 The taxonomy biomarkers of IBD patients

To identify the specific bacterial taxa (from phylum to genus level) among different groups, LEfSe analysis was applied in the 16S rRNA profile (Figure 3A). Proteobacteria at the phylum level and *Escherichia-Shigella*, *Enterococcus*, *Abiotrophia*, and *Granulicatella* at the genus level were significantly increased in IBD patients. To further explore the differences between CD and UC patients, we analyzed paired samples from each group, and the results showed that *Escherichia-Shigella* specifically increased in CD patients, while *Enterococcus* was specific to UC patients (Supplementary Figure 2A). *Faecalibacterium*, *Agathobacter*, *Roseburia*, *Christensenellaceae R-7 group*, *Ruminococcus*, *Ruminococcus torques group*, *Holdemanella*, *Eubacterium hallii group*, and *Fusicatenibacter* were specific bacterial taxa in the healthy control (CTL) group. *Roseburia*, *Christensenellaceae R-7 group*, and *Holdemanella* were identified in both CD and UC patients paired with healthy participants (Supplementary Figure 2B). The striking differences and interactions between bacterial communities were investigated by NetworkX (Figure 3B). *Abiotrophia* was specifically identified in CD patients, while *Enterococcus* co-occurred between CD and

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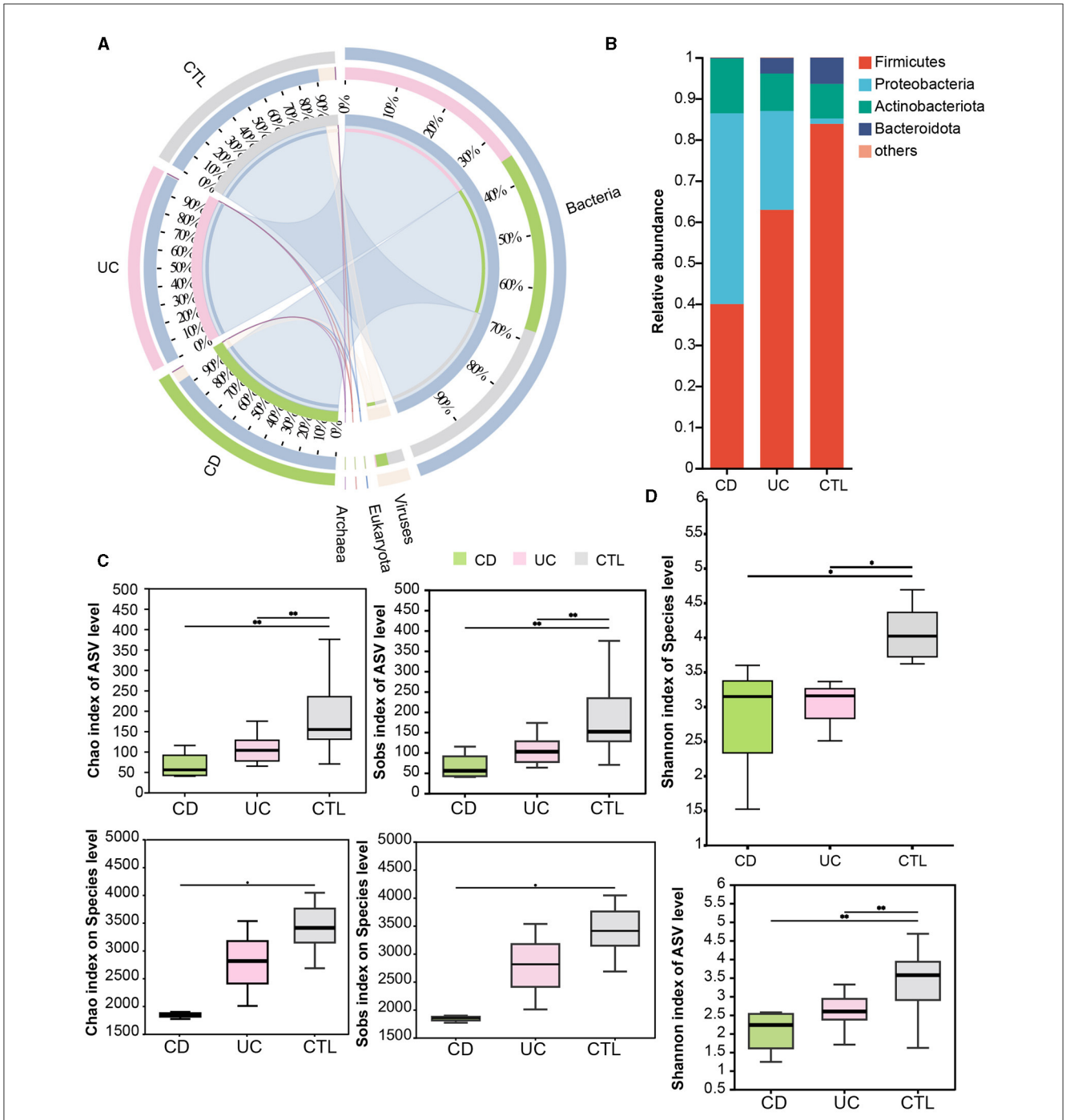


FIGURE 1
The composition and alpha diversity in participants. **(A)** The circoplot of microbial composition at the domain level. **(B)** The barplot of composition at the phylum level. **(C)** The difference of richness in microbial species. **(D)** The difference of the Shannon index in microbial species. The upper panel shows results from 16S rRNA sequencing and the lower panel from metagenomics analysis both in **(C, D)**. The Wilcoxon test was conducted, and * indicated $p < 0.05$, ** indicated $p < 0.01$. CD means Crohn's Disease patients, UC means Ulcerative Colitis patients, and CTL means healthy participants.

UC patients, and *Escherichia-Shigella* interactions were higher among the three groups. In the CTL group, *Christensenellaceae R-7 group*, *Fusicatenibacter*, *Dialister*, *Ruminococcus*, *Holdemanella*, *UCG-002*, and *Fusicatenibacter* were specifically identified. Furthermore, the *Christensenellaceae R-7 group*, *Fusicatenibacter*, and *Holdemanella* were identified as the core gut microbes in healthy participants by Venn analysis (Figure 4A). In addition, metagenome sequencing showed distinct viral profiles in IBD patients, including *Kagunavirus*, *Sukhumvitvirus*, and *Jerseyvirus*, that emerged as the predominant viral genera in CD patients, while our analysis did not reveal any specific viral signatures at the genus level in UC patients (Figure 4B).

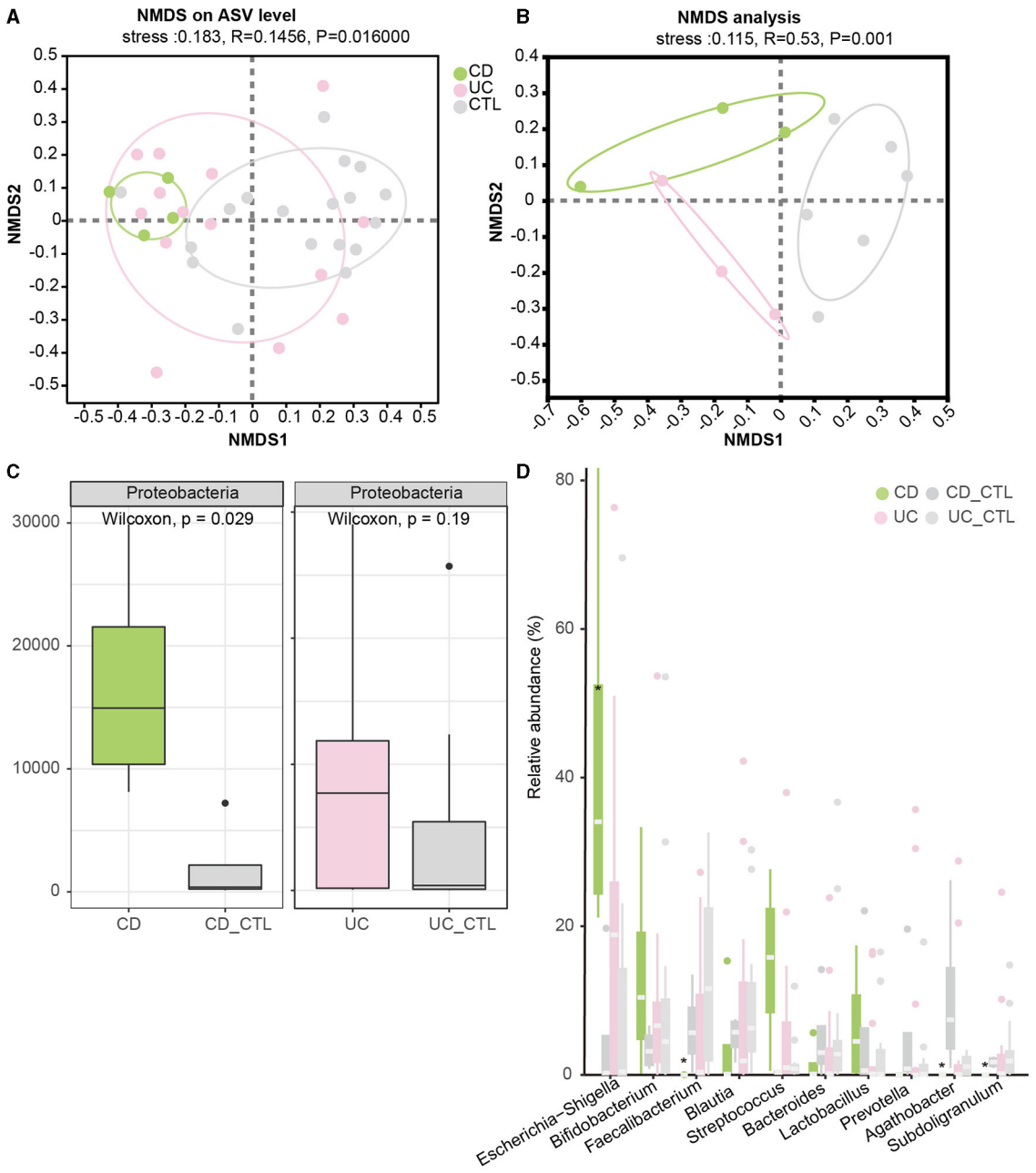


FIGURE 2
Beta diversity among groups and the significant dominant bacterias. **(A, B)** Depict the NMDS results in 16S rRNA sequencing and metagenome sequencing, respectively. **(C)** The boxplot demonstrates the difference in Proteobacteria. The left panel compares CD patients, while the right panel compares UC patients. **(D)** The boxplot of the top 10 bacteria at the genus level, with * indicating a significant difference ($p < 0.05$). CD_CTL and UC_CTL were healthy controls for CD and UC patients, respectively.

These results highlighted the divergent viral landscapes and dysbiosis of the gut microbiome in these two forms of IBD.

3.5 Functional analysis and virulence factor profiles based on metagenomics

To elucidate functional disparities, we employed the KEGG database in the metagenome profile. The NMDS analysis at the KEGG level 3 (stress = 0.044) revealed significant functional differentiation among the studied groups (Figure 5A). To focus on the influence of the immune system, the Venn diagram illustrated an enrichment of natural killer cell-mediated cytotoxicity in CD patients (Figure 5B). We then examined pathways specifically implicated in human diseases. Notably, the pathway bacterial invasion of epithelial cells and reactive oxygen species were prominently enriched in CD patients, while glutathione metabolism was enriched in UC patients (Figure 5C). Additionally, the virulence factor database (VFDB) was used

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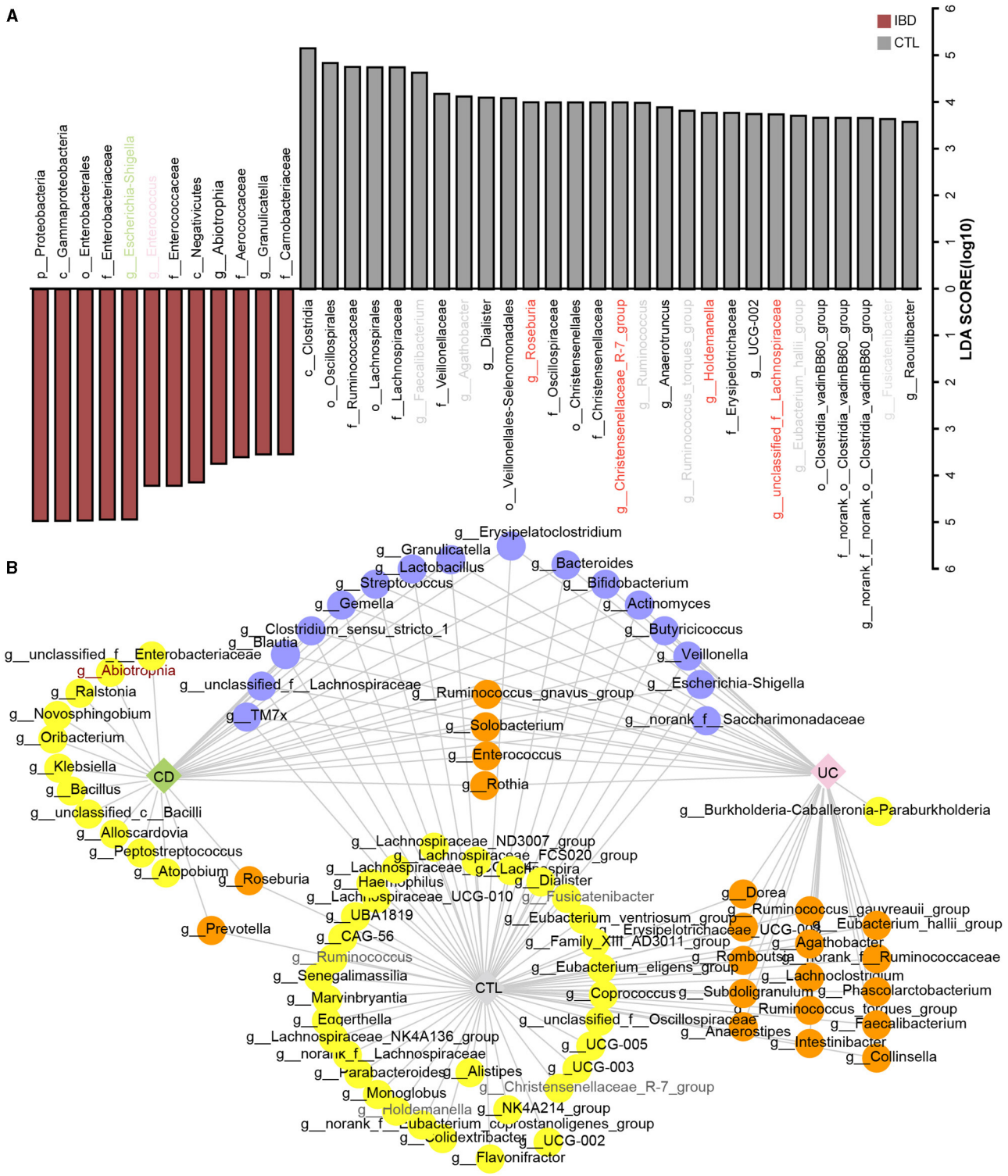


FIGURE 3
The taxonomy biomarkers across different groups. **(A)** The results of LEfSe analysis in the 16S rRNA profile highlight distinct microbial markers between IBD and CTL. The specific bacterial genera are colored with the same color as **(A)**, while red color means both identified in CD and UC when paired with healthy participants. The threshold of the LDA score is 3.5, and it is the same with Supplementary Figures 2A, B. **(B)** The co-occurrence network is derived from 16S rRNA sequencing. In this network, diamonds denote different group classifications, whereas green, pink, and gray correspond to CD, UC, and CTL, respectively, as indicated. Dots represent various bacterial genera, with color coding as follows: yellow represents bacteria unique to one group, orange represents bacteria associated with both CD and UC patients, and purple represents bacteria common across all three groups.

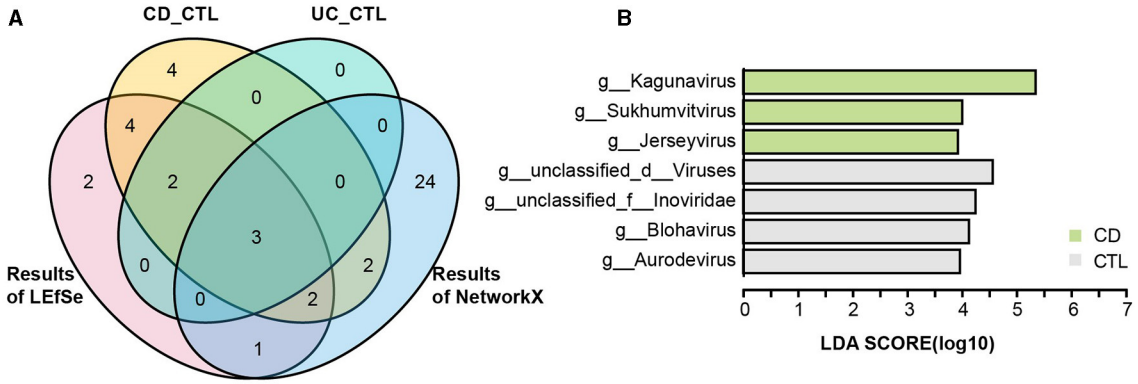


FIGURE 4
The taxonomy biomarkers of the CTL group and viral taxa. **(A)** A Venn diagram illustrates the distinct and shared significant genus-level microbes in the healthy participants. CD_CTL (yellow) and UC_CTL (turquoise) refer to the genera that have significantly increased in the healthy control participants paired with individuals affected by Crohn's disease (CD) and ulcerative colitis (UC), respectively, as shown in Supplementary Figure 2. The LEfSe results (red) indicate the genera that are significantly increased in healthy participants compared to those with inflammatory bowel disease (IBD), as presented in Figure 3A. The NetworkX results (blue) represent the specific microbial genera in the co-occurrence network of healthy participants, detailed in Figure 3B. **(B)** The LEfSe analysis results highlight differential viral taxa based on metagenomic sequencing profiles across groups. The LDA score is > 4, and the color scheme for this bar is consistent with Figure 1 to ensure consistency in visuals.

to assess the virulence of unique target proteins, characterizing significant distance values across three groups (Figure 5D). Remarkably, phenazine biosynthesis, implicated in host tissue damage, and MsrAB, associated with oxidative stress resistance, emerged as unique virulence factors in UC patients. In contrast, virulence factors in CD patients appeared predominantly related to the uptake and utilization of essential minerals, which is crucial for bacterial survival and proliferation (Figure 5E).

To reveal the complexity of microbial interactions within the gut microbiome, the Spearman correlation was performed between the microbiome at the genus level and various virulence factors (Figure 6). Lipooligosaccharide (LOS) (CVF494) demonstrated the most extensive and closest interactions with others. Chu (VF0227), which is specific to CD patients, showed interactions with bacteria, including *Escherichia* and *Subdoligranulum* genera. Within the bacterial community, *Enterococcus* and *Ruminococcus* exhibited the highest degree of interaction with virulence factors. Interestingly, our analysis revealed that only a limited number of viruses, six in total, showed interactions with virulence factors. Among these viruses, *Kagunavirus*, identified as a virus specific to CD patients, was a notable example of such interactions.

4 Discussion

In this case-control study, we investigated microbial dysbiosis in patients with IBD. Our findings demonstrate a marked decrease in both microbial richness and community diversity in these patients. Specifically, the Proteobacteria phylum was significantly more abundant in patients with CD. At the genus level, *Enterococcus* and *Escherichia-Shigella* were identified as biomarkers in IBD patients, with specific prevalence in UC and CD patients, respectively. In contrast, the *Christensenellaceae R-7 group*, *Fusicatenibacter*, and *Holdemanella* were more common in healthy controls. Functional analysis indicated distinct metabolic pathways between the two forms of IBD, pointing to the varied viral profiles in CD and UC patients. These results highlight the complex intricacies of microbial interactions within the gut microbiome.

Our findings reveal significant alterations in the gut microbiota of IBD patients and confirmed a notable decrease in microbial richness and diversity (Alam et al., 2020; Xu et al., 2022). A decrease in alpha diversity, critical for maintaining gut homeostasis, might compromise the mucosal barrier, thus impairing the gut's defensive mechanisms (Leibovitzh et al., 2022). Moreover, a noteworthy finding is the increased Proteobacteria phylum, highlighting its potential role in IBD pathogenesis (Mukhopadhya et al., 2012; Wiredu Ocansey et al., 2023) and suggesting a distinct microbial signature in these conditions. This observation is consistent with prior studies that reinforce the importance of Proteobacteria in CD patients (Vester-Andersen et al., 2019). In this study, no significant difference in microbial richness and diversity between CD and UC patients was identified, despite a previous study reporting inconsistent results regarding substantially lower species richness in CD patients compared to UC patients (Alam et al., 2020). Among newly diagnosed patients, alpha diversity showed no significant differences between UC and CD patients (Rausch et al., 2023), and the consistency in the microbial community at the early stages of the diseases might explain these inconsistent findings. These variations highlight the complex and multifaceted nature of microbial profiles in IBD.

The LEfSe analysis identified specific bacterial taxa associated with CD and UC patients, providing insights into the distinct microbial landscapes of these diseases. The decrease in commensal bacteria, such as the *Christensenellaceae R-7 group*, *Fusicatenibacter*, and *Holdemanella*, alongside an increase in *Enterococcus* and *Escherichia-Shigella*, suggests a progression toward a more pro-inflammatory gut environment. *Escherichia-Shigella* and *Enterococcus*, which are most prevalent in patients with IBD, may cause intestinal inflammation (Chen et al., 2014; Wiredu Ocansey et al., 2023). In an animal study, *Enterococcus*

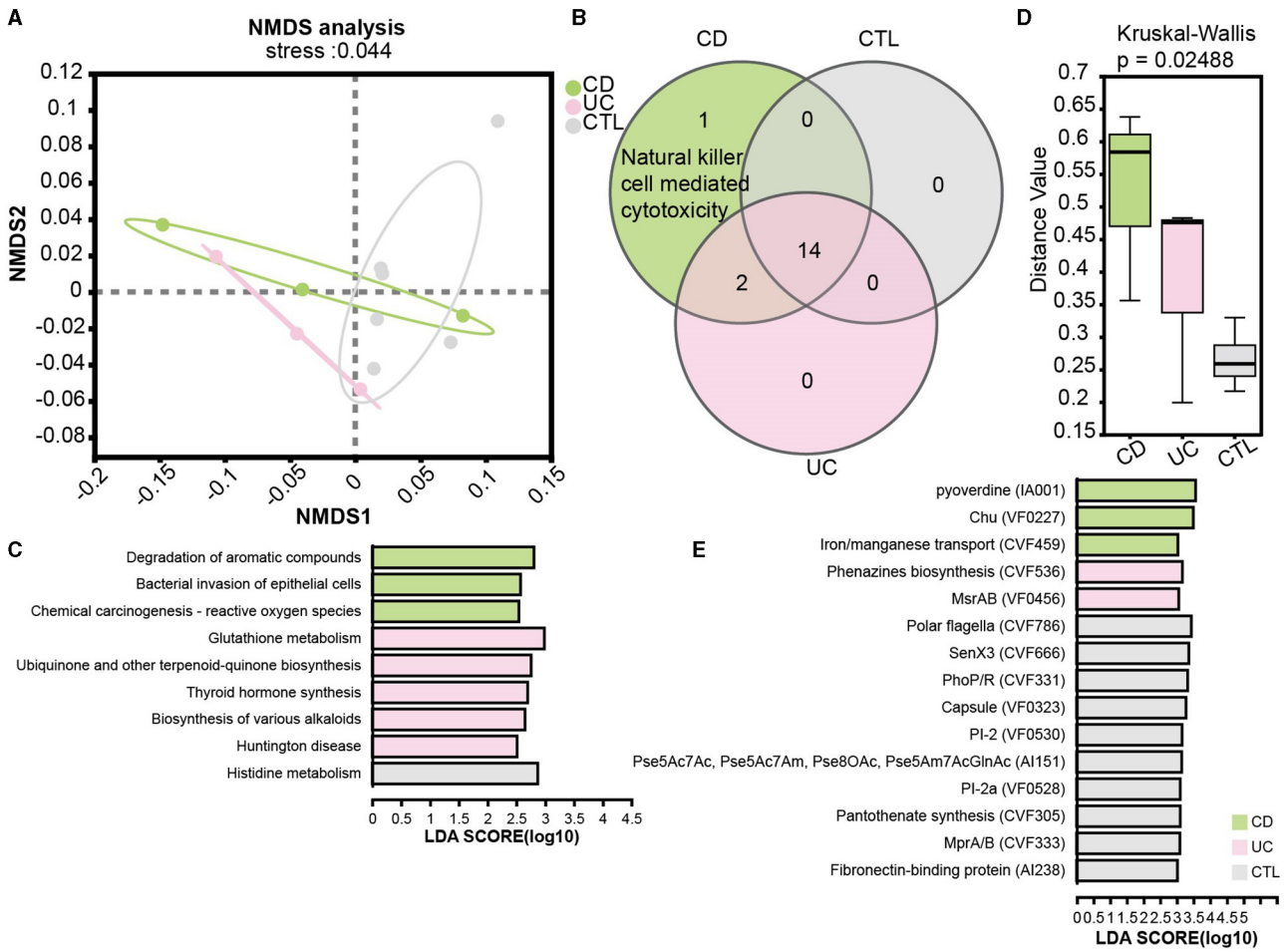


FIGURE 5
Functional analysis in patients and the significant virulence factors. **(A)** NMDS ordination plot based on KEGG orthologous group level 3. **(B)** A Venn plot for KEGG pathways specific in the immune system. **(C)** LEfSe analysis of KEGG functional predictions in human diseases. **(D)** The barplot of differences in community structure among different groups and Bray Curtis distance algorithm to calculate the distance value. **(E)** The LEfSe results showed significant virulence factors, LDA score is > 3. CD means Crohn's disease patients, UC means ulcerative colitis patients, and CTL means healthy participants in the metagenome profile.

and *Escherichia-Shigella* were connected with a high-protein diet and negatively correlated with downregulated genes involved in innate immunity (Mu et al., 2016). The co-occurrence patterns of these two microbiomes might be related to the dietary patterns of participants. *Enterococcus* also stimulates apoptosis of intestinal epithelial cells deprived of the protective mucus layer (Golińska et al., 2013) and might contribute to the pathway of "bacterial invasion of epithelial cells." Meanwhile, the *Christensenellaceae R-7 group* could be the cause of the increased *Escherichia-Shigella* levels and was also identified as a biomarker in the feces of healthy participants with high abundance (Cai et al., 2023). *Fusicatenibacter*, *Faecalibacterium,* and *Ruminococcus* are healthy gut-associated butyrate-producing bacteria known to be beneficial to host immunity (Maruyama et al., 2022; Malan-Müller et al., 2023), and butyrate is an important regulator that reduces mucosal inflammation and strengthens the epithelial defense barrier (Canani et al., 2011). Furthermore, *Holdemanella* was positively correlated with C18-3OH, a compound known for its anti-inflammatory properties (Pujo et al., 2021). *Abiotrophia* was previously shown to increase in healthy participants in a study in Saudi Arabia (Masoodi et al., 2020), which is inconsistent with our study. The genus *Abiotrophia* consists only of *Abiotrophia defectiva* and is related to a higher incidence of endocarditis among IBD patients (Wong et al., 2017; Bhardwaj et al., 2018), indicating a potential role in modulating gut inflammation. These alterations in microbial composition and diversity could potentially contribute to the pathogenesis and clinical manifestations of IBD. Moreover, the core biomarkers identified are consistent across different ethnic groups, indicating that the microbial signatures linked to IBD may possess a degree of universality.

Functional analysis suggests different pathogenic mechanisms. A previous study also revealed that CD patients showed a 3-fold higher level of NK activity than that detected in UC patients (Van Tol et al., 1992). Additionally, the enrichment of reactive oxygen species in CD patients mediates the activation of NK cells (Sun et al., 2020). On the other hand, glutathione metabolism was also significantly enriched in IBD patients (Santoru et al., 2017). The oxidized glutathione content in the mucosa displayed a significant

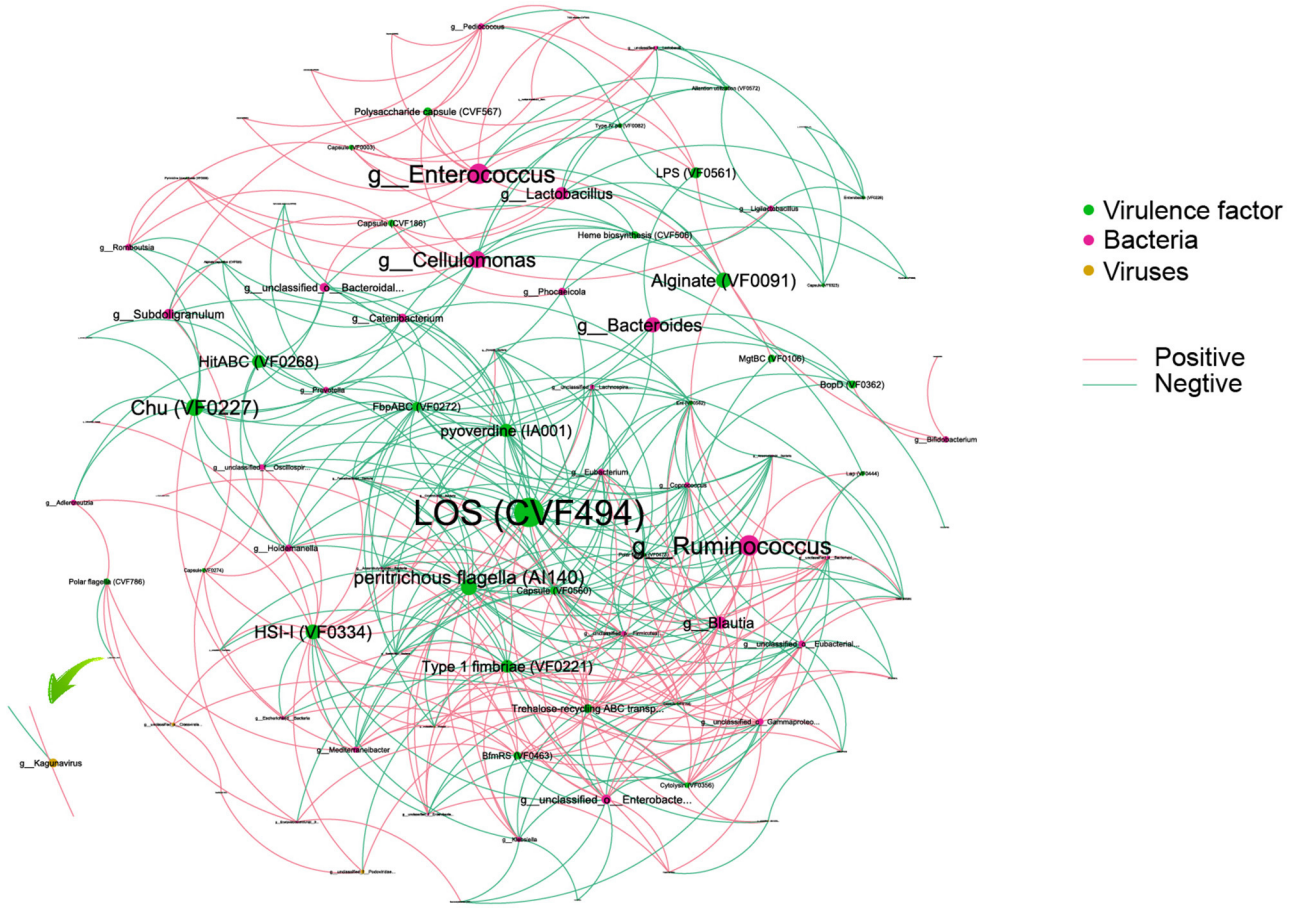


FIGURE 6
An interaction among bacteria, virus, and virulence factors. Different colors of dots represent the Bacteria (green), virulence factor (red), and viruses (yellow). The size of the dots represents the degree of interaction. The pink line represents a positive association, and the green line represents a negative association.

positive correlation with both clinical and histological indicators of disease severity among patients with UC (Iantomasi et al., 1994). Furthermore, the potential causality of oxidative stress and the underlying biological mechanisms in CD was identified (Xu et al., 2023). This finding suggests that, while oxidative stress is implicated in both UC and CD patients, its role may differ slightly between the two conditions.

The analysis of the viral profile revealed a notable trend where viruses were significantly more abundant in CD patients than in UC patients, with three specific viruses showing a marked increase in CD patients. This observation, which is inconsistent with previous findings (Zuo et al., 2019), may be due to variability in the mucosal virome, potentially influenced by differences in sampling sites or techniques. Moreover, a significant relationship between *Kagunavirus* and polar flagella (CVF786), with a notable increase in healthy participants, was identified ($R = -0.71$, $p = 0.01$). The surface components of flagella, including pili and capsular polysaccharides, act as microbial-associated molecular patterns that regulate a cellular protease-dependent signaling cascade. This cascade produces various cytokines and chemokines, alleviating inflammation and enhancing intestinal epithelial function (Liu et al., 2020), and potentially enables beneficial bacteria to colonize niche environments within the gut. This negative correlation suggests that the presence of the virus might inhibit the motility and colonization capabilities of healthy bacteria in CD patients. This pattern highlights the complexities of host-microbe interactions and suggests *Kagunavirus* as a potential disease marker, warranting further investigation into its mechanisms.

The analysis of the interaction between the microbiome and the virulence factor revealed that the majority of linkages persisted between bacteria and virulence factors, with the *Enterococcus* genus appearing to have a substantial impact on inflammatory processes (Golińska et al., 2013). A notable positive correlation ($R = 0.61$, $p = 0.036$) was identified between the *Escherichia* genus and Chu (VF0227), a heme permease protein. This relationship held particular importance due to the role of Chu (VF0227) in iron acquisition and utilization, potentially facilitating the proliferation of adherent-invasive *Escherichia coli* within the inflamed intestines of human hosts (Abdelhalim et al., 2020). This positive regulatory effect might drive the colonization of adherent-invasive *Escherichia coli*, exacerbating intestinal inflammation. The interaction between *Escherichia coli* and Chu (VF0227) highlighted a crucial element of microbial survival and virulence within the host environment.

In summary, our study offered comprehensive insights into the altered microbial composition, interactions, and functionalities in IBD patients, with the case-control approach controlling for potential confounders. These findings significantly enhanced our understanding of the relationship between the gut microbiome and IBD pathogenesis. Additionally, the main biomarkers identified were consistent across various ethnic groups, indicating that the microbial signatures associated with IBD might exhibit a degree of universality, transcending diverse geographical backgrounds. However, this study has certain limitations, notably the need for a larger sample size to reinforce our findings. Future research could benefit from longitudinal studies to further elucidate the dynamic role of the gut microbiota in the pathogenesis process of IBD.

Data availability statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beiing GSA: Institute of Genomics, Chinese Academy of Sciences (CRA015053).

Ethics statement

The studies involving humans were approved by Ethics Committee of the Qinghai Provincial Traditional Chinese Medicine Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HW: Methodology, Supervision, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Visualization. YW: Data curation, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Investigation, Methodology, Project administration. LY: Data curation, Software, Visualization, Writing – review & editing, Investigation, Validation. JF: Data curation, Software, Writing – review & editing, Investigation, Methodology. ST: Data curation, Formal analysis, Software, Visualization, Writing – review & editing. LC: Data curation, Formal analysis, Visualization, Writing – review & editing, Investigation. WH: Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. JL: Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. XW: Investigation, Writing – review & editing, Writing – original draft, Conceptualization, Funding acquisition, Methodology, Project administration, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1375804/full#supplementary-material

References

Abdelhalim, K. A., Uzel, A., and Ünal, N. G. (2020). The role of major virulence factors and pathogenicity of adherent-invasive *Escherichia coli* in patients with Crohn's disease. *Gastroenterol. Rev.* 15, 279–288. doi: 10.5114/pg.2020.93235

Alam, M. T., Amos, G. C. A., Murphy, A. R. J., Murch, S., Wellington, E. M. H., and Arasaradnam, R. P. (2020). Microbial imbalance in inflammatory bowel disease patients at different taxonomic levels. *Gut Pathog.* 12:1. doi: 10.1186/s13099-019-0341-6

Basha, O. M., Hafez, R. A., Salem, S. M., Anis, R. H., and Hanafy, A. S. (2023). Impact of gut Microbiome alteration in Ulcerative Colitis patients on disease severity and outcome. *Clin. Exp. Med.* 23, 1763–1772. doi: 10.1007/s10238-022-00917-x

Bhardwaj, R. G., Al-Khabbaz, A., and Karched, M. (2018). Cytokine induction of peripheral blood mononuclear cells by biofilms and biofilm supernatants of *Granulicatella* and *Abiotrophia* spp. *Microbial. Pathog.* 114, 90–94. doi: 10.1016/j.micpath.2017.11.037

Cai, X., Zhou, N., Zou, Q., Peng, Y., Xu, L., Feng, L., et al. (2023). Integration of taxa abundance and occurrence frequency to identify key gut bacteria correlated to clinics in Crohn's disease. *BMC Microbiol.* 23:247. doi: 10.1186/s12866-023-02999-3

Canani, R. B., Costanzo, M. D., Leone, L., Pedata, M., Meli, R., and Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World J. Gastroenterol.* 17, 1519–1528. doi: 10.3748/wjg.v17.i12.1519

Cao, Z., Sugimura, N., Burgermeister, E., Ebert, M. P., Zuo, T., and Lan, P. (2022). The gut virome: a new microbiome component in health and disease. *eBioMedicine* 81:104113. doi: 10.1016/j.ebiom.2022.104113

Chen, L., Wang, W., Zhou, R., Ng, S. C., Li, J., Huang, M., et al. (2014). Characteristics of fecal and mucosa-associated microbiota in Chinese patients with inflammatory bowel disease. *Medicine* 93:e51. doi: 10.1097/MD.0000000000000051

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

GBD 2017 Inflammatory Bowel Disease Collaborators (2020). The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol. Hepatol.* 5, 17–30. doi: 10.1016/S2468-1253(19)30333-4

Golińska, E., Tomusiak, A., Gosiewski, T., Wiecek, G., Machul, A., Mikołajczyk, D., et al. (2013). Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease. *World J. Gastroenterol.* 19, 3562–3572. doi: 10.3748/wjg.v19.i23.3562

He, P., Yu, L., Tian, F., Zhang, H., Chen, W., and Zhai, Q. (2022). Dietary patterns and gut microbiota: the crucial actors in inflammatory bowel disease. *Adv. Nutr.* 13, 1628–1651. doi: 10.1093/advances/nmac029

Iantomasi, T., Marraccini, P., Favilli, F., Vincenzini, M. T., Ferretti, P., and Tonelli, F. (1994). Glutathione metabolism in Crohn`s disease. *Biochem. Med. Metabol. Biol.* 53, 87–91. doi: 10.1006/bmmb.1994.1062

Jansen, D., Falony, G., Vieira-Silva, S., Simsek, C., Marcelis, T., Caenepeel, C., et al. (2023). Community types of the human gut virome are associated with endoscopic outcome in ulcerative colitis. *J. Crohn's Colitis* 17, 1504–1513. doi: 10.1093/ecco-jcc/jjad061

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucl. Acids Res.* 28, 27–30. doi: 10.1093/nar/28.1.27

Kaplan, G. G., and Windsor, J. W. (2021). The four epidemiological stages in the global evolution of inflammatory bowel disease. *Nat. Rev. Gastroenterol. Hepatol.* 18, 56–66. doi: 10.1038/s41575-020-00360-x

Leibovitzh, H., Lee, S.-H., Xue, M., Raygoza Garay, J. A., Hernandez-Rocha, C., Madsen, K. L., et al. (2022). Altered gut microbiome composition and function are associated with gut barrier dysfunction in healthy relatives of patients with Crohn's disease. *Gastroenterology* 163, 1364–1376.e10. doi: 10.1053/j.gastro.2022.07.004

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, Z., Chen, Y., and Ke, H. (2024). Investigating the causal relationship between gut microbiota and Crohn's disease: a mendelian randomization study. *Gastroenterology* 166, 354–355. doi: 10.1053/j.gastro.2023.08.047

Liu, B., Zheng, D., Zhou, S., Chen, L., and Yang, J. (2022). VFDB 2022: a general classification scheme for bacterial virulence factors. *Nucl. Acids Res.* 50, D912–D917. doi: 10.1093/nar/gkab1107

Liu, C., Cui, Y., Li, X., and Yao, M. (2021). Microeco : an R package for data mining in microbial community ecology. *FEMS Microbiol. Ecol.* 97:fiaa255. doi: 10.1093/femsec/fiaa255

Liu, Q., Yu, Z., Tian, F., Zhao, J., Zhang, H., Zhai, Q., et al. (2020). Surface components and metabolites of probiotics for regulation of intestinal epithelial barrier. *Microbial. Cell Factor.* 19, 1–11. doi: 10.1186/s12934-020-1289-4

Ma, T., Wan, M., Liu, G., Zuo, X., Yang, X., and Yang, X. (2023). Temporal trends of inflammatory bowel disease burden in China from 1990 to 2030 with comparisons to Japan, South Korea, the European Union, the United States of America, and the World. *Clin. Epidemiol.* 15, 583–599. doi: 10.2147/CLEP.S402718

Malan-Müller, S., Valles-Colomer, M., Palomo, T., and Leza, J. C. (2023). The gut-microbiota-brain axis in a Spanish population in the aftermath of the COVID-19 pandemic: microbiota composition linked to anxiety, trauma, and depression profiles. *Gut Microbes* 15:2162306. doi: 10.1080/19490976.2022.2162306

Maruyama, S., Matsuoka, T., Hosomi, K., Park, J., Nishimura, M., Murakami, H., et al. (2022). Classification of the occurrence of dyslipidemia based on gut bacteria related to barley intake. *Front. Nutr.* 9:812469. doi: 10.3389/fnut.2022.812469

Masoodi, I., Alshanqeeti, A., Alyamani, E., Allehibi, A., Alqutub, A., Alsayari, K., et al. (2020). Microbial dysbiosis in irritable bowel syndrome: a single-center metagenomic study in Saudi Arabia: microbial dysbiosis in IBS. *JGH Open* 4:12313. doi: 10.1002/jgh3.12313

Massimino, L., Palmieri, O., Facoetti, A., Fuggetta, D., Spanò, S., Lamparelli, L. A., et al. (2023). Gut virome-colonising *Orthohepadnavirus* genus is associated with ulcerative colitis pathogenesis and induces intestinal inflammation *in vivo*. *Gut* 72, 1838–1847. doi: 10.1136/gutjnl-2022-328375

Mu, C., Yang, Y., Luo, Z., Guan, L., and Zhu, W. (2016). The colonic microbiome and epithelial transcriptome are altered in rats fed a high-protein diet compared with a normal-protein diet. *J. Nutr.* 146, 474–483. doi: 10.3945/jn.115.223990

Mukhopadhya, I., Hansen, R., El-Omar, E. M., and Hold, G. L. (2012). IBD-what role do Proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* 9, 219–230. doi: 10.1038/nrgastro.2012.14

Ning, L., Zhou, Y. L., Sun, H., Zhang, Y., Shen, C., Wang, Z., et al. (2023). Microbiome and metabolome features in inflammatory bowel disease via multi-omics integration analyses across cohorts. *Nat. Commun.* 14:7135. doi: 10.1038/s41467-023-42788-0

Park, J., and Cheon, J. H. (2021). Incidence and prevalence of inflammatory bowel disease across Asia. *Yonsei Med. J.* 62, 99–108. doi: 10.3349/ymj.2021.62.2.99

Pujo, J., Petitfils, C., Faouder, P. L., Eeckhaut, V., Payros, G., Maurel, S., et al. (2021). Bacteria-derived long chain fatty acid exhibits anti-inflammatory properties in colitis. *Gut* 70, 1088–1097. doi: 10.1136/gutjnl-2020-321173

Rausch, P., Ellul, S., Pisani, A., Bang, C., Tabone, T., Marantidis Cordina, C., et al. (2023). Microbial dynamics in newly diagnosed and treatment Naïve IBD patients in the mediterranean. *Inflamm. Bowel Dis.* 29, 1118–1132. doi: 10.1093/ibd/izad004

Raygoza Garay, J. A., Turpin, W., Lee, S. H., Smith, M. I., Goethel, A., Griffiths, A. M., et al. (2023). Gut microbiome composition is associated with future onset of Crohn's disease in healthy first-degree relatives. *Gastroenterology* 165, 670–681. doi: 10.1053/j.gastro.2023.05.032

Rehman, A., Rausch, P., Wang, J., Skieceviciene, J., Kiudelis, G., Bhagalia, K., et al. (2016). Geographical patterns of the standing and active human gut microbiome in health and IBD. *Gut* 65, 238–248. doi: 10.1136/gutjnl-2014-308341

Santoru, M. L., Piras, C., Murgia, A., Palmas, V., Camboni, T., Liggi, S., et al. (2017). Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. *Sci. Rep.* 7:9523. doi: 10.1038/s41598-017-10034-5

Shao, B., Yang, W., and Cao, Q. (2022). Landscape and predictions of inflammatory bowel disease in China: China will enter the Compounding Prevalence stage around 2030. *Front. Publ. Health* 10:1032679. doi: 10.3389/fpubh.2022.1032679

Sun, L., Wang, X., Saredy, J., Yuan, Z., Yang, X., and Wang, H. (2020). Innate-adaptive immunity interplay and redox regulation in immune response. *Redox Biol.* 37:101759. doi: 10.1016/j.redox.2020.101759

Van Tol, E. A. F., Verspaget, H. W., Peña, A. S., and Lamers, C. B. H. W. (1992). Normal inflammatory bowel disease mucosa conceals alterations in natural killer cell activity. *Scand. J. Gastroenterol.* 27, 999–1005. doi: 10.3109/00365529209028129

Vester-Andersen, M. K., Mirsepasi-Lauridsen, H. C., Prosberg, M. V., Mortensen, C. O., Träger, C., Skovsen, K., et al. (2019). Increased abundance of proteobacteria in aggressive Crohn's disease seven years after diagnosis. *Sci. Rep.* 9:13473. doi: 10.1038/s41598-019-49833-3

Wang, R., Li, Z., Liu, S., and Zhang, D. (2023). Global, regional and national burden of inflammatory bowel disease in 204 countries and territories from 1990 to 2019: a systematic analysis based on the Global Burden of Disease Study 2019. *Br. Med. J. Open* 13:e065186. doi: 10.1136/bmjopen-2022-065186

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Berlin: Springer.

Wiredu Ocansey, D. K., Hang, S., Yuan, X., Qian, H., Zhou, M., Valerie Olovo, C., et al. (2023). The diagnostic and prognostic potential of gut bacteria in inflammatory bowel disease. *Gut Microbes* 15:2176118. doi: 10.1080/19490976.2023.2176118

Wong, Y. J., Tan, B. X., Chlebicki, M. P., and Schwender, B. J. (2017). Abiotrophia defectiva: a rare gut pathogen resulting endocarditis in inflammatory bowel disease. *J. Microbil. Infect. Dis.* 7, 217–219. doi: 10.5799/jmid.369281

Xu, S., Li, X., Zhang, S., Qi, C., Zhang, Z., Ma, R., et al. (2023). Oxidative stress gene expression, DNA methylation, and gut microbiota interaction trigger Crohn's disease: a multi-omics Mendelian randomization study. *BMC Med.* 21:179. doi: 10.1186/s12916-023-02878-8

Xu, X., Ocansey, D. K. W., Hang, S., Wang, B., Amoah, S., Yi, C., et al. (2022). The gut metagenomics and metabolomics signature in patients with inflammatory bowel disease. *Gut Pathog.* 14:26. doi: 10.1186/s13099-022-00499-9

Yang, H., Zhou, R., Bai, X., Guo, M., Ruan, G., Wang, L., et al. (2022). Trend and geographic variation in incidence and prevalence of inflammatory bowel disease in regions across China: a nationwide employee study between 2013 and 2016. *Front. Med.* 9:251. doi: 10.3389/fmed.2022.900251

Zhang, X., and Gérard, P. (2022). Diet-gut microbiota interactions on cardiovascular disease. *Comput. Struct. Biotechnol. J.* 20, 1528–1540. doi: 10.1016/j.csbj.2022.03.028

Zhang, Y., Wei, Y., Tang, D., Lu, J., Zhang, N., Hu, Y., et al. (2023). Association of major dietary patterns and different obesity phenotypes in Southwest China: the China Multi-Ethnic Cohort (CMEC) Study. *Eur. J. Nutr.* 62, 465–476. doi: 10.1007/s00394-022-02997-7

Zuo, T., Lu, X. J., Zhang, Y., Cheung, C. P., Lam, S., Zhang, F., et al. (2019). Gut mucosal virome alterations in ulcerative colitis. *Gut* 68, 1169–1179. doi: 10.1136/gutjnl-2018-318131

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Exploring of gut microbiota features in dyslipidemia and chronic coronary syndrome patients undergoing coronary angiography

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Chronic coronary syndrome (CCS) has a high mortality rate, and dyslipidemia is a major risk factor. Atherosclerosis, a cause of CCS, is influenced by gut microbiota dysbiosis and its metabolites. The objective of this study was to study the diversity and composition of gut microbiota and related clinical parameters among CCS patients undergoing coronary angiography and dyslipidemia patients in comparison to healthy volunteers in Thailand. CCS patients had more risk factors and higher inflammatory markers, high-sensitivity C-reactive protein (hs-CRP) than others. The alpha diversity was lower in dyslipidemia and CCS patients than in the healthy group. A significant difference in the composition of gut microbiota was observed among the three groups. The relative abundance of Proteobacteria, Fusobacteria, Enterobacteriaceae, *Prevotella,* and *Streptococcus* was significantly increased while *Roseburia*, *Ruminococcus,* and *Faecalibacterium* were lower in CCS patients. In CCS patients, Lachnospiraceae, Peptostreptococcaceae, and *Pediococcus* were positively correlated with hs-CRP. In dyslipidemia patients, *Megasphaera* was strongly positively correlated with triglyceride (TG) level and negatively correlated with high-density lipoprotein cholesterol (HDL-C). The modification of gut microbiota was associated with changes in clinical parameters involved in the development of coronary artery disease (CAD) in CCS patients.

KEYWORDS

coronary artery disease, dyslipidemia, gut microbiome, gut microbiota, chronic coronary syndrome, cardiovascular disease

Introduction

Cardiovascular disease (CVD) is the world's leading cause of mortality and one of Thailand's significant health issues. This disease has several risk factors, including dyslipidemia (Hedayatnia et al., 2020), diabetes mellitus (Abdul-Ghani et al., 2017), hypertension (Dustan, 2000), obesity, insulin resistance, and metabolic syndrome (Tune et al., 2017). Currently, lifestyle changes like exercising, eating healthily, and consuming less salt to lower blood pressure can help to reduce the risk factors of CVD. These changes can be combined with blood-sugar-lowering drugs, statins, and antihypertensive drugs. However, morbidity and mortality rates, which are still very high all over the world, including Thailand, are impacted by CVD.

Even though there are other coronary artery disease (CAD) risk factors, dyslipidemia is a major one (Yusuf et al., 2004). Acute and chronic coronary syndromes are two subtypes of CAD that nare distinguished by the timing of the onset of the signs and symptoms. A phrase used to describe CAD as a chronic, progressive condition that can be stabilized is chronic coronary syndrome (CCS) (Knuuti et al., 2020).

More than 1,100 different bacterial species have been linked to numerous symptoms and diseases, including cancer, diabetes mellitus, obesity, and CVD (Afzaal et al., 2022), according to earlier research. Since metabolic syndrome is a cardiovascular condition, eating the proper amount of healthful food can both prevent and treat it. The majority of gastrointestinal tract microorganisms cannot be cultured. The genes of the microorganism, also known as the microbiome, must therefore be examined to determine the type and quantity of these microorganisms.

Gut microbiota is a microorganism that lives in the human digestive tract. The quantity and type of microorganisms in the gastrointestinal tract are determined and influenced by several external factors such as geographic origin, age, genetics, diet, and use of prebiotics and antibiotics (Thursby and Juge, 2017; Fan and Pedersen, 2021). The gastrointestinal tract contains a variety of microorganisms such as Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria, in which their mechanisms of action and habitats are different. Crucial gastrointestinal bacteria that have been presented since birth include *Lactobacillus* and *Bifidobacterium* (Yang et al., 2019). Nowadays people pay attention to the diversity of gut microbiota, which affects the immune system, nutrient metabolism, and the stabilization of the lining of the gastrointestinal tract (gut epithelium). The gut microbiota is also responsible for digesting certain nutrients that cannot be digested in the stomach and also helps to synthesize vitamins and hormones in the body. The body and the gut microbiome have a complicated interaction through metabolites, including short-chain fatty acids (SCFAs), bile acid (BA), and trimethylamine-*N*-oxide (TMAO). Several organs may be affected by these metabolites' effects. Atherosclerosis, a cause of CVD, is influenced by gut microbiota and metabolites (Pieczynska et al., 2020).

Recent studies have highlighted the role of gut dysbiosis in several diseases, including atherosclerosis and many CVDs. These microorganisms are inherited from the mother at birth, as well as from the environment, food, and medicine (Santacroce et al., 2021). The changing intake of macronutrients leads to metabolic syndrome, if there is an imbalance of microorganisms (dysbiosis) in the gastrointestinal tract. Alterations in gut microbiota will lead to an increase in various diseases and consuming a low-fiber, high-fat, and high-sugar diet affects the digestive system including the gut ecosystem. The increase of the abundance of lipopolysaccharides (LPS)-producing gram-negative bacteria, such as *Escherichia, Shigella, Veillonella, Haemophilus,* and *Klebsiella*, increased with the severity of CAD in contrast to the bacteria that produce butyric acids, such as Ruminococcaceae and Lachnospiraceae, decreased the onset of CAD. Moreover, several types of gut microbiota, including those from Bacteroidetes phylum, *Roseburia intestinalis, Faecalibacterium prausnitzii*, and *Eubacterium rectale*, are also protective factors against CAD (Jonsson and Bäckhed, 2017; Liu H. et al., 2019; Liu et al., 2020).

Sequencing of the 16S rRNA gene is a clustering-independent widely used technique in microbiome studies to analyze the composition and diversity of bacterial communities. This technique provides insights into the diversity, distribution, and relative abundance of bacteria, which are essential for understanding the role of microbiota in various environments, including the human body, soil, water, and other ecological systems (Lane et al., 1985; Wensel et al., 2022).

To the best of our knowledge, no study has been conducted in Thailand to determine if the gut microbiome is associated with the parameters of dyslipidemia and CCS patients undergoing coronary angiography. The purpose of this exploratory study is to identify potential relationships among relative gut microbiota composition and related parameters in patients with dyslipidemia and CCS patients undergoing coronary angiography in Thailand.

Materials and methods

Subject enrollment

Ninety-one patients between the ages of 35 and 70 who were admitted to Chulabhorn Hospital's cardiovascular center between February and June of 2023 were enlisted. Using statistical matching techniques with age and sex, participants were separated into three groups: CCS patients undergoing coronary angiography, patients with cardiovascular risk factors, dyslipidemia, and healthy volunteers. This study protocol was approved by the ethics committee of Chulabhorn Hospital. All individuals provided written informed consent before participating in the study. The Schematic diagram showing the research workflow is in Figure 1.

Inclusion criteria

In patients with CCS undergoing coronary angiography group, they must meet the following criteria: (1) patients with stable anginal symptoms (2) patients <1 year after an acute coronary syndrome (ACS) with stabilized symptoms after revascularization (3) CCS patients >1 year after initial diagnosis or revascularization (4) asymptomatic patients in whom CAD is found after screening (Knuuti et al., 2020). Coronary angiography was used to confirm the diagnosis of CCS in those with ≥70% stenosis in coronary arteries larger than 2.5 mm in one view; ≥ 50% stenosis in coronary arteries in two views; and ≥ 50% stenosis in the left main coronary artery (Jiangping et al., 2013); and they must assent by signing the consent forms.

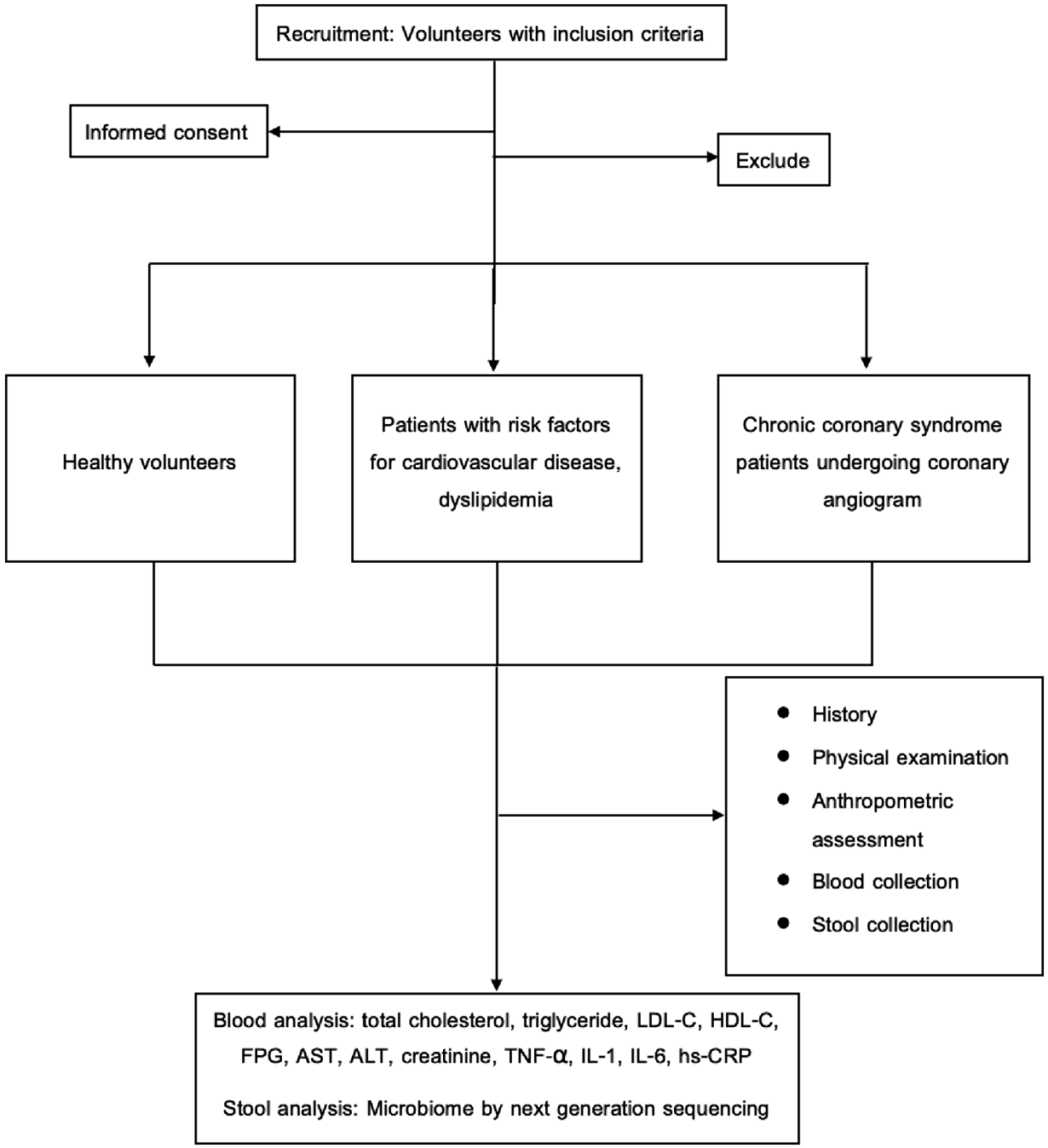


FIGURE 1
Schematic diagram showing the research workflow. ALT, alanine transaminase; AST, aspartate transaminase; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IL-1, interleukin-1; IL-6, interleukin-6; LDL-C, low-density lipoprotein cholesterol; TNF-α, tumor necrosis factor-alpha.

In the group of dyslipidemia patients, they must only have one risk factor for CVD, dyslipidemia (Total cholesterol (TC) ≥ 200 mg/dL or triglyceride (TG) ≥ 150 mg/dL or low-density lipoprotein cholesterol (LDL-C) ≥ 160 mg/dL or patients on treatment) (Lin et al., 2018), without type 2 diabetes mellitus (T2DM) (fasting blood glucose (FBS) ≥ 126 mg/dL for 2 times separately or patients on treatment) (American Diabetes Association, 2014), hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg at least 2 different days or patients on treatment) (Williams et al., 2019), obesity (body mass index ≥30 kg/m2) (Samadoulougou et al., 2020), and metabolic syndrome [at least 3 of the following risk factors: waist circumference ≥ 90 cm for men and ≥ 80 cm for women, blood pressure ≥ 130/85 mmHg or on antihypertensive drugs, TG ≥ 150 mg/dL or on medication, high-density lipoprotein cholesterol (HDL-C) ≤ 40 mg/dL for men and ≤ 50 mg/dL for women and FBS ≥100 mg/dL or on medication] (Huang, 2009). Additionally, none of the aforementioned diseases must exist among the group of healthy volunteers.

Exclusion criteria

Patients who met the following criteria were excluded: (1) kidney disease (estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m2 for more than 3 months), liver disease (Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) > 5 times normal or jaundice), cancer, intestinal diseases such as inflammatory bowel disease, thyroid dysfunction, immunodeficiency such as human immunodeficiency virus (HIV); (2) use antibiotics, immunosuppressants, probiotics supplement, synbiotics, herbal supplements, antacids or laxatives within 4 weeks before participating in the study; (3) history of gastrointestinal disease and other infections within 4 weeks; (4) smoking; (5) alcoholism; (6) pregnant or lactating; (7) Covid-19 infection within 4 weeks.

Sample size calculation

The sample size calculation is based on "Hypothesis testing and power calculations for taxonomic-based human microbiome data," in this research, the value of Alpha (α) = 5%, power = 80%, and the function of the number of sequence reads is equal to 50,000 with three groups. A total of 25 volunteers were obtained and it was determined that the dropout rate of 20%, resulting in a total of 30 volunteers per group, or 90 volunteers in total (La Rosa et al., 2012).

Sample collection and high-throughput sequencing

Each patient's blood was collected to evaluate FBS, hemoglobin A1C (HbA1C), TC, TG, HDL-C, LDL-C, AST, ALT, creatinine, and high-sensitivity C-reactive protein (hs-CRP). The fresh stool was collected in DNA/RNA shield fecal collection tubes (Zymo Research, CA, United States) 1 day before an appointment and immediately frozen at-20°C for 48 h before further analysis. Using the QIAamp Stool Mini kit (Qiagen, United States), DNA was extracted from stool samples. Nanodrop and electrophoresis were used to evaluate the quantity and quality of DNA. Using 515\u00B0F and 806R primers and 2X KAPA hot-start ready mix, the V4 hypervariable region of the 16S rRNA gene was amplified by PCR. The PCR conditions included an initial denaturation at 94°C for 3 min, followed by 25 cycles of 98°C for 20 s., 55°C for 30 s., 72°C for 30 s., and a final extension step at 72°C for 5 min. The 16S amplicons were purified using AMPure XP beads and indexed using Nextera XT index kit, followed by 8 cycles of the aforementioned PCR condition. Finally, the PCR products were cleaned and pooled for cluster generation and 250-bp paired-end read sequencing on the Illumina® MiSeq™.

Sequencing data analysis

QIIME 22019.10 was used for microbiome bioinformatics (Bolyen et al., 2019). The raw sequence data was demultiplexed using the q2-demux plugin, and reads with expected errors (maxEE) higher than 3.0 were discarded by denoising software, DADA2 (via q2-dada2). A phylogeny was constructed using the SEPP q2-plugin, placing short sequences into sepp-refs-gg-13-8.qza reference phylogenetic tree. Alpha-diversity metric, beta-diversity metric, and Principle Coordinate Analysis (PCoA) were estimated using q2-diversity after samples were rarefied (subsampled without replacement) to a minimum read. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier against the Greengenes 13_8 99% operational taxonomic units (OTUs) reference sequences. Statistical tests of alpha and beta diversity were performed using Kruskal-Wallis and permutational multivariate analysis of variance (PERMANOVA) (number of permutations = 999), respectively. Moreover, the significantly differential abundance analysis of microbiota was conducted using LEfSe (Segata et al., 2011) via the algorithm module on the Galaxy platform at http://huttenhower.sph.harvard.edu/galaxy. First, nonparametric factorial Kruskal-Wallissum-rank tests were applied to choose features differentially distributed among classes ($p < 0.05$). The linear discriminant analysis (LDA) model was used to estimate their effect sizes and supported by 30-fold bootstrapping (cutoff = logarithmic LDA score of ≥2.0). In addition, significant *p*-values associated with the microbial significantly differential features by LEfSe were corrected for multiple hypothesis testing using the Benjamini and Hochberg false discovery rate correction.

Statistical analysis and visualization

Stata/SE 16.1 software (StataCorp LP, College Station, TX, United States) was used to analyze the statistical data. Statistics were considered significant when *p*-value <0.05. All study variables were subjected to descriptive statistics analysis, which was provided as frequency (%) for categorical data and mean ± standard deviation (SD) or median for nonnormal quantitative data. One-way analysis of variance (ANOVA) statistic and *post hoc* analysis using the Scheffe test with *p*-value <0.05 were utilized if the distribution of the quantitative data, such as age and laboratory results, was normal. The Kruskal-Wallis test and *post hoc* Mann–Whitney U test with *p*-value <0.017 were used if the data distribution was not normal.

The following Spearman's correlation coefficient was analyzed; (1) anthropometric measurements, including BMI and waist circumference (2) physical examination; blood pressure and (3) blood tests; FBS, HbA1C, TC, TG, LDL-C, HDL-C, AST, ALT, and hs-CRP. Correlation heat map visualization was performed using the ggplot2 R package. A *p*-value <0.05 was considered statistically significant and was labeled in the figure. In addition, the phylogenetic heat tree was visualized using the metacoder R package.

Results

Clinical characteristics

Ninety-one patients were included and divided into three groups: CCS patients undergoing coronary angiography, dyslipidemia patients, and healthy volunteers, with 30, 32, and 29 patients in each group, respectively. To be mentioned, 96 patients were recruited but the microbiota data of one dyslipidemia patient and four CCS patients

were missed because stool samples could not be collected. So, we examined 91 samples of the gut microbiota. The patients were 43.96% female, with a mean age of 57.57 ± 8.35 years, and 26.37% had hypertension. There was no statistically significant difference in age or gender among the groups. Baseline characteristics are shown in Table 1.

The average age of the CCS patients was 59.60 ± 9.04 years and 56.67% of them had a history of CAD. In comparison to other groups, more patients had obesity, low HDL-C level, metabolic syndrome, hypertension, and diabetes mellitus at 43.33, 43.33, 33.33, 80, and 30% with statistically significant, respectively. Additionally, the levels of FBS, HbA1C, and hs-CRP were higher in this group than in others with statistical significance.

In dyslipidemia patients, the mean age was 57.44 ± 8.88 years. Diabetes mellitus and hypertension patients were omitted. There were 9.38% of the patients with impaired fasting glucose in this group however the average of FBS and HbA1C was in the normal range. TC and LDL-C were higher than others with statistical significance.

Unexpectedly, TC and LDL-C were found to be lowest in CCS patients compared to patients with dyslipidemia and healthy volunteers at 144.33 ± 41.24 and 77.11 ± 34.44 vs. 202.93 ± 43.67 and 129.96 ± 40.78 vs. 178.28 ± 22.28 and 111.70 ± 21.97, respectively. According to this study, 96.67% of CCS patients utilized statins, with a high-intensity statin rate of 93.10%. All patients took statins for a minimum of three months. Patients with dyslipidemia used statins at a rate of 90.63% with only high-intensity statins at 37.93%.

Diversity of the gut microbiota

A bacterial diversity analysis was performed to characterize the microbial community. Chao1 index was estimated to reflect the number of OTUs in a sample, the values of which are positively correlated with the species richness of the sample. Moreover, Shannon indicates it reflects the averaging or uniformity of the abundance of different species in a sample, which is positively correlated with the species diversity. Alpha diversity analyses, including the values of Chao1 and Shannon indexes, are comprehensive indicators of species richness and uniformity in community ecology (Figures 2A,B). From both Chao1 and Shannon indexes, there was a trend that showed lower diversity in dyslipidemia patients and CCS patients than in healthy volunteers.

Beta diversity refers to species differences between different environmental communities, and it also can be used to evaluate the overall heterogeneity of the species or the environmental community. Weighted and Unweighted UniFac distances were used in this study to determine the similarities and differences in the composition structure between the microbial communities. Principal coordinates analysis illustrated a statistically significant difference (PERMANOVA, $p < 0.01$) in the clustering of fecal samples between the gut microbiome of the dyslipidemia patients, CCS patients, and healthy volunteers (Figures 2C,D). The different colors in the PCoA analysis represent different samples, and the different shapes represent different groups. The closer the sample distance is, the more similar the microbial composition between the samples is, and the smaller the difference is.

Assessment of bacterial taxonomic composition of CCS and dyslipidemia patients and healthy volunteers

At the phylum level, among all three groups, the most common bacterial phyla were Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. The relative abundance of Proteobacteria was increased in CCS patients (the percentage of Proteobacteria in CCS patients, dyslipidemia patients, and healthy volunteers: 8.22, 4.79, and 4.16, respectively). Moreover, the relative abundance of Fusobacteria was also increased in CCS patients (the percentage of Fusobacteria in CCS patients, dyslipidemia patients, and healthy volunteers: 1.48, 1.07, and 0.89, respectively). On the other hand, in CCS patients, Actinobacteria and Verrucomicrobia had the lowest relative abundance proportion among the three groups. In terms of other phyla, there was no difference. The proportion of Firmicutes and Bacteroidetes was higher in the group of dyslipidemia patients than in the other groups (Figure 3A).

At the family level, when the gut microbiota of the three groups was analyzed at this level, Lachnospiraceae and the Bacteroidaceae families were shown to be the two most common families. Compared to other groups, Prevotellaceae, Enterobacteriaceae and Streptococcaceae families were more prevalent proportionately in CCS patients (Figure 3B).

At the genus level, the highest abundance in each group belonged to the genus *Bacteroides* (the percentage of *Bacteroides* in CCS patients, dyslipidemia patients, and healthy volunteers: 19.02, 19.15, and 21.50, respectively). In the group of CCS patients, *Prevotella* and *Streptococcus* genera were more prevalent in abundance than other groups, with statistical significance following from *Bacteroides* genus (Figure 3C). Moreover, the relative abundance of *Roseburia* (the percentage of *Roseburia* in CCS patients, dyslipidemia patients and healthy volunteers: 3.17, 5.67, and 4.71, respectively), *Ruminococcus* (the percentage of *Ruminococcus* in CCS patients, dyslipidemia patients and healthy volunteers: 2.40, 4.54, and 3.79, respectively) and *Faecalibacterium* genera (the percentage of *Faecalibacterium* in CCS patients, dyslipidemia patients and healthy volunteers: 6.20, 6.98 and 7.08, respectively) were the lowest in CSS patients' group than in the others.

At the species level, from the pairwise phylogenetic heat tree, the abundance of *Streptococcus, Veillonella dispar,* and *Prevotella copri* was higher in CCS patients than in healthy volunteers with statistical significance. On the other side, fewer *Roseburia faecis*, *Subdoligranulum* var*iabile, Bifidobacterium,* and *Anaerostipes* were detected in this group (Figure 3D).

The characteristic of bacterial microbiota of CCS patients undergoing coronary angiography

In CCS patients, the relative abundance of Prevotellaceae, Enterobacteriaceae, Streptococcaceae, Clostridiaceae, and Paraprevotellaceae families had significantly risen at the family level compared with healthy volunteers, according to the linear discriminant analysis effect size (LEfSe). At the genus level, the LEfSe revealed that *Prevotella, Streptococcus, Phascolarctobacterium, Dorea, Paraprevotella,* and *Veillonella* genera had significantly higher relative

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TABLE 1 Clinical characteristics of the patients ($N = 91$) in this study.

Parameters	Total ($n = 91$)	CCS ($n = 30$)	DLP ($n = 32$)	Healthy ($n = 29$)	p-value
Age (years)	57.57 ± 8.35	59.60 ± 9.04	57.44 ± 8.88	55.62 ± 6.60	0.066[b]
Male (%)	51 (56.04)	20 (66.67)	16 (50.00)	15 (51.72)	0.356[c]
BMI (kg/m^2)	24.10 ± 4.07	24.63 ± 3.47	25.19 ± 5.45	22.37 ± 1.78	0.003[b]
Waist circumference (cm)	82.32 ± 9.76	88.20 ± 8.92	81.25 ± 10.59	77.43 ± 6.04	<0.001[b]
History of CAD (%)	17 (18.68)	17 (56.67)	0 (0.00)	0 (0.00)	<0.001[c]
Medication					
Antiplatelets	30 (32.97)	30 (100.00)	0 (0.00)	0 (0.00)	<0.001[c]
Antihypertensive drugs	24 (26.37)	24 (80.00)	0 (0.00)	0 (0.00)	<0.001[c]
Oral antidiabetic drugs	7 (7.69)	7 (23.33)	0 (0.00)	0 (0.00)	<0.001[d]
Statins	58 (63.74)	29 (96.67)	29 (90.63)	0 (0.00)	<0.001[d]
Statin intensity					<0.001[d]
Low intensity	2 (3.45)	0 (0.00)	2 (6.90)	0 (0.00)	
Moderate intensity	18 (31.03)	2 (6.90)	16 (55.17)	0 (0.00)	
High intensity	38 (65.52)	27 (93.10)	11 (37.93)	0 (0.00)	
Duration ≥ 3 months	54 (93.10)	29 (100.00)	25 (86.21)	0 (0.00)	0.112[d]
Obesity (%)	19 (21.11)	13 (43.33)	6 (19.35)	0 (0.00)	<0.001[c]
Abdominal obesity (%)*	21 (23.08)	13 (43.33)	5 (15.63)	3 (10.34)	0.005[c]
Hypertriglyceridemia (%)**	18 (19.78)	8 (26.67)	10 (31.25)	0 (0.00)	0.005[c]
Low HDL-C (%)[#]	18 (19.78)	13 (43.33)	5 (15.63)	0 (0.00)	<0.001[c]
Impaired fasting glucose (%)[@]	28 (30.77)	21 (70.00)	3 (9.38)	4 (13.79)	<0.001[c]
Metabolic syndrome (%)	10 (10.99)	10 (33.33)	0 (0.00)	0 (0.00)	<0.001[d]
Hypertension (%)	24 (26.37)	24 (80.00)	0 (0.00)	0 (0.00)	<0.001[c]
Diabetes mellitus (%)	9 (9.89)	9 (30.00)	0 (0.00)	0 (0.00)	<0.001[d]
Dyslipidemia (%)	49 (53.85)	17 (56.67)	32 (100.00)	0 (0.00)	<0.001[c]
Heart failure (%)	1 (1.10)	1 (3.33)	0 (0.00)	0 (0.00)	0.648[d]
Stroke (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1.000[d]
PAD (%)	1 (1.10)	1 (3.33)	0 (0.00)	0 (0.00)	0.648[d]
SBP (mmHg)	121.65 ± 11.88	124.23 ± 14.34	122.41 ± 10.12	118.14 ± 10.35	0.174[b]
DBP (mmHg)	73.99 ± 9.64	72.27 ± 7.76	73.59 ± 10.57	76.21 ± 10.20	0.283[a]
Laboratory data					
FBS (mg/dl)	99.74 ± 18.81	114.31 ± 23.98	95.59 ± 11.00	89.76 ± 8.53	<0.001[b]
HbA1C (mg/dL)	5.69 ± 0.75	6.04 ± 0.97	5.74 ± 0.50	5.28 ± 0.47	<0.001[b]
Total Cholesterol (mg/dL)	175.76 ± 44.19	144.33 ± 41.24	202.93 ± 43.67	178.28 ± 22.28	<0.001[b]
Triglyceride (mg/dL)	119.26 ± 74.86	135.83 ± 92.69	136.44 ± 76.38	83.17 ± 25.98	0.003[b]
LDL-C (mg/dL)	106.72 ± 39.97	77.11 ± 34.44	129.96 ± 40.78	111.70 ± 21.97	<0.001[b]
HDL-C (mg/dL)	56.31 ± 17.89	45.90 ± 15.08	59.84 ± 18.09	63.17 ± 15.88	<0.001[b]
Serum creatinine (mg/dL)	0.86 ± 0.22	0.98 ± 0.20	0.83 ± 0.21	0.77 ± 0.19	<0.001[b]
AST (IU/L)	22.90 ± 11.23	24.50 ± 11.96	25.19 ± 10.60	18.72 ± 10.29	<0.001[b]
ALT (IU/L)	23.22 ± 15.45	26.10 ± 12.79	25.59 ± 10.94	17.62 ± 20.43	<0.001[b]
hs-CRP (mg/dL)	2.36 ± 2.99	3.46 ± 4.26	2.03 ± 2.05	1.58 ± 1.80	0.043[b]

Categorical variables are expressed as frequency and percentage. Continuous variables are expressed as mean ± standard deviation. [a] One-way ANOVA, post hoc test analysis using Scheffe, significant when p-value < 0.05, [b] Kruskal-Wallis test, post hoc test analysis using Mann–Whitney U test, significant when p-value < 0.017 (0.05/3), [c] Chi square test, significant when p-value < 0.05, [d] Fisher's exact test, significant when p-value < 0.05, [+]Obesity, BMI ≥ 25 kg/m^2, *Abdominal obesity, waist circumference > 90 cm for male, waist circumference > 80 cm for female, **Hypertriglyceridemia = triglyceride ≥ 150 mg/dL, [#]Low HDL-C = HDL-C < 40 mg/dL for male, HDL-C < 50 mg/dL for female, [@]Impaired fasting glucose = FBS ≥ 100 mg/dL, ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CAD, coronary artery disease; CCS, chronic coronary syndrome patients; DBP, diastolic blood pressure; DLP, dyslipidemia patients; FBS, fasting blood sugar; HbA1C, hemoglobin A1C; healthy, healthy volunteers; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; IU/L, international units per liter; LDL-C, low-density lipoprotein cholesterol; mmHg, millimeters of mercury; mg/dL, milligrams per deciliter, ng/L, nanogram per liter; PAD, peripheral artery disease; SBP, systolic blood pressure.

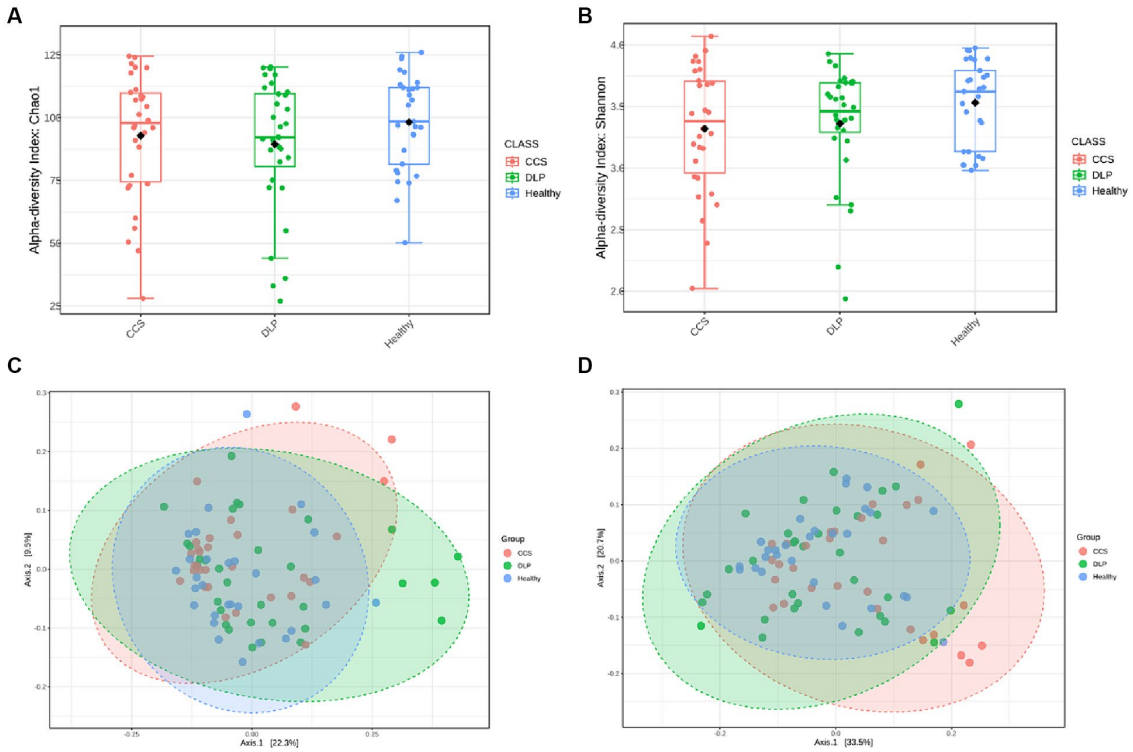


FIGURE 2
Analysis of alpha- and beta-diversity of microbial composition in the three patient groups. Diversity within bacterial communities was measured by the Chao1 index **(A)**, the Shannon diversity index **(B)** the principal coordinate analysis (PCoA) of beta diversity based on the unweighted UniFrac distance **(C)** and the weighted UniFrac distance **(D)**. Kruskal-Wallis H test was used in the statistical test of the alpha diversity (no statistically significant difference found). Permanova (Permutational multivariate analysis of variance) test was used in the statistical test of the beta diversity. Statistically significant differences found at $p < 0.001$. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

abundances in CCS patients compared to healthy volunteers (Figure 4A).

When comparing CCS patients with dyslipidemia patients, The result showed that the relative abundance of Prevotellaceae, Enterobacteriaceae, Streptococcaceae, Fusobacteriaceae, Clostridiaceae, Veillonellaceae, and Porphyromonadaceae families were higher in CCS patients (Figure 4C).

The characteristic of bacterial microbiota of dyslipidemia patients

In dyslipidemia patients, the relative abundance of Prevotellaceae, Enterobacteriaceae, and Fusobacteriaceae families had significantly risen at the family level compared with healthy volunteers, according to the LEfSe. At the genus level, *Prevotella, Clostridium,* and *Dorea* genera had significantly higher relative abundances in dyslipidemia patients compared to healthy volunteers (Figure 4B).

When we compared dyslipidemia patients with CCS patients, we found that the relative abundance of the Lachnospiraceae family was higher in dyslipidemia patients (Figure 4C).

Associations of risk factors with gut microbiome composition in CCS patients undergoing coronary angiography

We calculated the Spearman correlation coefficient between a range of clinical indicators that may be associated with the risk factors of CCS patients (Table 1) and gut microbiota at the genus level (Figure 5A). We found that Lachnospiraceae, Peptostreptococcaceae families, and *Pediococcus* genus were positively correlated with hs-CRP. *Weissella* genus was positively correlated with LDL-C while the *Sutterella* and *Roseburia* genera were negatively correlated with LDL-C.

Associations of risk factors with gut microbiome composition in dyslipidemia patients

In dyslipidemia patients, *Pseudobutyrivibrio, Catenibacterium, Weissella, Prevotella,* and *Anaerostipes* genera were positively correlated with body mass index (BMI) while *Bacteroides* and *Bifidobacterium* genera were negatively correlated with BMI. For this group's lipid profile, genus *Subdoligranulum* was positively correlated

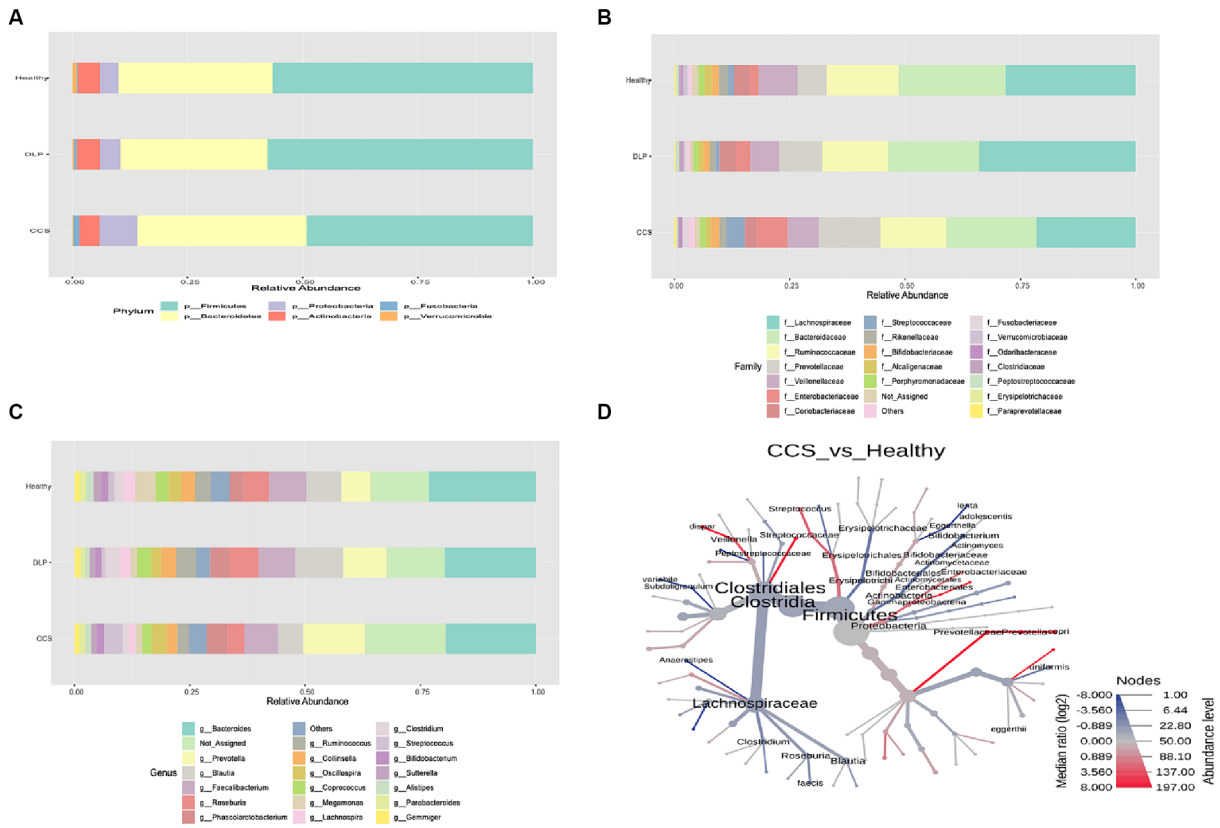


FIGURE 3
The relative abundance of bacterial taxonomic profile in the feces at the phylum **(A)** family **(B)** and genus **(C)** level. The phylogenetic heat tree **(D)** in comparison of bacterial microbiota between CCS and healthy groups shows the bacteria composition at the species level. The color of nodes and edges represents the mean change in operational taxonomic units (OTUs) richness at each taxonomic group, with red indicating greater richness in CCS and blue in healthy, while node size represents OTUs richness of each taxonomic group across the dataset. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

with TC level; genus *Megasphaera* was strongly positively correlated and genera *Collinsella* and *Catenibacterium* were also positively correlated with TG level; genus *Megasphaera* was negatively correlated with HDL-C; genus *Subdoligranulum* was positively correlated with LDL-C level and genera *Lactobacillus* and *Butyricimonas* were negatively correlated with LDL-C level (Figure 5B).

Discussion

This is the first investigation on gut microbiota and associated factors in CCS patients undergoing coronary angiography in Thailand. In this study, the gut microbiome differed among the three groups. There was a reduction in diversity in dyslipidemia patients and CCS patients' group, which correlated with other studies (Huttenhower et al., 2012; Fu et al., 2015; Sawicka-Smiarowska et al., 2021). This finding is consistent with other studies that patients suffering from various diseases have also reduced bacterial diversity, for instance, hypertension (Yang et al., 2015), Crohn's disease (Hansen et al., 2012), psoriatic arthritis (Scher et al., 2015), metabolic syndrome (Arora and Bäckhed, 2016), diabetes mellitus, and obesity (Pascale et al., 2019).

In this study, the composition pattern of the bacterial microbiota differed significantly between the group of CCS patients and healthy volunteers. The most prevalent bacterial phyla were Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, which correlated with other studies (Zheng et al., 2020). Alterations in the ratio of the major phyla Firmicutes to Bacteroidetes have been proposed as a potential CAD risk factor, suggesting that changes in the microbiome's composition may also contribute to the onset and progression of atherosclerosis and CAD (Emoto et al., 2016, 2017; Cui et al., 2017). Furthermore, it was established that a greater Firmicutes/Bacteroidetes ratio (F/B ratio) plays a significant impact on CAD patients (Cui et al., 2017). The F/B ratio is used to diagnose gut dysbiosis and the relationship between this ratio and numerous well-known cardiovascular risk factors, including age, sex, food, and BMI, has been demonstrated (Mariat et al., 2009; Holscher et al., 2015; Emoto et al., 2016; Canfora et al., 2017). However, in this study, dyslipidemia patients had the greatest F/B ratio whereas the CCS patients' group did not.

Numerous pathogenic genera, including *Escherichia, Salmonella, Vibrio, Yersinia*, and *Legionella*, are members of the phylum Proteobacteria. In our study, the relative abundance proportion of Proteobacteria was the highest in CCS patients. According to Shin et al. study, a higher relative abundance of Proteobacteria is associated with gut dysbiosis and many diseases (obesity, T2DM, and cancers) in human (Shin et al., 2015). Mainly Proteobacteria, particularly *Enterobacteriaceae* and some Firmicutes is the abundance of bacteria producing TMAO precursor (Via et al., 2019).

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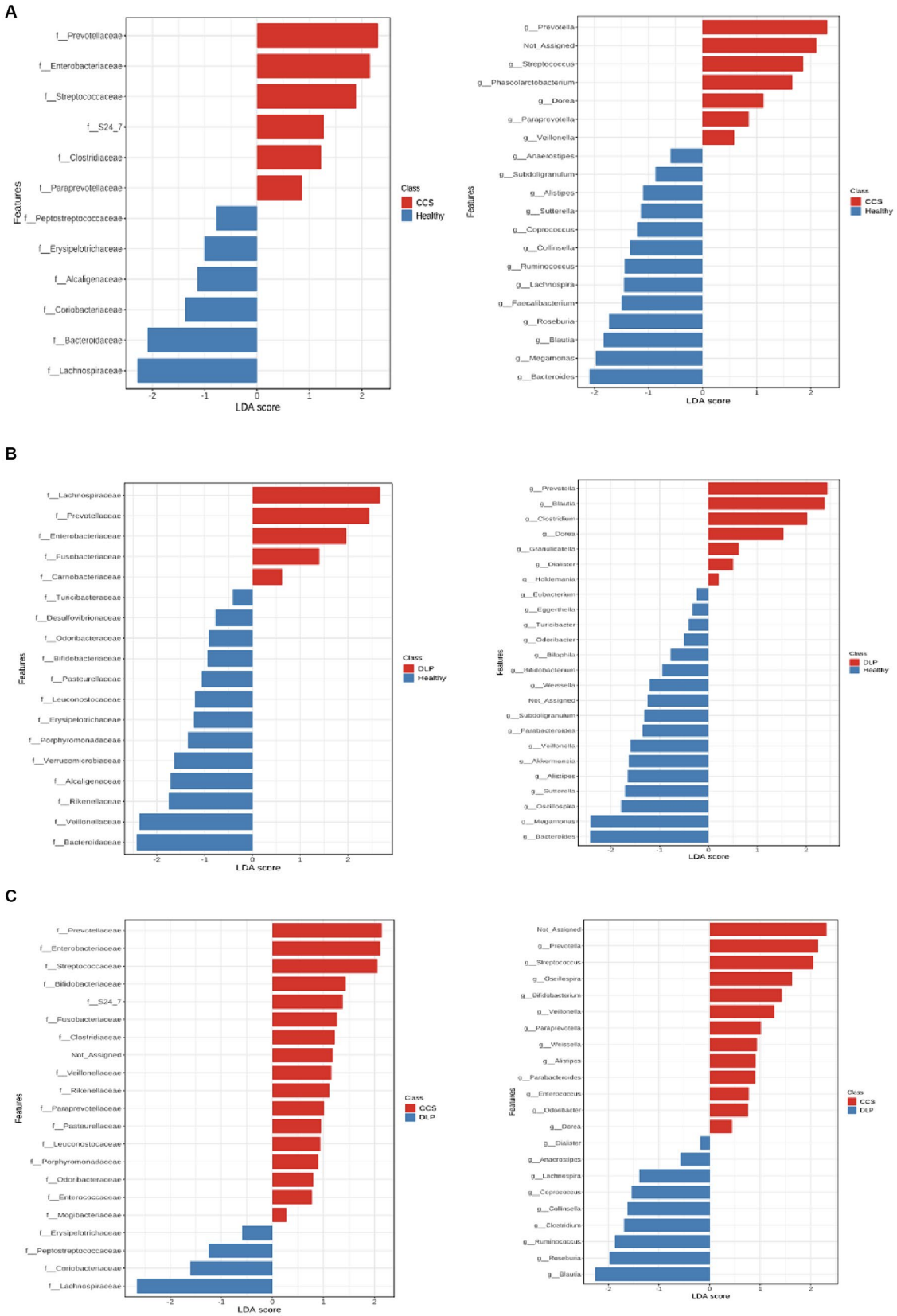


FIGURE 4
The linear discriminant analysis effect size (LEfSe) in comparison of bacterial microbiota between CCS patients and healthy volunteers **(A)**, dyslipidemia patients and healthy volunteers **(B)**, and CCS patients and dyslipidemia patients **(C)**. CCS, chronic coronary syndrome patients; DLP, dyslipidemia patients; Healthy, healthy volunteers.

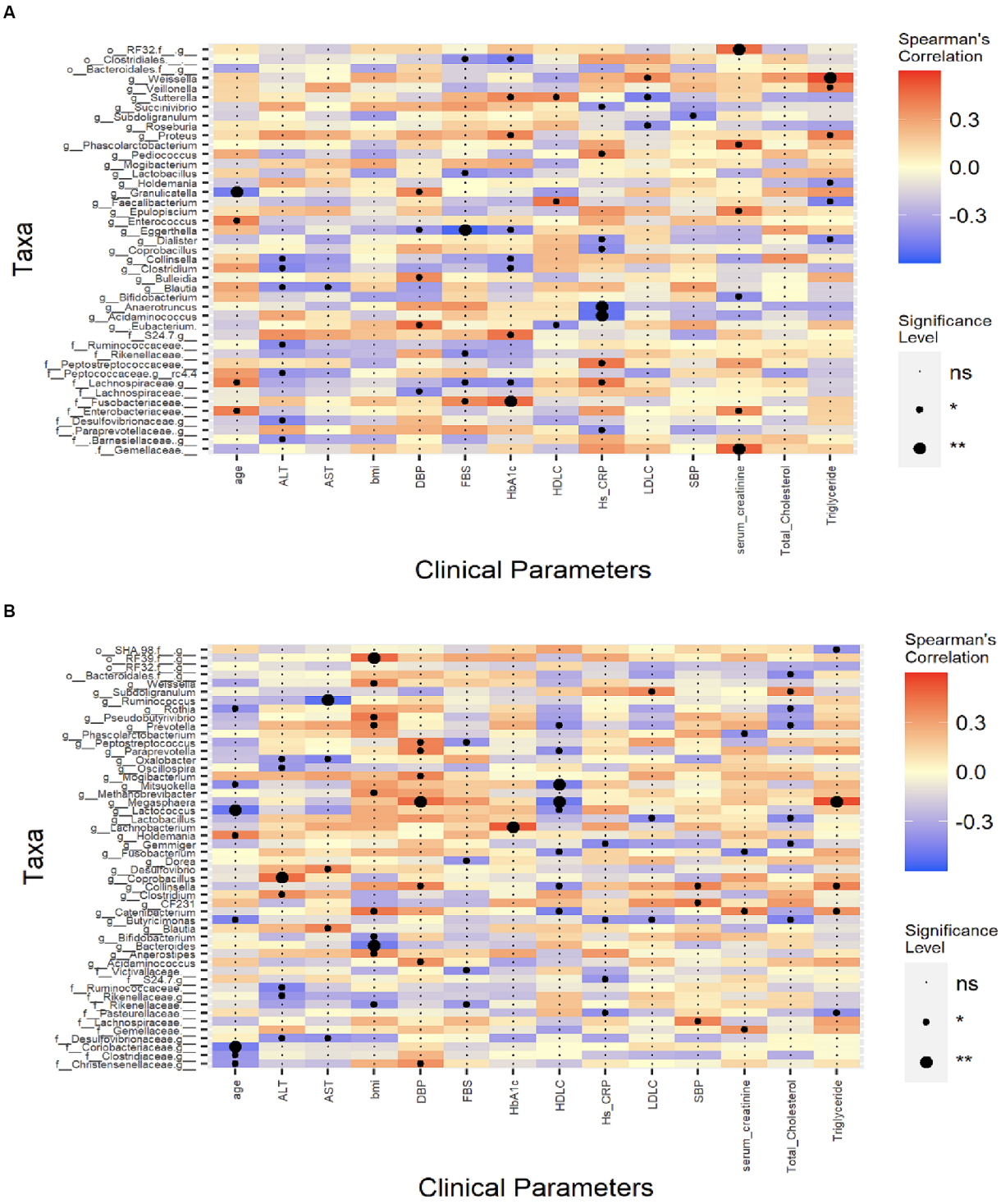


FIGURE 5
Spearman's correlation analysis between the clinical indexes and the microbiota. CCS patients undergoing coronary angiography group **(A)**, dyslipidemia patients' group **(B)**. The colour represents positive (red) or negative (blue) correlations, and $*p < 0.05$, $**p < 0.01$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA1C, hemoglobin A1C; hs-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure.

Fusobacteria proportion was increased in the group of CCS patients, however, Actinobacteria and Verrucomicrobia exhibited the lowest relative abundance proportion in CCS patients in comparison with others. This finding is consistent with the study by Zhang et al. (2019) and Cui et al. (2017). *Fusobacterium nucleatum* may initially cause periodontal disease, which then causes CAD due to

atherosclerosis via inflammation and lipid metabolism (Zhou et al., 2022). However, no research on the association between *Fusobacterium* and CAD has been published.

In addition, *Streptococcus, Veillonella,* and *Prevotella* genera were more common in abundance than other groups in the group of CCS patients, with statistical significance following from the *Bacteroides* genus. Previous studies have reported that *Prevotella* and *Streptococcus* genera had a close relationship with metabolic syndrome, atherosclerosis, and CAD (Abranches et al., 2009; Yakob et al., 2011; Madhogaria et al., 2022; Bazaz et al., 2023). It suggested that the changes in the abundance of *Streptococcus, Veillonella, Prevotella* were the characteristics of the bacterial microbiota of the CCS patients. The greatest associations were found for *Streptococcus anginosus* and *Streptococcus oralis*, according to Sayols-Baixeras et al.'s study of the correlation between *Streptococcus* spp. and subclinical coronary atherosclerosis (Sayols-Baixeras et al., 2023). The prevalence of various LPS-producing gram-negative bacteria, including *Escherichia, Shigella, Veillonella, Klebsiella,* and *Haemophilus* increased with the severity of the CAD, according to a study by Liu H. et al. (2019). High blood LPS levels were associated with a threefold increased risk of incident atherosclerosis (Mayr et al., 2006). *Veillonella* can be found in atherosclerosis plaque and is associated with cholesterol levels (Koren et al., 2011). In addition, *Veillonella* has a high relative abundance in CAD patients (Zhang et al., 2019; Bouzid et al., 2022). These findings suggested that the differences in bacterial abundance between the three groups may be linked to the progression of CAD and lipid metabolism in dyslipidemia patients.

The role of *Prevotella* in human health is controversial. *Prevotella* is a beneficial microbe, however, it is associated with chronic inflammation. *Prevotella* is linked to high complex carbohydrate diets from plants, fruits, and vegetables, whereas *Bacteroides* is connected to fat and protein diets (Wu et al., 2011). *Prevotella* can be found in healthy humans and is considered a commensal bacteria. According to De Filippis et al.'s research, vegetarians had the Mediterranean diet at a high level, which was connected with this bacteria strains and higher levels of SCFAs (De Filippis et al., 2016). According to Emoto et, *Lactobacillus, Streptococcus*, and *Enterococcus* increased in the gut microbiota of CAD patients whereas *Bacteroides* and *Prevotella* decreased (Emoto et al., 2016).

Prevotella strains' genetic diversity may help to explain the variations in how they react to dietary and health conditions in different patients (Ley, 2016). For example, the study of Italian people's gut microbiome showed that varying dietary choices could be responsible for *P. copri* strains with different functions, which resulted in different ways for human health (Filippis et al., 2019). *P. copri* increases the prevalence in non-Westernized people. They typically consume diets rich in fresh vegetables and fruits (Tett et al., 2019).

However, recent studies have connected increasing *Prevotella* abundance and certain strains to metabolic syndrome, obesity, hypertension, insulin resistance, non-alcoholic fatty liver disease (NAFLD), and low-grade systemic inflammation (Zhu et al., 2013; Pedersen et al., 2016; Madhogaria et al., 2022) due to augmentation mucosal helper T-cell (Th17) immune responses and stimulation epithelial cells to produce interleukin-1 (IL-1), IL-8, IL-6 and IL-23 (Larsen, 2017).

Prevotella copri was higher in CCS patients than in healthy volunteers in our study, which is correlated with many studies. Numerous diseases are linked with this microorganism to chronic inflammatory processes; for example, rheumatoid arthritis, periodontitis, HIV infection, metabolic syndrome, inflammatory bowel disease, CAD, and cardiac valve calcification. This bacterium has a role in the development of rheumatoid arthritis (RA) and is an immune-relevant (Pianta et al., 2017). In RA patients, gut dysbiosis may have a role in the early stage of RA as shown by the enrichment of *P. copri* (Alpizar-Rodriguez et al., 2019). Moreover, *P. copri* showed a high degree of genetic and functional diversity depending on the lifestyle of the patients and was associated with worse arthritis in these patients (Nii et al., 2023). In CVD patients, *P. copri* may be a major risk factor, particularly in cardiac valve calcification patients. This microorganism and LDL-C have a positive correlation, which likely supports its pro-inflammatory effects. It is a potential key pathogen implicated in CVDs because of its roles in immunity and inflammation (Liu Z. et al., 2019). Moreover, *P. intermedia, P. nigrescens* were periodontopathic bacteria in atherosclerotic plaques (Gaetti-Jardim et al., 2009).

In this investigation, *Faecalibacterium* genera were lower in the CSS patients' group than in the others. This finding is correlated with Zhu et al. (2016), *Faecalibacterium, Subdoligranulum, Roseburia*, and *Eubacterium rectale* were decreased. There is a significant anti-inflammatory property of *Faecalibacterium* (Machiels et al., 2014).

The Bacteroides genus had the highest relative abundance when we compared the healthy volunteers' group to the other groups, and the relative abundance of the Roseburia genus was higher in the healthy volunteers' group than in the other groups in our study. Due to their ability to produce SCFAs, prior research indicated that Bacteroides and Bifidobacterium have a certain protective effect on metabolism and are primarily protective bacteria for CCS (Hoving et al., 2018; Kazemian et al., 2020; Abdi et al., 2022). In addition, Roseburia has been linked to improved glucose intolerance and weight loss in mice and in patients with atherosclerosis with comparatively high amounts of Collinsella, whereas the normal control group has a substantially larger abundance of Roseburia and Eubacterium (Zhu et al., 2018; Liu H. et al., 2019). A study by Liu H. et al. (2019) revealed that the prevalence of bacteria that produced butyric acid, such as Lachnospiraceae and Ruminococcaceae, decreased with the progression of CAD, this findings are correlated with our study that Lachnospiraceae and Ruminococcacea families were less proportion in CCS patients when compared to others.

SCFAs are metabolites of the fermentation of complex carbohydrates. Members of phylum Bacteroidetes can produce butyrate and acetate, whereas phylum Firmicutes can produce butyrate. SCFAs have a favorable correlation with *Roseburia, Bacteroides* spp., and *Eubacterium rectale*. They have been proposed as a protective effect on CCS patients and SCFA producers have decreased in some CCS patients. Additionally, they improve health by boosting the immunological response of the host, preserving the integrity of the intestinal barrier by controlling the expression of tight junction proteins, and reducing blood lipid levels by preventing the production of cholesterol (David et al., 2014; Krishnan et al., 2015; Chambers et al., 2018). SCFAs play a protective role in atherosclerosis, while LPS stimulates body inflammation and accelerates the formation of atherosclerosis (Vinolo et al., 2011).

When butyrate-producing bacteria disappear, the gut barrier may become damaged, making it easier for microbial toxins like LPS to leak and cause inflammation by binding to Toll-like receptors. Patients

with CAD have been found to have a higher LPS biosynthesis in the microbiome, which is connected to insulin resistance and abdominal obesity (Trøseid et al., 2013; Brandsma et al., 2019).

The synthesis of TMAO, a strong risk factor for the development of CAD, involves dietary choline, betaine, phosphatidylcholine, L-carnitine, and lecithin which are obtained from a variety of sources, including egg, fish, red meat, soybean, peanuts (Tang et al., 2013; Zhu et al., 2016). By producing choline and the intermediary molecule trimethylamine (TMA), the gut bacteria also contribute to the synthesis of TMAO. The capacity of the gut microbiota to synthesize choline via the phospholipase D (PLD) enzyme has just recently been discovered. The flavin-containing monooxygenase (FMO) enzyme metabolizes the microbiome-derived TMA molecule into TMAO in hepatocytes (Phillips et al., 1995). Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes are the TMA producers. The production of foam cells is stimulated by the impairment of cholesterol metabolism in macrophages caused by TMAO-dependent activation of macrophage scavenger receptors and CD36 expression. The higher the TMAO production, the higher the CAD risk (Wang et al., 2011). In the context of increased intestinal permeability, TMAO is also linked to C-reactive protein (CRP), endothelial dysfunction, and elevated blood levels of the LPS endotoxin. It can also cause platelet hyperreactivity, which has an impact on the CAD progression (Al-Obaide et al., 2017).

The LDL-C level was lowest in CCS patients. The reason for the differences in LDL-C levels was that the majority of patients (96.67%) were treated with statins. The treatment with statins is associated with a lower prevalence of gut microbiota dysbiosis (Vieira-Silva et al., 2020). In mice study, statins could moderate gut microbiota by increasing the abundance of *Bacteroides, Butyricimonas,* and *Mucispirillum* (Kim et al., 2019). Moreover, the clinical response to statin therapy in individuals with CAD is associated with the gut microbiota. For instance, poor statin response is associated with a significant reduction in the number of beneficial bacteria (*Akkermansia muciniphila* and *Lactobacillus*) and an increase in the number of bacteria (Holdemanella and Facecallibacterium) (Wang et al., 2021). Statins have also been linked to anti-inflammatory and immunomodulatory properties (Lee et al., 2016).

The genus *Megasphaera* was strongly positively correlated with TG level and negatively correlated with HDL-C. López-Montoya et al. (2022) investigation confirmed our results, showing *Megasphaera* and *Escherichia-Shigella* were highly associated with atherogenic dyslipidemia patients, defined as both hypertriglyceridemia and low HDL-C. Moreover, one study has shown that patients with symptomatic stroke had an altered gut microbiota and defined *Megasphaera* as opportunistic pathogens (Zeng et al., 2019). In obesity and overweight patients, *Megasphaera* significantly increased relative abundance and correlated with low physical activity (Palmas et al., 2021). *Megasphaera* is involved in a mechanism that generates ammonia, which has negative consequences (Anand et al., 2016).

Hs-CRP can help in identifying chronic inflammation. Periodontal disease was linked to CAD and low-grade inflammation, increased CRP, and fibrinogen, according to De Oliveira et al.'s research (De Oliveira et al., 2010). There is a correlation between hs-CRP and CAD severity in CCS patients (Habib and Masri, 2013). Patients with myocardial infarction with elevated hs-CRP level (≥ 2 mg/L) were at higher risk of major adverse cardiovascular events and death (Carrero et al., 2019). In CCS patients of our study, the Lachnospiraceae, Peptostreptococcaceae families, and *Pediococcus* genus were positively correlated with hs-CRP, a finding that has not been reported before.

This study had some limitations, one of which was that the functional roles of the gut microbiota were not examined using a shotgun sequencing approach yielded more information due to its broader gene list and capacity to profile metabolic pathways. Moreover, the cross-sectional nature of this study makes it difficult to determine a causal relationship between changes in the gut microbiome and the disease. Other possible confounding variables that might have influenced the study's findings were obesity, impaired fasting glucose, and polypharmacy.

Conclusion

In conclusion, this study demonstrated a significant difference in the composition of gut microbiota between CCS patients, dyslipidemia patients, and healthy volunteers. The alpha diversity was lower in CCS and dyslipidemia patients than in healthy volunteers. The relative abundance of Proteobacteria, Fusobacteria, *Prevotella,* and *Streptococcus* was significantly increased while *Roseburia*, *Ruminococcus,* and *Faecalibacterium* were lower in CCS patients. In dyslipidemia patients, *Megasphaera* was strongly positively correlated with TG level and negatively correlated with HDL-C. Modification of gut microbiota is associated with changes in clinical parameters involved in the development of CAD in CCS patients.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject/; PRJNA1000984.

Ethics statement

The studies involving humans were approved by Ethics Committee of Chulabhorn Hospital and Srinakharinwirot University research ethics committees (IEC No: 174/2564, issued 22 July 2022 and SWUEC/E/M-100/2565E, issued 22 February 2023, respectively). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

WL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. PP: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – review & editing. VM: Conceptualization, Validation, Visualization, Writing – review & editing. DS: Conceptualization, Data curation, Methodology, Project administration, Visualization, Writing – review & editing. EE:

Conceptualization, Data curation, Supervision, Validation, Visualization, Writing – review & editing. MT: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Conflict of interest

PP is employed by Mod Gut Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abdi, M., Hadi Esmaeili, G. G., and Ranjbar, R. (2022). Lactobacilli and Bifidobacterium as anti-atherosclerotic agents. *Iran. J. Basic Med. Sci.* 25, 934–946. doi: 10.22038/IJBMS.2022.63860.14073

Abdul-Ghani, M., DeFronzo, R. A., Del Prato, S., Chilton, R., Singh, R., and Ryder, R. E. J. (2017). Cardiovascular disease and type 2 diabetes: has the Dawn of a new era arrived? *Diabetes Care* 40, 813–820. doi: 10.2337/dc16-2736

Abranches, J., Zeng, L., Bélanger, M., Rodrigues, P. H., Simpson-Haidaris, P. J., Akin, D., et al. (2009). Invasion of human coronary artery endothelial cells by *Streptococcus Mutans* OMZ175: *S. mutans* endothelial cell invasion. *Oral Microbiol. Immunol.* 24, 141–145. doi: 10.1111/j.1399-302X.2008.00487.x

Afzaal, M., Saeed, F., Shah, Y. A., Hussain, M., Rabail, R., Socol, C. T., et al. (2022). Human gut microbiota in health and disease: unveiling the relationship. *Front. Microbiol.* 13, 1–14. doi: 10.3389/fmicb.2022.999001

Al-Obaide, M., Singh, R., Datta, P., Rewers-Felkins, K., Salguero, M., Al-Obaidi, I., et al. (2017). Gut microbiota-dependent trimethylamine-N-oxide and serum biomarkers in patients with T2DM and advanced CKD. *J. Clin. Med.* 6:86. doi: 10.3390/jcm6090086

Alpizar-Rodriguez, D., Lesker, T. R., Gronow, A., Gilbert, B., Raemy, E., Lamacchia, C., et al. (2019). *Prevotella Copri* in individuals at risk for rheumatoid arthritis. *Ann. Rheum. Dis.* 78, 590–593. doi: 10.1136/annrheumdis-2018-214514

American Diabetes Association (2014). Diagnosis and classification of diabetes mellitus. *Diabetes Care* 37, S81–S90. doi: 10.2337/dc14-S081

Anand, S., Kaur, H., and Mande, S. S. (2016). Comparative in silico analysis of butyrate production pathways in gut commensals and pathogens. *Front. Microbiol.* 7:1945. doi: 10.3389/fmicb.2016.01945

Arora, T., and Bäckhed, F. (2016). The gut microbiota and metabolic disease: current understanding and future perspectives. *J. Intern. Med.* 280, 339–349. doi: 10.1111/joim.12508

Bazaz, R., Marriott, H. M., Wright, C., Chamberlain, J., West, L. E., Gelsthorpe, C., et al. (2023). Transient increase in atherosclerotic plaque macrophage content following *Streptococcus Pneumoniae* pneumonia in ApoE-deficient mice. *Front. Cell. Infect. Microbiol.* 13:1090550. doi: 10.3389/fcimb.2023.1090550

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Bouzid, F., Gtif, I., Alfadhli, S., Charfeddine, S., Ghorbel, W., Abdelhédi, R., et al. (2022). A potential Oral microbiome signature associated with coronary artery disease in Tunisia. *Biosci. Rep.* 42:BSR20220583. doi: 10.1042/BSR20220583

Brandsma, E., Kloosterhuis, N. J., Koster, M., Dekker, D. C., Gijbels, M. J. J., Van Der Velden, S., et al. (2019). A proinflammatory gut microbiota increases systemic inflammation and accelerates atherosclerosis. *Circ. Res.* 124, 94–100. doi: 10.1161/CIRCRESAHA.118.313234

Canfora, E. E., Van Der Beek, C. M., Hermes, G. D. A., Goossens, G. H., Jocken, J. W. E., Holst, J. J., et al. (2017). Supplementation of diet with Galacto-oligosaccharides increases Bifidobacteria, but not insulin sensitivity, in obese prediabetic individuals. *Gastroenterology* 153, 87–97.e3. doi: 10.1053/j.gastro.2017.03.051

Carrero, J. J., Franko, M. A., Obergfell, A., Gabrielsen, A., and Jernberg, T. (2019). Hs-CRP level and the risk of death or recurrent cardiovascular events in patients with myocardial infarction: a healthcare-based study. *J. Am. Heart Assoc.* 8:e012638. doi: 10.1161/JAHA.119.012638

Chambers, E. S., Preston, T., Frost, G., and Morrison, D. J. (2018). Role of gut microbiota-generated short-chain fatty acids in metabolic and cardiovascular health. *Curr. Nutr. Reports* 7, 198–206. doi: 10.1007/s13668-018-0248-8

Cui, L., Zhao, T., Haibing, H., Zhang, W., and Hua, X. (2017). Association study of gut Flora in coronary heart disease through high-throughput sequencing. *Biomed. Res. Int.* 2017, 1–10. doi: 10.1155/2017/3796359

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi: 10.1038/nature12820

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., et al. (2016). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut* 65, 1812–1821. doi: 10.1136/gutjnl-2015-309957

De Oliveira, C., Watt, R., and Hamer, M. (2010). Toothbrushing, inflammation, and risk of cardiovascular disease: results from Scottish health survey. *BMJ* 340:c2451. doi: 10.1136/bmj.c2451

Dustan, H. P. (2000). Hypertension. *J. Am. Coll. Cardiol.* 35, 10B–12B. doi: 10.1016/S0735-1097(00)80042-X

Emoto, T., Yamashita, T., Kobayashi, T., Sasaki, N., Hirota, Y., Hayashi, T., et al. (2017). Characterization of gut microbiota profiles in coronary artery disease patients using data mining analysis of terminal restriction fragment length polymorphism: gut microbiota could be a diagnostic marker of coronary artery disease. *Heart Vessel.* 32, 39–46. doi: 10.1007/s00380-016-0841-y

Emoto, T., Yamashita, T., Sasaki, N., Hirota, Y., Hayashi, T., So, A., et al. (2016). Analysis of gut microbiota in coronary artery disease patients: a possible link between gut microbiota and coronary artery disease. *J. Atheroscler. Thromb.* 23, 908–921. doi: 10.5551/jat.32672

Fan, Y., and Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 19, 55–71. doi: 10.1038/s41579-020-0433-9

Filippis, D., Francesca, E. P., Tett, A., Tarallo, S., Naccarati, A., De Angelis, M., et al. (2019). Distinct genetic and functional traits of human intestinal *Prevotella Copri* strains are associated with different habitual diets. *Cell Host Microbe* 25, 444–453.e3. doi: 10.1016/j.chom.2019.01.004

Fu, J., Bonder, M. J., Cenit, M. C., Tigchelaar, E. F., Maatman, A., Dekens, J. A. M., et al. (2015). The gut microbiome contributes to a substantial proportion of the variation in blood lipids. *Circ. Res.* 117, 817–824. doi: 10.1161/CIRCRESAHA.115.306807

Gaetti-Jardim, E., Marcelino, S. L., Feitosa, A. C. R., Romito, G. A., and Avila-Campos, M. J. (2009). Quantitative detection of periodontopathic Bacteria in atherosclerotic plaques from coronary arteries. *J. Med. Microbiol.* 58, 1568–1575. doi: 10.1099/jmm.0.013383-0

Habib, S. S., and Al Masri, A. (2013). Relationship of high sensitivity C-reactive protein with presence and severity of coronary artery disease. *Pak. J. Med. Sci.* 29, 1425–1429. doi: 10.12669/pjms.296.3302

Hansen, R., Russell, R. K., Reiff, C., Louis, P., McIntosh, F., Berry, S. H., et al. (2012). Microbiota of De-novo Pediatric IBD: increased Faecalibacterium Prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. *Am. J. Gastroenterol.* 107, 1913–1922. doi: 10.1038/ajg.2012.335

Hedayatnia, M., Asadi, Z., Zare-Feyzabadi, R., Yaghooti-Khorasani, M., Ghazizadeh, H., Ghaffarian-Zirak, R., et al. (2020). Dyslipidemia and cardiovascular disease risk among the MASHAD study population. *Lipids Health Dis.* 19:42. doi: 10.1186/s12944-020-01204-y

Holscher, H. D., Gregory Caporaso, J., Hooda, S., Brulc, J. M., Fahey, G. C., and Swanson, K. S. (2015). Fiber supplementation influences phylogenetic structure and functional capacity of the human intestinal microbiome: follow-up of a randomized controlled trial. *Am. J. Clin. Nutr.* 101, 55–64. doi: 10.3945/ajcn.114.092064

Hoving, L. R., Katiraei, S., Heijink, M., Pronk, A., Van Der Wee-Pals, L., Streefland, T., et al. (2018). Dietary mannan oligosaccharides modulate gut microbiota, increase Fecal bile acid excretion, and decrease plasma cholesterol and atherosclerosis development. *Mol. Nutr. Food Res.* 62:1700942. doi: 10.1002/mnfr.201700942

Huang, P. L. (2009). A comprehensive definition for metabolic syndrome. *Dis. Model. Mech.* 2, 231–237. doi: 10.1242/dmm.001180

Huttenhower, C., Gevers, D., Knight, R., Sahar Abubucker, H., Jonathan, B., Chinwalla, A. T., et al. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Jiangping, S., Zhe, Z., Wei, W., Yunhu, S., Jie, H., Hongyue, W., et al. (2013). Assessment of coronary artery stenosis by coronary angiography: a head-to-head comparison with pathological coronary artery anatomy. *Circ. Cardiovasc. Interv.* 6, 262–268. doi: 10.1161/CIRCINTERVENTIONS.112.000205

Jonsson, A. L., and Bäckhed, F. (2017). Role of gut microbiota in atherosclerosis. *Nat. Rev. Cardiol.* 14, 79–87. doi: 10.1038/nrcardio.2016.183

Kazemian, N., Mahmoudi, M., Halperin, F., Wu, J. C., and Pakpour, S. (2020). Gut microbiota and cardiovascular disease: opportunities and challenges. *Microbiome* 8:36. doi: 10.1186/s40168-020-00821-0

Kim, J., Lee, H., An, J., Song, Y., Lee, C.-K., Kim, K., et al. (2019). Alterations in gut microbiota by statin therapy and possible intermediate effects on Hyperglycemia and Hyperlipidemia. *Front. Microbiol.* 10:1947. doi: 10.3389/fmicb.2019.01947

Knuuti, J., Wijns, W., Saraste, A., Capodanno, D., Barbato, E., Funck-Brentano, C., et al. (2020). 2019 ESC guidelines for the diagnosis and Management of Chronic Coronary Syndromes. *Eur. Heart J.* 41, 407–477. doi: 10.1093/eurheartj/ehz425

Koren, O., Spor, A., Felin, J., Fåk, F., Stombaugh, J., Tremaroli, V., et al. (2011). Human Oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc. Natl. Acad. Sci.* 108, 4592–4598. doi: 10.1073/pnas.1011383107

Krishnan, S., Alden, N., and Lee, K. (2015). Pathways and functions of gut microbiota metabolism impacting host physiology. *Curr. Opin. Biotechnol.* 36, 137–145. doi: 10.1016/j.copbio.2015.08.015

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* 82, 6955–6959. doi: 10.1073/pnas.82.20.6955

Larsen, J. M. (2017). The immune response to Prevotella Bacteria in chronic inflammatory disease. *Immunology* 151, 363–374. doi: 10.1111/imm.12760

La Rosa, P. S., Brooks, J. P., Deych, E., Boone, E. L., Edwards, D. J., Wang, Q., et al. (2012). Hypothesis testing and power calculations for taxonomic-based human microbiome data. *PLoS One* 7:e52078. doi: 10.1371/journal.pone.0052078

Lee, S., Lee, Y., Kim, J., An, J., Kim, K., Lee, H., et al. (2016). Atorvastatin and rosuvastatin improve physiological parameters and alleviate immune dysfunction in metabolic disorders. *Biochem. Biophys. Res. Commun.* 478, 1242–1247. doi: 10.1016/j.bbrc.2016.08.101

Ley, R. E. (2016). Prevotella in the gut: choose carefully. *Nat. Rev. Gastroenterol. Hepatol.* 13, 69–70. doi: 10.1038/nrgastro.2016.4

Lin, C.-F., Chang, Y.-H., Chien, S.-C., Lin, Y.-H., and Yeh, H.-Y. (2018). Epidemiology of Dyslipidemia in the Asia Pacific region. *Int. J. Gerontol.* 12, 2–6. doi: 10.1016/j.ijge.2018.02.010

Liu, H., Chen, X., Xiaomin, H., Niu, H., Tian, R., Wang, H., et al. (2019). Alterations in the gut microbiome and metabolism with coronary artery disease severity. *Microbiome* 7:68. doi: 10.1186/s40168-019-0683-9

Liu, Z., Li, J., Liu, H., Tang, Y., Zhan, Q., Lai, W., et al. (2019). The intestinal microbiota associated with cardiac valve calcification differs from that of coronary artery disease. *Atherosclerosis* 284, 121–128. doi: 10.1016/j.atherosclerosis.2018.11.038

Liu, H., Zhuang, J., Tang, P., Li, J., Xiong, X., and Deng, H. (2020). The role of the gut microbiota in coronary heart disease. *Curr. Atheroscler. Rep.* 22:77. doi: 10.1007/s11883-020-00892-2

López-Montoya, P., Cerqueda-García, D., Rodríguez-Flores, M., López-Contreras, B., Villamil-Ramírez, H., Morán-Ramos, S., et al. (2022). Association of gut Microbiota with atherogenic Dyslipidemia, and its impact on serum lipid levels after bariatric surgery. *Nutrients* 14:3545. doi: 10.3390/nu14173545

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., and Eeckhaut, V. (2014). A decrease of the butyrate-producing species *Roseburia hominis* and *Faecalibacterium Prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 63, 1275–1283. doi: 10.1136/gutjnl-2013-304833

Madhogaria, B., Bhowmik, P., and Kundu, A. (2022). Correlation between human gut microbiome and diseases. *Inf. Med.* 1, 180–191. doi: 10.1016/j.imj.2022.08.004

Mariat, D., Firmesse, O., Levenez, F., Vd Guimarães, H., Sokol, J. D., Corthier, G., et al. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* 9:123. doi: 10.1186/1471-2180-9-123

Mayr, M., Kiechl, S., Tsimikas, S., Miller, E., Sheldon, J., Willeit, J., et al. (2006). Oxidized low-density lipoprotein autoantibodies, chronic infections, and carotid atherosclerosis in a population-based study. *J. Am. Coll. Cardiol.* 47, 2436–2443. doi: 10.1016/j.jacc.2006.03.024

Nii, T., Maeda, Y., Motooka, D., Naito, M., Matsumoto, Y., Ogawa, T., et al. (2023). Genomic repertoires linked with pathogenic potency of arthritogenic *Prevotella Copri* isolated from the gut of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* 82, 621–629. doi: 10.1136/ard-2022-222881

Palmas, V., Pisanu, S., Madau, V., Casula, E., Deledda, A., Cusano, R., et al. (2021). Gut microbiota markers associated with obesity and overweight in Italian adults. *Sci. Rep.* 11:5532. doi: 10.1038/s41598-021-84928-w

Pascale, A., Marchesi, N., Govoni, S., Coppola, A., and Gazzaruso, C. (2019). The role of gut microbiota in obesity, diabetes mellitus, and effect of metformin: new insights into old diseases. *Curr. Opin. Pharmacol.* 49, 1–5. doi: 10.1016/j.coph.2019.03.011

Pedersen, H. K., Gudmundsdottir, V., Nielsen, H. B., Hyotylainen, T., Nielsen, T., Jensen, B. A. H., et al. (2016). Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* 535, 376–381. doi: 10.1038/nature18646

Phillips, I. R., Dolphin, C. T., Clair, P., Hadley, M. R., Hutt, A. J., McCombie, R. R., et al. (1995). The molecular biology of the flavin-containing monooxygenases of Man. *Chem. Biol. Interact.* 96, 17–32. doi: 10.1016/0009-2797(94)03580-2

Pianta, A., Arvikar, S., Strle, K., Drouin, E. E., Wang, Q., Costello, C. E., et al. (2017). Evidence of the immune relevance of *Prevotella Copri*, a gut microbe, in patients with rheumatoid arthritis. *Arthritis Rheumatol.* 69, 964–975. doi: 10.1002/art.40003

Pieczynska, M. D., Yang, Y., Petrykowski, S., Horbanczuk, O. K., Atanasov, A. G., and Horbanczuk, J. O. (2020). Gut microbiota and its metabolites in atherosclerosis development. *Molecules* 25:594. doi: 10.3390/molecules25030594

Samadoulougou, S., Idzerda, L., Dault, R., Lebel, A., Cloutier, A.-M., and Vanasse, A. (2020). Validated methods for identifying individuals with obesity in health care administrative databases: a systematic review. *Obes. Sci. Pract.* 6, 677–693. doi: 10.1002/osp4.450

Santacroce, L., Man, A., Charitos, I. A., Haxhirexha, K., and Topi, S. (2021). Current knowledge about the connection between health status and gut microbiota from birth to elderly. *Front. Biosci. Landmark* 26:135. doi: 10.52586/4930

Sawicka-Smiarowska, E., Bondarczuk, K., Bauer, W., Niemira, M., Szalkowska, A., Raczkowska, J., et al. (2021). Gut microbiome in chronic coronary syndrome patients. *J. Clin. Med.* 10:5074. doi: 10.3390/jcm10215074

Sayols-Baixeras, S., Dekkers, K. F., Baldanzi, G., Jönsson, D., Hammar, U., Lin, Y. T., et al. (2023). Streptococcus species abundance in the gut is linked to subclinical coronary atherosclerosis in 8973 participants from the SCAPIS cohort. *Circulation* 148, 1–14. doi: 10.1161/CIRCULATIONAHA.123.063914

Scher, J. U., Ubeda, C., Artacho, A., Attur, M., Isaac, S., Reddy, S. M., et al. (2015). Decreased bacterial diversity characterizes the altered gut microbiota in patients with psoriatic arthritis, resembling dysbiosis in inflammatory bowel disease: gut microbiota in PsA. *Arthritis Rheumatol.* 67, 128–139. doi: 10.1002/art.38892

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shin, N.-R., Whon, T. W., and Bae, J.-W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Tang, W. H. W., Wang, Z., Levison, B. S., Koeth, R. A., Britt, E. B., Xiaoming, F., et al. (2013). Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N. Engl. J. Med.* 368, 1575–1584. doi: 10.1056/NEJMoa1109400

Tett, A., Huang, K. D., Asnicar, F., Fehlner-Peach, H., Pasolli, E., Karcher, N., et al. (2019). The *Prevotella Copri* complex comprises four distinct clades underrepresented in westernized populations. *Cell Host Microbe* 26, 666–679.e7. doi: 10.1016/j.chom.2019.08.018

Thursby, E., and Juge, N. (2017). Introduction to the human gut microbiota. *Biochem. J.* 474, 1823–1836. doi: 10.1042/BCJ20160510

Trøseid, M., Nestvold, T. K., Rudi, K., Thoresen, H., Nielsen, E. W., and Lappegård, K. T. (2013). Plasma lipopolysaccharide is closely associated with Glycemic control and abdominal obesity. *Diabetes Care* 36, 3627–3632. doi: 10.2337/dc13-0451

Tune, J. D., Goodwill, A. G., Sassoon, D. J., and Mather, K. J. (2017). Cardiovascular consequences of metabolic syndrome. *Transl. Res.* 183, 57–70. doi: 10.1016/j.trsl.2017.01.001

Via, D., Alessandro, G. G., Taverniti, V., Rondini, G., Velardi, I., Gambaro, V., et al. (2019). Urinary TMAO levels are associated with the taxonomic composition of the gut microbiota and with the choline Tma-lyase gene (cutC) Harbored by Enterobacteriaceae. *Nutrients* 12:62. doi: 10.3390/nu12010062

Vieira-Silva, S., Falony, G., Belda, E., Nielsen, T., Aron-Wisnewsky, J., Chakaroun, R., et al. (2020). Statin therapy is associated with lower prevalence of gut microbiota dysbiosis. *Nature* 581, 310–315. doi: 10.1038/s41586-020-2269-x

Vinolo, M. A. R., Rodrigues, H. G., Hatanaka, E., Sato, F. T., Sampaio, S. C., and Curi, R. (2011). Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J. Nutr. Biochem.* 22, 849–855. doi: 10.1016/j.jnutbio.2010.07.009

Wang, Z., Klipfell, E., Bennett, B. J., Koeth, R., Levison, B. S., DuGar, B., et al. (2011). Gut Flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 472, 57–63. doi: 10.1038/nature09922

Wang, L., Zhou, W., Guo, M., Hua, Y., Zhou, B., Li, X., et al. (2021). The gut microbiota is associated with clinical response to statin treatment in patients with coronary artery disease. *Atherosclerosis* 325, 16–23. doi: 10.1016/j.atherosclerosis.2021.03.007

Wensel, C. R., Pluznick, J. L., Salzberg, S. L., and Sears, C. L. (2022). Next-generation sequencing: insights to advance clinical investigations of the microbiome. *J. Clin. Invest.* 132:e154944. doi: 10.1172/JCI154944

Williams, B., Mancia, G., Spiering, W., Rosei, E. A., Azizi, M., Burnier, M., et al. (2019). 2018 ESC/ESH guidelines for the management of arterial hypertension. *Eur. Heart J.* 77, 71–159. doi: 10.5603/KP.2019.0018

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., et al. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334, 105–108. doi: 10.1126/science.1208344

Yakob, M., Söder, B., Meurman, J. H., Jogestrand, T., Nowak, J., and Söder, P.-Ö. (2011). Prevotella Nigrescens and *Porphyromonas Gingivalis* are associated with signs of carotid atherosclerosis in subjects with and without periodontitis: periodontal microorganisms and atherosclerosis. *J. Periodontal Res.* 46, 749–755. doi: 10.1111/j.1600-0765.2011.01398.x

Yang, B., Chen, Y., Catherine Stanton, R., Ross, P., Lee, Y.-K., Zhao, J., et al. (2019). Bifidobacterium and Lactobacillus composition at species level and gut microbiota diversity in infants before 6 weeks. *Int. J. Mol. Sci.* 20:3306. doi: 10.3390/ijms20133306

Yang, T., Santisteban, M. M., Rodriguez, V., Li, E., Ahmari, N., Carvajal, J. M., et al. (2015). Gut dysbiosis is linked to hypertension. *Hypertension* 65, 1331–1340. doi: 10.1161/HYPERTENSIONAHA.115.05315

Yusuf, S., Hawken, S., Ôunpuu, S., Dans, T., Avezum, A., Lanas, F., et al. (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 364, 937–952. doi: 10.1016/S0140-6736(04)17018-9

Zeng, X., Xuxuan Gao, Y., Peng, Q. W., Zhu, J., Tan, C., Xia, G., et al. (2019). Higher risk of stroke is correlated with increased opportunistic pathogen load and reduced levels of butyrate-producing Bacteria in the gut. *Front. Cell. Infect. Microbiol.* 9:4. doi: 10.3389/fcimb.2019.00004

Zhang, Y., Jun, X., Wang, X., Ren, X., and Liu, Y. (2019). Changes of intestinal bacterial microbiota in coronary heart disease complicated with nonalcoholic fatty liver disease. *BMC Genomics* 20:862. doi: 10.1186/s12864-019-6251-7

Zheng, Y.-Y., Ting-Ting, W., Liu, Z.-Q., Li, A., Guo, Q.-Q., Ma, Y.-Y., et al. (2020). Gut microbiome-based diagnostic model to predict coronary artery disease. *J. Agric. Food Chem.* 68, 3548–3557. doi: 10.1021/acs.jafc.0c00225

Zhou, J., Liu, L., Peiyao, W., Zhao, L., and Yafei, W. (2022). *Fusobacterium Nucleatum* accelerates atherosclerosis via macrophage-driven aberrant proinflammatory response and lipid metabolism. *Front. Microbiol.* 13:798685. doi: 10.3389/fmicb.2022.798685

Zhu, L., Baker, S. S., Gill, C., Liu, W., Alkhouri, R., Baker, R. D., et al. (2013). Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology* 57, 601–609. doi: 10.1002/hep.26093

Zhu, Q., Gao, R., Zhang, Y., Pan, D., Zhu, Y., Zhang, X., et al. (2018). Dysbiosis signatures of gut microbiota in coronary artery disease. *Physiol. Genomics* 50, 893–903. doi: 10.1152/physiolgenomics.00070.2018

Zhu, W., Gregory, J. C., Org, E., Buffa, J. A., Gupta, N., Wang, Z., et al. (2016). Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell* 165, 111–124. doi: 10.1016/j.cell.2016.02.011

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Clostridioides difficile infection is associated with differences in transcriptionally active microbial communities

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Clostridioides difficile infection (CDI) is responsible for around 300,000 hospitalizations yearly in the United States, with the associated monetary cost being billions of dollars. Gut microbiome dysbiosis is known to be important to CDI. To the best of our knowledge, metatranscriptomics (MT) has only been used to characterize gut microbiome composition and function in one prior study involving CDI patients. Therefore, we utilized MT to investigate differences in active community diversity and composition between CDI+ ($n = 20$) and CDI− ($n = 19$) samples with respect to microbial taxa and expressed genes. No significant (Kruskal-Wallis, $p > 0.05$) differences were detected for richness or evenness based on CDI status. However, clustering based on CDI status was significant for both active microbial taxa and expressed genes datasets (PERMANOVA, $p \leq 0.05$). Furthermore, differential feature analysis revealed greater expression of the opportunistic pathogens *Enterocloster bolteae* and *Ruminococcus gnavus* in CDI+ compared to CDI− samples. When only fungal sequences were considered, the family Saccharomycetaceae expressed more genes in CDI−, while 31 other fungal taxa were identified as significantly (Kruskal-Wallis $p \leq 0.05$, log(LDA) ≥ 2) associated with CDI+. We also detected a variety of genes and pathways that differed significantly (Kruskal-Wallis $p \leq 0.05$, log(LDA) ≥ 2) based on CDI status. Notably, differential genes associated with biofilm formation were expressed by *C. difficile*. This provides evidence of another possible contributor to *C. difficile*'s resistance to antibiotics and frequent recurrence *in vivo*. Furthermore, the greater number of CDI+ associated fungal taxa constitute additional evidence that the mycobiome is important to CDI pathogenesis. Future work will focus on establishing if *C. difficile* is actively producing biofilms during infection and if any specific fungal taxa are particularly influential in CDI.

KEYWORDS

***Clostridioides difficile*, metatranscriptomics, fungi, human microbiome, mycobiome**

1 Introduction

Hospital-acquired *Clostridioides difficile* infection (CDI) accounts for approximately 300,000 hospitalizations per year (Bobo et al., 2011) with mortality rates as high as 16.7% during outbreaks (Dubberke et al., 2008), and it has surpassed methicillin-resistant *Staphylococcus aureus* as the highest incidence hospital-acquired infection in the United States (Lessa et al., 2015), all resulting in billions of dollars in annual healthcare costs (Dubberke and Olsen, 2012). Antibiotics remain the most important risk factor for CDI due to their mechanism of action lacking species-level specificity, with their broad impact on gut ecology creating an intestinal dysbiosis in which *C. difficile* has a selective advantage for population growth (Aslam et al., 2005; Shivashankar et al., 2014). Antibiotics are also the most common intervention for CDI, with unacceptably high recurrence rates of 15%–30% after first treatment, representing another manifestation of their causal influence in creating gut microbial environments that promote this disease state (Choi et al., 2011). These observations underscore the importance of gut dysbiosis in the pathogenesis of CDI, which has implications both for disease prevention and disease treatment. A more precise understanding of the dysbiosis of CDI may, therefore, impact the prevention and treatment of this disease.

The majority of microbiome studies on CDI have used amplicons (16S rRNA and/or ITS) to characterize the dominant microbial communities in CDI (Antharam et al., 2013; Sangster et al., 2016; Lamendella et al., 2018; Zuo et al., 2018). However, the studies that relied solely on 16S rRNA data were unable to comment on the possible role of fungi in the pathogenesis of CDI. More recent investigations, including several by our team, suggest that fungi may have a role in CDI (Lamendella et al., 2018; Markey et al., 2018; Stewart et al., 2019). These data suggest that pre-colonization of mice with *C. albicans* lowers their susceptibility to CDI (Markey et al., 2018). Several human cohort studies also demonstrate the consistent presence of fungi, especially *C. glabrata*, in patients with CDI, while CDI− patients with diarrhea and comparable antibiotic exposures lack this fungal enrichment (Lamendella et al., 2018; Stewart et al., 2019). Furthermore, while predictive functional tools exist, namely PICRUSt2 (Douglas et al., 2020) and Tax4Fun2 (Wemheuer et al., 2020), 16S rRNA and ITS data do not allow for the direct detection of expressed genes, and neither of those tools provide any information on which of the predictive functions were likely being expressed.

Shotgun metagenomics can directly detect genes, but it does not distinguish between genes that were expressed vs. those that were only present. In contrast, metatranscriptomics (MT) both overcomes limitations associated with amplicon sequencing and allows us to focus on transcriptionally active microbes, as well as the genes they were expressing. Therefore, we once more utilized MT sequencing to characterize microbial communities within the context of CDI. In this study, we sought to examine potential differences in overall community diversity and identify differentially active microbes and differentially expressed functions based on CDI status. Additionally, building on previous work, we were able to link specific genes and pathways to fungal expression. Despite only having information on CDI status, we were able to detect multiple differences in active community composition and function, which seem to have remained consistent regardless of possible confounding variables.

2 Materials and methods

2.1 Sample collection

The Institutional Review Board at the University of Virginia approved this study (IRB-HSR# 21646) with waiver of consent, as samples were de-identified remnants. Diarrheal samples from CDI+ ($n = 20$) and CDI− ($n = 20$) patients collected by the University of Virginia Medical Center between September 2019 and August 2020. CDI was diagnosed based on the presence of a conserved sequence of the *tcdB* gene, as determined by Xpert® *C. difficile* assay (Cepheid, Sunnyvale, CA, United States). To be included in the study, patients had to be at least 18 years old. Samples were stored in a −80°C freezer after collection until further processing.

2.2 RNA extraction, library preparation, and sequencing

RNA was extracted from samples using the ZymoBIOMICS RNA Miniprep Kit (Zymo Research, Irvine, CA, United States) according to the manufacturer's protocol with the following exceptions: 1 volume of lysis buffer was used, the optional DNase I treatment was completed, and extracts were eluted with 50 uL of DNase/RNase free water. After extraction, quantification was conducted using an Invitrogen Qubit 4 Fluorometer and Qubit RNA High Sensitivity Assay Kit (ThermoFisher Scientific, Waltham, MA, United States).

RNA was then processed to make MT libraries using the NEBNext Ultra II RNA Library Prep Kit for use with Illumina (New England Biolabs, Ipswich, MA, United States), which utilizes a random priming approach for reverse transcription. The protocol specifically for use with purified mRNA was followed. Depletion of rRNA was not performed.

Libraries were quality checked using an Agilent 2100 Bioanalyzer and DNA High Sensitivity kit. Results from these steps were used to pool samples in an equimolar ratio. The pool was then gel purified using a 2% agarose gel and the Qiagen QIAquick gel extraction kit (Qiagen, Germantown, MD, United States). Following purification, the pool was sequenced using an Illumina NextSeq 550 platform to produce 2 × 150 bp reads by Wright Labs LLC (Huntingdon, PA, United States).

2.3 Bioinformatics and statistical analyses

2.3.1 Quality checking and sequence annotation

Quality was checked in the raw sequence data using VSEARCH (Rognes et al., 2016). Following initial quality evaluation, fastp (Chen et al., 2018) was used to filter the data with a sliding window of 4 with a minimum average Phred Q score of 20 in which a window not meeting the average would result in the window as well as the remainder of the sequence being dropped. Sequences shorter than 90 bp after filtering were discarded.

Kraken2 (Wood et al., 2019) was subsequently used to taxonomically annotate the remaining sequences with a database, including its standard libraries, as well as fungi. Species-level annotations were then collated into a table for use with downstream analyses. Counts for the species *Homo sapiens* were excluded to avoid

human contamination impacting results. Another table was created by subsetting the full species table to only include fungal species.

The Kraken2-annotated sequences, except for those identified as *Homo sapiens*, were paired with PEAR (Zhang et al., 2014) and dereplicated with VSEARCH. Emapper v2.0 (Huerta-Cepas et al., 2017) using the eggNOG 5.0 database (Huerta-Cepas et al., 2019) was run on the dereplicated sequences. Hits against KEGG Orthologs (KOs) were used to create a table of reads per kilobase (rpk) values for downstream analysis. During table creation, the counts were rpk normalized by dividing sequence occurrences by three times the respective length of the protein they were annotated as (to convert from amino acid length to nucleotide length) and multiplying the quotient by 1,000. The full KOs table was subsetted to create another table containing only annotations of fungal sequences.

Additionally, a fifth dataset containing predicted metabolites based on the paired filtered data was created for use with LEfSe analysis (Segata et al., 2011). The filtered paired sequences were used with HUMAnN3 (Beghini et al., 2021) to generate UniRef90 annotations as input for MelonnPan (Mallick et al., 2019) predicted metabolite analysis using their pre-trained model (Franzosa et al., 2019). The "Unmapped" row was removed from the table, and UniRef90 counts were converted to relative abundances prior to MelonnPan analysis.

In total, five datasets were generated and used for all subsequent analyses: total active species, fungal active species, total expressed genes, fungal expressed genes, and predicted metabolites. Of the five, total active species and total expressed genes were used with all subsequent analyses, while the other three were only used for differential feature analysis. One sample (73) was omitted from all analyses due to yielding fewer than 500,000 raw sequences.

2.3.2 Alpha diversity analysis

Alpha diversity was calculated by subsampling the active microbial taxa and expressed genes dataset tables at 10 different depths, ranging from 99,000 to 990,000 for the active microbial species dataset (Supplementary Figure S1) and 590 to 5,900 for the expressed genes dataset (Supplementary Figure S2). A single sample (68) was omitted from alpha diversity analyses with the microbial taxa dataset to facilitate a higher rarefaction depth as opposed to having to use a maximum depth of 117,600 instead to retain it. Twenty iterations were performed at each depth to obtain average alpha diversity values for the different metrics [Observed Features and Pielou's Evenness (Pielou, 1966)]. Averages for the greatest depth were used to see if any alpha diversity metrics differed significantly based on CDI status (Kruskal-Wallis, $p \leq 0.05$) with QIIME2 (Bolyen et al., 2019).

2.3.3 Beta diversity analysis

Beta diversity analyses were conducted after the tables had first undergone counts per million normalization to mitigate differences between samples based on sequencing depth. The Bray-Curtis distance metric (Sørensen, 1948) was used to create a distance matrix for both datasets. The resulting distance matrices were visualized as Principal Coordinates Analysis plots with 95% confidence intervals around the centroids using the ggordiplot package (Quensen, 2020) through R (R Core Team, 2023). Statistical differences between sample groupings based on CDI status were evaluated as well (PERMANOVA, $p \leq 0.05$) through QIIME2.

2.3.4 Differential feature analysis

Differential feature analysis was performed using LEfSe (Segata et al., 2011) to identify features (genes, pathways, predicted metabolites, and taxa) that had significantly different abundances based on CDI status. For all datasets, the table was normalized with the counts per million (CPM) method. Only features identified as having significantly differential abundance (Kruskal-Wallis, $p \leq 0.05$) with a log(LDA) score of at least 2.0 were considered to be enriched, with the exception of the predicted metabolites dataset in which features only had to differ significantly based on Kruskal-Wallis. Levene's test was performed using the rstatix package (Kassambara, 2020) in R with CPM-normalized values for differential features identified within the active microbial species dataset, as several of the most differential taxa seemed to be highly variable.

SparseDossa2 (Ma et al., 2021) was used to simulate 1,000 tables with various minimum fold change differences for the active microbial taxa and expressed genes datasets in order to assess the corresponding change required to achieve 80% power for LEfSe to detect significant features. Each simulated table consisted of 39 samples, split into two groups of sizes 19 and 20, and was based on a SparseDossa2 model fitted for the respective data type.

For every simulation, the active microbial taxa simulated tables used 17 of the 346 species (5%) that were present in at least 30% of the samples with a minimum average relative abundance of 0.01% to model differential features and 76 of the 1,515 KOs (5%) meeting those requirements were used for each simulation based on the expressed genes dataset. Simulated samples had an average depth of 10,000,000 for both the active microbial taxa and expressed genes datasets. All simulated tables were CPM-normalized prior to being used as input for LEfSe and subject to the same criteria as the observed data to be considered differential. Differential features associated with the expected group were counted as true positives.

2.3.5 Differential contributors analysis

The stratified gene contribution table was subsetted to include only genes of interest. The rpk-normalized values were then converted to relative abundances such that for each sample, the values of the taxa contributing to the gene(s) of interest summed to 100. Wilcoxon rank sum tests were then used to assess the significance of differences in relative contribution based on CDI status.

2.3.6 Machine learning

Random forest models were created to assess how well the features identified as differential by LEfSe could be used to predict CDI status compared to models trained on the full datasets. The models were generated with Scikit-learn in Python based on 500 decision trees (Pedregosa et al., 2011). They were evaluated using k-fold cross-validation with five repetitions of 10 folds. The datasets used for model creation include the full species datasets and the species identified as differential by LEfSe. Those two datasets were used to generate additional models after excluding *Clostridioides difficile*. Models were also generated using the full genes dataset and the genes identified as differential. All datasets were subject to CPM normalization prior to model creation. Feature importance was evaluated using Gini importance, and the 10 most important features were plotted for all models.

2.3.7 CoNet analysis

Cooccurrence networks for the active microbial taxa datasets were generated with CoNet (Faust and Raes, 2016) in Cytoscape (Shannon et al., 2003), using automatic thresholds for Spearman correlation (Spearman Rank Correlation Coefficient, 2008), Bray-Curtis (Sørensen, 1948), and Kullback–Leibler (Kullback and Leibler, 1951), with an edge selection parameter of 100 and a minimum occurrence of 10 samples for a feature to be considered. The *p*-values from the individual methods were combined with the Brown method (Brown, 1975), and Benjamini-Hochberg (Benjamini and Hochberg, 1995) p-value correction was performed with a cutoff of 0.05 for significance. Networks were created for CDI+ Total Active Species and CDI− Total Active Species datasets and then juxtaposed using CytoGEDEVO (Malek et al., 2016).

3 Results

3.1 Description of sequencing results

In total, 40 samples were subjected to MT sequencing, yielding 759,555,378 raw sequences, and after quality filtration, 639,700,992 sequences remained. Of those samples, 39 (CDI + =20, CDI− = 19) had enough data for downstream analysis after quality filtering (range 942,194–82,265,454 sequences per sample, Figure 1).

3.2 Richness and evenness

Pielou's evenness values within the expressed genes dataset tended to be higher in CDI− samples compared to the CDI+ cohort, though the difference was not significant (Table 1). Overall, alpha diversity was variable among samples but did not differ significantly between CDI+ and CDI− for the active microbial taxa or expressed genes datasets based on either the Pielou's evenness or Observed Features metrics (Figure 2).

3.3 Differential active microbial communities

Significant clustering based on CDI status was observed for both active taxa and expressed gene datasets (PERMANOVA, $p = 0.005$ and

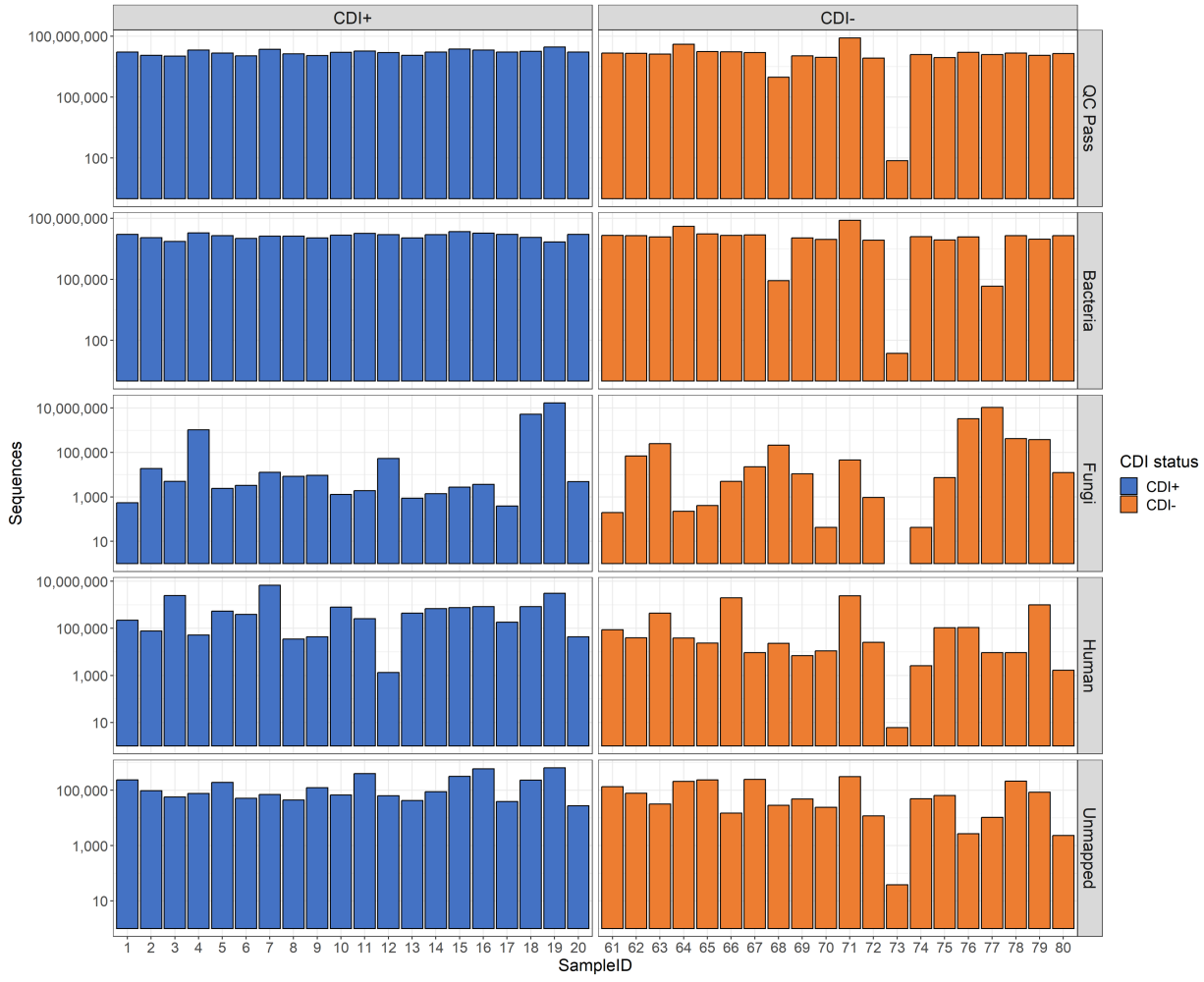


FIGURE 1
Bar plots of sequencing results. The number of sequences retained after quality control (QC Pass), annotated as Bacteria (Bacteria), annotated as Fungi (Fungi), annotated as *Homo sapiens* (Human), and unidentified by Kraken2 (Unmapped) are shown. Raw sequence counts are shown on the y-axis. The x-axis shows individual samples. Each patient was associated with a single sample.

TABLE 1 Alpha diversity results for the Observed Features and Pielou's evenness metrics.

Metric	Dataset	CDI− mean	CDI+ mean	H	Kruskal-Wallis *p*-value
Observed features	Total active species	1188.278 (±91.799)	1431.865 (±98.996)	2.585	0.108
Observed features	Total expressed genes	1297.142 (±68.824)	1245.733 (±56.513)	0.967	0.325
Pielou's evenness	Total active species	0.403 (±0.034)	0.464 (±0.014)	1.969	0.161
Pielou's evenness	Total expressed genes	0.838 (±0.023)	0.831 (±0.011)	3.651	0.056

Significance was evaluated using Kruskal-Wallis tests through QIIME2. Mean values with standard error are reported.

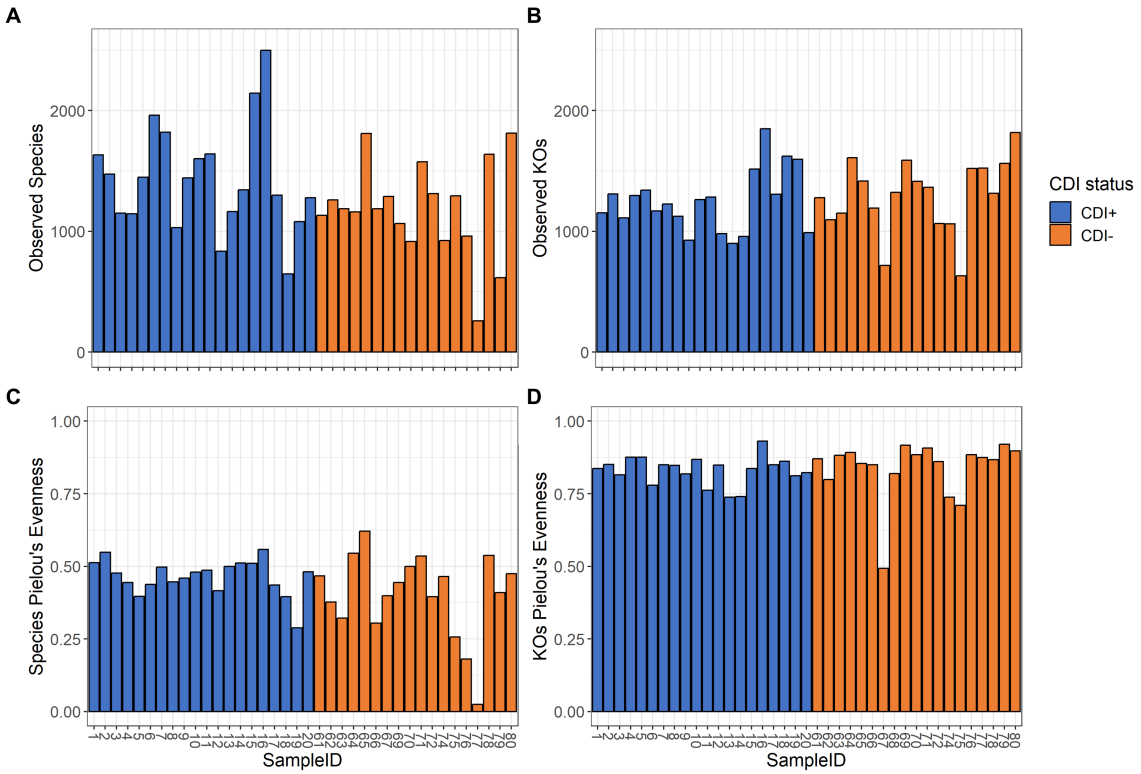


FIGURE 2
Alpha diversity values for the active composition and expressed genes datasets based on the Observed Features and Pielou's Evenness metrics per sample. Samples are colored based on CDI status. Sample 68 was omitted from the active microbial species analyses to allow for a greater maximum rarefaction depth. **(A)** Observed Features within the active microbial species dataset. **(B)** Observed Features within the expressed genes dataset. **(C)** Pielou's evenness within the active microbial species dataset. **(D)** Pielou's evenness within the expressed genes dataset. Alpha diversity did not differ significantly (Kruskal-Wallis, $p \leq 0.05$) based on CDI status within either dataset by either metric.

$p = 0.013$, respectively; Figure 3). Subsequent LEfSe analysis identified the microbial taxa and KEGG Orthologs (KOs) driving differential clustering.

The commensal bacteria *Clostridium butyricum*, *Lacticaseibacillus rhamnosus*, and *Roseburia intestinalis* were the most differentially active microbial species in CDI− (Figure 4). However, variability in the expression of all three taxa within CDI− was relatively high (Supplementary Table S1). In contrast, though its corresponding LDA score was lower, *Lactiplantibacillus plantarum* was more consistently active in the CDI− samples (Supplementary Table S1).

Multiple butyrate producers were identified as being more active in CDI+, including *Anaerostipes hadrus* (Allen-Vercoe et al., 2012), *Coproccocus comes* (Louis and Flint, 2009), and *Roseburia hominis* (Louis and Flint, 2009), and all three were found to express genes within the Butanoate Metabolism pathway (ko00650) in this study. However, the taxa *Clostridioides difficile*, *Enterocloster bolteae*, and *Ruminococcus gnavus* were the three most differentially active species in CDI+ (Kruskal-Wallis, $p \leq 0.05$, $\log(\text{LDA}) \geq 3.0$), with *C. difficile* being the most differential (Supplementary Figure S3). *Clostridium scindens* was also identified as being more active in CDI+.

When our microbial taxa table was subsetted to include only fungal taxa, the family Saccharomycetaceae was more active in CDI− samples (Figure 5). In contrast, 31 fungal taxa were more active in CDI+ (Supplementary Table S2). Several differential genes (Supplementary Table S3) and pathways (Supplementary Table S4) were also identified within the fungi functional gene datasets. However, we recovered relatively low numbers of fungal sequences (Supplementary Figure S4).

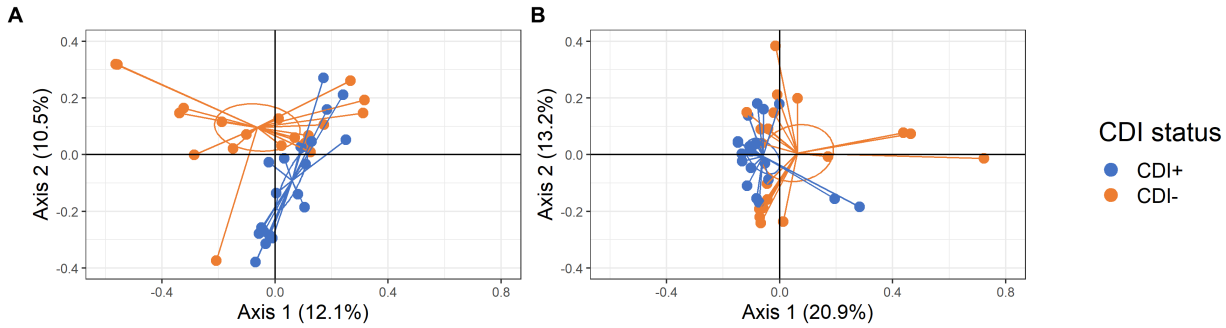


FIGURE 3
PCoA plots of samples colored by CDI status with centroids shown within 95% confidence interval ellipses based on Bray-Curtis distances calculated with active microbial species **(A)** and expressed genes **(B)**.

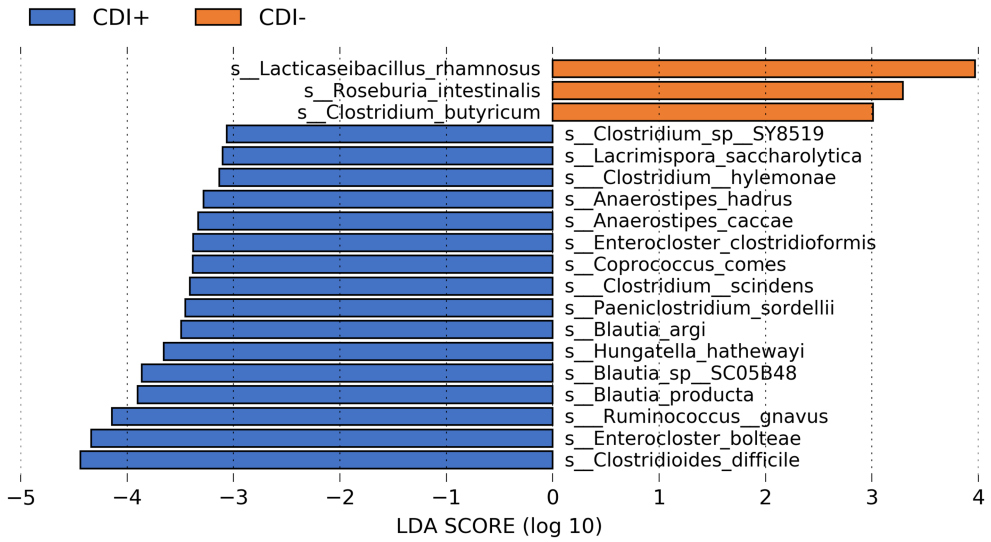


FIGURE 4
Bar plot of LEfSe results showing highly differential active species (LDA ≥ 3.0, Kruskal-Wallis $p \leq 0.05$) based on CDI status. See Supplementary Table S1 for all active taxa identified as differential by LEfSe.

More differential genes were identified within the CDI+ cohort in the total expressed genes dataset (CDI+ genes = 99, CDI− genes = 31), including several that were especially of interest due to their involvement with biofilm formation or sporulation and their expression by *C. difficile* (Table 2). However, a greater number of pathways were expressed associated as differential in CDI− (Supplementary Table S5). Interestingly, 22 of the genes more expressed in CDI+ code for ribosomal subunits (Supplementary Table S6). The most significantly different contributors to those genes were taxa significantly more active in CDI+ (Supplementary Table S7).

Clostridioides difficile was one of the taxa found to be significantly more critical to the expression of differential rRNA genes in CDI+ (Supplementary Table S7). It was also found to be a significantly more important contributor to the expression of three differential KOs related to spore formation in CDI+ (Supplementary Table S8). Likewise, *Enterocloster bolteae* contributed to the differential expression of two of those KOs (Supplementary Table S8). Similarly, though it was not associated with differential expression of those three spore formation KOs, *Ruminoccocus gnavus* expressed genes relating to spore formation as well. *Clostridioides difficile* also constituted a greater proportion of the expression of genes relating to biofilm formation in CDI+, specifically K03073 (*SecE*), K03075 (*SecG*), and K03666 (*hfq*; Supplementary Tables S9, S10). *Clostridioides difficile* and *R. gnavus* were also significantly more important to the expression of genes within the differential Flagellar Assembly (ko02040) in CDI+ (Supplementary Table S11). Additionally, *C. difficile* was identified as expressing the *tcdB* gene in three CDI+ samples (13, 19, and 5), albeit very few sequences were associated with both (1, 2, and 5 sequences respectively).

Random forest modeling was used to help evaluate the consistency of features identified by LEfSe for differentiating samples based on CDI status, regardless of possible confounding factors. The performance of those models indicates that both microbial taxa and expressed genes identified by LEfSe differed consistently between

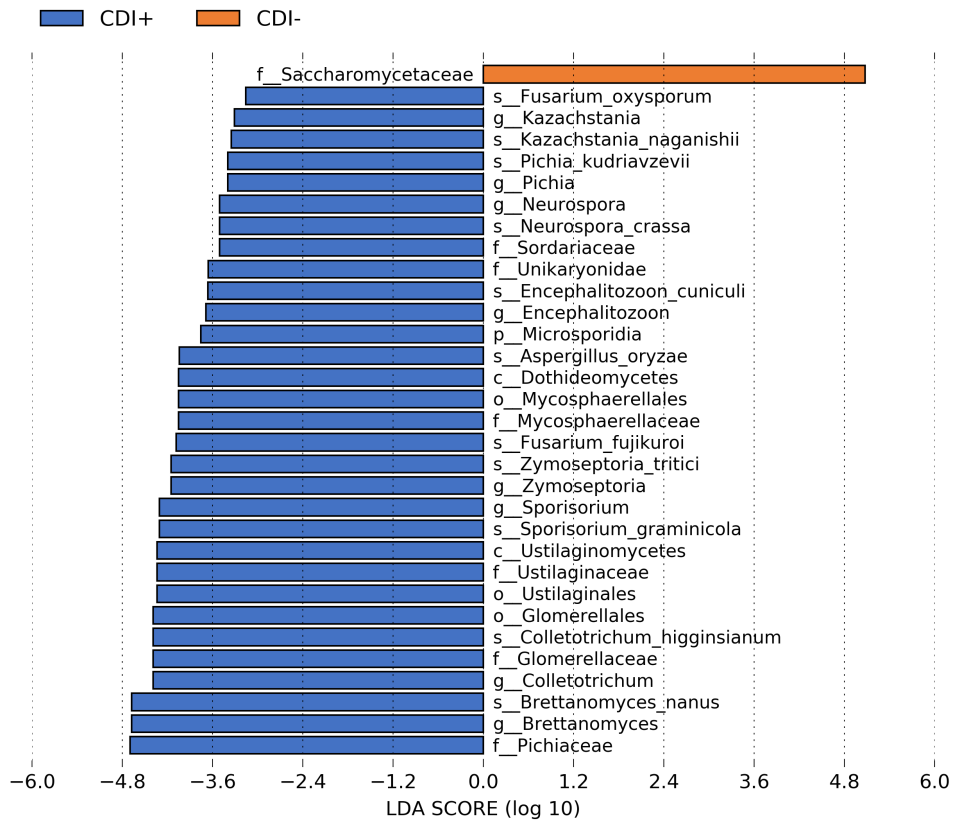


FIGURE 5
Bar plot of LEfSe results showing differential active fungal taxa (LDA ≥ 2.0, Kruskal-Wallis $p \leq 0.05$) based on CDI status.

TABLE 2 Differential CDI+ KEGG Orthologs (KOs) of interest due to their potential to facilitate CDI recurrence.

KO	Reason	Total Samples	Samples associated with *C. difficile* expression
K03073: preprotein translocase subunit SecE	Biofilm formation	38	13
K03075: preprotein translocase subunit SecG	Biofilm formation	36	15
K03666: host factor-I protein	Biofilm formation	28	7
K06334: spore coat protein JC	Sporulation	29	10
K06412: stage V sporulation protein G	Sporulation	27	14
K06418: small acid-soluble spore protein A (major alpha-type SASP)	Sporulation	29	21

The KOs listed here were all expressed by *Clostridioides difficile* in at least one sample. The number of samples that the KO was present in is reported in the "Total Samples" column, and the number of samples that it was identified as being expressed by *C. difficile* is reported in the "Samples Associated with *C. difficile* Expression" column.

CDI+ and CDI−. Both random forest models using differential features (genes or species) had average accuracies of 84.2% (Table 3). As expected, the differential species model excluding *C. difficile* performed worse, but it still had a 9% greater average accuracy than the random forest model that was generated using all species. Likewise, the genes model had a 7% increase in average accuracy between the dataset containing all genes and the dataset containing only genes identified as differentially expressed by LEfSe. Additionally, eight of the 10 best predictors for the full species dataset were also identified as being predictive of CDI status by LEfSe (Figure 6). The best predictors for the other models are shown in Supplementary Figures S5–S9.

3.4 Cross domain interactions

Co-occurrence network analysis helped us model potential transkingdom interactions between bacteria and fungi with respect to CDI status. The networks revealed negative relationships between fungi and bacterial taxa in both CDI− and CDI+ networks. In the CDI− network, 18 fungi were present, with *Nakaseomyces glabratus* (formerly *Candida glabrata*) having negative correlations with 57 taxa, 55 of which were bacteria (Figure 7). In contrast, *N. glabratus* was also entirely absent in the CDI+ network (Figure 7). Furthermore, the CDI+ network only had two fungi, *Saccharomyces cerevisiae* and *Debaryomyces hansenii*.

TABLE 3 Random forest classification accuracy.

Model input	Average accuracy
All microbial species	69.5%
LEfSe identified differential species	84.2%
All microbial species without *Clostridioides difficile*	70.2%
LEfSe identified differential species without *C. difficile*	78.5%
All genes	77.2%
LEfSe identified differential genes	84.2%

Random forest modeling was performed using the scikit-learn package with Python.

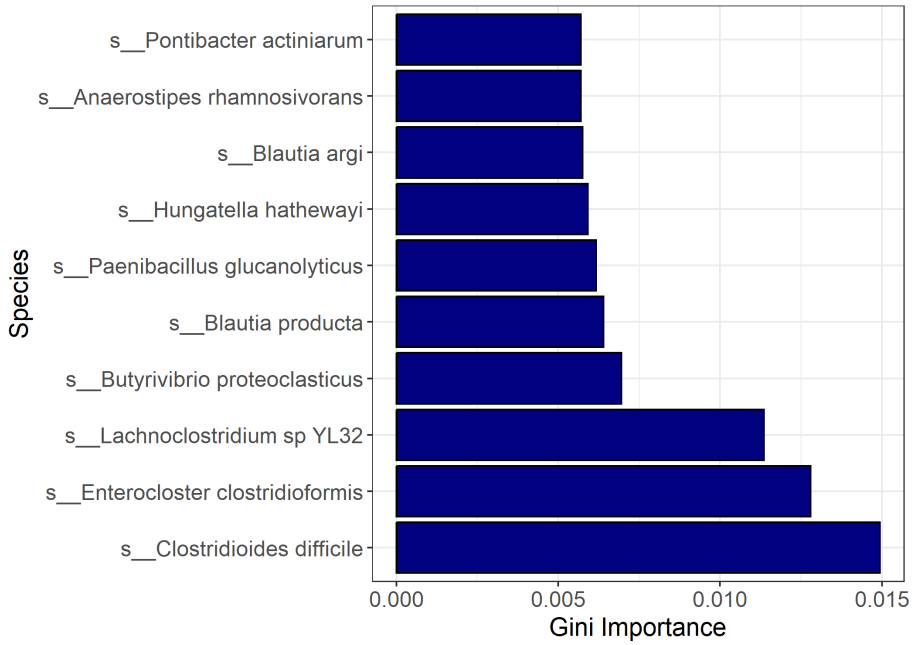


FIGURE 6
Bar plot of most important features for classification with random forest based on the full active microbial taxa dataset. Feature importance was measured by the decrease in impurity after splitting by the feature (Gini Importance). *Pontibacter actiniarum* and *Butyrivibrio proteoclasticus* were not identified as differential (LDA ≥ 2.0, Kruskal-Wallis $p \leq 0.05$) by LEfSe.

3.5 Predictive metabolic modeling

Several predicted metabolites ($n = 16$) were found to be significantly more abundant (Kruskal-Wallis, $p \leq 0.05$; Supplementary Table S12) in CDI+ patients, including caproic acid (Figure 8). In addition, a secondary bile acid, lithocholic acid, was also predicted to be more abundant in CDI+. Fewer predicted metabolites ($n = 6$) were associated with CDI–.

4 Discussion

4.1 Microbiome differences

Although previous studies have noted significant differences in alpha diversity, those studies utilized 16S rRNA and ITS data to characterize microbial communities (Antharam et al., 2013; Lamendella et al., 2018; Zuo et al., 2018). To the best of our knowledge, this study is the first to examine possible alpha diversity differences based on CDI status using MT data. However, these results are aligned with another study that compared CDI− diarrheal communities to CDI+ communities that also did not observe significant differences between those groups (Antharam et al., 2013).

Still, similar to other CDI microbiome studies (Lamendella et al., 2018; Zuo et al., 2018; Stewart et al., 2019) overall community composition and gene expression differed significantly based on CDI status. The recapitulation of this finding is notable because these results indicate that both the active community members and the functions they express differ with respect to CDI status.

The species most strongly associated with CDI− samples are beneficial to human health. Specifically, both *Clostridium butyricum* and *Roseburia intestinalis* are butyrate producers (Hayashi et al., 2021; Nie et al., 2021). Similarly, the use of *Lacticaseibacillus rhamnosus* as a probiotic has been associated with increased butyrate production (Berni Canani et al., 2016; Lin et al., 2020; Carucci et al., 2022), and *Clostridium* and *Roseburia* genera are contained in a newly

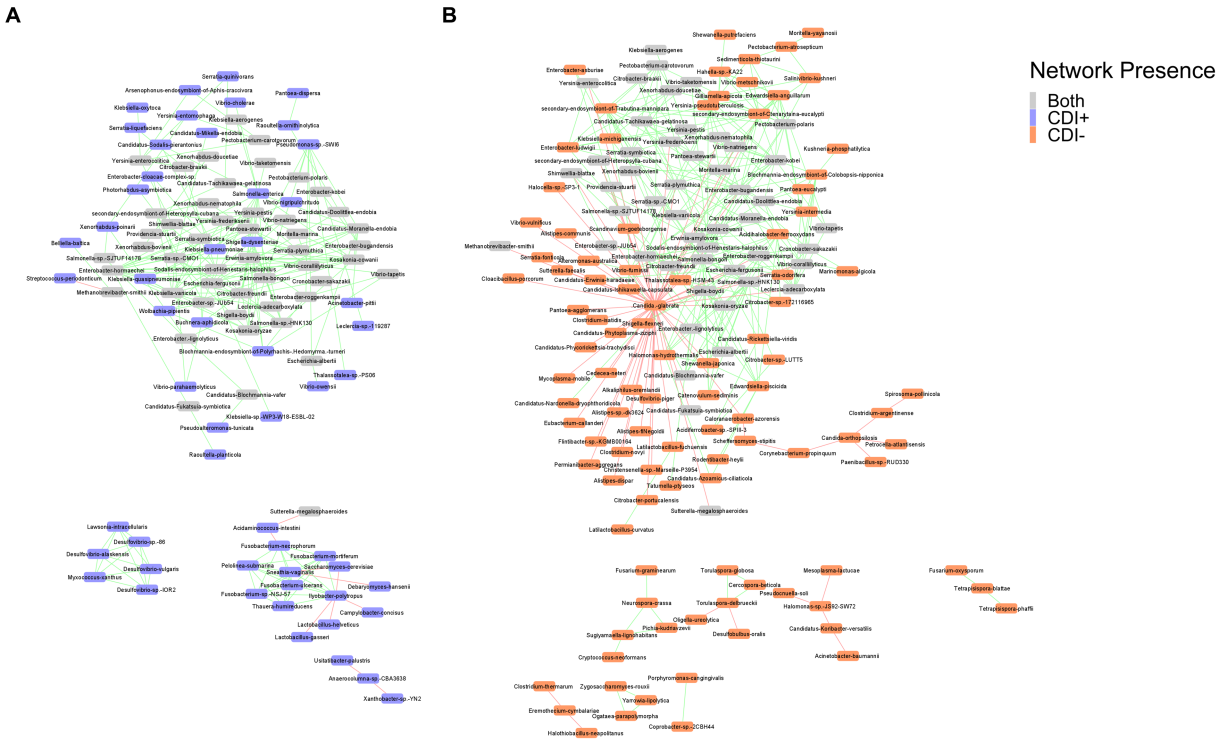


FIGURE 7
Co-occurrence networks for CDI+ **(A)** and CDI− **(B)**. Nodes are labeled by species name and colored by network presence. Edges showing positive correlations between the connected nodes are shown in green, and edges showing negative correlations are shown in red. Pairs of nodes that were only connected to each other were omitted from the final networks.

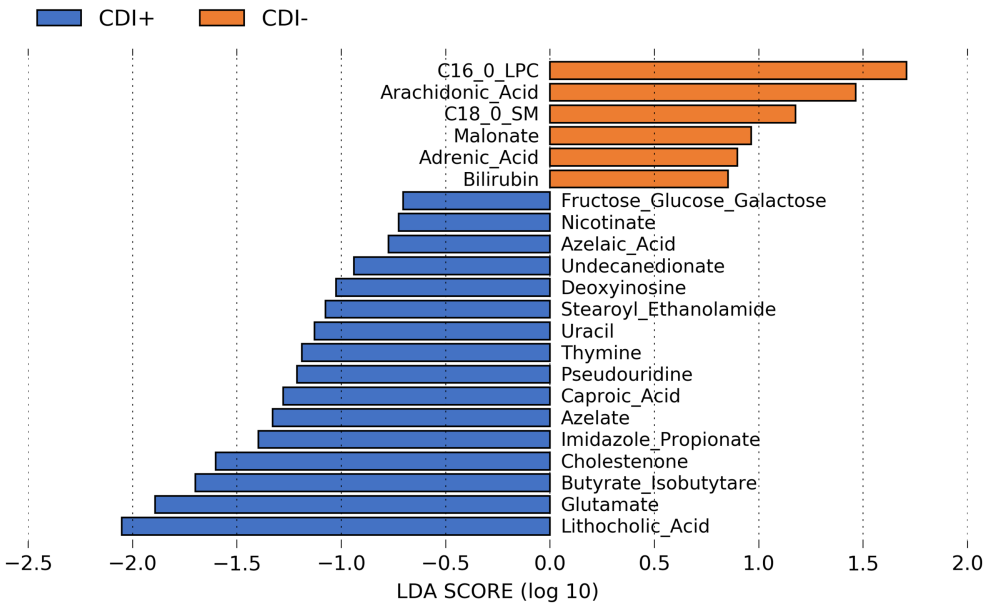


FIGURE 8
LEfSe bar plot showing differentially abundant predicted metabolites resulting from MelonnPan analysis (Kruskal-Wallis $p \leq 0.05$) based on CDI status.

FDA-approved fecal microbiota capsule (SER-109) for use against recurrent CDI (Khanna et al., 2016). In the context of CDI, butyrate has been found to reduce inflammation and improve intestinal barrier function following *C. difficile* colonization (Fachi et al., 2019). *Roseburia intestinalis* was most abundant in the healthy cohort in a study examining the gut microbiome of children based on CDI and

hospitalization (IC vs. non-IC) status (Mohandas et al., 2020). Additionally, multiple studies have found *C. butryicum* promotes resistance to *C. difficile* infection (Hagihara et al., 2021; Hayashi et al., 2021), and it has been investigated as a possible treatment for CDI (Oka et al., 2018; Lee et al., 2022). Likewise, studies have found evidence administering *Lactiplantibacillus plantarum* as a probiotic may help prevent CDI (Klarin et al., 2008; Kujawa-Szewieczek et al., 2015; Dudzicz et al., 2018). Therefore, the higher activity of *C. butryicum* and *L. plantarum* in CDI− samples could have helped protect those patients against acquiring *C. difficile*.

Multiple butyrate producers were also identified as more active in CDI+. Although previous research has shown that butyrate was significantly reduced in CDI cohorts compared to healthy controls (Pensinger et al., 2023), another study did not find a significant difference in butyrate producers (based on 16S rRNA analysis) in their diarrheal controls compared to CDI, while their healthy cohort had significantly more butyrate producers compared to their CDI and diarrheal control groups (Antharam et al., 2013). Therefore, since both CDI+ and CDI− groups in this study were comprised of diarrheal samples, differential gene expression attributed to butyrate producers in both is consistent with previous findings.

Interestingly, *Clostridium scindens* was also identified as being more active in CDI+. *Clostridium scindens* had previously been associated with resistance to CDI as a result of producing secondary bile acids (Buffie et al., 2015; Greathouse et al., 2015), but resistance may be contingent on the resulting concentration of deoxycholate (Dubois et al., 2019). Still, *C. scindens* has also been found to inhibit *C. difficile* growth independent of bile acid production (Aguirre et al., 2021). Instead, the authors of that study suggested resistance could be due to competition for nutrients with *C. difficile* (Aguirre et al., 2021). Therefore, though unexpected, if nutrient conditions were conducive to *C. difficile* growth in our CDI+ patients, increased activity of *C. scindens* could have also been favored.

Besides *Clostridioides difficile*, the other species most strongly associated with CDI+ are also known pathogens. *Enterocloster bolteae* has been previously noted to be an opportunistic pathogen and are also correlated with the expression of inflammatory genes (Pandit et al., 2021). Similarly, *Ruminococcus gnavus* has been found to produce a glucorhamnan that causes inflammation (Henke et al., 2019). Additionally, *Ruminoccocus gnavus* was previously associated with CDI in another study that found several operational taxonomic units (OTUs) belonging to this species were more abundant in their CDI IBD group compared to the non-CDI IBD group and the healthy subjects (Sokol et al., 2017). Notably, *Ruminococcus gnavus* is a mucin degrader, and a previous study found *C. difficile* grown in the presence of MUC2 with mucin-degrading taxa also present had increased expression of genes associated with flagella (Engevik et al., 2021).

The larger number of fungal taxa associated with CDI+ aligns with previous studies that found fungal taxa were more abundant in CDI patients (Sangster et al., 2016; Lamendella et al., 2018; Stewart et al., 2019). Therefore, this study provides further evidence that the mycobiome may have an important impact on the pathogenesis of CDI. For instance, *Encephalitozoon cuniculi*, a species of fungi enriched in CDI+ patients, is a known opportunistic pathogen (Moretto et al., 2015). Conversely, the use of *Saccharomyces boulardii,* which is a part of the family Saccharomycetaceae (more active in CDI− patients), as a probiotic seems to reduce CDI-associated diarrhea (Goldenberg et al., 2017).

The relatively large number of differential KEGG Orthologs associated with ribosomal subunits was likely due to the taxa expressing them being more transcriptionally active in CDI+ samples because of other reasons, rather than a driving reason for increased activity themselves. Higher expression of genes coding for ribosomes has previously been correlated with an increase in activity for the microbes expressing them, though not consistently (Blazewicz et al., 2013). Still, the relatively greater contribution of CDI+ differential taxa to these genes provides another line of evidence that they were more active in CDI+.

Clostridioides difficile is known to form spores during infection (Paredes-Sabja et al., 2014), which fits with the observation of it as a significantly more important contributor to the expression of three differential KOs related to spore formation in CDI+ in this study. Likewise, *Enterocloster bolteae* can form spores (Magdy Wasfy et al., 2023) and contributed to the differential expression of two of those KOs (Supplementary Table S8). Similarly, though *Ruminoccocus gnavus* had historically been considered to be non-spore forming, more recent work has demonstrated that it can form spores under certain conditions (Crost et al., 2023). Therefore, it is possible that members of all three taxa could form spores in response to antibiotics, which may help explain their presence in CDI+ samples, as antibiotics are often used to treat CDI.

Both *SecE* and *SecG* have previously been proposed to be involved with *C. difficile* biofilm (Poquet et al., 2018). *SecE* and *SecG* have also been associated with biofilm formation in *Staphylococcus aureus* (Resch et al., 2005) and *Bifidobacterium longum* (Zhang et al., 2023) respectively. Therefore, although we did not recapture our previous finding of differential biofilm pathways (Stewart et al., 2019), the observed overall increased expression of *hfq*, *SecE* and *SecG* and their expression by *C. difficile* in this study, as well as other genes relating to biofilm formation, provides further *in vivo* evidence that biofilm formation is involved in the pathogenesis of CDI. However, *hfq*, *SecE*, and *SecG* are also components of other pathways, and of the 21 KOs expressed by *C. difficile* that are associated with at least one biofilm formation pathway, only two are associated solely with biofilm formation (Supplementary Table S10). Therefore, future work is needed to confirm *in vivo* biofilm formation. Still, biofilm formation, if occurring, could help protect *C. difficile* from antibiotics and consequently, contribute to CDI's high reoccurrence rate.

Two of the four pathways more expressed in CDI+ [Ethylbenzene Degradation (ko00642) and Flagellar Assembly (ko02040)] were also identified as being differentially expressed in our prior study (Stewart et al., 2019). Notably, the production of flagella has been associated with pathogen colonization (Rossez et al., 2015), and *C. difficile*, *E. bolteae*, and *Parabacteroides distasonis* were significantly more important contributors to genes within that pathway in CDI+ (Supplementary Table S11). Like *E. bolteae*, *P. distasonis* is also an opportunistic pathogen (Ezeji et al., 2021). Therefore, the enrichment of the flagellar assembly pathway here was driven by the increased activity of potentially pathogenic bacteria.

Correlations between bacteria and fungi within CDI+ samples have been observed in other studies (Lamendella et al., 2018; Stewart et al., 2019). However, Lamendella et al., 2018 found no correlations between fungi and bacteria in CDI− (Lamendella et al., 2018), and Stewart et al., 2019 had only one unidentified fungi with a significant correlation to an unidentified *Bacteroides* OTU (Stewart et al., 2019).

Networks in both were based on ITS data, so the use of MT data here instead is a likely contributor to the differences in the prevalence of fungi in the CDI− network. Still, despite evidence of fungi's importance to CDI pathogenesis, co-occurrence analysis itself did not identify a significant correlation between fungal species and *C. difficile* in this study or in either of those previous studies (Lamendella et al., 2018; Stewart et al., 2019).

Caproic acid was previously detected in 41% of CDI+ fecal samples according to a gas chromatography study used to predict *C. difficile* (Levett, 1984). In contrast, lithocholic acid, also associated with CDI+ in this study, has previously been found to inhibit the growth of *C. difficile* (Kang et al., 2019). However, it is possible that lithocholic acid could have been more abundant in our CDI+ samples but still below a concentration that would have been lethal to *C. difficile*, and if so, then it is also possible that lithocholic acid could have induced biofilm formation, as was the case with deoxycholate in a previous study (Dubois et al., 2019). This possibility is supported by our observance of *C. difficile* expressing multiple genes related to biofilm formation.

Still, it should be noted that tools like MelonnPan are predictive metabolic modeling tools, and it is unknown whether the lithocholic acid metabolite was truly more abundant in our CDI+ samples. Consequently, directly measuring metabolites of interest like lithocholic acid and caproic acid, in conjunction with microbiome analysis, would be valuable to further understanding how the abundance of metabolites previously associated with CDI relates to microbiome composition and function.

4.2 Limitations

Unfortunately, no information about antibiotic usage or clinical history was reported for the individuals used in this study. Previous work has shown that antibiotic usage in conjunction with CDI has a large impact on the host's overall microbial community (Lamendella et al., 2018). Specifically, in that study, the fidaxomicin and metronidazole groups had significant differences in alpha and beta diversity between CDI+ and CDI−, while the vancomycin group did not (Lamendella et al., 2018). Additionally, different taxa were detected as being significantly differential between CDI+/− depending on the antibiotic class (Lamendella et al., 2018). Still, another study did not find a significant difference in alpha diversity within their CDI+ and CDI− groups when split by antibiotics exposure (Antharam et al., 2013). However, it is possible that a differential usage of antibiotics could have impacted our results. Consequently, we limited much of our discussion to comparing the results obtained herein to previous CDI studies.

The failure to consistently capture sequences associated with both *C. difficile* and the gene used for diagnosis (*tcdB*) could also indicate our sequencing depth was not sufficient to fully capture all expressed genes in our samples. Additionally, due to the cost associated with MT, our sample size was smaller than some previous studies. Though, *post hoc* power analysis using SparseDossa2 showed we had reasonable (>80%) power to detect changes corresponding to at least 7.5 log2 fold change in the active species dataset and 4.5 log2 fold in the expressed genes dataset with LEfSe.

4.3 Conclusions and future directions

Despite antibiotic usage being a potentially large confounding variable, this work has contributed great value in characterizing active microbes and their associated functions in the context of CDI. To the best of our knowledge, MT analysis had only been applied to human samples associated with this disease state in a previous study by our group, in which it was used to identify differential genes and pathways (Stewart et al., 2019). Therefore, this study represents one of the first applications of MT to characterize differences in overall community diversity between CDI+ and CDI− cohorts. Although alpha diversity did not differ significantly, beta diversity for active species and expressed genes differentiated CDI+ and CDI− cohorts.

Subsequent differential feature analysis identified the specific genes and taxa driving those overall differences, revealing significant increases in the activity of several beneficial bacteria in CDI− and increased activity of taxa associated with inflammation in CDI+. Although the previous finding of differential biofilm formation pathways (Stewart et al., 2019) was not recaptured, several specific genes associated with biofilm formation were identified as differentially expressed in CDI+, and *C. difficile* was identified as a contributor to them, serving as additional evidence for the role of biofilm formation in CDI pathogenesis. Biofilm formation during infection would help explain CDI's high recurrence rate, and if that is the case, treatments designed to target biofilms specifically could be effective for preventing reoccurrence.

Additionally, multiple fungal taxa were identified as being more transcriptionally active in CDI patients, which provides additional support for the potential importance of the mycobiome to CDI. Notably, *E. cuniculi* can cause inflammation, and increased inflammation due to it or other fungi could be one mechanism by which severe CDI could occur irrespective of bacterial toxin production. However, coverage for both *E. cuniculi* and fungi overall in this study tended to be low. Determining if specific fungal taxa are important for preventing or enabling CDI could facilitate the use of additional treatment methods. The low proportion of CDI+ samples that had *tcdB* identified within them suggests that greater sequencing coverage would also be valuable for characterizing the functional capabilities of these microbial communities. Therefore, future work should include deeper metatranscriptomics sequencing in order to increase coverage of the entire microbial community.

Increasing fungal sequences specifically, either through overall increased sequencing depth or targeting sequencing, would also aid better understanding if fungal activity contributes to the pathogenesis of CDI. Likewise, the development of methods to increase the proportion of fungal nucleic acids recovered would similarly help facilitate investigations of fungi's role in CDI. Additionally, future iterations of the CDI status model could theoretically be trained to differentiate non-*C. difficile* diarrhea with colonization from clinically-relevant *C. difficile* infection, as a means to reduce unnecessary anti-*C. difficile* antibiotic use (Polage et al., 2015). Metabolomics data would also be invaluable for further exploring the role that previously identified metabolites of interest play in CDI.

Data availability statement

Raw sequencing data are available from NCBI's Short Read Archive under BioProject ID PRJNA1035947.

Ethics statement

The studies involving humans were approved by Institutional Review Board at the University of Virginia. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because samples were de-identified remnants.

Author contributions

JC: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. JL: Investigation, Writing – review & editing. JW: Resources, Supervision, Writing – review & editing. PK: Formal analysis, Writing – review & editing. MK: Formal analysis, Writing – original draft, Writing – review & editing. CB: Formal analysis, Writing – original draft, Writing – review & editing. CK: Conceptualization, Writing – review & editing. GM: Conceptualization, Writing – review & editing. DS: Conceptualization, Writing – original draft, Writing – review & editing. RL: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

JC, JL, JW and RL was employed by Wright Labs LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1398018/full#supplementary-material

References

Aguirre, A. M., Yalcinkaya, N., Wu, Q., Swennes, A., Tessier, M. E., Roberts, P., et al. (2021). Bile acid-independent protection against Clostridioides difficile infection. *PLoS Pathog.* 17:e1010015. doi: 10.1371/journal.ppat.1010015

Allen-Vercoe, E., Daigneault, M., White, A., Panaccione, R., Duncan, S. H., Flint, H. J., et al. (2012). Anaerostipes hadrus comb. nov., a dominant species within the human colonic microbiota; reclassification of *Eubacterium hadrum* Moore et al. 1976. *Anaerobe* 18, 523–529. doi: 10.1016/j.anaerobe.2012.09.002

Antharam, V. C., Li, E. C., Ishmael, A., Sharma, A., Mai, V., Rand, K. H., et al. (2013). Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhea. *J. Clin. Microbiol.* 51, 2884–2892. doi: 10.1128/JCM.00845-13

Aslam, S., Hamill, R. J., and Musher, D. M. (2005). Treatment of *Clostridium difficile*-associated disease: old therapies and new strategies. *Lancet Infect. Dis.* 5, 549–557. doi: 10.1016/S1473-3099(05)70215-2

Beghini, F., McIver, L. J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., et al. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *eLife* 10:e65088. doi: 10.7554/eLife.65088

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Berni Canani, R., Sangwan, N., Stefka, A. T., Nocerino, R., Paparo, L., Aitoro, R., et al. (2016). *Lactobacillus rhamnosus* GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. *ISME J.* 10, 742–750. doi: 10.1038/ismej.2015.151

Blazewicz, S. J., Barnard, R. L., Daly, R. A., and Firestone, M. K. (2013). Evaluating rRNA as an indicator of microbial activity in environmental communities: limitations and uses. *ISME J.* 7, 2061–2068. doi: 10.1038/ismej.2013.102

Bobo, L. D., Dubberke, E. R., and Kollef, M. (2011). *Clostridium difficile* in the ICU. *Chest* 140, 1643–1653. doi: 10.1378/chest.11-0556

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Brown, M. B. (1975). 400: a method for combining non-independent, one-sided tests of significance. *Biometrics* 31, 987–992. doi: 10.2307/2529826

Buffie, C. G., Bucci, V., Stein, R. R., McKenney, P. T., Ling, L., Gobourne, A., et al. (2015). Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nature* 517, 205–208. doi: 10.1038/nature13828

Carucci, L., Nocerino, R., Paparo, L., De Filippis, F., Coppola, S., Giglio, V., et al. (2022). Therapeutic effects elicited by the probiotic Lacticaseibacillus rhamnosus GG in children with atopic dermatitis. The results of the ProPAD trial. *Pediatr. Allergy Immunol.* 33:e13836. doi: 10.1111/pai.13836

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi: 10.1093/bioinformatics/bty560

Choi, H. K., Kim, K. H., Lee, S. H., and Lee, S. J. (2011). Risk factors for recurrence of *Clostridium difficile* infection: effect of vancomycin-resistant enterococci colonization. *J. Korean Med. Sci.* 26, 859–864. doi: 10.3346/jkms.2011.26.7.859

Crost, E. H., Coletto, E., Bell, A., and Juge, N. (2023). *Ruminococcus gnavus*: friend or foe for human health. *FEMS Microbiol. Rev.* 47:fuad014. doi: 10.1093/femsre/fuad014

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Dubberke, E. R., Butler, A. M., Reske, K. A., Agniel, D., Olsen, M. A., D'Angelo, G., et al. (2008). Attributable outcomes of endemic *Clostridium difficile*–associated disease in nonsurgical patients. *Emerg. Infect. Dis.* 14, 1031–1038. doi: 10.3201/eid1407.070867

Dubberke, E. R., and Olsen, M. A. (2012). Burden of *Clostridium difficile* on the healthcare system. *Clin. Infect. Dis.* 55, S88–S92. doi: 10.1093/cid/cis335

Dubois, T., Tremblay, Y. D. N., Hamiot, A., Martin-Verstraete, I., Deschamps, J., Monot, M., et al. (2019). A microbiota-generated bile salt induces biofilm formation in *Clostridium difficile*. *NPJ Biofilms Microbiomes* 5:14. doi: 10.1038/s41522-019-0087-4

Dudzicz, S., Kujawa-Szewieczek, A., Kwiecień, K., Więcek, A., and Adamczak, M. (2018). *Lactobacillus plantarum* 299v reduces the incidence of *Clostridium difficile* infection in nephrology and transplantation Ward—results of one year extended study. *Nutrients* 10:1574. doi: 10.3390/nu10111574

Engevik, M. A., Engevik, A. C., Engevik, K. A., Auchtung, J. M., Chang-Graham, A. L., Ruan, W., et al. (2021). Mucin-degrading microbes release monosaccharides that chemoattract Clostridioides difficile and facilitate colonization of the human intestinal mucus layer. *ACS Infect Dis* 7, 1126–1142. doi: 10.1021/acsinfecdis.0c00634

Ezeji, J. C., Sarikonda, D. K., Hopperton, A., Erkkila, H. L., Cohen, D. E., Martinez, S. P., et al. (2021). *Parabacteroides distasonis*: intriguing aerotolerant gut anaerobe with emerging antimicrobial resistance and pathogenic and probiotic roles in human health. *Gut Microbes* 13:1922241. doi: 10.1080/19490976.2021.1922241

Fachi, J. L., Felipe, J. S., Pral, L. P., da Silva, B. K., Corrêa, R. O., de Andrade, M. C. P., et al. (2019). Butyrate protects mice from *Clostridium difficile*-induced colitis through an HIF-1-dependent mechanism. *Cell Rep.* 27, 750–761.e7. doi: 10.1016/j.celrep.2019.03.054

Faust, K., and Raes, J. (2016). CoNet app: inference of biological association networks using Cytoscape. *F1000Res* 5:1519. doi: 10.12688/f1000research.9050.2

Franzosa, E. A., Sirota-Madi, A., Avila-Pacheco, J., Fornelos, N., Haiser, H. J., Reinker, S., et al. (2019). Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat. Microbiol.* 4, 293–305. doi: 10.1038/s41564-018-0306-4

Goldenberg, J. Z., Yap, C., Lytvyn, L., Lo, C. K.-F., Beardsley, J., Mertz, D., et al. (2017). Probiotics for the prevention of *Clostridium difficile*-associated diarrhea in adults and children. *Cochrane Database Syst. Rev.* 2017:CD006095. doi: 10.1002/14651858.CD006095.pub4

Greathouse, K. L., Harris, C. C., and Bultman, S. J. (2015). Dysfunctional families: Clostridium scindens and secondary bile acids inhibit the growth of *Clostridium difficile*. *Cell Metab.* 21, 9–10. doi: 10.1016/j.cmet.2014.12.016

Hagihara, M., Ariyoshi, T., Kuroki, Y., Eguchi, S., Higashi, S., Mori, T., et al. (2021). *Clostridium butyricum* enhances colonization resistance against Clostridioides difficile by metabolic and immune modulation. *Sci. Rep.* 11:15007. doi: 10.1038/s41598-021-94572-z

Hayashi, A., Nagao-Kitamoto, H., Kitamoto, S., Kim, C. H., and Kamada, N. (2021). The butyrate-producing bacterium *Clostridium butyricum* suppresses Clostridioides difficile infection via neutrophil- and antimicrobial cytokine–dependent but GPR43/109a-independent mechanisms. *J. Immunol.* 206, 1576–1585. doi: 10.4049/jimmunol.2000353

Henke, M. T., Kenny, D. J., Cassilly, C. D., Vlamakis, H., Xavier, R. J., and Clardy, J. (2019). *Ruminococcus gnavus*, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. *Proc. Natl. Acad. Sci.* 116, 12672–12677. doi: 10.1073/pnas.1904099116

Huerta-Cepas, J., Forslund, K., Coelho, L. P., Szklarczyk, D., Jensen, L. J., von Mering, C., et al. (2017). Fast genome-wide functional annotation through Orthology assignment by eggNOG-mapper. *Mol. Biol. Evol.* 34, 2115–2122. doi: 10.1093/molbev/msx148

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., et al. (2019). eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.* 47, D309–D314. doi: 10.1093/nar/gky1085

Kang, J. D., Myers, C. J., Harris, S. C., Kakiyama, G., Lee, I.-K., Yun, B.-S., et al. (2019). Bile acid 7α-dehydroxylating gut bacteria secrete antibiotics that inhibit *Clostridium difficile*: role of secondary bile acids. *Cell Chem Biol* 26, 27–34.e4. doi: 10.1016/j.chembiol.2018.10.003

Kassambara, A. (2020). rstatix: Pipe-friendly Framework for Basic Statistical Tests in R. Available at: https://github.com/kassambara/rstatix (Accessed 21 January 2021).

Khanna, S., Pardi, D. S., Kelly, C. R., Kraft, C. S., Dhere, T., Henn, M. R., et al. (2016). A novel microbiome therapeutic increases gut microbial diversity and prevents recurrent *Clostridium difficile* infection. *J Infect Dis* 214, 173–181. doi: 10.1093/infdis/jiv766

Klarin, B., Wullt, M., Palmquist, I., Molin, G., Larsson, A., and Jeppsson, B. (2008). *Lactobacillus plantarum* 299v reduces colonisation of *Clostridium difficile* in critically ill patients treated with antibiotics. *Acta Anaesthesiol. Scand.* 52, 1096–1102. doi: 10.1111/j.1399-6576.2008.01748.x

Kujawa-Szewieczek, A., Adamczak, M., Kwiecień, K., Dudzicz, S., Gazda, M., and Więcek, A. (2015). The effect of *Lactobacillus plantarum* 299v on the incidence of *Clostridium difficile* infection in high risk patients treated with antibiotics. *Nutrients* 7, 10179–10188. doi: 10.3390/nu7125526

Kullback, S., and Leibler, R. A. (1951). On information and sufficiency. *Ann. Math. Stat.* 22, 79–86. doi: 10.1214/aoms/1177729694

Lamendella, R., Wright, J. R., Hackman, J., McLimans, C., Toole, D. R., Rubio, W. B., et al. (2018). Antibiotic treatments for *Clostridium difficile* infection are associated with distinct bacterial and fungal community structures. *mSphere* 3:17. doi: 10.1128/mSphere.00572-17

Lee, J.-C., Chiu, C.-W., Tsai, P.-J., Lee, C.-C., Huang, I.-H., Ko, W.-C., et al. (2022). *Clostridium butyricum* therapy for mild-moderate Clostridioides difficile infection and the impact of diabetes mellitus. *Biosci Microbiota Food Health* 41, 37–44. doi: 10.12938/bmfh.2021-049

Lessa, F. C., Mu, Y., Bamberg, W. M., Beldavs, Z. G., Dumyati, G. K., Dunn, J. R., et al. (2015). Burden of *Clostridium difficile* infection in the United States. *N. Engl. J. Med.* 372, 825–834. doi: 10.1056/NEJMoa1408913

Levett, P. N. (1984). Detection of *Clostridium difficile* in faeces by direct gas liquid chromatography. *J. Clin. Pathol.* 37, 117–119. doi: 10.1136/jcp.37.2.117

Lin, R., Sun, Y., Mu, P., Zheng, T., Mu, H., Deng, F., et al. (2020). *Lactobacillus rhamnosus* GG supplementation modulates the gut microbiota to promote butyrate production, protecting against deoxynivalenol exposure in nude mice. *Biochem. Pharmacol.* 175:113868. doi: 10.1016/j.bcp.2020.113868

Louis, P., and Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* 294, 1–8. doi: 10.1111/j.1574-6968.2009.01514.x

Ma, S., Ren, B., Mallick, H., Moon, Y. S., Schwager, E., Maharjan, S., et al. (2021). A statistical model for describing and simulating microbial community profiles. *PLoS Comput. Biol.* 17:e1008913. doi: 10.1371/journal.pcbi.1008913

Magdy Wasfy, R., Mbaye, B., Borentain, P., Tidjani Alou, M., Murillo Ruiz, M. L., Caputo, A., et al. (2023). Ethanol-producing Enterocloster bolteae is enriched in chronic hepatitis B-associated gut dysbiosis: a case–control Culturomics study. *Microorganisms* 11:2437. doi: 10.3390/microorganisms11102437

Malek, M., Ibragimov, R., Albrecht, M., and Baumbach, J. (2016). CytoGEDEVO—global alignment of biological networks with Cytoscape. *Bioinformatics* 32, 1259–1261. doi: 10.1093/bioinformatics/btv732

Mallick, H., Franzosa, E. A., Mclver, L. J., Banerjee, S., Sirota-Madi, A., Kostic, A. D., et al. (2019). Predictive metabolomic profiling of microbial communities using amplicon or metagenomic sequences. *Nat. Commun.* 10:3136. doi: 10.1038/s41467-019-10927-1

Markey, L., Shaban, L., Green, E. R., Lemon, K. P., Mecsas, J., and Kumamoto, C. A. (2018). Pre-colonization with the commensal fungus *Candida albicans* reduces murine susceptibility to *Clostridium difficile* infection. *Gut Microbes* 9, 497–509. doi: 10.1080/19490976.2018.1465158

Mohandas, S., Soma, V. L., Tran, T. D. B., Sodergren, E., Ambooken, T., Goldman, D. L., et al. (2020). Differences in gut microbiome in hospitalized immunocompetent vs. immunocompromised children, including those with sickle cell disease. *Front. Pediatr.* 8:446. doi: 10.3389/fped.2020.583446

Moretto, M. M., Harrow, D. I., Hawley, T. S., and Khan, I. A. (2015). Interleukin-12-producing CD103+ CD11b− CD8+ dendritic cells are responsible for eliciting gut intraepithelial lymphocyte response against encephalitozoon cuniculi. *Infect. Immun.* 83, 4719–4730. doi: 10.1128/iai.00820-15

Nie, K., Ma, K., Luo, W., Shen, Z., Yang, Z., Xiao, M., et al. (2021). *Roseburia intestinalis*: a beneficial gut organism from the discoveries in genus and species. *Front. Cell. Infect. Microbiol.* 11:757718. doi: 10.3389/fcimb.2021.757718

Oka, K., Osaki, T., Hanawa, T., Kurata, S., Sugiyama, E., Takahashi, M., et al. (2018). Establishment of an endogenous *Clostridium difficile* rat infection model and evaluation of the effects of *Clostridium butyricum* MIYAIRI 588 probiotic strain. *Front. Microbiol.* 9:1264. doi: 10.3389/fmicb.2018.01264

Pandit, L., Cox, L. M., Malli, C., D'Cunha, A., Rooney, T., Lokhande, H., et al. (2021). *Clostridium bolteae* is elevated in neuromyelitis optica spectrum disorder in India and shares sequence similarity with AQP4. *Neurol Neuroimmunol Neuroinflamm* 8:907. doi: 10.1212/NXI.0000000000000907

Paredes-Sabja, D., Shen, A., and Sorg, J. A. (2014). *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends Microbiol.* 22, 406–416. doi: 10.1016/j.tim.2014.04.003

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., et al. (2011). Scikit-learn: machine learning in python. *J. Mach. Learn. Res.* 12, 2825–2830.

Pensinger, D. A., Fisher, A. T., Dobrila, H. A., Van Treuren, W., Gardner, J. O., Higginbottom, S. K., et al. (2023). Butyrate differentiates permissiveness to Clostridioides difficile infection and influences growth of Diverse *C. difficile* isolates. *Infect. Immun.* 91:e0057022. doi: 10.1128/iai.00570-22

Pielou, E. C. (1966). The measurement of diversity in different types of biological collections. *J. Theor. Biol.* 13, 131–144. doi: 10.1016/0022-5193(66)90013-0

Polage, C. R., Gyorke, C. E., Kennedy, M. A., Leslie, J. L., Chin, D. L., Wang, S., et al. (2015). Overdiagnosis of *Clostridium difficile* infection in the molecular test era. *JAMA Intern. Med.* 175, 1792–1801. doi: 10.1001/jamainternmed.2015.4114

Poquet, I., Saujet, L., Canette, A., Monot, M., Mihajlovic, J., Ghigo, J.-M., et al. (2018). *Clostridium difficile* biofilm: Remodeling metabolism and cell surface to build a sparse and heterogeneously aggregated architecture. *Front. Microbiol.* 9:2084. doi: 10.3389/fmicb.2018.02084

Quensen, J. (2020). ggordiplots. Available at: https://github.com/jfq3/ggordiplots (Accessed 31 May 2021).

R Core Team (2023). R: A language and environment for statistical computing. Available at: https://www.R-project.org/

Resch, A., Rosenstein, R., Nerz, C., and Götz, F. (2005). Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Appl. Environ. Microbiol.* 71, 2663–2676. doi: 10.1128/AEM.71.5.2663-2676.2005

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Rossez, Y., Wolfson, E. B., Holmes, A., Gally, D. L., and Holden, N. J. (2015). Bacterial flagella: twist and stick, or dodge across the kingdoms. *PLoS Pathog.* 11:e1004483. doi: 10.1371/journal.ppat.1004483

Sangster, W., Hegarty, J. P., Schieffer, K. M., Wright, J. R., Hackman, J., Toole, D. R., et al. (2016). Bacterial and fungal microbiota changes Distinguish *C. difficile* infection from other forms of Diarrhea: results of a prospective inpatient study. *Front. Microbiol.* 7:789. doi: 10.3389/fmicb.2016.00789

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shivashankar, R., Khanna, S., Kammer, P. P., Scott Harmsen, W., Zinsmeister, A. R., Baddour, L. M., et al. (2014). Clinical predictors of recurrent *Clostridium difficile* infection in out-patients. *Aliment. Pharmacol. Ther.* 40, 518–522. doi: 10.1111/apt.12864

Sokol, H., Jegou, S., McQuitty, C., Straub, M., Leducq, V., Landman, C., et al. (2017). Specificities of the intestinal microbiota in patients with inflammatory bowel disease and *Clostridium difficile* infection. *Gut Microbes* 9, 55–60. doi: 10.1080/19490976.2017.1361092

Sørensen, T. J. (1948). *A method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of the vegetation on Danish commons*. København: I kommission hos E. Munksgaard.

Spearman Rank Correlation Coefficient (2008). *The concise Encyclopedia of statistics*, (New York, NY: Springer), 502–505.

Stewart, D. B., Wright, J. R., Fowler, M., McLimans, C. J., Tokarev, V., Amaniera, I., et al. (2019). Integrated meta-omics reveals a fungus-associated bacteriome and distinct functional pathways in Clostridioides difficile infection. *mSphere* 4:19. doi: 10.1128/mSphere.00454-19

Wemheuer, F., Taylor, J. A., Daniel, R., Johnston, E., Meinicke, P., Thomas, T., et al. (2020). Tax4Fun2: prediction of habitat-specific functional profiles and functional redundancy based on 16S rRNA gene sequences. *Environ Microbiome* 15:11. doi: 10.1186/s40793-020-00358-7

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. bioRxiv [Preprint], bioRxiv:762302.

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics* 30, 614–620. doi: 10.1093/bioinformatics/btt593

Zhang, T., Liu, Z., Zhao, J., Zhang, H., Chen, W., Li, H., et al. (2023). Multi-omics analysis of the biofilm forming mechanism of *Bifidobacterium longum*. *LWT* 188:115415. doi: 10.1016/j.lwt.2023.115415

Zuo, T., Wong, S. H., Cheung, C. P., Lam, K., Lui, R., Cheung, K., et al. (2018). Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in *Clostridium difficile* infection. *Nat. Commun.* 9:3663. doi: 10.1038/s41467-018-06103-6

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Genetic insights into gut microbiota and risk of prostatitis: a Mendelian randomization study

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Background: The dysbiosis of gut microbiota (GM) is considered a contributing factor to prostatitis, yet the causality remains incompletely understood.

Methods: The genome-wide association study (GWAS) data for GM and prostatitis were sourced from MiBioGen and FinnGen R10, respectively. In the two-sample Mendelian randomization (MR) analysis, inverse variance weighting (IVW), MR-Egger, weighted median, simple mode, weighted mode, and maximum likelihood (ML) methods were utilized to investigate the causal relationship between GM and prostatitis. A series of sensitivity analysis were conducted to confirm the robustness of the main results obtained from the MR analysis.

Results: According to the IVW results, genus *Sutterella* (OR: 1.37, 95% CI: 1.09–1.71, $p = 0.006$) and genus *Holdemania* (OR: 1.21, 95% CI: 1.02–1.43, $p = 0.028$) were associated with an increased risk of prostatitis. The phylum Verrucomicrobia (OR: 0.76, 95% CI: 0.58–0.98, $p = 0.033$) and genus *Parasutterella* (OR: 0.84, 95% CI: 0.70–1.00, $p = 0.045$) exhibited a negative association with prostatitis, indicating a potential protective effect. Sensitivity analysis showed that these results were not affected by heterogeneity and horizontal pleiotropy. Furthermore, the majority of statistical methods yielded results consistent with those of the IVW analysis.

Conclusions: In this study, we identified two GM taxon that might be protective against prostatitis and two GM taxon that could increase the risk of developing prostatitis. These findings could potentially provide a valuable theoretical basis for the future development of preventive and therapeutic strategies for prostatitis.

KEYWORDS

prostatitis, gut microbiota, Mendelian randomization, causal relationship, genome-wide association study

1 Introduction

Prostatitis, characterized by inflammation or swelling of the prostate gland, is a disease predominantly affecting adult males of all age groups and emerges as the most common urological diagnosis among men under the age of 50 (Yebes et al., 2023). Approximately half of all men are estimated to encounter symptoms of prostatitis at some stage in their lives (Naber and Weidner, 2000). Among all prostatitis cases, more than 90%—95% are diagnosed as chronic nonbacterial prostatitis (CNP), representing 25% of all visits to urological clinics worldwide (Khan et al., 2017). Moreover, a study involving 3,000 patients aged 20–59 revealed that prostatitis patients are increasingly younger, with 78%

of them under the age of 40, and the prevalence of the condition rises with age (Liang et al., 2004). Patients with chronic prostatitis (CP) often manifest symptoms such as pelvic pain, voiding dysfunction, sexual disorders, and psychosocial distress (Verze et al., 2016). The clinical management of CP commonly involves the use of antibiotics, alpha-blockers, phytotherapy, and hormonal therapy (Khan et al., 2017). Nevertheless, treating CP poses a significant challenge for urologists due to its controversial etiology. In addition, the application of these traditional medicines can increase the risk of gastrointestinal problems, allergies and kidney damage (Heta and Robo, 2018). Therefore, there is a demand for innovative therapeutic strategies beyond conventional medicines.

The intestine serves as the primary site for digestion and absorption, while also functioning as the largest immune organ (Thomson et al., 2020). The gut microbiota (GM) is a diverse group of microorganisms that reside in the human gastrointestinal tract, comprising about 40 trillion microbes representing over 1,000 microbial species. Recent studies have extensively explored the association between GM and human diseases (Illiano et al., 2020). Its crucial role in maintaining both local and systemic tissue homeostasis has garnered recognition (Gensollen et al., 2016). Moreover, the metabolic by-products and ligands produced by gut commensal bacteria possess the capacity to regulate and fine-tune the innate and adaptive immune system's development and functionality, thereby aiding in defense against infections induced by diverse pathogens (Abt et al., 2012).

The concept of the gut-prostate axis was initially introduced in 2005, indicating the close relationship between the prostate and the intestine, particularly during the treatment of prostatitis (Abascal et al., 2005). Shoskes et al. (2016a,b) reported that the GM of CP patients exhibited lower alpha diversity and higher counts of *Varibaculum*. Additionally, Konkol et al. (2019) observed an increase in Odoribacter, Clostridiaceae, and Rikenellaceae, along with a decrease in Lactobacillus, Lachnospiraceae, and Bacteroides uniformis in the GM of rats with CP.

To date, however, the existence of a causal relationship between GM and prostatitis remains uncertain due to potential confounding factors and the possibility of reverse causation. Mendelian randomization (MR) presents as an emerging genetic epidemiological approach that uses summary data from genome-wide association study (GWAS) as instrumental variables to statistically deduce causality from an exposure to an outcome (Smith and Ebrahim, 2003). The MR approach shares a conceptual similarity with randomized controlled trials, differing only in that patients are allocated based on their DNA genotypes. In addition, the MR approach can overcome the methodological constraints of conventional observational studies, rendering the results more robust and reliable (Emdin et al., 2017). As the number of large-scale GWASs increases rapidly and more data is available, the statistical power of MR analysis is improving significantly (Kurilshikov et al., 2021; Verma et al., 2024).

In this study, GM taxa were selected as the exposure and prostatitis as the outcome for MR analysis, aiming to investigate the causal relationship and lay a theoretical basis for further exploration of the mechanisms underlying prostatitis. Furthermore, understanding the role of specific GM taxa and their impact on prostatitis has the potential to advance biomarker identification and precision medicine for this disease.

2 Methods

2.1 Study design and assumptions of MR

To assess the causal links between gut microbiota and prostatitis, a two-sample MR analysis was conducted. The flowchart of the MR analysis is shown in Figure 1. Additionally, the MR analysis met the following three assumptions in order to minimize bias and produce reliable results: (1) Correlation assumption: the selected IVs are closely related to gut microbiota taxa; (2) Independence assumption: the IVs and confounding factors were independent of each other; (3) Exclusive assumption: the included IVs affected prostatitis only through GM taxa (Davies et al., 2018).

2.2 Data sources and selection of IVs

2.2.1 Exposure data of GM

Kurilshikov et al. (2021) evaluated the relationship between genetic variation and GM by conducting 16S rRNA gene sequencing profiles and gathering genotyping data from 18,340 individuals, based on the MiBioGen consortium (https://mibiogen.gcc.rug.nl/). All the participants of the MiBioGen consortium was of European ancestry, sourced from 25 cohorts in 11 different nations. By examining 211 taxa (phylum, class, order, family, and genus), the GWAS study obtained 122,110 variant sites, which allowed for the analysis of GM taxonomic variance among various populations. In this study, the lowest taxonomic level was genus.

A comprehensive inspection of quality was conducted out on the SNPs in order to obtain reliable Instrumental Variables (IVs) that would ensure data robustness and results accuracy. (1) Selection of IVs at a significance level of $p < 1 \times 10^{-5}$ was implemented to ensure a comprehensive outcome (Jia et al., 2019; Lv et al., 2021). (2) To diminish linkage disequilibrium (LD) among the SNPs, LD-clumping was performed ($r^2 < 0.001$, window size = 10,000 kb) on all IVs. SNPs that did not adhere to the assumption were removed. (3) The *F*-statistic for each bacterial taxa was calculated to assess the strength of the Ivs ($F = \beta^2/se^2$) (Liu et al., 2023). To minimize weak instrument bias, IVs with *F* statistic <10 were excluded (Burgess et al., 2011).

2.2.2 Outcome data of prostatitis

The GWAS summary statistics for prostatitis were sourced from FinnGen Release10 (https://r10.finngen.fi/). Samples gathered by the Finnish Biological Bank's National Network were used in the FinnGen study. Genomic data was integrated with prostatitis data, comprising 4,160 cases and 130,139 controls, involving a total of 21,311,942 SNPs. The study's participants were all of European ancestry. Prostatitis diagnosis was based on the International Classification of Diseases (ICD) codes. Specifically, in ICD-10, it is denoted as N41, and in ICD-9, as 601.

In order to avoid the impact of alleles on the association between GM and prostatitis, exposure data and outcome data were harmonized by removing palindrome SNPs.

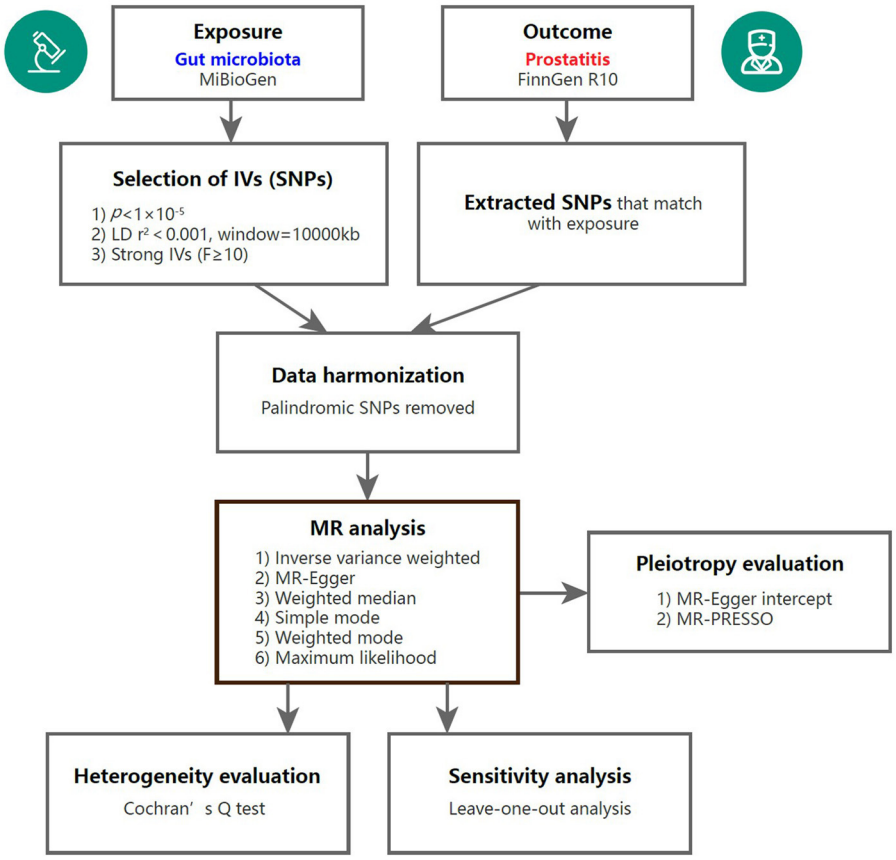


FIGURE 1
Overview of the MR analysis process. IV, instrumental variable; SNP, single nucleotide polymorphism; MR, Mendelian randomization; MR-PRESSO, Mendelian randomization pleiotropy residual sum and outlier.

2.3 Statistical analysis

All statistical analysis was performed using the "TwoSampleMR" package and the "MR-PRESSO" package in R (version 4.3.2). The "forest" package was used for drawing the forest plot. Some figures have been optimized to enhance clarity of display (Chen et al., 2022). Results with $p < 0.05$ were considered statistically significant.

2.3.1 MR analysis

The association between GM and prostatitis was assessed using six statistical methods: inverse variance weighted (IVW), MR-Egger, weighted median, simple mode, weighted mode and maximum likelihood (ML). IVW was used as the main analysis due to its high statistical power (Hemani et al., 2018). MR-Egger, weighted median, simple mode and weighted mode were applied as additional methods for MR analysis (Bowden et al., 2015, 2016). Furthermore, the ML was applied due to its minimal bias, particularly in small sample sizes. To determine the size of the causal effect, odds ratio (OR) and 95% confidence intervals (CI) were calculated.

2.3.2 Sensitivity analysis

Heterogeneity was assessed through Cochrane's *Q*-test and *Q*-values with a $p < 0.05$ indicated the presence of heterogeneity. The global test from the MR-PRESSO estimator and the MR-Egger intercept method were used to assess pleiotropy, with $p < 0.05$ in IVs being considered as evidence of horizontal pleiotropy. To further increase the overall robustness of our results, the leave-one-out analysis was conducted to identify any significant link influenced by a single SNP (Xiang et al., 2021).

When the IVW result was statistically significant and there was no horizontal pleiotropy or heterogeneity, the GM taxa was thought to be associated with a higher risk of prostatitis.

3 Results

3.1 Selection of IVs related to gut microbiota

After LD-clumping and palindromic SNPs removal, 2,740 SNPs were finally identified as IVs related to 211 GM taxa for prostatitis ($p < 1 \times 10^{-5}$). All SNPs demonstrated sufficient validity (*F* statistic ranged from 14.59 to 88.43, all $F > 10$), indicating that the estimation of causal effects in our study was unlikely to be

Microbiota	Methods	nSNP	OR (95%CI)	p
Sutterella (genus)	Inverse variance weighted	12	1.37 (1.09−1.71)	0.006
	Maximum likelihood	12	1.38 (1.09−1.73)	0.007
	MR Egger	12	2.94 (1.12−7.73)	0.053
	Simple mode	12	1.45 (0.81−2.60)	0.232
	Weighted median	12	1.42 (1.02−1.97)	0.037
	Weighted mode	12	1.46 (0.83−2.58)	0.219
Holdemania (genus)	Inverse variance weighted	15	1.21 (1.02−1.43)	0.028
	Maximum likelihood	15	1.22 (1.03−1.44)	0.022
	MR Egger	15	1.24 (0.73−2.11)	0.449
	Simple mode	15	1.35 (0.92−1.98)	0.152
	Weighted median	15	1.22 (0.97−1.54)	0.097
	Weighted mode	15	1.34 (0.95−1.89)	0.116
Verrucomicrobia (phylum)	Inverse variance weighted	12	0.76 (0.58−0.98)	0.033
	Maximum likelihood	12	0.76 (0.61−0.94)	0.010
	MR Egger	12	0.93 (0.46−1.87)	0.845
	Simple mode	12	1.05 (0.59−1.86)	0.880
	Weighted median	12	0.86 (0.63−1.15)	0.306
	Weighted mode	12	1.02 (0.64−1.61)	0.950
Parasutterella (genus)	Inverse variance weighted	14	0.84 (0.70−1.00)	0.045
	Maximum likelihood	14	0.84 (0.70−1.00)	0.055
	MR Egger	14	0.92 (0.57−1.50)	0.753
	Simple mode	14	0.81 (0.55−1.19)	0.306
	Weighted median	14	0.88 (0.69−1.12)	0.299
	Weighted mode	14	0.89 (0.63−1.25)	0.509

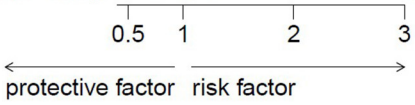


FIGURE 2
MR results of casual links between specific gut microbiota taxa and prostatitis risk. nSNP, number of single nucleotide polymorphism.

influenced by weak IVs. The key information of selected IVs was detailed in Supplementary Table S1.

3.2 Results of Mendelian randomization analysis

Following MR analysis, we obtained insights into the correlation between 211 GM taxa and prostatitis, with detailed data presented in Supplementary Table S2. Based on the results, we identified four GM taxa associated with the risk of prostatitis, comprising one phylum and four genera (Figure 2). At the biological phylum classification level, the IVW method results indicated that Verrucomicrobia exhibited an association with a reduced risk of prostatitis (OR: 0.76, 95% CI: 0.58–0.98, $p = 0.033$). Moving to the biological genus classification level, the MR estimates from the IVW method revealed that *Sutterella* and *Holdemania* were positively correlated with the risk of prostatitis (OR: 1.37, 95% CI: 1.09–1.71, $p = 0.006$ for *Sutterella*; OR: 1.21, 95% CI: 1.02–1.43, $p = 0.028$ for *Holdemania*), while *Parasutterella* exhibited a negative association with the risk of prostatitis (OR: 0.84, 95% CI: 0.70–1.00, $p = 0.045$). In the MR analysis of three genera, the direction and effect size of other five estimators were similar to IVW (Figure 2).

In both the *Sutterella* and *Holdemania* groups, scatter plots showed that an increased SNP effect was related to a higher probability of prostatitis (Figure 3). In addition, a decreased incidence of prostatitis was associated with larger SNP effects in the *Parasutterella* group. The fitted lines of the scatter plot for the Verrucomicrobia group showed inconsistent directions for several estimators. The weighted mode and simple mode showed a positive correlation, but the remaining estimators revealed a negative correlation.

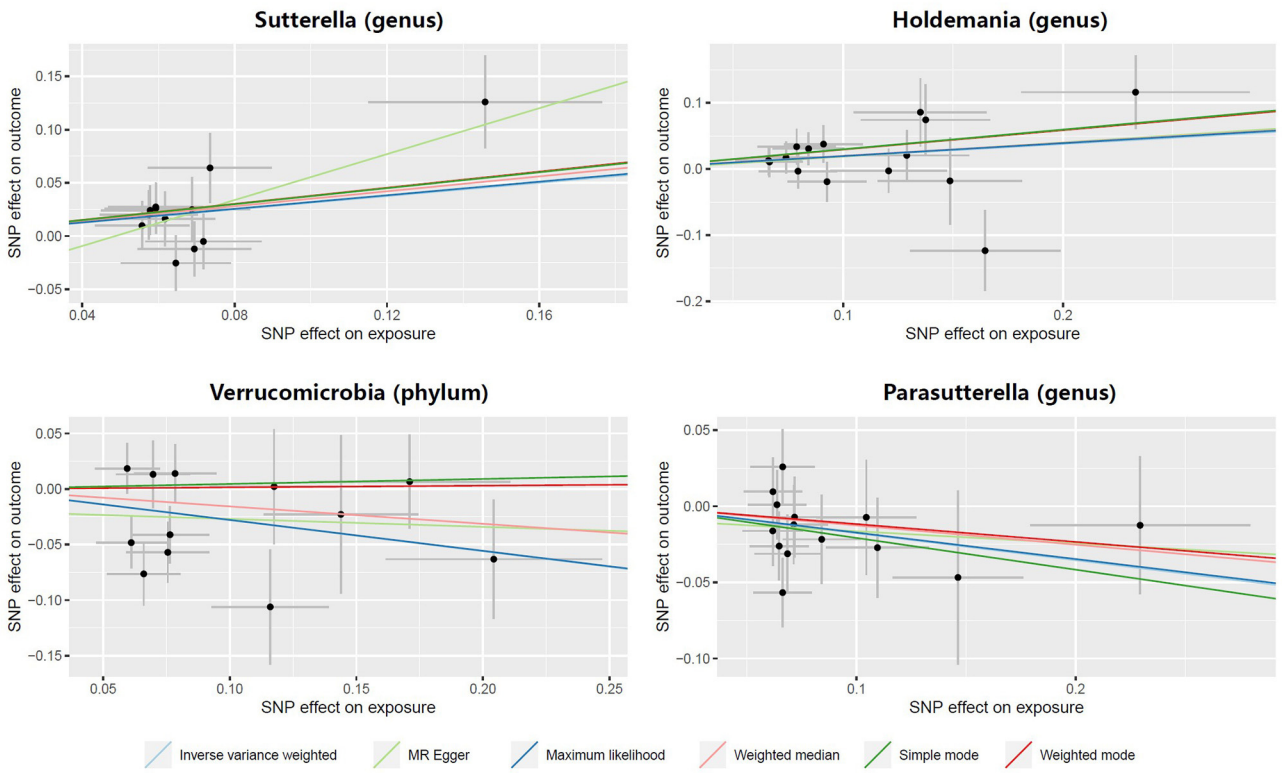


FIGURE 3
Scatter plots illustrating the causal effect of gut microbiota on prostatitis.

3.3 Results of sensitivity analysis

After sensitivity analysis, the effect of accurate MR results on prostatitis in one phylum and three genera was confirmed. Egger test showed no significant horizontal pleiotropy in Verrucomicrobia (phylum, $p = 0.539$), *Sutterella* (genus, $p = 0.141$), *Holdemania* (genus, $p = 0.927$) and *Parasutterella* (genus, $p = 0.677$) for prostatitis (Table 1). Moreover, MR-PRESSO was used to further verify the statistically significant MR results to ensure the reliability of MR Egger regression. The results of global test confirmed the absence of horizontal pleiotropy in Verrucomicrobia ($p = 0.106$), *Sutterella* ($p = 0.410$), *Holdemania* ($p = 0.427$) and *Parasutterella* ($p = 0.766$) groups.

Meanwhile, there was no evidence of heterogeneity in Verrucomicrobia (IVW: $p = 0.085$; MR Egger: $p = 0.071$), *Sutterella* (IVW: $p = 0.417$; MR Egger: $p = 0.554$), *Holdemania* (IVW: $p = 0.389$; MR Egger: $p = 0.318$) and *Parasutterella* (IVW: $p = 0.760$; MR Egger: $p = 0.704$) for prostatitis (Table 2). The results of leave-one-out analysis further validated the robustness of the data (Figure 4). The IVW results were considered reliable in the absence of pleiotropy and heterogeneity. Thus, Verrucomicrobia (phylum), *Sutterella* (genus), *Holdemania* (genus), and *Parasutterella* (genus) were identified as causally related to the risk of prostatitis.

4 Discussion

Prostatitis is a major health problem plaguing adult men worldwide, and it can lead to a serious decline in patients' quality of life or even induce psychological disorders. Meanwhile, the etiology and pathological mechanisms of prostatitis are complex and heterogeneous (Maeda et al., 2023). Liu et al. (2022) attempted to explore the pathogenesis behind CP and conducted a multi-omics analysis using a rat model, which ultimately verified the existence of the gut-prostate axis. The GM, as the second human genome, influencing host epigenetics through multiple mechanisms, including DNA methylation programming. It has garnered significant attention in recent years for its role in the pathogenesis of various diseases (Human Microbiome Jumpstart Reference Strains Consortium et al., 2010). Researches have revealed its significant influence on the human immune system through mechanisms such as increasing immune cell populations, synthesizing short-chain fatty acids (SCFAs), enhancing oral tolerance, and modulating inflammation (Samuelson et al., 2015). Differences in GM abundance between prostatitis patients and healthy controls have been observed in numerous studies, although findings across current experiments remain inconsistent (Shoskes et al., 2016a,b; Konkol et al., 2019; Liu et al., 2021). To the best of our knowledge, this is the first MR analysis utilizing genetic data

TABLE 1 Assessment of horizontal pleiotropy in the casual association.

Microbiota	nSNP	*F*-statistics	*p*-values (Global test)	Intercept (Egger test)	*p*-values (Egger test)
Sutterella (genus)	12	21.05	0.410	−0.053	0.141
Holdemania (genus)	15	21.86	0.427	−0.002	0.927
Verrucomicrobia (phylum)	12	21.96	0.106	−0.020	0.539
Parasutterella (genus)	14	22.06	0.766	−0.009	0.677

TABLE 2 Assessment of heterogeneity using Cochrane's *Q*-test.

Microbiota	*Q*-values		*p*-values	
	IVW	MR Egger	IVW	MR Egger
Sutterella (genus)	11.315	8.766	0.417	0.554
Holdemania (genus)	14.841	14.830	0.389	0.318
Verrucomicrobia (phylum)	17.843	17.149	0.085	0.071
Parasutterella (genus)	9.173	8.991	0.760	0.704

IVW, inverse variance weighted.

to investigate a potential causal relationship between the GM and prostatitis.

In earlier reports, higher levels of Odoribacter, Clostridiaceae, Varibaculum, Rikenellaceae, Peptococcaceae, and Allobaculum were observed in the intestines of individuals with chronic nonbacterial prostatitis as well as in rat models, with a concomitant decrease in Lactobacillus, Bacteroides, and Lachnospiraceae (Shoskes et al., 2016a,b; Konkol et al., 2019). Following this, in a multi-omics analysis conducted on a rat model of inflammatory prostate, Liu et al. found an increase in the relative abundances of Bacteroidales, Corynebacteriaceae, Corynebacterium, Prevotella, Prevotellaceae, and Rikenellaceae RC9, alongside a decrease in the relative abundance of Firmicutes, Bacilli, Coprococcus, Clostridiales, Clostridia, Turicibacteraceae, and Lachnospiracea (Liu et al., 2021, 2022). In particular, the variation in abundance of Bacteroidales and Firmicutes is significant. It is evident that there exists substantial heterogeneity in the outcomes of these studies, with some even presenting conflicting conclusions. We believe that this may stem from differences in species/ethnicity among subjects, modeling methodologies, and microbial examination techniques. This study provides insight from a genetic standpoint, indicating that increased abundance of *Sutterella* and *Holdemania* poses a risk for prostatitis, while Verrucomicrobia and *Parasutterella* might offer protective effects against the development of the condition. The research findings regarding *Sutterella* and *Parasutterella* are consistent with a recent study (Shen et al., 2024). Notably, the other two types of GM taxon have received little attention in previously reported studies on the subject.

Members of the genus *Sutterella*, as prevalent symbionts in the gastrointestinal tract, demonstrate an ability to adhere to intestinal mucosal epithelial cells. *In vitro* experiments have indicated their role in maintaining intestinal barrier function while exhibiting a mild pro-inflammatory capacity (Hiippala et al., 2016). During co-culture with intestinal epithelial cells, members of the genus *Sutterella* have been observed to induce the production of IL-8, which is considered a potential pro-inflammatory mechanism (Kaakoush, 2020). Furthermore, a theoretical viewpoint posits that *Sutterella* may compromise the effectiveness of the intestine antibacterial immune response, specifically by limiting the ability to contain intracellular bacteria, rather than directly inducing inflammation (Hansen et al., 2019). The genus *Holdemania* belongs to the phylum Firmicutes and is a relatively atypical genus. It can be found in various environments, including the gastrointestinal tract of animals. Some observational studies have also suggested a significant increase in the abundance of *Holdemania* in the intestines of patients with various inflammatory diseases such as hepatitis, neuroinflammation, and pulmonary inflammation (Zhang et al., 2023). It is speculated that changes in its abundance may affect the integrity of the intestinal barrier, facilitating the translocation of microbial products into the bloodstream, thereby triggering inflammatory responses. However, the actual role of such bacterium in the pathogenesis of prostatitis remains unexplored in research reports. The phylum Verrucomicrobia encompasses various beneficial intestinal bacteria, whose outer membrane proteins can protect interactions between cells. Members of the phylum Verrucomicrobia are believed to play a significant role in enhancing host immune responses and metabolic functions (van Niftrik and Devos, 2017). Research revealed that their metabolic products may be involved in the reduction of the inflammatory factor TNF-α and its membrane receptor TNF-R2, which may be related to the protective capacity of the phylum Verrucomicrobia against prostatitis. (Zhang et al., 2022). Regarding the genus *Parasutterella*, its bacterial metabolites haloperidol glucuronide and PPs 7-keto deoxycholic acid are considered important signaling molecules in the gut-prostate axis, promoting remission of chronic non-bacterial prostatitis in rats (Yu et al., 2022). Another study found a positive correlation between an elevation in the abundance of *Parasutterella* and serum testosterone levels (Chu et al., 2020). These microorganisms indirectly mediate the host's immune-inflammatory response by modulating hormone synthesis. Such effects have also been observed in the chronic intestinal inflammation of patients with irritable bowel syndrome (Chen et al., 2018).

While this study has shed light on the role of the gut microbiota in the pathogenesis of prostatitis, it is equally important to pay attention to the local microbial community within the urogenital tract. Wu et al. evaluated the microbiota composition in the urethral secretions and expressed prostatic secretions (EPS) of 33 male patients with CP and compared them with samples from

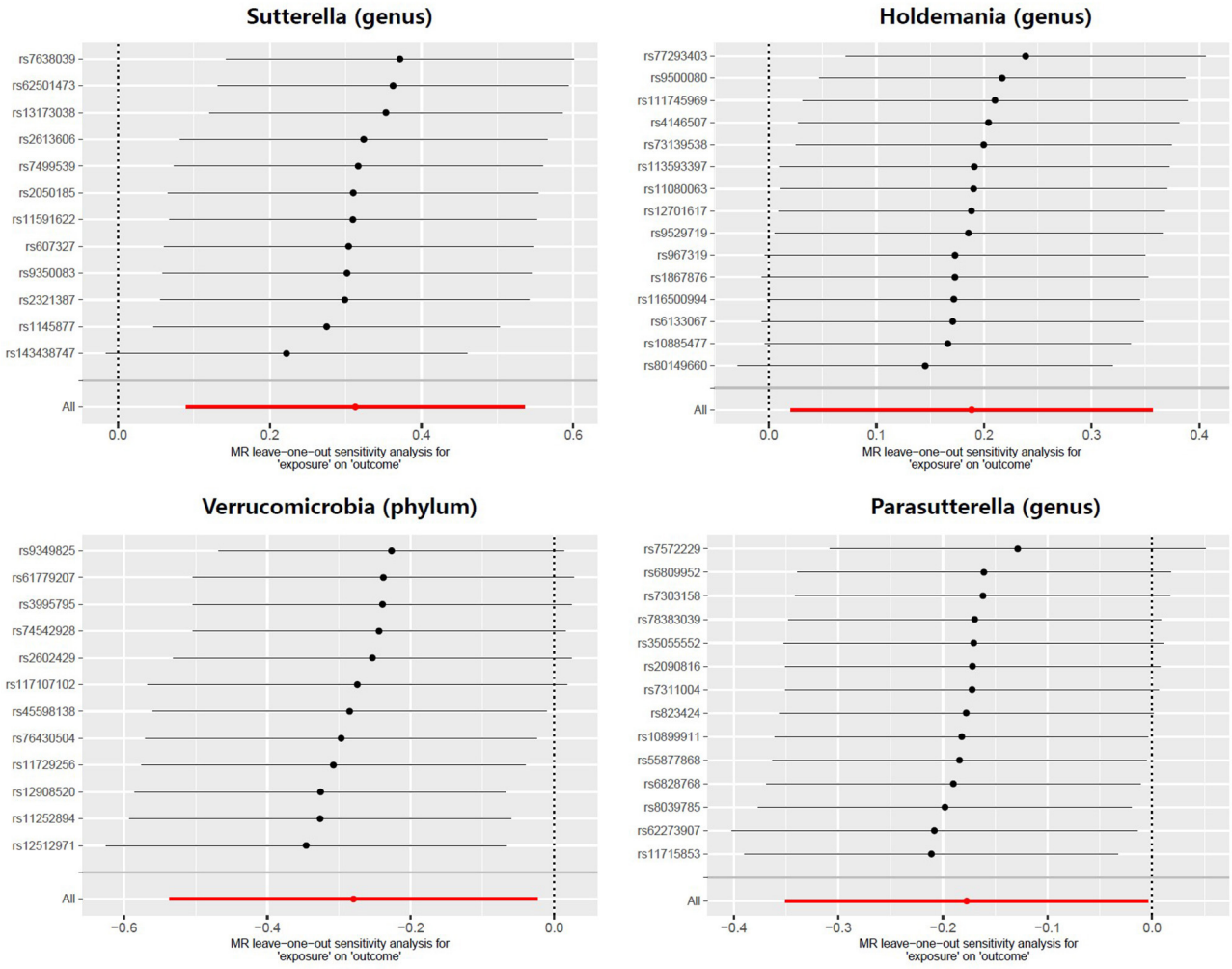


FIGURE 4
Leave-one-out analysis for the effect of individual SNPs on the correlation between the GM taxa and the risk of prostatitis. The horizontal axis represents the effect of the GM on the risk of prostatitis after excluding individual SNP. The vertical axis represents the individual SNP that have been excluded. The red line represents the overall effect of all SNPs. The dots represent the odds ratio values, the horizontal lines represent the 95% confidence intervals.

30 healthy individuals. The results revealed significant differences between the experimental and control groups (Wu et al., 2020). Similarly, Shoskes et al. (2016a) confirmed that in patients with CP, 17 GM taxon, including Clostridia and Bacteroidia, were over-represented, while five were under-represented. Streptococcus, Escherichia, and Pseudomonas are considered common local genera implicated in benign diseases, including chronic prostatitis. This may be related to their greater involvement in prostatic metabolism (Mjaess et al., 2023). It is noteworthy that microbial migration may also be a potential mechanism. Some researchers speculate, based on the similarity between the fecal and seminal microbiota of patients with CP, that live bacteria may migrate from the intestine to the genitourinary tract, becoming part of the local microbiota (Wang et al., 2023).

The metabolites and bioactive factors produced by GM may also act as exacerbators or suppressors of deleterious changes triggered by the local microbiome, thereby exerting an impact on prostatitis. For instance, *Sutterella* has been identified as a driving factor in the progression of diabetes, which, due to its weakening effect on immune function, may serve as a promoter of inflammation initiated by local pathogens in the prostate (Liu and Dong, 2024).

Additionally, while viruses, fungi, and archaea did not yield positive results in this study, their role as minor constituents of the gut microbiota in potentially regulating prostatic metabolism cannot be overlooked and requires further clarification. This study has several noteworthy strengths. Firstly, unlike previous research where stool samples were collected post-episode, this study establishes the temporal sequence between GM colonization and the onset of prostatitis. MR analysis is a method for exploring causality because it eliminates potential confounders and reverse causality, helping to improve the stability of our results. Secondly, the genetic variation of GM taxa utilized in the study was derived from the largest available GWAS meta-analysis, ensuring the strength and reliability of the instrumental variables selected in the MR analysis. Thirdly, any potential horizontal pleiotropy

was identified and addressed through the use of MR-Egger regression intercepts and MR-PRESSO tests. Sensitivity analysis was conducted to assess the robustness of the results and provide statistical evidence of potential bias.

When interpreting the findings of this study, several limitations need to be considered in account. First of all, the causal relationships between GM and prostatitis at the species level could not be identified because the exposure dataset only included data at the genus taxonomic level. Species and subspecies within any genus may exert different or even opposite effects on host metabolism. In Wang et al.'s study on the pathogenic microbial community of prostatitis, although Gammaproteobacteria and Betaproteobacteria both belong to Proteobacteria, they exhibit opposite distribution trends and clinical significance (Wang et al., 2023). Therefore, it is necessary to conduct more comprehensive research on the impact of species within genera on prostatic metabolism. Secondly, the categorization of prostatitis subtypes was not available within the FinnGen consortium database, which limited our ability to perform subgroup analyses. The etiology, pathogens, pathogenesis, and clinical features of prostatitis vary among different types. Further subgroup analysis based on specific subtypes may yield more accurate results. Thirdly, population stratification may still cause interference even if the bulk of participants in the GWAS meta-analysis for both GM data and prostatitis data were of European ancestry. The generalization of the present findings to other ethnic groups was constrained. Future MR research on this subject should be conducted in populations outside of Europe to reconfirm the results. Finally, we were unable to perform correlation analysis of GM abundance between individuals with prostatitis and healthy controls.

5 Conclusions

The results of this two-sample MR study indicate that Verrucomicrobia and *Parasutterella* may be protective against prostatitis, whereas *Sutterella* and *Holdemania* may be positively associated with the risk of prostatitis. Further studies are needed to more comprehensively understand the possible beneficial or harmful effects of these GM taxa on prostatitis as well as the underlying mechanisms. Confirming the possible effect of prostatitis on GM is also important, even though there isn't much evidence to support it.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

PQ: Data curation, Investigation, Writing – original draft. YH: Investigation, Project administration, Writing – review & editing. HS: Formal analysis, Software, Visualization, Writing – review & editing. DJ: Conceptualization, Methodology, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1389715/full#supplementary-material

References

Abascal, K., Ganora, L., and Yarnell, E. (2005). The effect of freeze-drying and its implications for botanical medicine: a review. *Phytother. Res.* 19, 655–660. doi: 10.1002/ptr.1651

Abt, M. C., Osborne, L. C., Monticelli, L. A., Doering, T. A., Alenghat, T., Sonnenberg, G. F., et al. (2012). Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity* 37, 158–170. doi: 10.1016/j.immuni.2012.04.011

Bowden, J., Davey Smith, G., and Burgess, S. (2015). Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int. J. Epidemiol.* 44, 512–525. doi: 10.1093/ije/dyv080

Bowden, J., Davey Smith, G., Haycock, P. C., and Burgess, S. (2016). Consistent estimation in Mendelian randomization with some invalid instruments using a weighted median estimator. *Genet. Epidemiol.* 40, 304–314. doi: 10.1002/gepi.21965

Burgess, S., Thompson, S. G., and CRP CHD Genetics Collaboration (2011). Avoiding bias from weak instruments in Mendelian randomization studies. *Int. J. Epidemiol.* 40, 755–764. doi: 10.1093/ije/dyr036

Chen, T., Liu, Y., and Huang, L. (2022). ImageGP: an easy-to-use data visualization web server for scientific researchers. *iMeta* 1:e5. doi: 10.1002/imt2.5

Chen, Y. J., Wu, H., Wu, S. D., Lu, N., Wang, Y. T., Liu, H. N., et al. (2018). *Parasutterella*, in association with irritable bowel syndrome and intestinal chronic inflammation. *J. Gastroenterol. Hepatol.* 33, 1844–1852. doi: 10.1111/jgh.14281

Chu, W., Zhai, J., Xu, J., Li, S., Li, W., Chen, Z. J., et al. (2020). Continuous light-induced PCOS-like changes in reproduction, metabolism, and gut microbiota in Sprague-Dawley rats. *Front. Microbiol.* 10:3145. doi: 10.3389/fmicb.2019.03145

Davies, N. M., Holmes, M. V., and Davey Smith, G. (2018). Reading Mendelian randomisation studies: a guide, glossary, and checklist for clinicians. *BMJ* 362:k601. doi: 10.1136/bmj.k601

Emdin, C. A., Khera, A. V., and Kathiresan, S. (2017). Mendelian randomization. *JAMA* 318, 1925–1926. doi: 10.1001/jama.2017.17219

Gensollen, T., Iyer, S. S., Kasper, D. L., and Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science* 352, 539–544. doi: 10.1126/science.aad9378

Hansen, I. S., Baeten, D. L. P., and den Dunnen, J. (2019). The inflammatory function of human IgA. *Cell. Mol. Life Sci.* 76, 1041–1055. doi: 10.1007/s00018-018-2976-8

Hemani, G., Zheng, J., Elsworth, B., Wade, K. H., Haberland, V., Baird, D., et al. (2018). The MR-base platform supports systematic causal inference across the human phenome. *Elife* 7:e34408. doi: 10.7554/eLife.34408.012

Heta, S., and Robo, I. (2018). The side effects of the most commonly used group of antibiotics in periodontal treatments. *Med. Sci.* 6:6. doi: 10.3390/medsci6010006

Hiippala, K., Kainulainen, V., Kalliomäki, M., Arkkila, P., and Satokari, R. (2016). Mucosal prevalence and interactions with the epithelium indicate commensalism of *Sutterella* spp. *Front. Microbiol.* 7:1706. doi: 10.3389/fmicb.2016.01706

Human Microbiome Jumpstart Reference Strains Consortium, Nelson, K. E., Weinstock, G. M., Highlander, S. K., Worley, K. C., Creasy, H. H., et al. (2010). A catalog of reference genomes from the human microbiome. *Science* 328, 994–999. doi: 10.1126/science.1183605

Illiano, P., Brambilla, R., and Parolini, C. (2020). The mutual interplay of gut microbiota, diet and human disease. *FEBS J.* 287, 833–855. doi: 10.1111/febs.15217

Jia, J., Dou, P., Gao, M., Kong, X., Li, C., Liu, Z., et al. (2019). Assessment of causal direction between gut microbiota-dependent metabolites and cardiometabolic health: a bidirectional Mendelian randomization analysis. *Diabetes* 68, 1747–1755. doi: 10.2337/db19-0153

Kaakoush, N. O. (2020). *Sutterella* species, IgA-degrading bacteria in ulcerative colitis. *Trends Microbiol.* 28, 519–522. doi: 10.1016/j.tim.2020.02.018

Khan, F. U., Ihsan, A. U., Khan, H. U., Jana, R., Wazir, J., Khongorzul, P., et al. (2017). Comprehensive overview of prostatitis. *Biomed. Pharmacother.* 94, 1064–1076. doi: 10.1016/j.biopha.2017.08.016

Konkol, Y., Keskitalo, A., Vuorikoski, H., Pietilä, S., Elo, L. L., Munukka, E., et al. (2019). Chronic nonbacterial prostate inflammation in a rat model is associated with changes of gut microbiota that can be modified with a galactoglucomannan-rich hemicellulose extract in the diet. *BJU Int.* 123, 899–908. doi: 10.1111/bju.14553

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., Demirkan, A., et al. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Liang, C. Z., Zhang, X. J., Hao, Z. Y., Yang, S., Wang, D. B., Shi, H. Q., et al. (2004). An epidemiological study of patients with chronic prostatitis. *BJU Int.* 94, 568–570. doi: 10.1111/j.1464-410X.2004.05002.x

Liu, J., Liu, L., Zhang, G., and Peng, X. (2021). Poria cocos polysaccharides attenuate chronic nonbacterial prostatitis by targeting the gut microbiota: comparative study of Poria cocos polysaccharides and finasteride in treating chronic prostatitis. *Int. J. Biol. Macromol.* 189, 346–355. doi: 10.1016/j.ijbiomac.2021.08.139

Liu, J., Wang, Y., Zhang, G., Liu, L., and Peng, X. (2022). Multi-omics analysis reveals changes in the intestinal microbiome, transcriptome, and methylome in a rat model of chronic non-bacterial prostatitis: indications for the existence of the gut-prostate axis. *Front. Physiol.* 12:753034. doi: 10.3389/fphys.2021.753034

Liu, M., Zhang, Y., Wu, J., Gao, M., Zhu, Z., Chen, H., et al. (2023). Causal relationship between kidney stones and gut microbiota contributes to the gut-kidney axis: a two-sample Mendelian randomization study. *Front. Microbiol.* 14:1204311. doi: 10.3389/fmicb.2023.1204311

Liu, X., and Dong, Q. (2024). Associations between gut microbiota and three prostate diseases: a bidirectional two-sample Mendelian randomization study. *Sci. Rep.* 14:4019. doi: 10.1038/s41598-024-54293-5

Lv, W. Q., Lin, X., Shen, H., Liu, H. M., Qiu, X., Li, B. Y., et al. (2021). Human gut microbiome impacts skeletal muscle mass via gut microbial synthesis of the short-chain fatty acid butyrate among healthy menopausal women. *J. Cachexia Sarcopenia Muscle* 12, 1860–1870. doi: 10.1002/jcsm.12788

Maeda, K., Shigemura, K., and Fujisawa, M. (2023). A review of current treatments for chronic prostatitis/chronic pelvic pain syndrome under the UPOINTS system. *Int. J. Urol.* 30, 431–436. doi: 10.1111/iju.15149

Mjaess, G., Karam, A., Roumeguère, T., Diamand, R., Aoun, F., McVary, K., et al. (2023). Urinary microbiota and prostatic diseases: the key for the lock? A systematic review. *Prostate Cancer Prostatic Dis.* 26, 451–460. doi: 10.1038/s41391-022-00602-w

Naber, K. G., and Weidner, W. (2000). Chronic prostatitis-an infectious disease? *J. Antimicrob. Chemother.* 46, 157–161. doi: 10.1093/jac/46.2.157

Samuelson, D. R., Welsh, D. A., and Shellito, J. E. (2015). Regulation of lung immunity and host defense by the intestinal microbiota. *Front. Microbiol.* 6:1085. doi: 10.3389/fmicb.2015.01085

Shen, C., Chen, Z., Zhang, W., Chen, X., Zheng, B., Shi, C., et al. (2024). Preliminary study of the effect of gut microbiota on the development of prostatitis. *BMC Med. Genomics* 17:35. doi: 10.1186/s12920-024-01812-y

Shoskes, D. A., Altemus, J., Polackwich, A. S., Tucky, B., Wang, H., and Eng, C. (2016a). The urinary microbiome differs significantly between patients with chronic prostatitis/chronic pelvic pain syndrome and controls as well as between patients with different clinical phenotypes. *Urology* 92, 26–32. doi: 10.1016/j.urology.2016.02.043

Shoskes, D. A., Wang, H., Polackwich, A. S., Tucky, B., Altemus, J., Eng, C., et al. (2016b). Analysis of gut microbiome reveals significant differences between men with chronic prostatitis/chronic pelvic pain syndrome and controls. *J. Urol.* 196, 435–441. doi: 10.1016/j.juro.2016.02.2959

Smith, G. D., and Ebrahim, S. (2003). 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int. J. Epidemiol.* 32, 1–22. doi: 10.1093/ije/dyg070

Thomson, C. A., Nibbs, R. J., McCoy, K. D., and Mowat, A. M. (2020). Immunological roles of intestinal mesenchymal cells. *Immunology* 160, 313–324. doi: 10.1111/imm.13191

van Niftrik, L., and Devos, D. P. (2017). Editorial: planctomycetes-verrucomicrobia-chlamydiae bacterial superphylum: new model organisms for evolutionary cell biology. *Front. Microbiol.* 8:1458. doi: 10.3389/fmicb.2017.01458

Verma, S. S., Gudiseva, H. V., Chavali, V. R. M., Salowe, R. J., Bradford, Y., Guare, L., et al. (2024). A multi-cohort genome-wide association study in African ancestry individuals reveals risk loci for primary open-angle glaucoma. *Cell* 187, 464–480.e10. doi: 10.1016/j.cell.2023.12.006

Verze, P., Cai, T., and Lorenzetti, S. (2016). The role of the prostate in male fertility, health and disease. *Nat. Rev. Urol.* 13, 379–386. doi: 10.1038/nrurol.2016.89

Wang, S., Zang, M., Yang, X., Lv, L., Chen, L., Cui, J., et al. (2023). Gut microbiome in men with chronic prostatitis/chronic pelvic pain syndrome: profiling and its predictive significance. *World J. Urol.* 41, 3019–3026. doi: 10.1007/s00345-023-04587-6

Wu, Y., Jiang, H., Tan, M., and Lu, X. (2020). Screening for chronic prostatitis pathogens using high-throughput next-generation sequencing. *Prostate* 80, 577–587. doi: 10.1002/pros.23971

Xiang, K., Wang, P., Xu, Z., Hu, Y. Q., He, Y. S., Chen, Y., et al. (2021). Causal effects of gut microbiome on systemic lupus erythematosus: a two-sample Mendelian randomization study. *Front. Immunol.* 12:667097. doi: 10.3389/fimmu.2021.667097

Yebes, A., Toribio-Vazquez, C., Martinez-Perez, S., Quesada-Olarte, J. M., Rodriguez-Serrano, A., Álvarez-Maestro, M., et al. (2023). Prostatitis: a review. *Curr. Urol. Rep.* 24, 241–251. doi: 10.1007/s11934-023-01150-z

Yu, J., Hu, Q., Liu, J., Luo, J., Liu, L., Peng, X., et al. (2022). Metabolites of gut microbiota fermenting Poria cocos polysaccharide alleviates chronic nonbacterial prostatitis in rats. *Int. J. Biol. Macromol.* 209(Pt B), 1593–1604. doi: 10.1016/j.ijbiomac.2022.04.029

Zhang, Q., Zhou, J., Zhang, X., Mao, R., and Zhang, C. (2023). Mendelian randomization supports causality between gut microbiota and chronic hepatitis B. *Front. Microbiol.* 14:1243811. doi: 10.3389/fmicb.2023.1243811

Zhang, W., Zhang, S., Zhao, F., Du, J., and Wang, Z. (2022). Causal relationship between gut microbes and cardiovascular protein expression. *Front. Cell. Infect. Microbiol.* 12:1048519. doi: 10.3389/fcimb.2022.1048519

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Role of N-acetylkynurenine in mediating the effect of gut microbiota on urinary tract infection: a Mendelian randomization study

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Introduction: This study explored the causal connections between gut microbiota (GM), urinary tract infection (UTI), and potential metabolite mediators using Mendelian randomization (MR).

Methods: We utilized summary statistics from the most comprehensive and extensive genome-wide association studies (GWAS) available to date, including 196 bacterial traits for GM, 1,091 blood metabolites, 309 metabolite ratios, alongside UTI data from ukb-b-8814 and ebi-a-GCST90013890. Bidirectional MR analyses were conducted to investigate the causal links between GM and UTI. Subsequently, two MR analyses were performed to identify the potential mediating metabolites, followed by a two-step MR analysis to quantify the mediation proportion.

Results: Our findings revealed that out of the total 15 bacterial traits, significant associations with UTI risk were observed across both datasets. Particularly, taxon *g_Ruminococcaceae UCG010* displayed a causal link with a diminished UTI risk in both datasets (ukb-b-8814: odds ratio [OR] = 0.9964, 95% confidence interval [CI] = 0.9930–0.9997, P = 0.036; GCST90013890: OR = 0.8252, 95% CI = 0.7217–0.9436, P = 0.005). However, no substantial changes in *g_Ruminococcaceae UCG010* due to UTI were noted (ukb-b-8814: β = 0.51, P = 0.87; ebi-a-GCST90013890: β = −0.02, P = 0.77). Additionally, variations in 56 specific metabolites were induced by *g_Ruminococcaceae UCG010*, with N-acetylkynurenine (NAK) exhibiting a causal correlation with UTI. A negative association was found between *g_Ruminococcaceae UCG010* and NAK (OR: 0.8128, 95% CI: 0.6647–0.9941, P = 0.044), while NAK was positively associated with UTI risk (OR: 1.0009; 95% CI: 1.0002–1.0016; P = 0.0173). Mediation analysis revealed that the association between *g_Ruminococcaceae UCG010* and UTI was mediated by NAK with a mediation proportion of 5.07%.

Discussion: This MR study provides compelling evidence supporting the existence of causal relationships between specific GM taxa and UTI, along with potential mediating metabolites.

KEYWORDS

gut microbiota, urinary tract infection, blood metabolites, N-acetylkynurenine, Mendelian randomization analysis

1 Introduction

Urinary tract infection (UTI), a prevalent inflammatory condition affecting the urinary system, is primarily caused by uropathogenic *Escherichia coli* (UPEC) (Ji, 2005). The characteristics of the pathogen include high rates of incidence and recurrence, and increased antibiotic resistance (Tandogdu and Wagenlehner, 2016; Medina and Castillo-Pino, 2019; Faine et al., 2022). More than 50% of women experience at least one UTI during their lifetime (Foxman, 2014). Following antibiotic treatment, 20–30% of women experience a relapse within six months (Foxman, 2002), with approximately half of the recurrent strains derived from the initial infection (Silverman et al., 2013). Antibiotics, while effective in treating UTI, can also increase the risk of recurrence, potentially by causing disruptions in the GM (Köves et al., 2017). The existing understanding of its pathogenesis primarily focuses on the ascending infection pathway of intestinal bacteria. Pathogenic bacteria are excreted from the intestines into the feces, colonize around the urethra or vagina, and then ascend through the urethra to the bladder, triggering an infection. In addition, it is closely associated with factors such as mucosal immunity, estrogen levels, bacterial fimbriae and virulence factors. However, it remains unclear whether additional mechanisms exist through which gut bacteria can influence the recurrence of urinary tract infections.

Growing evidence suggests that the interaction between the gut and bladder, known as the gut-bladder axis, plays a crucial role in the pathogenesis of UTI (Yang et al., 2022). The gut-bladder axis implies that individuals with UTI often show an imbalance in the GM composition (Vervoort et al., 2015; Paalanne et al., 2018), and alterations in the GM can enhance susceptibility to recurrent UTI. Studies have revealed that uropathogens from the gut can repeatedly enter the urethra (Lee et al., 2014), and an increased abundance of pathogenic bacteria is a risk factor for bacteriuria after kidney transplantation (Magruder et al., 2019). Nevertheless, Worby et al. (2022b) have yielded conflicting conclusions, they discovered the influencing factor of recurrent UTI is not necessarily an increase in uropathogenic abundance, but rather a decrease in the diversity and richness of the GM. Nonetheless, owing to the influence of antibiotics and diet, it is challenging to ascertain whether dysbiosis of the GM is a consequence of UTI or an indicator of increased disease susceptibility.

The concept of gut-bladder axis also suggests that gut microbiota indirectly influence the host immune system through its metabolites, thereby exacerbating inflammation in the distal bladder (Worby et al., 2022a). For example, short-chain fatty acids (SCFAs) can modulate immune cell function and enhance the integrity of the intestinal barrier, thereby reducing the risk of urinary tract infections (Martin-Gallausiaux et al., 2021). Trimethylamine-N-oxide (TMAO) promotes the release of inflammatory factors during infection and increases the pathogenicity of *E. coli* in bladder cells (Wu et al., 2023). Iron participates in the replication of *E. coli* and in the host's nutritional immune defense (Butler-Laporte et al., 2023). In addition, blood metabolites such as serum procalcitonin (Pecile et al., 2004), C-reactive protein (Shaikh et al., 2020), and serum calcitonin gene-related peptide (Lamot et al., 2022), play a crucial role in clinical diagnosis, which can be used as biomarkers for acute pyelonephritis in children. However, our current understanding does not clarify how the interaction between the GM and metabolites influences susceptibility to recurrent urinary tract infections.

Mendelian randomization (MR) analysis uses genetic variation as instrumental variables (IVs) to investigate the causal relationship between exposure and outcome. This method offers better control over confounding factors and sample size compared to clinical research, while also being more cost-effective. Moreover, to assess the causal relationship between GM and UTI, as well as the potential role of blood metabolites in this relationship, this study utilized statistical data from genome-wide association studies (GWAS) for MR analysis to clarify their relationship. Our study attempted to identify specific genera of bacteria or blood metabolites that may influence the occurrence of UTI, providing new perspectives for further mechanistic research, clinical diagnosis, and drug treatment.

2 Materials and methods

2.1 Overall design

The overarching design of the proposed study is illustrated in **Figure 1**. This investigation examined the bidirectional causal association between GM and UTI using a two-sample Mendelian randomization (TSMR) approach. To unravel the potential underlying mechanisms, we performed MR analyses to examine the relationship between the GM and serum metabolites. Subsequently, another MR analysis was carried out to explore the link between serum metabolites and UTI. To further probe the causal pathway from gut microbiota to UTI, a two-step MR design was employed for mediation analysis, specifically to investigate whether serum metabolites act as mediators in this pathway.

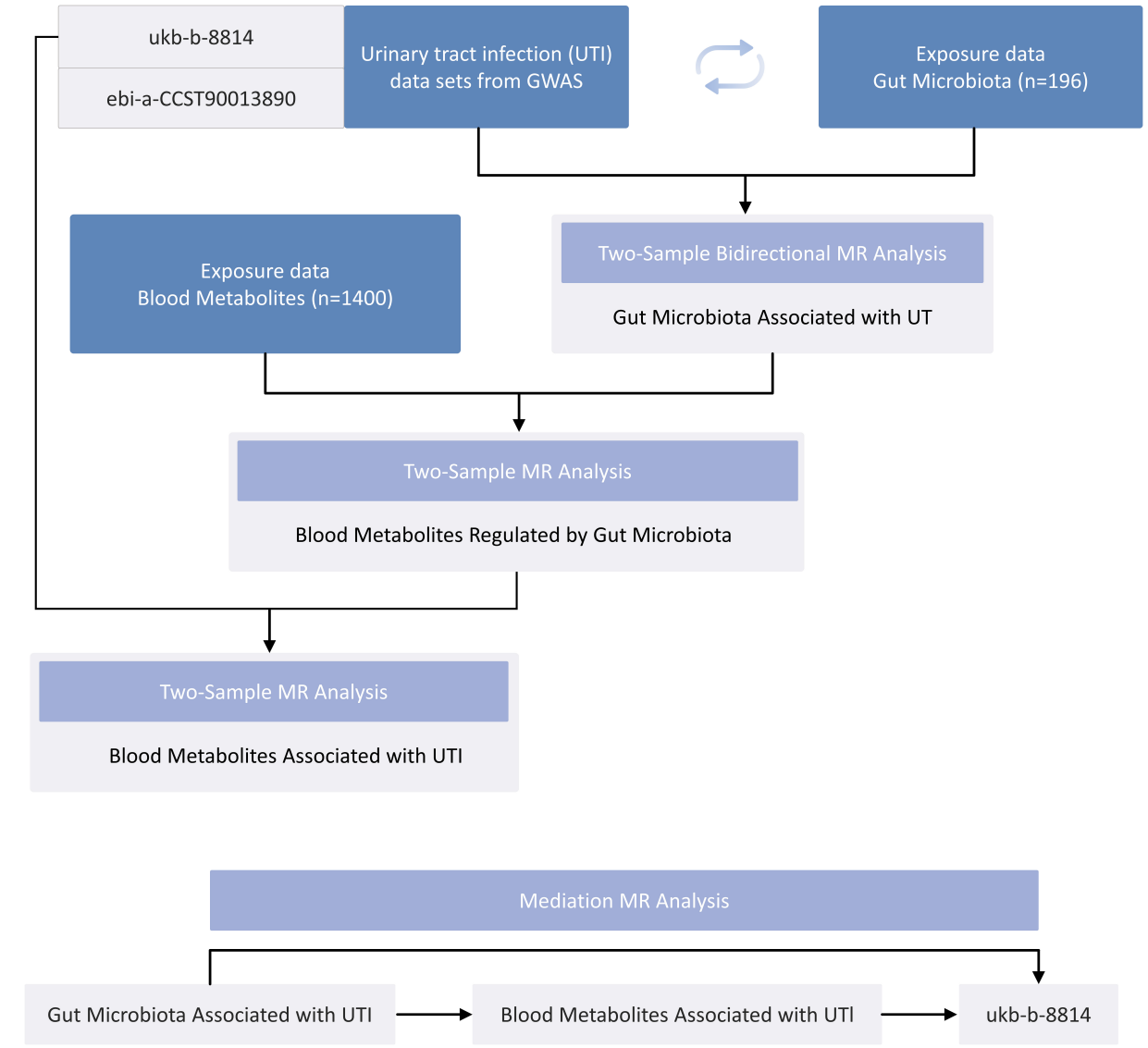


FIGURE 1
A flowchart detailing the Mendelian randomization study design.

2.2 Data sources

Genetic variants correlated with the GM in this study were sourced from the MiBioGen Consortium's extensive GWAS meta-analysis (van der Velde et al., 2019; Kurilshikov et al., 2021), encompassing 18,340 individuals across 24 cohorts, primarily of European descent (n = 13,266). GWAS data on tract infection were acquired by the publicly available GWAS catalog (ebi-a-GCST90013890) (Mbatchou et al., 2021) and the UK Biobank (ukb-b-8814). Summary-level statistical data for 1,091 blood metabolites and 309 metabolite ratios were derived from a comprehensive meta-analysis of the GWAS genomic atlas of the plasma metabolome, which prioritizes the metabolites implicated in human diseases (Chen et al., 2023). The data sources are presented in **Table 1**. Given that our study relied on publicly available summary data, there was no extra need for further ethical approval or consent procedures.

2.3 TSMR design

In our analysis, single nucleotide polymorphisms (SNPs) are IVs. The three core assumptions of MR that must hold, to strengthen causal inference claims, are as follows (refer to **Figure 2**; Emdin et al., 2017): (1) Relevance: the genetic variants (SNPs) used as IVs must be associated with the exposure of interest. The selection criteria detailed below directly address this assumption by ensuring that only significantly associated SNPs are selected. (2) Independence: the IVs must not be connected to any confounders of the exposure-outcome relationship. The use of SNPs as IVs inherently supports this assumption, since genetic variants are randomly assorted at conception, independent of confounders that may affect the outcome studied later in life. (3) Exclusivity: the IVs influence the outcome exclusively through their association with the exposure, not through other pathways. The methodology employs various statistical methods for sensitivity

TABLE 1 Details of the genome-wide association studies and datasets utilized in our analyses.

Trait	GWAS ID	Consortium	Sample size	Year
Urinary tract infection	ukb-b-8814	MRC-IEU	463010	2018
Urinary tract infection	ebi-a-GCAT90013890 aaZGGCST90013890	NA	397867	2021
Human gut microbiome	NA	MiBioGen	18340	2021
1,091 blood metabolites and 309 metabolite ratios	NA	CLSA	8299	2023

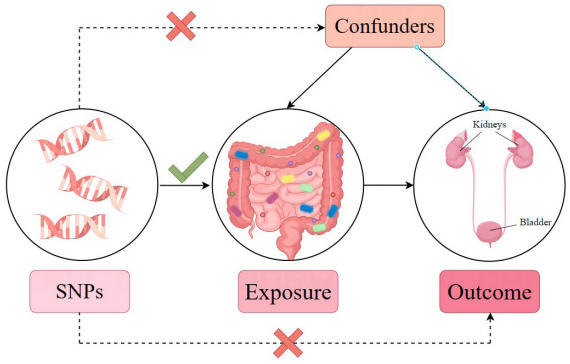


FIGURE 2
Mendelian randomization model and three key assumptions of a Mendelian randomization analysis.

analysis to detect and adjust for pleiotropy, supporting the exclusivity assumption. Furthermore, the robustness of the causal inference claims is further supported by multiple MR methods (inverse variance-weighted [IVW], MR-Egger regression, weighted median, simple mode, and weighted mode) to assess causal associations from different angles, providing a comprehensive view of the causal effect. Sensitivity analyses, including Cochran's Q test for heterogeneity, MR-Egger intercept for horizontal pleiotropy, and leave-one-out analysis, were performed to detect and correct for potential biases or influential outliers. Through this streamlined methodology and assumption verification, we aimed to enhance the credibility and accuracy of our causal inference, providing robust methodological backing for our conclusions.

2.4 Selection of IVs

To obtain qualified IVs, the corresponding selection criteria were utilized. Initially, IVs were selected relying on a significance level of $P < 1.0 \times 10^{(-5)}$. This threshold ensures that only SNPs strongly associated with the exposure are considered, reducing the risk of selecting IVs by chance. Additionally, a linkage disequilibrium (LD) threshold of $R^2 < 0.001$ and a clumping distance of 10,000 kb were applied using 1000 Genomes EUR data. This step minimizes the likelihood of selecting SNPs that are in LD with each other, ensuring that each SNP represents an independent source of genetic variation. Then, F-statistics were calculated to verify the robust connection between SNPs with an F-statistic of > 10 were considered to have a significant association with the exposure (Palmer et al., 2012; Gill et al., 2019; Levin et al., 2020). This criterion ensures that the selected IVs are strong instruments, reducing the risk of weak instrument bias, which can invalidate MR assumptions and conclusions.

2.5 Statistical analysis

This study employed multiple methodologies, including IVW (Burgess et al., 2015), MR-Egger regression (Bowden et al., 2015), weighted median (Bowden et al., 2016), simple mode, and weighted model (Hartwig et al., 2017), to assess causal associations. The IVW method is considered the primary analysis method due to its ability to offer accurate effect estimates and being commonly adopted as the predominant approach in the majority of MR analyses.

Diverse methods have been introduced for sensitivity analysis. First, Cochran's Q test was used to assess IV heterogeneity, with a *P*-value of > 0.05 indicating no heterogeneity (Burgess et al., 2015). Second, the MR-Egger intercept method was used to quantify the heterogeneity effects among the genetic instruments. A *P*-value of less than 0.05, indicating a potential bias in the IVW estimate, could be attributed to horizontal pleiotropy. Additionally, MR-PRESSO detected anomalies and possible horizontal pleiotropy with a global *P*-value of less than 0.05, suggesting the existence of horizontal pleiotropy (Verbanck et al., 2018). Third, a leave-one-out sensitivity test identified potential heterogeneous SNPs (Burgess et al., 2017). Finally, funnel and forest plots were created for the direct identification of pleiotropy.

Statistical analyses were conducted using R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria). The MR analyses were conducted using the TSMR (version 0.5.7) and MR-PRESSO (version 1.0) R packages (Hemani et al., 2018; Verbanck et al., 2018).

3 Results

3.1 Gut microbiota association with UTI through two-sample bidirectional MR analysis

A thorough evaluation was conducted within the framework of MR analysis, and the outcomes were visually represented in a heatmap (Gu et al., 2014; **Supplementary Figure 1**). The

analysis revealed significant associations between seven bacterial traits across diverse taxonomic levels and UTI risk in the UKB dataset (Figures 3A–G). Among these traits, *c_Clostridia* (odds ratio [OR] = 0.9966, 95% confidence interval [CI]: 0.9938–0.9993, P = 0.016) and *g_Ruminococcaceae UCG010* (OR = 0.9964, 95% CI: 0.9930–0.9997, P = 0.036) were identified as protective factors. In contrast, *g_Ruminococcus2* (OR = 1.0026, 95% CI: 1.0001–1.0051, P = 0.038), *f_Bacteroidales S24 7group* (OR = 1.0027, 95% CI: 1.0006–1.0047, P = 0.012), *g_Clostridium sensu stricto1* (OR = 1.0047, 95% CI: 1.0000–1.0094, P = 0.048), and *g_Bacteroides* or *f_Bacteroidaceae* (OR = 1.0039, 95% CI: 1.0010–1.0068, P = 0.009, for both) were identified as risk factors. The detailed results are presented in Supplementary Table 1. Importantly, the P-values for pleiotropy and heterogeneity analyses consistently exceeded 0.05, indicating the absence of significant heterogeneity or pleiotropy issues. Moreover, these results were considered reliable and were supported by sensitivity analyses, which ruled out pleiotropy concerns.

The causal effects of the GM on UTI were investigated further using an additional dataset (ebi-a-GCST90013890) (Supplementary Figure 2), Using the IVW method, one order and two genera exhibited positive associations, whereas six genera showed negative associations with UTI (Supplementary Table 2). Interestingly, *g_Ruminococcaceae UCG010* was the only bacterial trait that overlapped between the two sets of results (Figure 4), indicating the potential involvement of specific bacterial traits in the onset of UTI.

Additionally, reverse MR analysis was conducted. Notably, *g_Ruminococcaceae UCG010* exhibited no significant changes attributed to UTI (ukb-b-8814: β = 0.51, P = 0.87; ebi-a-GCST90013890: β = −0.02, P = 0.77). Furthermore, no further evidence of a causal effect for UTI on the other taxa identified in the above results. When analyzing the ukb-b-8814 dataset, eight features (one class, one order, one family, and five genera) were significantly regulated. Similarly, the analysis of the ebi-a-GCST90013890 dataset revealed seven features with evident changes, including one family, and six genera.

3.2 Regulation of multiple metabolites by *Ruminococcaceae* UCG010

Recognizing the potential significance of metabolites in the interplay between GM and UTI, we conducted extensive MR analysis to unravel their intricate relationships. We discovered that *Ruminococcaceae UCG010* co-regulates 56 specific metabolites. Among these, 24 metabolites showed an upward regulatory trend and 32 showed a downward trend. Noteworthy metabolites, including sulfate of piperine metabolite C18H21NO3 (P = 0.0040), 1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P = 0.0029), 9,10-DiHOME (P = 0.0061), 13-HODE + 9-HODE (p = 0.0087), N-palmitoyl-sphinganine (P = 0.0049), and 12,13-DiHOME (P = 0.0093), displayed a significant positive correlation with *Ruminococcaceae UCG010*. In contrast, Pseudouridine (P = 0.0058), 2-hydroxy-3-methylvalerate (p = 0.0033), and N2, N5-diacetylornithine (P = 0.0036) were significantly negatively correlated with *Ruminococcaceae UCG010*. Further details of the remaining metabolites are displayed in Supplementary Table 3.

3.3 Association of N-acetylkynurenine (NAK) with increased risk of UTI

To delve into the relationship of causality between the 56 metabolites and UTI, we conducted MR analysis using the GWAS dataset (ukb-b-8814). IVW analysis revealed that only NAK acid (OR: 1.0009, 95% CI: 1.0002–1.0016, P = 0.0173) was significantly associated with an increased risk of UTI (Figure 5). This finding suggests that the increased risk of UTI associated with elevated levels of *Ruminococcaceae UCG010* may be partly attributed to the upregulation of NAK.

3.4 Mediation analysis of gut microbiome, NAK, and UTI

To explore the mediating role of N-acetylkynurenine, we calculated its indirect effects and proportions. The overall effect size was indicated by the β value between *Ruminococcaceae UCG010* and UTI, determined through TSMR. The indirect effect was derived by multiplying the β value of *Ruminococcaceae UCG010* to NAK with the β value of NAK to UTI. The direct effect was calculated by subtracting the indirect effect from the overall effect. Following these computations, the mediation effect manifested as −0.00018 (95% CI: −0.0419–0.0415), with a mediated proportion of 5.07%.

4 Discussion

In light of our awareness, this study emerges as the primary instance of being the first to delve deeply into the potential causal association between GM, blood metabolites, and urinary tract infections. Additionally, for the first time, we discovered a negative correlation between N-acetylkynurenine and *Ruminococcaceae UCG010*. These findings suggest that *Ruminococcaceae UCG010* plays a protective role against UTI and that the onset of UTI does not alter the abundance of this specific bacterial group. Our observations identified 56 metabolites associated with *Ruminococcaceae UCG010*. Further analysis revealed that this bacterium downregulated N-acetylkynurenine levels, subsequently contributing to UTI development. This finding provides theoretical support for the existence and mechanism of the gut-bladder axis from a genetic perspective.

In our study, the genus *Ruminococcaceae UCG010*, a member of the *Ruminococcaceae* family, emerged as a potential risk factor for UTI. Consistent with prior studies (Worby et al., 2022b), the abundance of *Ruminococcaceae* decreased in patients with recurrent UTI, suggesting that *Ruminococcus* could serve as a potential marker for dysbiosis in recurrent cystitis (Graziani et al., 2022). The *f_Ruminococcaceae* is also known to be significantly affected by chronic infections (Martinez et al., 2022; Tran et al., 2023) and the administration of antibiotics (Ross et al., 2016). Although direct evidence linking *Ruminococcaceae UCG010* to UTI remains elusive, some studies have proposed that a higher abundance of this bacterium in the GM may correlate with decreased risks of type 2 diabetes (Lyu et al., 2023), non-alcoholic fatty liver disease (Tsai et al., 2020), and obesity (Nseir et al.,

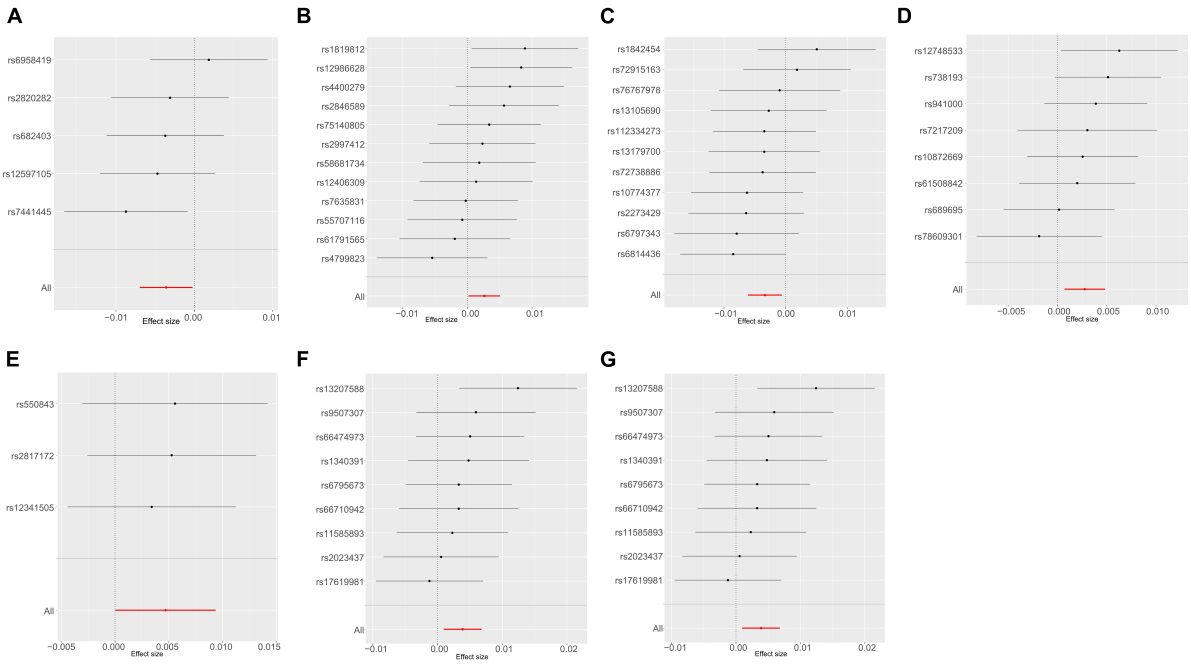


FIGURE 3
Mendelian randomization analyses show causal effects of gut microbiota on urinary tract infection using the ukb-b-8814 dataset. **(A–G)** Forest plots of 7 bacterial traits with *P*-value < 0.05 by IVW method. **(A)** genus *Ruminococcaceae* UCG010; **(B)** genus *Ruminococcus2*; **(C)** class Clostridia; **(D)** family Bacteroidales S24 7group; **(E)** genus *Clostridium* sensu stricto1; **(F)** genus *Bacteroides*; **(G)** family *Bacteroidaceae*.

2015), each being a risk factor for UTI. The mechanism involved in UTI could be that *Ruminococcaceae UCG010* produces SCFAs (Balmer et al., 2020). Hess discovered that acetate accumulates at the site of infection and regulates the inflammatory process by encoding the metabolic and functional reshaping of memory CD8+ T cells. Supplementation with acetate improved the symptoms of bladder inflammation and immune suppression caused by UPEC infection (Sturov et al., 2022). The production of butyrate in the intestine promotes the differentiation of Tregs, enhances epithelial barrier integrity, and inhibits proinflammatory responses (Siddiqui and Cresci, 2021). Decreased butyrate abundance is connected to heightened susceptibility to infections (He et al., 2022). In addition, *Ruminococcaceae* are involved in bile acid (BA) metabolism (Pickard et al., 2017), they can decompose cholesterol, generate secondary bile acids, and influence the formation of urinary stones (Zhou et al., 2023). A humanized dyslipidemia mouse model demonstrated that *Ruminococcaceae UCG010* reduced BA synthesis (Xu et al., 2023). Interestingly, we also discovered that *Ruminococcus2*, which belongs to the *Ruminococcaceae* family, is a risk factor for UTI. This indicates that despite their taxonomic similarity, different genera or species within the same family can have different functions and characteristics. These factors may be influenced by the genomic and metabolic capabilities. Further experiments and observations are required to obtain a more detailed understanding of the functions of these microorganisms and their effects on human health.

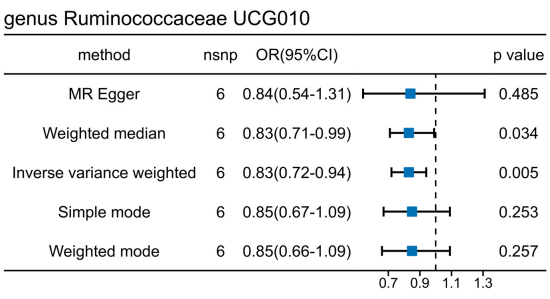


FIGURE 4
Mendelian randomization analyses show causal effects of *g_Ruminococcaceae* UCG010 on urinary tract infection using the ebi-a-GCST90013890 dataset.

As a major degradation product of N-acetyl tryptophan (Agus et al., 2018), NAK has been found to be an activator of aryl hydrocarbon receptor (AhR) (Rael et al., 2018). NAK inhibits macrophage activation by activating AhR, thereby reducing the secretion of interleukin-6 (IL-6) and chemokine-10 (CXCL-10). Macrophages, IL-6, and CXCL-10 are closely related to inflammatory responses. M1 macrophages primarily mediate tissue damage and initiate inflammatory responses, whereas M2 macrophages principally inhibit granulation and scar formation (Han et al., 2019; Kuhn et al., 2023). Transition from M1 to M2 can be observed during the progression of acute to chronic urinary tract infection (Hreha et al., 2020). However, premature inhibition of M1 could compromise the bactericidal function of the bladder. IL-6 is an early marker that rapidly increases during inflammation (Rashid et al., 2021). Furthermore, IL-6 deficient mice demonstrated heightened UPEC load, decreased antimicrobial peptide release, and heightened mortality (Khalil et al., 2000). Clinical investigations have documented a considerable rise in CXCL-10 in the urine of

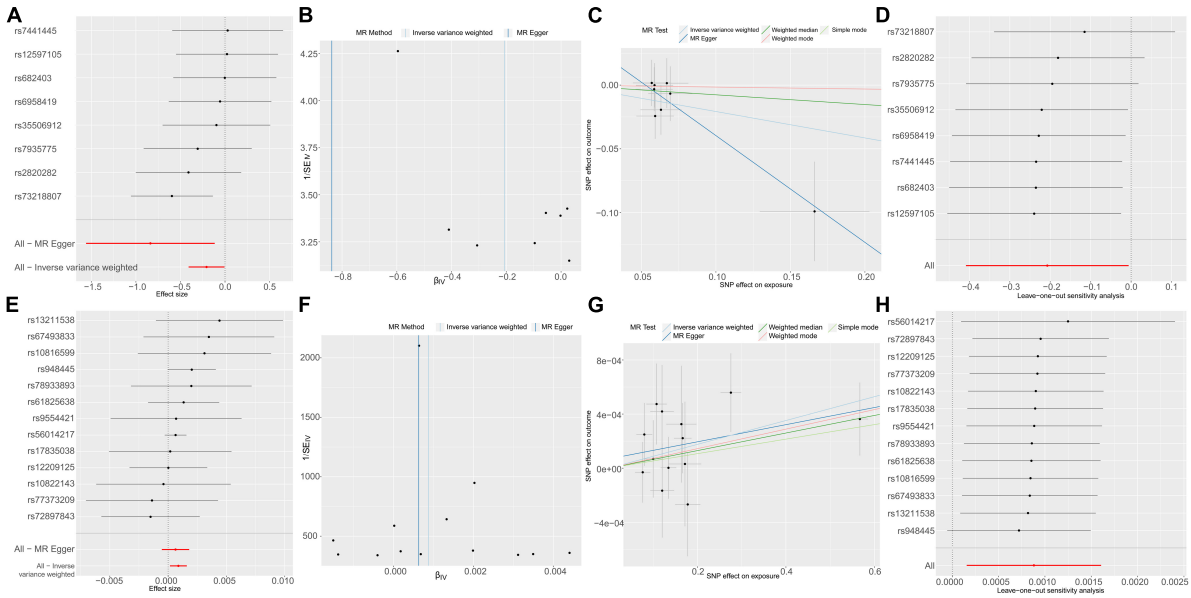


FIGURE 5
Causal effects of *g_Ruminococcaceae* UCG010 on N-acetylkynurenine **(A–D)** and N-acetylkynurenine on UTI **(E–H)**. **(A)** Forest plot. **(B)** Funnel plot to assess heterogeneity. **(C)** Scatter plots of genetic associations with *g_Ruminococcaceae* UCG010 against the genetic associations with N-acetylkynurenine. **(D)** Leave-one-out sensitivity analysis of SNPs associated with *g_Ruminococcaceae* UCG010 and their risk of N-acetylkynurenine. **(E)** Forest plot. **(F)** Funnel plot to assess heterogeneity. **(G)** Scatter plots of genetic associations with N-acetylkynurenine against the genetic associations with UTI. **(H)** Leave-one-out sensitivity analysis of SNPs associated with N-acetylkynurenine and their risk of UTI.

patients with UTI, with levels decreasing following antibiotic administration. CXCL-10 drives inflammation by activating T cell chemotaxis and endothelial adhesion, as well as by enhancing cell lysis mediated by natural killer cells (Platten et al., 2019). This suggests that NAK may inhibit the body's inflammatory response and thereby affect pathogen clearance.

Our mediation analyses further supported the genetic evidence for a link between GM and UTI. To the best of our knowledge, no prior study has directly linked *Ruminococcaceae UCG010* to N-acetylkynurenine. Furthermore, it is imperative to acknowledge the inherent limitations and potential sources of bias within MR studies, including our own. MR analyses rely on several key assumptions: the genetic variants employed as IVs are associated with the exposure but not with any confounders of the exposure-outcome relationship, and these variants influence the outcome solely through the exposure. While we have strived to select IVs with strong associations and minimal linkage disequilibrium to mitigate pleiotropy and bias, residual confounding due to unmeasured or inadequately measured variables, and potential violation of these assumptions may still affect the interpretation of our findings. However, a growing number of research have explored the involvement of the GM in tryptophan metabolism pathways, including indole-uracil, serotonin, and aromatic amino acid metabolism pathways (Xue et al., 2023). Recently, the involvement of *Ruminococcus* species in tryptophan metabolism has been confirmed (Coletto et al., 2022). *Ruminococcaceae UCG010* is a major producer of SCFAs, and its metabolite butyrate can influence the activity of intestinal epithelial cells (IELs) indoleamine 2,3-dioxygenase (IDO) (Martin-Gallausiaux et al., 2018). During UPEC infection, local IDO levels in the bladder increase, promoting indole-uracil production, and inhibiting neutrophil chemotaxis (Loughman et al., 2016). Studies have shown that kynurenine, a product of Try degradation, can serve as a key signaling molecule to activate AhR and promote the Treg-macrophage axis in suppressing T-cell dysfunction (Campesato et al., 2020). Additionally, research has found associations between Try metabolites and infectious diseases. Kynurenines exhibit antimicrobial activity and directly influence the proliferation of gut microbiota (Notarangelo et al., 2014). Experiments have demonstrated that the host's AhR receptor can qualitatively and quantitatively perceive the quorum sensing signal molecules secreted by Pseudomonas aeruginosa at various stages of infection and coordinate host defense functions based on the infection status (Moura-Alves et al., 2019). N-acetylkynurenine is an AhR agonist. AhR participates in inflammatory responses and immune tolerance, regulates the mucosal barrier function, and maintains intestinal homeostasis (Su et al., 2022). Deficiency of the AhR repressor leads to fewer IELs, which can cause intestinal infection and inflammation (Gao et al., 2022). By virtue of our findings and previous literature, we speculate that *Ruminococcaceae UCG010* may reduce the production of N-acetyl-kynurenine through the tryptophan acid pathway, inhibiting AhR-mediated inflammatory responses in macrophages and other cells, thereby affecting the occurrence and progression of tract infections.

Our MR analysis revealed that more than 30 gut microbial taxa were causally associated with UTI. Similar to other studies, patients with UTI exhibit gut dysbiosis, such as decreased levels of *Lactobacillus* and *Bifidobacteria*, along with an increased abundance of conditionally pathogenic enterobacteria and *Clostridium* (Stepanova et al., 2018). However, most of the gut microbial taxa identified in our study have rarely been reported to be associated with UTI in previously published literatures. To

investigate the mechanism underlying this causal relationship, we interpreted it from several perspectives. (1) An imbalance in the GM facilitates the colonization of pathogenic bacteria (Bowden et al., 2016), enhancing their adhesion and toxicity. During UPEC infection, the TLR4/NF-κB cell pathway is activated and the expression of the host cell phosphatase transporter protein Pituitary specific transcription factor 1 is upregulated. This mediates the escape of UPEC from the vacuoles to the cytoplasm, thereby evading a systemic immune response (Pang et al., 2022). (2) In this study, the bacterial genera that had causal relationships were mostly those that produced SCFAs, such as *Bacteroidales*, *Turicibacter*, and so on. The metabolic products of fatty acids produced by gut microbiota also exert direct antibacterial effects. They suppress the expression of virulence genes in *E. coli*, thereby decreasing the adhesion and motility of extraintestinal pathogenic *E. coli* (ExPEC). In contrast, SCFAs can freely diffuse inside and outside bacterial cell membranes, causing pH disturbances between cells and thus inhibiting bacterial growth (Buffie and Pamer, 2013). In patients with recurrent UTI, gut dysbiosis is often accompanied by a reduction in SCFAs levels. In particular, the absence of bacteria that produce butyrate, an important component of SCFAs, is notable (Worby et al., 2022b). Butyrate plays a critical role in maintaining the intestinal barrier integrity, improving intestinal inflammation, and promoting immune regulation (Sturov et al., 2022). Therefore, dysbiosis of the gut microbiota, which results in a decrease in SCFAs, may increase the risk of urinary tract infections. (3) Urinary tract infection is closely relevant to the intestinal barrier function, which is to prevent the entry of harmful substances, such as bacteria, toxins, and antigens, from the intestine into the bloodstream. ExPECs have been demonstrated to establish specific interactions with the epithelial barrier of the intestine (Poole et al., 2017), and to induce dysfunction of the intestinal barrier prior to the onset of disease (Nagpal and Yadav, 2017). antibiotic treatment can disrupt the structure and function of tight junction proteins, leading to tight junction dysfunction and increased intestinal permeability (Feng et al., 2019), which facilitates the translocation of antigens and toxic substances into systemic circulation, thereby contributing to the development of chronic inflammation (Sharapatov et al., 2021).

This study encompasses several limitations that warrant careful consideration for a comprehensive understanding of its scope and implications of its findings. Firstly, the GWAS analysis conducted leveraged data primarily derived from a European demographic, inherently limiting the extrapolation of our findings to diverse populations, including those characterized by distinct genetic compositions and environmental exposures. This demographic focus raises questions about the universality of our conclusions and underscores the imperative for inclusive research involving a broader spectrum of ethnicities to ensure global applicability. Secondly, the reliance on aggregated summary data for UTI cases in our study precluded the possibility of subgroup analyses, notably those differentiated by sex and age. This reliance significantly hampers our understanding of UTI dynamics across different demographics, as it masks potential variations in disease susceptibility and progression that are crucial for personalized medical management approaches. Thirdly, our investigation into the gut microbiome was restricted to the genus level, constrained by the taxonomic resolution provided in the GM dataset. This limitation prevented us from delving into the potentially more informative specie-level analysis, which could offer finer insights into microbiota-UTI interactions. The ability to analyze at the species level could reveal nuanced microbial behaviors and their specific roles in urinary tract infections, marking a critical area for future exploration. To address these limitations and advance our comprehension of the intricate interplay among GM, their metabolites, and UTI, future research should aim for a more inclusive and detailed approach. This includes expanding the genetic and environmental diversity of study populations, enhancing the taxonomic resolution of microbiome analysis, and implementing rigorous methods to control for confounders and biases. Furthermore, a more nuanced application and critical evaluation of MR methods are essential to refine our causal inferences. Such comprehensive efforts are pivotal for unraveling the intricate mechanisms underlying UTI and tailoring effective prevention and treatment strategies across varied population segments.

Previous clinical studies have revealed a correlation between GM and UTI. It is challenging to establish a causal relationship, due to various confounding factors. Our research finds that gut microbiota and blood metabolites can influence the onset and progression of UTI. With the increasing prevalence of antibiotic resistance, there is an urgent demand for novel approaches to treating UTI. Besides conventional treatments targeting pathogens, our study proposes a new investigation of possibility, namely the microbial therapy. Gut microbiota can serve as potential biological markers for UTI and directly affect their recurrence and prognosis. Focusing on gut microbiota and blood metabolites, we can delve into deeper mechanism studies and drug intervention clinical trials. Biological therapies such as fecal microbiota transplantation (FMT) and probiotics have initiated preliminary clinical studies, but these treatments possess limitations. FMT is a non-targeted therapy and commercially available probiotics consist mostly of single strains or a mixture of several strains, none of which were specifically developed for the characteristics of UTI pathology. Notably, our study identified protective commensal bacterial groups against UTI, paving the way for the development of personalized microbiome-based therapeutic strategies.

5 Conclusion

Our findings, obtained through mediation analysis, indicated that *Ruminococcaceae UCG010* can act as a direct protective agent against UTI and indirectly reduce the occurrence of UTI by reducing N-acetylkynurenine levels. Consequently, this study provides independent evidence supporting the association between the composition of the gut microbiota and the risk of developing UTI.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

YH: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. CH: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. CL: Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. XY: Data curation, Formal analysis, Writing – review & editing, Validation. JW: Formal analysis, Funding acquisition, Validation, Writing – review & editing. LG: Formal analysis, Writing – review & editing. RY: Formal analysis, Methodology, Writing – review & editing. BL: Formal analysis, Supervision, Writing – review & editing. XZ: Conceptualization, Data curation, Project administration, Writing – review & editing. WH: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1384095/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Heatmap of MR results.

SUPPLEMENTARY FIGURE 2
Mendelian randomization analyses show causal effects of gut microbiota on UTI using the ebi-a-GCST90013890 dataset.

References

Agus, A., Planchais, J., and Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell Host Microbe* 23, 716–724. doi: 10.1016/j.chom.2018.05.003

Balmer, M., Ma, E., Thompson, A., Unterstab, G., Lötscher, J., Dehio, P., et al. (2020). Memory CD8+ T Cells balance pro- and anti-inflammatory activity by reprogramming cellular acetate handling at sites of infection. *Cell Metab.* 32, 457–467.e5. doi: 10.1016/j.cmet.2020.07.004

Bowden, J., Davey Smith, G., and Burgess, S. (2015). Mendelian randomization with invalid instruments: Effect estimation and bias detection through Egger regression. *Int. J. Epidemiol.* 44, 512–525.

Bowden, J., Davey Smith, G., Haycock, P., and Burgess, S. (2016). Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet. Epidemiol.* 40, 304–314. doi: 10.1002/gepi.21965

Buffie, C. G., and Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nat. Rev. Immunol.* 13:790. doi: 10.1038/nri3535

Burgess, S., Bowden, J., Fall, T., Ingelsson, E., and Thompson, S. G. (2017). Sensitivity analyses for robust causal inference from mendelian randomization analyses with multiple genetic variants. *Epidemiology* 28, 30–42. doi: 10.1097/EDE.0000000000000559

Burgess, S., Scott, R. A., Timpson, N. J., Davey Smith, G., Thompson, S. G., and Epic-InterAct Consortium. (2015). Using published data in Mendelian randomization: A blueprint for efficient identification of causal risk factors. *Eur. J. Epidemiol.* 30, 543–552. doi: 10.1007/s10654-015-0011-z

Butler-Laporte, G., Farjoun, Y., Chen, Y., Hultström, M., Liang, K. Y. H., Nakanishi, T., et al. (2023). Increasing serum iron levels and their role in the risk of infectious diseases: A Mendelian randomization approach. *Int. J. Epidemiol.* 52, 1163–1174. doi: 10.1093/ije/dyad010

Campesato, L. F., Budhu, S., Tchaicha, J., Weng, C. H., Gigoux, M., Cohen, I. J., et al. (2020). Blockade of the AHR restricts a Treg-macrophage suppressive axis induced by L-Kynurenine. *Nat. Commun.* 11:4011. doi: 10.1038/s41467-020-17750-z

Chen, Y., Lu, T., Pettersson-Kymmer, U., Stewart, I., Butler-Laporte, G., Nakanishi, T., et al. (2023). Genomic atlas of the plasma metabolome prioritizes metabolites implicated in human diseases. *Nat. Genet.* 55, 44–53. doi: 10.1038/s41588-022-01270-1

Coletto, E., Latousakis, D., Pontifex, M., Crost, E., Vaux, L., Perez Santamarina, E., et al. (2022). The role of the mucin-glycan foraging *Ruminococcus gnavus* in the communication between the gut and the brain. *Gut Microbes* 14:2073784. doi: 10.1080/19490976.2022.2073784

Emdin, C., Khera, A., and Kathiresan, S. (2017). Mendelian randomization. *JAMA* 318, 1925–1926. doi: 10.1001/jama.2017.17219.

Faine, B., Rech, M., Vakkalanka, P., Gross, A., Brown, C., Harding, S., et al. (2022). High prevalence of fluoroquinolone-resistant UTI among US emergency department patients diagnosed with urinary tract infection, 2018-2020. *Acad. Emerg. Med.* 29, 1096–1105. doi: 10.1111/acem.14545

Feng, Y., Huang, Y., Wang, Y., Wang, P., Song, H., and Wang, F. (2019). Antibiotics induced intestinal tight junction barrier dysfunction is associated with microbiota dysbiosis, activated NLRP3 inflammasome and autophagy. *PLoS One* 14:e0218384. doi: 10.1371/journal.pone.0218384

Foxman, B. (2002). Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *Am. J. Med.* 113, 5–13. doi: 10.1016/S0002-9343(02)01054-9

Foxman, B. (2014). Urinary tract infection syndromes: Occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect. Dis. Clin. North Am.* 28, 1–13. doi: 10.1016/j.idc.2013.09.003

Gao, S., Khan, M., Kalsoom, F., Liu, Z., Chen, Y., and Chen, Z. (2022). Role of gene regulation and inter species interaction as a key factor in gut microbiota adaptation. *Arch. Microbiol.* 204:342. doi: 10.1007/s00203-022-02935-5

Gill, D., Efstathiadou, A., Cawood, K., Tzoulaki, I., and Dehghan, A. (2019). Education protects against coronary heart disease and stroke independently of cognitive function: Evidence from Mendelian randomization. *Int. J. Epidemiol.* 48, 1468–1477. doi: 10.1093/ije/dyz200

Graziani, C., Laterza, L., Talocco, C., Pizzoferrato, M., Simone, N. D., D'Ippolito, S., et al. (2022). Intestinal permeability and dysbiosis in female patients with recurrent cystitis: A pilot study. *J. Pers. Med.* 12:1005. doi: 10.3390/jpm12061005

Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). Circlize Implements and enhances circular visualization in R. *Bioinformatics* 30, 2811–2812. doi: 10.1093/bioinformatics/btu393

Han, H. I., Skvarca, L. B., Espiritu, E. B., Davidson, A. J., and Hukriede, N. A. (2019). The role of macrophages during acute kidney injury: Destruction and repair. *Pediatr. Nephrol.* 34, 561–569. doi: 10.1007/s00467-017-3883-1

Hartwig, F. P., Davey Smith, G., and Bowden, J. (2017). Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. *Int. J. Epidemiol.* 46, 1985–1998. doi: 10.1093/ije/dyx102

He, Z., Ma, Y., Yang, S., Zhang, S., Liu, S., Xiao, J., et al. (2022). Gut microbiota-derived ursodeoxycholic acid from neonatal dairy calves improves intestinal homeostasis and colitis to attenuate extended-spectrum β-lactamase-producing enteroaggregative *Escherichia coli* infection. *Microbiome* 10:79. doi: 10.1186/s40168-022-01269-0

Hemani, G., Zheng, J., Elsworth, B., Wade, K. H., Haberland, V., Baird, D., et al. (2018). The MR-Base platform supports systematic causal inference across the human phenome. *Elife* 7:e34408. doi: 10.7554/eLife.34408

Hreha, T. N., Collins, C. A., Daugherty, A. L., Griffith, J. M., Hruska, K. A., and Hunstad, D. A. (2020). Androgen-influenced polarization of activin a-producing macrophages accompanies post-pyelonephritic renal scarring. *Front. Immunol.* 11:1641. doi: 10.3389/fimmu.2020.01641

Ji, A. (2005). [Epidemiology and etiology of urinary tract infections in the community. Antimicrobial susceptibility of the main pathogens and clinical significance of resistance]. *Enfermedades Infecciosas Microbiol. Clin.* 23(Suppl. 4), 3–8. doi: 10.1157/13091442

Khalil, A., Tullus, K., Bartfai, T., Bakhiet, M., Jaremko, G., and Brauner, A. (2000). Renal cytokine responses in acute *Escherichia coli* pyelonephritis in IL-6-deficient mice. *Clin. Exp. Immunol.* 122, 200–206. doi: 10.1046/j.1365-2249.2000.01377.x

Köves, B., Cai, T., Veeratterapillay, R., Pickard, R., Seisen, T., Lam, T. B., et al. (2017). Benefits and harms of treatment of asymptomatic bacteriuria: A systematic review and meta-analysis by the european association of urology urological infection guidelines panel. *Eur. Urol.* 72, 865–868. doi: 10.1016/j.eururo.2017.07.014

Kuhn, H. W., Hreha, T. N., and Hunstad, D. A. (2023). Immune defenses in the urinary tract. *Trends Immunol.* 44, 701–711. doi: 10.1016/j.it.2023.07.001

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., Demirkan, A., et al. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Lamot, M., Miler, M., Nikolac Gabaj, N., Lamot, L., Milošević, M., Harjaček, M., et al. (2022). Serum calprotectin is a valid biomarker in distinction of bacterial urinary tract infection from viral respiratory illness in children under 3 years of age. *Front. Pediatr.* 10:768260. doi: 10.3389/fped.2022.768260

Lee, J. R., Muthukumar, T., Dadhania, D., Toussaint, N. C., Ling, L., Pamer, E., et al. (2014). Gut microbial community structure and complications after kidney transplantation: A pilot study. *Transplantation* 98, 697–705. doi: 10.1097/TP.0000000000000370

Levin, M. G., Judy, R., Gill, D., Vujkovic, M., Verma, S. S., Bradford, Y., et al. (2020). Genetics of height and risk of atrial fibrillation: A Mendelian randomization study. *PLoS Med.* 17:e1003288. doi: 10.1371/journal.pmed.1003288

Loughman, J. A., Yarbrough, M. L., Tiemann, K. M., and Hunstad, D. A. (2016). Local generation of kynurenines mediates inhibition of neutrophil chemotaxis by uropathogenic *Escherichia coli*. *Infect. Immun.* 84, 1176–1183. doi: 10.1128/IAI.01202-15

Lyu, B., Ma, J., Bai, Y., and Feng, Z. (2023). Casual effects of gut microbiota on risk of infections: A two-sample Mendelian randomization study. *Front. Microbiol.* 14:1284723. doi: 10.3389/fmicb.2023.1284723

Magruder, M., Sholi, A. N., Gong, C., Zhang, L., Edusei, E., Huang, J., et al. (2019). Gut uropathogen abundance is a risk factor for development of bacteriuria and urinary tract infection. *Nat. Commun.* 10:5521. doi: 10.1038/s41467-019-13467-w

Martinez, E., Taminiau, B., Rodriguez, C., and Daube, G. (2022). Gut microbiota composition associated with clostridioides difficile colonization and infection. *Pathogens* 11:781. doi: 10.3390/pathogens11070781

Martin-Gallausiaux, C., Larraufie, P., Jarry, A., Béguet-Crespel, F., Marinelli, L., Ledue, F., et al. (2018). Butyrate produced by commensal bacteria down-regulates indolamine 2,3-dioxygenase 1 (IDO-1) expression via a dual mechanism in human intestinal epithelial cells. *Front. Immunol.* 9:421934. doi: 10.3389/fimmu.2018.02838

Martin-Gallausiaux, C., Marinelli, L., Blottière, H., Larraufie, P., and Lapaque, N. (2021). SCFA: Mechanisms and functional importance in the gut. *Proc. Nutr. Soc.* 80, 37–49. doi: 10.1017/S0029665120006916

Mbatchou, J., Barnard, L., Backman, J., Marcketta, A., Kosmicki, J., Ziyatdinov, A., et al. (2021). Computationally efficient whole-genome regression for quantitative and binary traits. *Nat. Genet.* 53, 1097–1103. doi: 10.1038/s41588-021-00870-7

Medina, M., and Castillo-Pino, E. (2019). An introduction to the epidemiology and burden of urinary tract infections. *Therap. Adv. Urol.* 11:175628721983217. doi: 10.1177/1756287219832172

Moura-Alves, P., Puyskens, A., Stinn, A., Klemm, M., Guhlich-Bornhof, U., Dorhoi, A., et al. (2019). Host monitoring of quorum sensing during *Pseudomonas aeruginosa* infection. *Science* 366:eaaw1629. doi: 10.1126/science.aaw1629

Nagpal, R., and Yadav, H. (2017). Bacterial translocation from the gut to the distant organs: An overview. *Ann. Nutr. Metab.* 71(Suppl. 1), 11–16. doi: 10.1159/000479918

Notarangelo, F. M., Wilson, E. H., Horning, K. J., Thomas, M. A. R., Harris, T. H., Fang, Q., et al. (2014). Evaluation of kynurenine pathway metabolism in Toxoplasma gondii-infected mice: Implications for schizophrenia. *Schizophr. Res.* 152:261. doi: 10.1016/j.schres.2013.11.011

Nseir, W., Farah, R., Mahamid, M., Sayed-Ahmad, H., Mograbi, J., Taha, M., et al. (2015). Obesity and recurrent urinary tract infections in premenopausal women: A retrospective study. *Int. J. Infect. Dis.* 41, 32–35. doi: 10.1016/j.ijid.2015.10.014

Paalanne, N., Husso, A., Salo, J., Pieviläinen, O., Tejesvi, M., Koivusaari, P., et al. (2018). Intestinal microbiome as a risk factor for urinary tract infections in children. *Eur. J. Clin. Microbiol. Infect. Dis.* 37, 1881–1891. doi: 10.1007/s10096-018-3322-7

Palmer, T. M., Lawlor, D. A., Harbord, R. M., Sheehan, N. A., Tobias, J. H., Timpson, N. J., et al. (2012). Using multiple genetic variants as instrumental variables for modifiable risk factors. *Stat. Methods Med. Res.* 21, 223–242. doi: 10.1177/0962280210394459

Pang, Y., Cheng, Z., Zhang, S., Li, S., Li, X., Li, X., et al. (2022). Bladder epithelial cell phosphate transporter inhibition protects mice against uropathogenic *Escherichia coli* infection. *Cell Rep.* 39:110698. doi: 10.1016/j.celrep.2022.110698

Pecile, P., Miorin, E., Romanello, C., Falleti, E., Valent, F., Giacomuzzi, F., et al. (2004). Procalcitonin: A marker of severity of acute pyelonephritis among children. *Pediatrics* 114, e249–e254. doi: 10.1542/peds.114.2.e249

Pickard, J. M., Zeng, M. Y., Caruso, R., and Núñez, G. (2017). Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunol. Rev.* 279, 70–89. doi: 10.1111/imr.12567

Platten, M., Nollen, E. A. A., Röhrig, U. F., Fallarino, F., and Opitz, C. A. (2019). Tryptophan metabolism as a common therapeutic target in cancer, neurodegeneration and beyond. *Nat. Rev. Drug Discov.* 18, 379–401. doi: 10.1038/s41573-019-0016-5

Poole, N. M., Green, S. I., Rajan, A., Vela, L. E., Zeng, X.-L., Estes, M. K., et al. (2017). Role for FimH in Extraintestinal Pathogenic *Escherichia coli* Invasion and Translocation through the intestinal epithelium. *Infect. Immun.* 85:e00581-17. doi: 10.1128/IAI.00581-17

Rael, L. T., Bar-Or, R., Banton, K. L., Mains, C. W., Roshon, M., Tanner, A. H., et al. (2018). The anti-inflammatory effect of LMWF5A and N-acetyl kynurenine on macrophages: Involvement of aryl hydrocarbon receptor in mechanism of action. *Biochem. Biophys. Rep.* 15:61. doi: 10.1016/j.bbrep.2018.06.006

Rashid, M. H., Sparrow, N. A., Anwar, F., Guidry, G., Covarrubias, A. E., Pang, H., et al. (2021). Interleukin-6 mediates delirium-like phenotypes in a murine model of urinary tract infection. *J. Neuroinflamm.* 18:247. doi: 10.1186/s12974-021-02304-x

Ross, C. L., Spinler, J. K., and Savidge, T. C. (2016). Structural and functional changes within the gut microbiota and susceptibility to *Clostridium difficile* infection. *Anaerobe* 41:37. doi: 10.1016/j.anaerobe.2016.05.006

Shaikh, N., Borrell, J., Evron, J., and Leeflang, M. (2020). Procalcitonin, C-reactive protein, and erythrocyte sedimentation rate for the diagnosis of acute pyelonephritis in children. *Cochrane Database Syst. Rev.* 9:CD009185. doi: 10.1002/14651858.CD009185.pub3

Sharapatov, Y., Turgunov, Y., and Lavrinenko, A. (2021). Pathogenic mechanisms of acute obstructive pyelonephritis. *Open Access Maced. J. Med. Sci.* 9, 124–128.

Siddiqui, M., and Cresci, G. (2021). The immunomodulatory functions of butyrate. *J. Inflammat. Res.* 14, 6025–6041. doi: 10.2147/JIR.S300989

Silverman, J. A., Schreiber, H. L., Hooton, T. M., and Hultgren, S. J. (2013). From physiology to pharmacy: Developments in the pathogenesis and treatment of recurrent urinary tract infections. *Curr. Urol. Rep.* 14, 448–456. doi: 10.1007/s11934-013-0354-5

Stepanova, N., Tolstanova, G., Sergiychuk, T., and Akulenko, I. (2018). Intestinal barrier dysfunction in hyperoxaluria women with recurrent pyelonephritis. *Eur. Urol. Suppl.* 17:e473. doi: 10.1016/S1569-9056(18)31175-8

Sturov, N. V., Popov, S. V., Zhukov, V. A., Lyapunova, T. V., Rusanova, E. I., and Kobylyanu, G. N. (2022). Intestinal microbiota correction in the treatment and prevention of urinary tract infection. *Turk. J. Urol.* 48:406. doi: 10.5152/tud.2022.22119

Su, X., Gao, Y., and Yang, R. (2022). Gut microbiota-derived tryptophan metabolites maintain gut and systemic homeostasis. *Cells* 11:2296. doi: 10.3390/cells11152296

Tandogdu, Z., and Wagenlehner, F. (2016). Global epidemiology of urinary tract infections. *Curr. Opin. Infect. Dis.* 29, 73–79. doi: 10.1097/QCO.0000000000000228

Tran, N., Chaidee, A., Surapinit, A., Yingklang, M., Roytrakul, S., Charoenlappanit, S., et al. (2023). Chronic *Strongyloides stercoralis* infection increases presence of the

Ruminococcus torques group in the gut and alters the microbial proteome. *Sci. Rep.* 13:4216. doi: 10.1038/s41598-023-31118-5

Tsai, M., Liu, Y., Lin, C., Wang, C., Wu, Y., Yong, C., et al. (2020). Gut microbiota dysbiosis in patients with biopsy-proven nonalcoholic fatty liver disease: A cross-sectional study in Taiwan. *Nutrients* 12:820. doi: 10.3390/nu12030820

van der Velde, K., Imhann, F., Charbon, B., Pang, C., van Enckevort, D., Slofstra, M., et al. (2019). MOLGENIS research: Advanced bioinformatics data software for non-bioinformaticians. *Bioinformatics* 35, 1076–1078. doi: 10.1093/bioinformatics/bty742

Verbanck, M., Chen, C. Y., Neale, B., and Do, R. (2018). Detection of widespread horizontal pleiotropy in causal relationships inferred from Mendelian randomization between complex traits and diseases. *Nat. Genet.* 50, 693–698. doi: 10.1038/s41588-018-0099-7

Vervoort, J., Xavier, B. B., Stewardson, A., Coenen, S., Godycki-Cwirko, M., Adriaenssens, N., et al. (2015). Metagenomic analysis of the impact of nitrofurantoin treatment on the human faecal microbiota. *J. Antimicrob. Chemother.* 70, 1989–1992. doi: 10.1093/jac/dkv062

Worby, C. J., Olson, B., Dodson, K., Earl, A., and Hultgren, S. (2022a). Establishing the role of the gut microbiota in susceptibility to recurrent urinary tract infections. *J. Clin. Investig.* 132:e158497. doi: 10.1172/JCI158497

Worby, C. J., Schreiber, H. L., Straub, T. J., Van Dijk, L. R., Bronson, R. A., Olson, B. S., et al. (2022b). Longitudinal multi-omics analyses link gut microbiome dysbiosis with recurrent urinary tract infections in women. *Nat. Microbiol.* 7, 630–639. doi: 10.1038/s41564-022-01107-x

Wu, R., Kumawat, A. K., and Demirel, I. (2023). Trimethylamine N-Oxide (TMAO) mediates increased inflammation and colonization of bladder epithelial cells during a uropathogenic *E. coli* infection in vitro. *Pathogens* 12:523. doi: 10.3390/pathogens12040523

Xu, H., Fang, F., Wu, K., Song, J., Li, Y., Lu, X., et al. (2023). Gut microbiota-bile acid crosstalk regulates murine lipid metabolism via the intestinal FXR-FGF19 axis in diet-induced humanized dyslipidemia. *Microbiome* 11:262. doi: 10.1186/s40168-023-01709-5

Xue, C., Li, G., Zheng, Q., Gu, X., Shi, Q., Su, Y., et al. (2023). Tryptophan metabolism in health and disease. *Cell Metab.* 35, 1304–1326. doi: 10.1016/j.cmet.2023.06.004

Yang, H. J., Kim, D. S., Lee, K. W., and Kim, Y. H. (2022). The urinary microbiome; axis crosstalk and short-chain fatty acid. *Diagnostics* 12:3119. doi: 10.3390/diagnostics12123119

Zhou, Z., Feng, D., Shi, D., Gao, P., Wang, L., and Wu, Z. (2023). Untargeted and targeted metabolomics reveal bile acid profile changes in rats with ethylene glycol-induced calcium oxalate nephrolithiasis. *Chem. Biol. Interact.* 381:110570. doi: 10.1016/j.cbi.2023.110570

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COVID-19-associated encephalopathy: connection between neuroinflammation and microbiota-gut-brain axis

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While neurological complications of COVID-19, such as encephalopathy, are relatively rare, their potential significant impact on long-term morbidity is substantial, especially given the large number of infected patients. Two proposed hypotheses for the pathogenesis of this condition are hypoxia and the uncontrolled release of proinflammatory cytokines. The gut microbiota plays an important role in regulating immune homeostasis and overall gut health, including its effects on brain health through various pathways collectively termed the gut–brain axis. Recent studies have shown that COVID-19 patients exhibit gut dysbiosis, but how this dysbiosis can affect inflammation in the central nervous system (CNS) remains unclear. In this context, we discuss how dysbiosis could contribute to neuroinflammation and provide recent data on the features of neuroinflammation in COVID-19 patients.

KEYWORDS

encephalopathy, gut microbiome, COVID-19, gut-brain axis, probiotic, cytokine, inflammation, butyrate

1 Introduction

COVID-19, caused by the SARS-CoV-2 virus, is linked to various neurological complications, including COVID-19 encephalopathy, which is often observed in severe cases, particularly in older patients with acute respiratory distress syndrome and multiple organ failure (Ellul et al., 2020; Abenza Abildúa et al., 2021; Garg et al., 2021; Pilotto et al., 2021; Akimoto et al., 2022). The Global COVID-19 Neuro Research Coalition defines COVID-19 encephalopathy as necessitating a COVID-19 diagnosis, temporal correlation with symptoms, exclusion of other diseases, and differentiation between primary brain disease and secondary brain dysfunction related to COVID-19 severity (Michael et al., 2023).

Studies on the human microbiome have revealed its crucial role in immune system development, combating pathogens, toxin elimination, metabolic functions, and overall health (Gu et al., 2020; Villapol, 2020). Research indicates that COVID-19 patients exhibit reduced gut microbiome diversity and abundance, with an increase in opportunistic pathogens and a decrease in beneficial microbiota, correlating with respiratory issues and neuropsychiatric symptoms (Wu et al., 2021). The complex connection between the gut and brain, known as the microbiota-gut-brain axis (MGBA), involves interactions through intestinal cells, the enteric nervous system (ENS), metabolic pathways, and neuroendocrine mechanisms, as

supported by numerous clinical studies (Cryan et al., 2019; Morais et al., 2021).

The neurological manifestations linked to COVID-19 are driven by inflammatory responses that inundate the brain via proinflammatory factors, causing damage to neural cells and resulting in brain ischemia (Otani et al., 2023). The gut microbiota plays an important role in controlling inflammation through direct and indirect mechanisms (Maciel-Fiuza et al., 2023). However, there is very little information about the role of neuroinflammation in COVID-19 encephalopathy and the potential role of the gut microbiota in regulating inflammation. With this review, we aim to elucidate this gap to provide possible new therapeutic options and understand the pathophysiology more comprehensively.

2 Neuroinflammation in COVID-19 encephalopathy

2.1 Symptoms and pathogenesis

More than half of hospitalized patients exhibit symptoms such as dizziness, altered mental status, ataxia, and cognitive dysfunction, with encephalopathy recognized as the most prevalent neurological symptom of COVID-19 (Graham et al., 2022). Currently, two main hypotheses regarding the pathogenesis of COVID-19 encephalopathy have been established: the hypoxic hypothesis and the inflammatory cytokine-based hypothesis (Ahmad and Rathore, 2020).

2.2 Hypoxic hypothesis

Systemic hypoxia resulting from COVID-19 infection can lead to metabolic deficiencies, causing global brain dysfunction. COVID-19 disrupts respiratory function, impairing gas exchange in the lungs and potentially inducing a hypoxic state (Vengalil et al., 2023). The metabolic processes of brain tissue are dependent on oxygen, and insufficient oxygen levels impede the oxidation of glucose for the production of ATP. An insufficient amount of energy can result in impaired neuronal function and ultimately cellular death, thereby playing a role in the development of COVID-19 encephalopathy (Karuppan et al., 2021).

The interaction between the brain and lungs, known as the brain-lung crosstalk axis, is a crucial but often overlooked mechanism that has significant implications for ventilatory management in the development of brain complications associated with COVID-19. Decreased systemic oxygen levels can impact the oxygenation of brain tissue, potentially leading to secondary brain injury. Concurrently, disturbances in lung function can disrupt the delicate equilibrium between oxygen and carbon dioxide levels, which are pivotal for maintaining cerebral homeostasis (Robba et al., 2021). Such disruptions can result in alterations in cerebral blood flow, leading to either ischemic or hyperemic conditions within the brain (Battaglini et al., 2020). These vascular changes may eventually culminate in cerebral edema and impairment of cerebral autoregulation. Notably, postmortem examinations of COVID-19 patients have consistently revealed acute hypoxic damage to both the cerebrum and cerebellum in the absence of evidence suggesting direct brain invasion or encephalitis (Solomon et al., 2020).

2.3 The cytokine-based hypothesis

The second mechanism involves the uncontrolled proliferation and secretion of cytokines triggered by COVID-19 infection (Perrin et al., 2021). This idea was supported by the findings of a clinical trial by Dirk Reinhold et al., who reported that COVID-19 is not primarily a neuroinflammatory disease; however, it does impact the brain indirectly by activating inflammatory pathways outside of the brain (Reinhold et al., 2023). The release of cytokines can trigger a series of reactions, which can weaken the integrity of the endothelial lining and disrupt the blood–brain barrier (BBB) (Perrin et al., 2021). In the blood of COVID-19 patients, researchers have found significantly increased levels of cytokines such as interleukin-6 (IL-6) and the astroglial marker S100B. Elevated levels of S100B are associated with increased permeability of the BBB, while elevated levels of IL-6 suggest CRS in the brain. After crossing the BBB, IL-6 triggers a positive feedback mechanism, stimulating adjacent immune cells to release additional cytokines such as IL-1β, TNF-α, and IFN-γ (Vengalil et al., 2023). These proinflammatory cytokines further activate brain-resident macrophages, perpetuating a secondary inflammatory response in brain tissue. This ongoing inflammation triggers the release of more cytokines, intensifying the positive feedback loop. Thus, monocytes and macrophages cross the BBB and initiate reactive gliosis, a process characterized by the proliferation of reactive glial cells, leading to scarring and the onset of encephalopathy.

A study conducted in France (Strasbourg) focused on COVID-19 patients who exhibited severe neurological symptoms, including confusion, cerebellar ataxia, agitation, tremor, and impaired consciousness (Vengalil et al., 2023). Laboratory evaluations were performed to assess CRS markers, such as IL-6, C-reactive protein, ferritin, and lactate dehydrogenase. In more than 50% of the patients, the highest levels of these markers occurred simultaneously with the emergence of neurological symptoms. Furthermore, an increase in the permeability of the BBB, as evidenced by elevated levels of S100B protein during CRS, was observed. Notably, reverse transcription PCR assays were unable to detect SARS-CoV-2 in cerebrospinal fluid (CSF). This indicates that the neurological symptoms were not caused by viral encephalitis but rather by CRS induced by COVID-19 infection (Perrin et al., 2021). The successful alleviation of CRS encephalopathy through the administration of steroids and intravenous immune globulin is a promising therapeutic strategy (Afshar et al., 2020; Pilotto et al., 2020).

In another clinical case, a man who was initially admitted with COVID-19 symptoms was later readmitted due to acute encephalopathy (Jang et al., 2020). Despite the absence of hypoxia, nasopharyngeal PCR confirmed SARS-CoV-2 infection. Upon readmission, the patient exhibited confusion, emesis, anorexia, and tremors, along with brain MRI findings suggestive of ischemia. Notably, CSF analysis did not detect SARS-CoV-2, ruling out encephalitis. The onset of encephalopathy correlated with increased levels of the inflammatory marker C-reactive protein, indicating a profound inflammatory reaction driven by innate immunity. This cytokine-associated toxicity, or cytokine storm, likely contributes to the development of encephalopathy through a cascade of proinflammatory cytokines crossing the BBB and triggering reactive gliosis, ultimately leading to brain scarring.

Despite elevated cytokine levels in COVID-19 patients, the virus interferes with the production of interferons, particularly type 1

interferon (IFN-I), which plays a crucial role in combating viral infections (Anjum et al., 2021). Although IFN-I is inhibited in lung tissue, its impact on brain tissue remains unclear. A case study involving a 39-year-old COVID-19 patient treated with IFN-β showed cognitive deficits despite recovery from respiratory failure, suggesting that the antiviral function of IFN-I may not extend to the brain (Umapathi et al., 2020).

Another study assessing CSF biomarkers in COVID-19 patients with neurological symptoms indicative of encephalopathy revealed elevated levels of neurofilament light chain (Nfl), indicating axonal injury (Edén et al., 2021). This suggests that neurological complications in COVID-19 patients may result from various mechanisms, including hypoxia-induced axonal injury.

In a study involving a sample of 175 individuals diagnosed with COVID-19, serum samples revealed increased levels of brain injury biomarkers, such as neurofilament light chain and glial fibrillary acidic protein (GFAP), in a manner that was dependent on the severity of the disease (Franke et al., 2021; Rachel et al., 2023). The observed elevation remained consistent even after a four-month period of observation, suggesting the presence of persistent brain injury. Increased levels of proinflammatory cytokines and autoantibodies against brain proteins, such as myelin-associated glycoproteins, were found to be correlated with these biomarkers (Rachel et al., 2023). Notably, a similar positive association between proinflammatory cytokines and biomarkers of brain injury was identified in a control group comprising 45 individuals diagnosed with influenza. This finding implies that the observed increases in these biomarkers may be associated with the severity of the infection rather than being specific to a particular virus (Rachel et al., 2023).

Certain patients with COVID-19 encephalopathy exhibit increased CSF concentrations of proinflammatory cytokines, such as interleukin 6, interleukin 8, and macrophage inflammatory protein-1 beta (MIP-1β). These findings may be associated with assessments of BBB integrity (Bartley et al., 2021; Rachel et al., 2023). Nevertheless, the occurrence of cytokines and disruption of the BBB extend beyond particular neurological phenotypes or abnormalities observed on brain imaging. Furthermore, there is a dearth of comparative studies involving control groups, such as COVID-19 patients who do not exhibit neurological symptoms (Douaud et al., 2022). There is limited evidence of SARS-CoV-2 in CSF (Abenza Abildúa et al., 2021; Rachel et al., 2023) (Figure 1).

In certain instances, there has been evidence supporting a humoral autoimmune mechanistic model (Rachel et al., 2023). In a limited number of cases, 23% of adolescents who were infected with SARS-CoV-2 and experienced neuropsychiatric complications displayed intrathecal antibodies against SARS-CoV-2 and neuronal autoantibodies on anatomical immunostaining. These individuals did not meet the criteria for encephalitis and had normal imaging and laboratory results (Starkey et al., 2017; Rachel et al., 2023). It is worth mentioning that a patient who exhibited a positive response to immunotherapy had antibodies specifically targeting transcription factor 4 that were isolated (Rachel et al., 2023). In three patients, severe myoclonus, the most prevalent movement disorder in patients with COVID-19 infection, and somnolence were observed in another case series. These patients had normal imaging and CSF levels (Helms et al., 2020; Rachel et al., 2023). An enhancement was noted subsequent to the administration of corticosteroids and/or plasma exchange, or both (Rachel et al., 2023).

Brain imaging associated with encephalopathy generally reveals normal findings, although it may sometimes display leptomeningeal enhancement, indicating inflammation. More severe cases, particularly in the intensive care setting, might show leukoencephalopathic alterations or microhemorrhages. These changes reflect damage to the white matter or small brain hemorrhages, often linked to the effects of severe COVID-19 or its treatments (Singh et al., 2022; Rachel et al., 2023). Another unusual finding, cytotoxic lesions in the splenium of the corpus callosum, has been reported in some cases, potentially resulting from cytokine-mediated glutamate release (Hosp et al., 2021; Huo et al., 2022; Rachel et al., 2023).

A meta-analysis conducted by Pyeong Hwa Kim et al. included 1,394 COVID-19 patients who underwent neuroimaging from 17 studies. Among these patients, 3.4% had COVID-19-related neuroimaging findings. The most common abnormality was related to the olfactory bulb and was observed in 23.1% of the patients. Among cerebral findings, white matter abnormalities were the most frequent (17.6%), followed by acute/subacute ischemic infarction (16.0%) and encephalopathy (13.0%). Critically ill patients had a significantly greater proportion of COVID-19-related neuroimaging findings than did other patients (Kim et al., 2021).

Functional imaging studies, such as positron emission tomography (PET) or functional magnetic resonance imaging (fMRI), can reveal abnormal patterns of brain metabolism or perfusion in COVID-19 patients with neurological symptoms. Frontoparietal or frontotemporal patterns are among the most commonly observed, suggesting that these regions might be more susceptible to the systemic effects of COVID-19 or related inflammatory responses (Liotta et al., 2020; Taquet et al., 2021; Rachel et al., 2023). Electroencephalography (EEG) in patients with COVID-19-related encephalopathy can reveal diffuse or focal slowing, which is indicative of brain dysfunction. In severe cases, patients might experience seizures or nonconvulsive status epilepticus, a critical condition requiring immediate intervention (Bartley et al., 2021; Rachel et al., 2023). CSF analysis is another valuable diagnostic tool. In COVID-19-related encephalopathy, CSF is often normal, suggesting a noninfectious cause. However, pleocytosis, an increase in white blood cells in the CSF, may indicate SARS-CoV-2-related or postinfective immune-mediated encephalitis. This condition requires further examination to exclude other possible infections caused by viruses or bacteria, as the treatment strategies can vary considerably (Xu et al., 2022; Rachel et al., 2023).

Neuroinflammation, characterized by inflammatory processes within the central nervous system (CNS), is frequently observed in encephalopathies associated with infectious diseases. A diverse range of pathogens, including viruses such as influenza and herpes, protozoa such as Toxoplasma and Plasmodium, and bacteria such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, have been implicated in eliciting neuroinflammatory responses (Rock et al., 2008; Gilden and Nagel, 2016; Brasil et al., 2017; Tapajós et al., 2019). The pathogenesis of neuroinflammation involves the mediation of various molecules, including cytokines, chemokines, reactive oxygen species, and others (Barbosa-Silva et al., 2021). Cytokines have detrimental effects on brain function, particularly impacting the hippocampus (Milatovic et al., 2003; Richwine et al., 2008). Specifically, interleukin-1β (IL-1β) decreases synaptic strength and long-term potentiation in rodent hippocampi, influencing neuronal

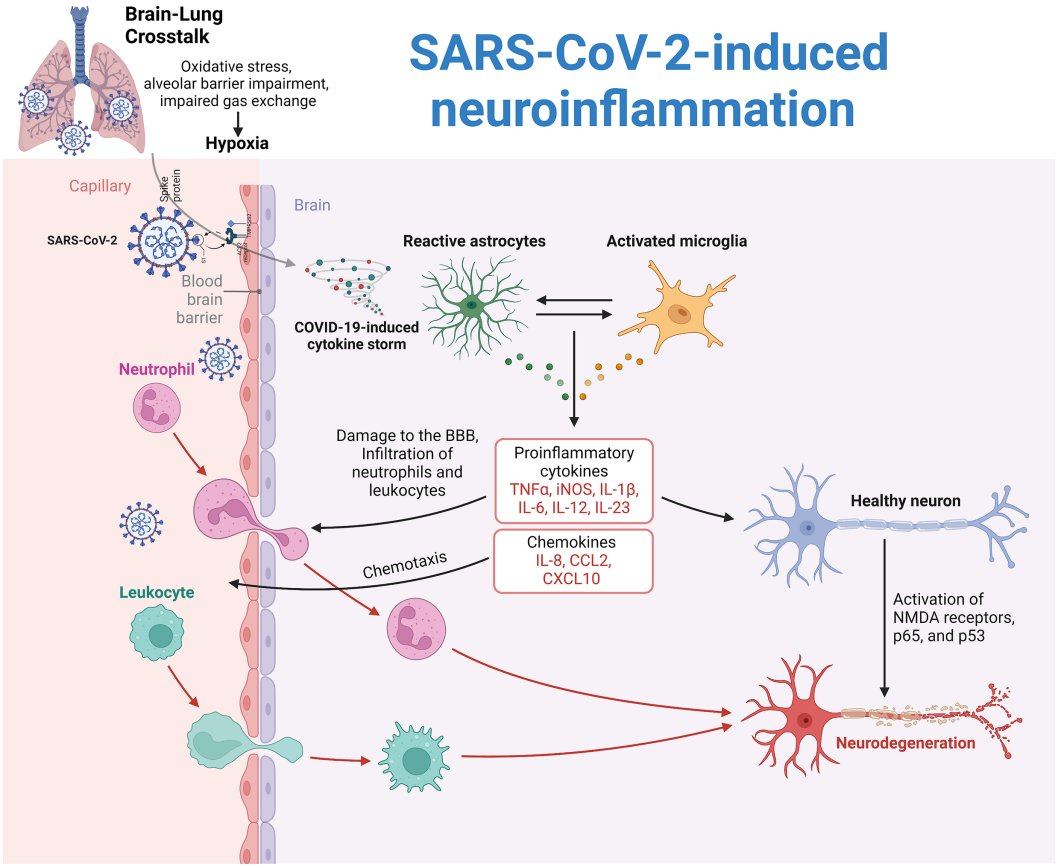


FIGURE 1
Pathogenesis of SARS-CoV-2-induced neuroinflammation. Two main theories explain the neuroinflammation observed in COVID-19 patients. The first theory is attributed to reduced systemic oxygenation, which leads to cerebral hypoxia and secondary brain damage, known as brain-lung crosstalk. The second theory involves the direct invasion of SARS-CoV-2, which triggers a hyperinflammatory response, causing a cytokine storm with inflammatory mediators such as IL-1β, IL-6, and TNF. This storm damages the blood–brain barrier (BBB), allowing immune cells and proinflammatory molecules to enter the brain. As a result, microglia and astrocytes become reactive. Microglia increase the production of reactive oxygen species, cytokines, and chemokines while reducing brain-derived neurotrophic factor (BDNF). Astrocytes increase glial fibrillary acidic protein (GFAP) and vimentin expression, leading to structural changes and reduced neurotransmitter recycling.

structure, synaptic adaptability, and memory and learning processes. In a study by Bellinger et al., interleukin-1β (IL-1β) was found to inhibit synaptic strength and reduce long-term potentiation in rodent hippocampi, thereby affecting neuronal structure, synaptic plasticity, and the processes underlying memory and learning (Lynch, 2002). Additionally, cytokines, notably IL-1β, hinder the signaling of brain-derived neurotrophic factor (BDNF), which is crucial for neuronal health (Tong et al., 2008). Furthermore, systemic administration of lipopolysaccharide (LPS) reduces the levels of BDNF, nerve growth factor (NGF), and neurotrophin-3, consequently affecting synaptic plasticity, memory, and neuronal survival (Guan and Fang, 2006) (Figure 2).

Glial reactivity also impacts neuronal cells, leading to a loss of support from glial cells. Astrocytes, for instance, regulate neurotransmitter levels such as gamma-aminobutyric acid (GABA), glutamate, and glycine in the synaptic space (Seifert et al., 2006). Astrogliosis, a consequence of glial reactivity, disrupts this regulation, resulting in glutamate toxicity (Heneka et al., 2015). Furthermore, glutamate toxicity can be exacerbated by the activation of indoleamine-2,3 dioxygenase (IDO), an enzyme expressed by microglia (Myint and Kim, 2014). Inflammatory mediators such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α modulate IDO activity. Additionally, IDO plays a role in tryptophan-serotonin availability, suggesting that proinflammatory cytokines contribute to neurotransmitter imbalances (Schmidt et al., 2013).

3 Role of the gut-brain axis in patients with COVID-19

Substantial evidence suggests that the SARS-CoV-2 virus can compromise the lining of the intestines and blood vessels within the brain through both direct and indirect mechanisms (Buzhdygan et al., 2020; Cardinale et al., 2020). Specifically, the virus relies on angiotensin-converting enzyme 2 (ACE2) receptors for cellular entry, which are abundant in the cells lining blood vessels in the brain and intestines (Hamming et al., 2004; Lu et al., 2020; Zhou et al., 2020). Studies have indicated that viruses can directly damage these cellular linings, induce inflammation, and trigger excessive immune responses known as cytokine storms, which can further exacerbate tissue damage (Elyaspour et al., 2021; Nicosia et al., 2021) (Figure 3).

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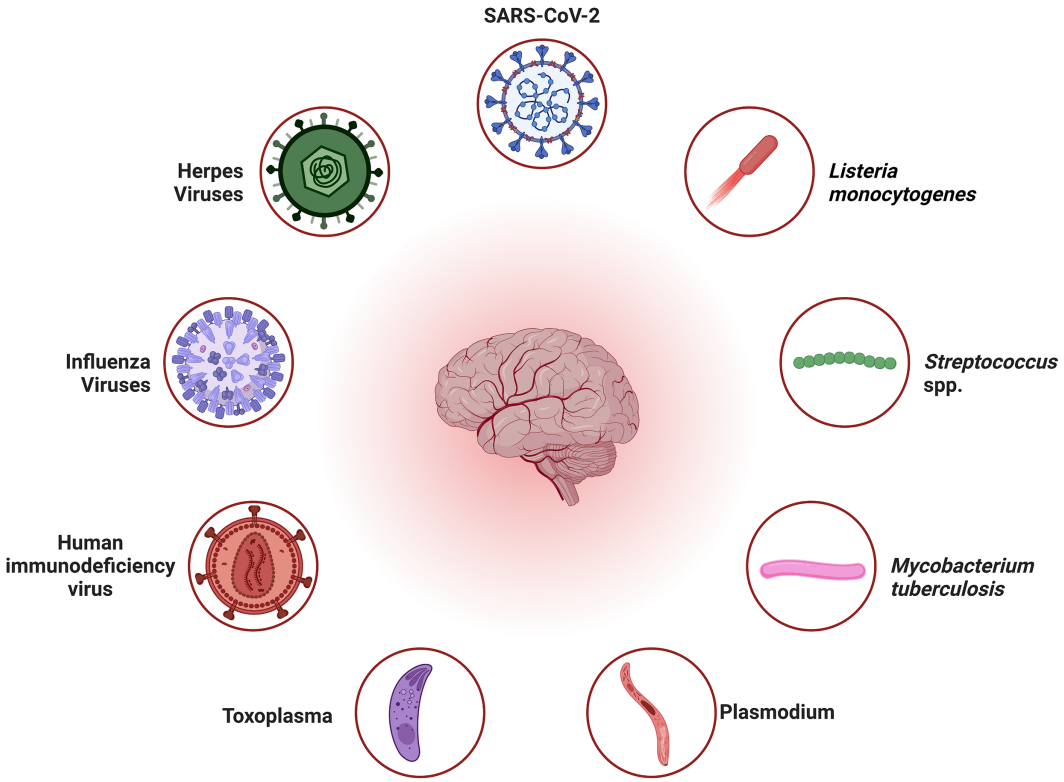


FIGURE 2
Overview of Pathogens that Cause Neuroinflammation. Neuroinflammation, a secondary inflammatory response in the brain, can occur as a result of peripheral infections caused by viruses, bacteria, or parasites. Inflammatory mediators that affect the brain endothelium and parenchyma as well as the subsequent response of brain cells to these mediators mediate this response.

The intestinal wall, which is rich in ACE2 receptors, is particularly susceptible to viral infiltration, leading to potential damage to the intestinal barrier and increased permeability (Cardinale et al., 2020). Additionally, COVID-19 frequently manifests with gastrointestinal disturbances, which can alter the balance of the gut microbiota toward a state that is proinflammatory and potentially harmful to neurological function (Kopel et al., 2020; Bernard-Raichon et al., 2022).

COVID-19 may cause substances with neurotoxic and neuroinflammatory properties to pass from the gut into the bloodstream due to a compromised intestinal barrier (Plummer et al., 2023). These substances may then enter the brain through weakened blood vessels, resulting in neuroinflammation caused by both direct viral damage and indirect effects of dysregulated immune responses (Plummer et al., 2023).

The previously established relationship between the gut microbiota and the CNS has been discussed as a potential explanation for the impact of SARS-CoV-2 on neurological functions (Monteleone et al., 2020). Reciprocal communication among the brain-gut-microbiota-immune axis is vital for maintaining immune equilibrium and the balance between Th17 and Treg cells (Luo et al., 2017; Pandiyan et al., 2019). Notably, the microbiota plays a significant role in this interplay and significantly influences these immune functions (Barnes and Powrie, 2009).

In the study by Viviani Mendes de Almeida et al., a notable increase in antibiotic-resistant Enterobacteriaceae strains was revealed in post-COVID-19 individuals compared to healthy controls. Additionally, reduced levels of short-chain fatty acids (SCFAs) are observed in fecal samples from post-COVID-19 patients, suggesting a potential link between altered gut microbiota and disease severity in COVID-19 patients (Mendes de Almeida et al., 2023).

SCFAs play a significant role not only in local effects within the colon and peripheral tissues but also in the intricate interplay between the gut and brain microbiota. The increased expression of monocarboxylate transporters (MCTs) in endothelial cells could facilitate the passage of SCFAs across the BBB, as evidenced by studies administering labeled SCFAs directly into the carotid artery of rats (Oldendorf, 1973; Vijay and Morris, 2014). Although research on the physiological levels of SCFAs in the brain is limited, all three SCFAs can be detected in human CSF, where they are present at higher concentrations than in peripheral blood (Li et al., 2017).

Furthermore, SCFAs appear to have a significant impact on the maintenance of BBB integrity. This is of utmost importance because it plays a critical role in regulating the transportation of nutrients and molecules into the brain, ultimately contributing to brain development and CNS homeostasis (Braniste et al., 2014). Research conducted on germ-free mice has shown that SCFAs control the production of tight

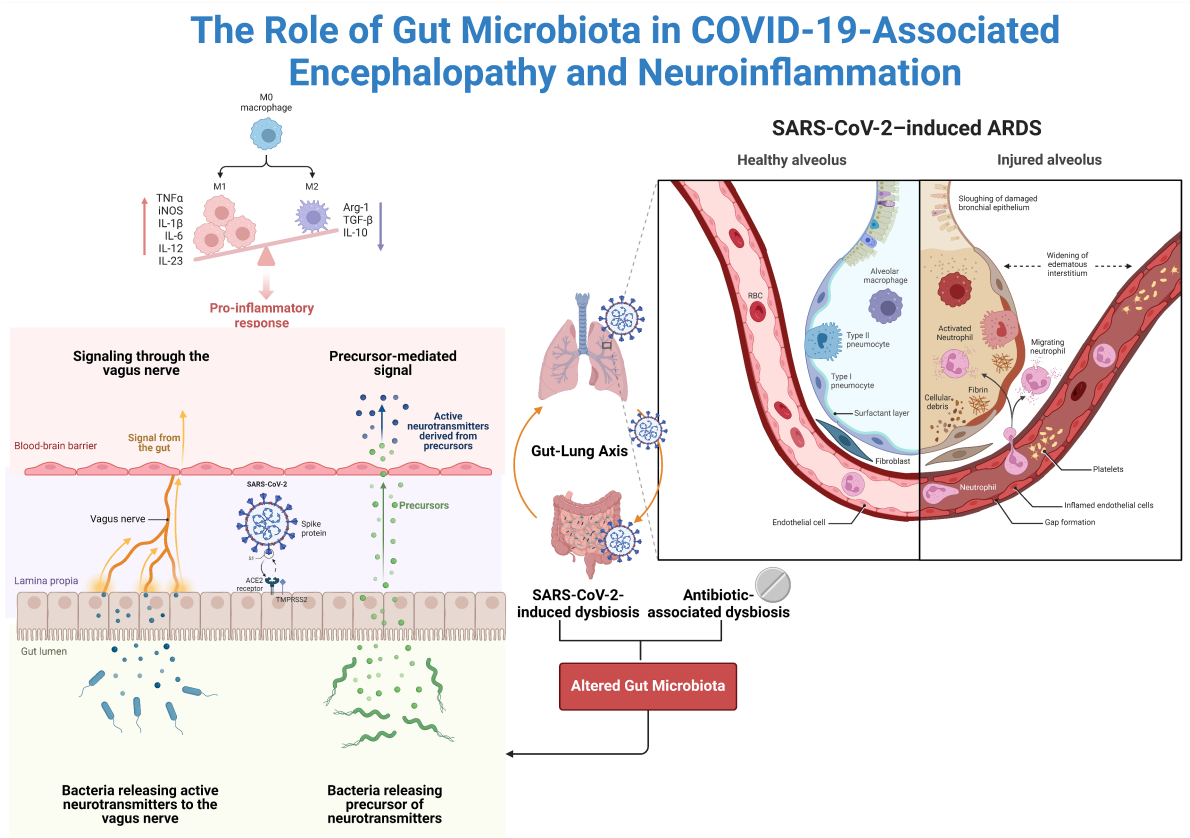


FIGURE 3
The Potential Impact of the Gut Microbiota on Neuroinflammation. Coronaviruses actively replicate in enterocytes since they highly express ACE2. The available data support a change in the microbiota in COVID-19 patients. Altered microbiota can cause intestinal barrier damage, systemic translocation of microbial products, and systemic inflammation. Additionally, the microbiota is connected to the brain via the vagus nerve and is referred to as the gut–brain axis.

junction proteins such as occludin and claudin, which in turn affect the permeability of the blood–brain barrier from the early stages of development to adulthood (Braniste et al., 2014). Nevertheless, the restoration of BBB integrity is achieved through the recolonization of these mice with complex microbiota or bacterial strains that produce SCFAs (Braniste et al., 2014).

Furthermore, previous studies conducted on cerebrovascular endothelial cells treated with propionate *in vitro* have indicated that SCFAs can alleviate the effects of LPS on vascular permeability (Hoyles et al., 2018). There is evidence indicating that SCFAs entering the CNS have neurological effects, although the specific mechanisms by which they work are still not fully understood. Animal studies have indicated a broad impact of SCFAs on various behavioral and neurological processes, potentially implicating them in significant stages of neurodegenerative and neurodevelopmental diseases (Silva et al., 2020).

Butyrate [also a short-chain fatty acid (SCFA)], an essential component in maintaining intestinal barrier integrity, plays a crucial role in this process (Candido et al., 1978). Furthermore, butyrate plays a crucial role in supporting the mucosal layer of the intestine. Additionally, it contributes to the process of histone acetylation, which alters the molecular composition of chromatin to facilitate transcription (Chriett et al., 2019). Moreover, butyrate has been shown to inhibit the expression of proinflammatory genes and the subsequent release of these genes induced by lipopolysaccharide (LPS) in endothelial cells. It has been proposed that the reduction in bacteria that produce butyrate may exacerbate the damage to gut epithelial cells caused by SARS-CoV-2, resulting from insufficient downregulation of these inflammatory mechanisms (Li et al., 2021).

Additionally, research has shown that butyrate can improve the body's natural immune response to viral infections by activating the Toll-like receptor signaling pathway. This results in increased levels of interleukin-1β, interferon regulatory factor-7, and interferon-alpha/beta receptor expression at both the mRNA and protein levels. Therefore, butyrate likely plays a role in the innate immune response to SARS-CoV-2 (Li et al., 2021).

LPS, an important constituent of the outer membrane of gram-negative bacteria in the gastrointestinal tract, induces systemic inflammation and stimulates microglia in the brain (Raetz and Whitfield, 2002; Brown, 2019). While elevated levels of this endotoxin have been detected in COVID-19 patients admitted to hospitals, its precise contribution to neuroinflammation in these patients is still unclear (Teixeira et al., 2021). Research conducted on rats has shown that LPS can directly enter the brain and bind to receptors on endothelial cells at the interface between the blood and brain. This process exacerbates damage to the endothelium caused by viruses and cytokines (Vargas-Caraveo et al., 2017). Importantly, LPS interferes with the BBB in specific areas of the brain, impacting regions that are

essential for complex cognitive processes such as the thalamus and frontal cortex (Jung and Haier, 2007; Vakhtin et al., 2014).

Once overshadowed by LPS, peptidoglycan (PGN) has emerged as a gram-positive counterpart contributing to systemic endotoxicity (Myhre et al., 2006). This shift is attributed to the increasing incidence of sepsis caused by gram-positive organisms (Mayr et al., 2014). PGN concentrations in blood plasma reflect intestinal permeability due to various conditions, such as ischemia and hemorrhagic shock (Tsunooka et al., 2004). Gut-derived PGN has been linked to inflammatory and autoimmune conditions, including multiple sclerosis, and has shown neurotoxic effects in animal models, triggering microglial and astrocytic nitric oxide production and leading to neuronal cell death (Boje and Arora, 1992; Buskila et al., 2005). Beyond its role in innate immunity, PGN has implications for neurodevelopment and associated disorders such as autism spectrum disorder (Arentsen et al., 2017; Gonzalez-Santana and Diaz, 2020). Elevated PGN concentrations have also been noted in hospitalized COVID-19 patients, potentially contributing to persistent neuroinflammation and neurotoxicity along the gut-brain axis (Plummer et al., 2023) (Figure 4).

4 Current and potential therapeutic options

The standard approach for managing encephalopathy in COVID-19 patients primarily involves supportive measures such as supplemental oxygen therapy and immune modulation (Wiersinga et al., 2020). Immune modulation therapy, including the administration of high-dose corticosteroids (intravenous methylprednisolone at a dosage of 500 mg to 1 g per day for 5 days) and intravenous immunoglobulin (at a dosage of 0.1–0.5 g per kg per day for 5–15 days), is prioritized over antiviral treatment due to the systemic inflammatory response induced by SARS-CoV-2 (Galeotti et al., 2020; Pilotto et al., 2020; Pugin et al., 2020). Additionally, repeated plasmapheresis has demonstrated efficacy in enhancing consciousness and reducing the levels of proinflammatory markers in the bloodstream (Dogan et al., 2020). Critically ill patients with encephalopathy are often managed in the intensive care unit (ICU), where mechanical ventilation may be necessary. Anti-epileptic drugs should be initiated both as abortive and prophylactic therapy in these patients if they exhibit altered mental status, seizures, or subtle muscular spasms (Shah et al., 2021). However, close monitoring for adverse effects and potential drug interactions is crucial, given the significant respiratory and cardiac risks associated with antiepileptic medications (Asadi-Pooya, 2020). The increased tendency for blood clot formation observed in COVID-19 patients can be effectively managed through the administration of anticoagulant drugs such as heparin. This treatment approach has been linked to improved outcomes, particularly in individuals with significantly elevated levels of a marker called D-dimer (Horie et al., 2020). Additionally, in COVID-19 patients not requiring mechanical ventilation, tocilizumab has been identified as a medication capable of lowering

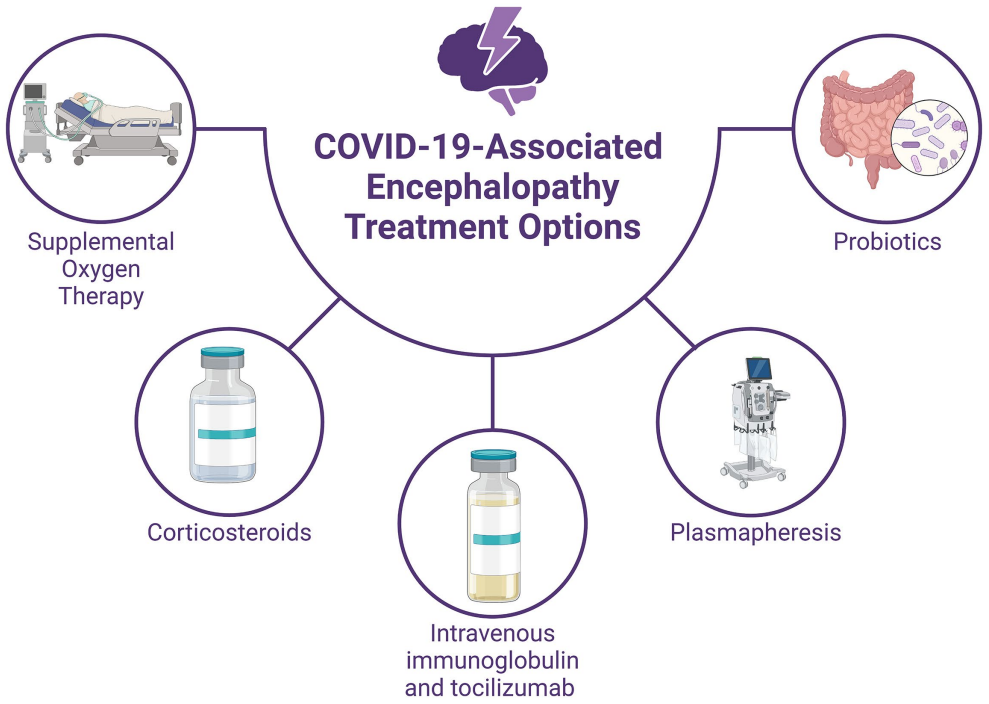


FIGURE 4
Treatment Options for COVID-19-associated Encephalopathy. Supportive therapy includes supplemental oxygen to address hypoxemia and immune modulation with high-dose corticosteroids and intravenous immunoglobulin to manage the systemic inflammatory response. Plasmapheresis can help reduce proinflammatory marker levels and improve consciousness in severe cases. Immunomodulation involves tocilizumab, which may reduce the need for mechanical ventilation in some patients but does not guarantee improved survival. Probiotic therapy with strains such as *Lactobacillus plantarum* and *Pediococcus acidilactici* has shown potential in reducing viral load and improving symptoms. Studies suggest that probiotic-supplemented patients may have shorter hospital stays and better outcomes.

the risk of progression to the need for mechanical ventilation or death. However, it should be noted that tocilizumab does not confer a survival benefit in these patients (Salama et al., 2021).

Another adjuvant therapeutic option can be probiotics, which are defined as beneficial microorganisms capable of surviving and proliferating within the gastrointestinal tract (Lehtoranta et al., 2014). They exert protective effects against a spectrum of pathogens while exhibiting varied benefits based on their specific strains. Through their interaction with the host immune system, probiotics elicit a response involving the production of both anti-and proinflammatory cytokines such as IL-10, IL-12, IL-17, and interferon-α (IFN-α) upon encountering infectious agents (Azad et al., 2018; Kalam and Balasubramaniam, 2024). This immunomodulatory action encompasses two pivotal mechanisms: an immunostimulatory effect activating IL-12 and promoting the activity of natural killer (NK) cells, T1 helper cells, and T2 helper cells (Th1 and Th2) against harmful pathogens and an immunoregulatory effect fostering the production of IL-10 and regulatory T cells (Tregs) by stimulating various immune cells, including Th-2 cells, B cells, dendritic cells (DCs), and monocytes, thereby orchestrating an adaptive immune response within the human host (Chiba et al., 2010; Kalam and Balasubramaniam, 2024).

A clinical study was conducted to evaluate the effectiveness of a combination of probiotics in outpatients who tested positive for COVID-19 (Kalam and Balasubramaniam, 2024). The mixture consisted of strains of *Lactobacillus plantarum* and *Pediococcus acidilactici* in equal proportions. All participants exhibited favorable tolerance to the probiotics during the trial. It is worth mentioning that the treatment group demonstrated a significant decrease in viral load in the nasopharyngeal region, as well as a reduction in both gastrointestinal (GI) and non-GI symptoms, compared to the placebo group (Kalam and Balasubramaniam, 2024). Interestingly, the individuals who received treatment exhibited elevated levels of antibodies specific to SARS-CoV-2 compared to those in the control group. However, no significant changes were observed in the composition of the fecal microbiota in either group. The effectiveness of probiotics may primarily depend on their interaction with the immune system of the host rather than the overall composition of the microbiota (Chiba et al., 2010; Kalam and Balasubramaniam, 2024).

Additionally, a study was conducted to evaluate the effectiveness of various strains of beneficial bacteria, including *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, in COVID-19 patients across a spectrum of disease severities. Patients received a dose of 1.0×10^7 CFU of probiotics alongside standard care (Kalam and Balasubramaniam, 2024). The results revealed that patients supplemented with probiotics exhibited improved clinical outcomes and a shortened duration of hospitalization compared to those receiving standard care alone (Zhang et al., 2021; Kalam and Balasubramaniam, 2024).

5 Conclusion

Encephalopathy associated with COVID-19 is characterized by neuroinflammation, which can manifest even without direct viral invasion into the central nervous system. Neuroinflammation, which is mediated by cytokines, chemokines, and reactive oxygen species, plays a central role in the pathogenesis of encephalopathy. COVID-19 induces encephalopathy primarily through two main mechanisms: hypoxia and the overproduction of cytokines.

SARS-CoV-2 also affects the intestinal epithelium, leading to gut dysbiosis, which can exacerbate neuroinflammation through the gut-brain axis. This altered balance in the gut microbiota may allow neuroinflammatory substances to enter the bloodstream and reach the brain.

Probiotics show potential for mitigating symptoms associated with COVID-19. Studies have shown improvements in immune modulation, reduced viral load, and improved clinical outcomes in COVID-19 patients who receive probiotics. However, further research is necessary to understand the mechanisms involved and optimize the use of probiotics in managing COVID-19-associated encephalopathy.

Author contributions

KD: Writing – original draft, Conceptualization. PP: Writing – original draft, Visualization. OK: Supervision, Writing – review & editing.

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Conflict of interest

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References

Abenza Abildúa, M. J., Atienza, S., Carvalho Monteiro, G., Erro Aguirre, M. E., Imaz Aguayo, L., Freire Álvarez, E., et al. (2021). Encephalopathy and encephalitis during acute SARS-CoV-2 infection. Spanish Society of Neurology COVID-19 registry. *Neurologia* 36, 127–134. doi: 10.1016/j.nrl.2020.11.013

Afshar, H., Yassin, Z., Kalantari, S., Aloosh, O., Lotfi, T., Moghaddasi, M., et al. (2020). Evolution and resolution of brain involvement associated with SARS-CoV-2 infection: a close clinical - Paraclinical follow up study of a case. *Mult. Scler. Relat. Disord.* 43:102216. doi: 10.1016/j.msard.2020.102216

Ahmad, I., and Rathore, F. A. (2020). Neurological manifestations and complications of COVID-19: a literature review. *J. Clin. Neurosci.* 77, 8–12. doi: 10.1016/j.jocn.2020.05.017

Akimoto, T., Hara, M., Tasaki, K., Kurosawa, Y., Nakamoto, T., Hirose, S., et al. (2022). Delayed encephalopathy after COVID-19: a case series of six patients. *Medicine* 101:e31029. doi: 10.1097/MD.0000000000031029

Anjum, F. R., Anam, S., Abbas, G., Mahmood, M. S., Rahman, S. U., Goraya, M. U., et al. (2021). Type I IFNs: a blessing in disguise or partner in crime in MERS-CoV-, SARS-CoV-, and SARS-CoV-2-induced pathology and potential use of type I IFNs in synergism with IFN-γ as a novel antiviral approach against COVID-19. *Viral Immunol.* 34, 321–329. doi: 10.1089/vim.2020.0085

Arentsen, T., Qian, Y., Gkotzis, S., Femenia, T., Wang, T., Udekwu, K., et al. (2017). The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. *Mol. Psychiatry* 22, 257–266. doi: 10.1038/mp.2016.182

Asadi-Pooya, A. A. (2020). Seizures associated with coronavirus infections. *Seizure* 79, 49–52. doi: 10.1016/j.seizure.2020.05.005

Azad, M. A. K., Sarker, M., and Wan, D. (2018). Immunomodulatory effects of probiotics on cytokine profiles. *Biomed. Res. Int.* 2018, 1–10. doi: 10.1155/2018/8063647

Barbosa-Silva, M. C., Lima, M. N., Battaglini, D., Robba, C., Pelosi, P., Rocco, P. R. M., et al. (2021). Infectious disease-associated encephalopathies. *Crit. Care* 25:236. doi: 10.1186/s13054-021-03659-6

Barnes, M. J., and Powrie, F. (2009). Regulatory T cells reinforce intestinal homeostasis. *Immunity* 31, 401–411. doi: 10.1016/j.immuni.2009.08.011

Bartley, C. M., Johns, C., Ngo, T. T., Dandekar, R., Loudermilk, R. L., Alvarenga, B. D., et al. (2021). Anti-SARS-CoV-2 and autoantibody profiles in the cerebrospinal fluid of 3 teenaged patients with COVID-19 and subacute neuropsychiatric symptoms. *JAMA Neurol.* 78, 1503–1509. doi: 10.1001/jamaneurol.2021.3821

Battaglini, D., Brunetti, I., Anania, P., Fiaschi, P., Zona, G., Ball, L., et al. (2020). Neurological manifestations of severe SARS-CoV-2 infection: potential mechanisms and implications of individualized mechanical ventilation settings. *Front. Neurol.* 11:845. doi: 10.3389/fneur.2020.00845

Bernard-Raichon, L., Venzon, M., Klein, J., Axelrad, J. E., Hussey, G. A., Sullivan, A. P., et al. (2022). Gut microbiome dysbiosis in antibiotic-treated COVID-19 patients is associated with microbial translocation and bacteremia. *Nat. Commun.* 13:5926. doi: 10.1038/s41467-022-33395-6

Boje, K. M., and Arora, P. K. (1992). Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. *Brain Res.* 587, 250–256. doi: 10.1016/0006-8993(92)91004-X

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., et al. (2014). The gut microbiota influences blood–brain barrier permeability in mice. *Sci. Transl. Med.* 6:263ra158. doi: 10.1126/scitranslmed.3009759

Brasil, L., Vieira, J. L. F., Araújo, E. C., Piani, P. P. F., Dias, R. M., Ventura, A., et al. (2017). Cognitive performance of children living in endemic areas for plasmodium vivax. *Malar. J.* 16:370. doi: 10.1186/s12936-017-2026-2

Brown, G. C. (2019). The endotoxin hypothesis of neurodegeneration. *J. Neuroinflammation* 16:180. doi: 10.1186/s12974-019-1564-7

Buskila, Y., Farkash, S., Hershfinkel, M., and Amitai, Y. (2005). Rapid and reactive nitric oxide production by astrocytes in mouse neocortical slices. *Glia* 52, 169–176. doi: 10.1002/glia.20217

Buzhdygan, T. P., DeOre, B. J., Baldwin-Leclair, A., Bullock, T. A., McGary, H. M., Khan, J. A., et al. (2020). The SARS-CoV-2 spike protein alters barrier function in 2D static and 3D microfluidic *in vitro* models of the human blood–brain barrier. *Neurobiol. Dis.* 146:105131. doi: 10.1016/j.nbd.2020.105131

Candido, E. P., Reeves, R., and Davie, J. R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* 14, 105–113. doi: 10.1016/0092-8674(78)90305-7

Cardinale, V., Capurso, G., Ianiro, G., Gasbarrini, A., Arcidiacono, P. G., and Alvaro, D. (2020). Intestinal permeability changes with bacterial translocation as key events modulating systemic host immune response to SARS-CoV-2: a working hypothesis. *Digest. Liver Dis.* 52, 1383–1389. doi: 10.1016/j.dld.2020.09.009

Chiba, Y., Shida, K., Nagata, S., Wada, M., Bian, L., Wang, C., et al. (2010). Well-controlled proinflammatory cytokine responses of Peyer's patch cells to probiotic *Lactobacillus casei*. *Immunology* 130, 352–362. doi: 10.1111/j.1365-2567.2009.03204.x

Chriett, S., Dąbek, A., Wojtala, M., Vidal, H., Balcerczyk, A., and Pirola, L. (2019). Prominent action of butyrate over β-hydroxybutyrate as histone deacetylase inhibitor, transcriptional modulator and anti-inflammatory molecule. *Sci. Rep.* 9:742. doi: 10.1038/s41598-018-36941-9

Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., et al. (2019). The microbiota-gut-brain Axis. *Physiol. Rev.* 99, 1877–2013. doi: 10.1152/physrev.00018.2018

Dogan, L., Kaya, D., Sarikaya, T., Zengin, R., Dincer, A., Akinci, I. O., et al. (2020). Plasmapheresis treatment in COVID-19-related autoimmune meningoencephalitis: case series. *Brain Behav. Immun.* 87, 155–158. doi: 10.1016/j.bbi.2020.05.022

Douaud, G., Lee, S., Alfaro-Almagro, F., Arthofer, C., Wang, C., McCarthy, P., et al. (2022). SARS-CoV-2 is associated with changes in brain structure in UK biobank. *Nature* 604, 697–707. doi: 10.1038/s41586-022-04569-5

Edén, A., Kanberg, N., Gostner, J., Fuchs, D., Hagberg, L., Andersson, L. M., et al. (2021). CSF biomarkers in patients with COVID-19 and neurologic symptoms: a case series. *Neurology* 96, e294–e300. doi: 10.1212/WNL.0000000000010977

Ellul, M. A., Benjamin, L., Singh, B., Lant, S., Michael, B. D., Easton, A., et al. (2020). Neurological associations of COVID-19. *Lancet Neurol.* 19, 767–783. doi: 10.1016/S1474-4422(20)30221-0

Elyaspour, Z., Zibaeenezhad, M. J., Razmkhah, M., and Razeghian-Jahromi, I. (2021). Is it all about endothelial dysfunction and thrombosis formation? The secret of COVID-19. *Clin. Appl. Thromb. Hemost.* 27:107602962110429. doi: 10.1177/10760296211042940

Franke, C., Ferse, C., Kreye, J., Reincke, S. M., Sanchez-Sendin, E., Rocco, A., et al. (2021). High frequency of cerebrospinal fluid autoantibodies in COVID-19 patients with neurological symptoms. *Brain Behav. Immun.* 93, 415–419. doi: 10.1016/j.bbi.2020.12.022

Galeotti, C., Kaveri, S. V., and Bayry, J. (2020). Intravenous immunoglobulin immunotherapy for coronavirus disease-19 (COVID-19). *Clin. Transl. Immunol.* 9:e1198. doi: 10.1002/cti2.1198

Garg, R. K., Paliwal, V. K., and Gupta, A. (2021). Encephalopathy in patients with COVID-19: a review. *J. Med. Virol.* 93, 206–222. doi: 10.1002/jmv.26207

Gilden, D., and Nagel, M. A. (2016). Neurological complications of human herpesvirus infections. *Neurology*, 316–322. doi: 10.1002/9781118777329.ch81

Gonzalez-Santana, A., and Diaz, H. R. (2020). Bacterial peptidoglycans from microbiota in neurodevelopment and behavior. *Trends Mol. Med.* 26, 729–743. doi: 10.1016/j.molmed.2020.05.003

Graham, E. L., Koralnik, I. J., and Liotta, E. M. (2022). Therapeutic approaches to the neurologic manifestations of COVID-19. *Neurotherapeutics* 19, 1435–1466. doi: 10.1007/s13311-022-01267-y

Gu, S., Chen, Y., Wu, Z., Chen, Y., Gao, H., Lv, L., et al. (2020). Alterations of the gut microbiota in patients with coronavirus disease 2019 or H1N1 influenza. *Clin. Infect. Dis.* 71, 2669–2678. doi: 10.1093/cid/ciaa709

Guan, Z., and Fang, J. (2006). Peripheral immune activation by lipopolysaccharide decreases neurotrophins in the cortex and hippocampus in rats. *Brain Behav. Immun.* 20, 64–71. doi: 10.1016/j.bbi.2005.04.005

Hamming, I., Timens, W., Bulthuis, M. L., Lely, A. T., Navis, G., and van Goor, H. (2004). Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J. Pathol.* 203, 631–637. doi: 10.1002/path.1570

Helms, J., Kremer, S., Merdji, H., Schenck, M., Severac, F., Clere-Jehl, R., et al. (2020). Delirium and encephalopathy in severe COVID-19: a cohort analysis of ICU patients. *Crit. Care* 24:491. doi: 10.1186/s13054-020-03200-1

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405. doi: 10.1016/S1474-4422(15)70016-5

Horie, S., McNicholas, B., Rezoagli, E., Pham, T., Curley, G., McAuley, D., et al. (2020). Emerging pharmacological therapies for ARDS: COVID-19 and beyond. *Intensive Care Med.* 46, 2265–2283. doi: 10.1007/s00134-020-06141-z

Hosp, J. A., Dressing, A., Blazhenets, G., Bormann, T., Rau, A., Schwabenland, M., et al. (2021). Cognitive impairment and altered cerebral glucose metabolism in the subacute stage of COVID-19. *Brain J. Neurol.* 144, 1263–1276. doi: 10.1093/brain/awab009

Hoyles, L., Snelling, T., Umlai, U.-K., Nicholson, J. K., Carding, S. R., Glen, R. C., et al. (2018). Microbiome–host systems interactions: protective effects of propionate upon the blood–brain barrier. *Microbiome* 6:55. doi: 10.1186/s40168-018-0439-y

Huo, S., Ferse, C., Bösl, F., Reincke, S. M., Enghard, P., Hinrichs, C., et al. (2022). Intravenous immunoglobulins for treatment of severe COVID-19-related acute encephalopathy. *J. Neurol.* 269, 4013–4020. doi: 10.1007/s00415-022-11152-5

Jang, K., Khatri, A., and Majure, D. T. (2020). COVID-19 leading to acute encephalopathy in a patient with heart transplant. *J. Heart Lung Transplant* 39, 853–855. doi: 10.1016/j.healun.2020.05.016

Jung, R. E., and Haier, R. J. (2007). The Parieto-frontal integration theory (P-FIT) of intelligence: converging neuroimaging evidence. *Behav. Brain Sci.* 30, 135–154. doi: 10.1017/S0140525X07001185

Kalam, N., and Balasubramaniam, V. (2024). Crosstalk between COVID-19 and the gut-brain axis: a gut feeling. *Postgrad. Med. J.*:qgae030. doi: 10.1093/postmj/qgae030

Karuppan, M. K. M., Devadoss, D., Nair, M., Chand, H. S., and Lakshmana, M. K. (2021). SARS-CoV-2 infection in the central and peripheral nervous system-associated morbidities and their potential mechanism. *Mol. Neurobiol.* 58, 2465–2480. doi: 10.1007/s12035-020-02245-1

Kim, P. H., Kim, M., Suh, C. H., Chung, S. R., Park, J. E., Kim, S. C., et al. (2021). Neuroimaging findings in patients with COVID-19: a systematic review and Meta-analysis. *Korean J. Radiol.* 22, 1875–1885. doi: 10.3348/kjr.2021.0127

Kopel, J., Perisetti, A., Gajendran, M., Boregowda, U., and Goyal, H. (2020). Clinical insights into the gastrointestinal manifestations of COVID-19. *Dig. Dis. Sci.* 65, 1932–1939. doi: 10.1007/s10620-020-06362-8

Lehtoranta, L., Pitkäranta, A., and Korpela, R. (2014). Probiotics in respiratory virus infections. *Eur. J. Clin. Microbiol.* 33, 1289–1302. doi: 10.1007/s10096-014-2086-y

Li, J., Richards, E. M., Handberg, E. M., Pepine, C. J., and Raizada, M. K. (2021). Butyrate regulates COVID-19-relevant genes in gut epithelial organoids from normotensive rats. *Hypertension (Dallas, Tex: 1979)* 77, e13–e16. doi: 10.1161/HYPERTENSIONAHA.120.16647

Li, R., Wang, H., Shi, Q., Wang, N., Zhang, Z., Xiong, C., et al. (2017). Effects of oral florfenicol and azithromycin on gut microbiota and adipogenesis in mice. *PLoS One* 12:e0181690. doi: 10.1371/journal.pone.0181690

Liotta, E. M., Batra, A., Clark, J. R., Shlobin, N. A., Hoffman, S. C., Orban, Z. S., et al. (2020). Frequent neurologic manifestations and encephalopathy-associated morbidity in Covid-19 patients. *Ann. Clin. Transl. Neurol.* 7, 2221–2230. doi: 10.1002/acn3.51210

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic characterization and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet (London, England).* 395, 565–574. doi: 10.1016/S0140-6736(20)30251-8

Luo, A., Leach, S. T., Barres, R., Hesson, L. B., Grimm, M. C., and Simar, D. (2017). The microbiota and epigenetic regulation of T helper 17/regulatory T cells: in search of a balanced immune system. *Front. Immunol.* 8:417. doi: 10.3389/fimmu.2017.00417

Lynch, M. A. (2002). Interleukin-1 beta exerts a myriad of effects in the brain and in particular in the hippocampus: analysis of some of these actions. *Vitam. Horm.* 64, 185–219. doi: 10.1016/S0083-6729(02)64006-3

Maciel-Fiuza, M. F., Muller, G. C., Campos, D. M. S., do Socorro Silva Costa, P., Peruzzo, J., Bonamigo, R. R., et al. (2023). Role of gut microbiota in infectious and inflammatory diseases. *Front. Microbiol.* 14:1098386. doi: 10.3389/fmicb.2023.1098386

Mayr, F. B., Yende, S., and Angus, D. C. (2014). Epidemiology of severe sepsis. *Virulence* 5, 4–11. doi: 10.4161/viru.27372

Mendes de Almeida, V., Engel, D. F., Ricci, M. F., Cruz, C. S., Lopes, Í. S., Alves, D. A., et al. (2023). Gut microbiota from patients with COVID-19 cause alterations in mice that resemble post-COVID symptoms. *Gut Microbes* 15:2249146. doi: 10.1080/19490976.2023.2249146

Michael, B. D., Walton, D., Westenberg, E., García-Azorín, D., Singh, B., Tamborska, A. A., et al. (2023). Consensus clinical guidance for diagnosis and Management of Adult COVID-19 encephalopathy patients. *J. Neuropsychiatry Clin. Neurosci.* 35, 12–27. doi: 10.1176/appi.neuropsych.22010002

Milatovic, D., Zaja-Milatovic, S., Montine, K. S., Horner, P. J., and Montine, T. J. (2003). Pharmacologic suppression of neuronal oxidative damage and dendritic degeneration following direct activation of glial innate immunity in mouse cerebrum. *J. Neurochem.* 87, 1518–1526. doi: 10.1046/j.1471-4159.2003.02120.x

Monteleone, G., Franzè, E., and Laudisi, F. (2020). Expression of receptors for SARS-CoV-2 in the gut of patients with inflammatory bowel disease. *Gut Liver* 14, 530–531. doi: 10.5009/gnl20112

Morais, L. H., Schreiber, H. L., and Mazmanian, S. K. (2021). The gut microbiota–brain axis in behaviour and brain disorders. *Nat. Rev. Microbiol.* 19, 241–255. doi: 10.1038/s41579-020-00460-0

Myhre, A. E., Aasen, A. O., Thiemermann, C., and Wang, J. E. (2006). PEPTIDOGLYCAN-AN ENDOTOXIN IN ITS OWN RIGHT? *Shock (Augusta, GA)* 25, 227–235. doi: 10.1097/01.shk.0000191378.55274.37

Myint, A. M., and Kim, Y. K. (2014). Network beyond IDO in psychiatric disorders: revisiting neurodegeneration hypothesis. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 48, 304–313. doi: 10.1016/j.pnpbp.2013.08.008

Nicosia, R. F., Ligresti, G., Caporarello, N., Akilesh, S., and Ribatti, D. (2021). COVID-19 vasculopathy: mounting evidence for an indirect mechanism of endothelial injury. *Am. J. Pathol.* 191, 1374–1384. doi: 10.1016/j.ajpath.2021.05.007

Oldendorf, W. H. (1973). Carrier-mediated blood–brain barrier transport of short-chain monocarboxylic organic acids. *Am. J. Phys.* 224, 1450–1453. doi: 10.1152/ajplegacy.1973.224.6.1450

Otani, K., Fukushima, H., and Matsuishi, K. (2023). COVID-19 delirium and encephalopathy: pathophysiology assumed in the first 3 years of the ongoing pandemic. *Brain Disord. (Amsterdam, Netherlands).* 10:100074. doi: 10.1016/j.dscb.2023.100074

Pandiyan, P., Bhaskaran, N., Zou, M., Schneider, E., Jayaraman, S., and Huehn, J. (2019). Microbiome dependent regulation of Tregs and Th17 cells in mucosa. *Front. Immunol.* 10:426. doi: 10.3389/fimmu.2019.00426

Perrin, P., Collongues, N., Baloglu, S., Bedo, D., Bassand, X., Lavaux, T., et al. (2021). Cytokine release syndrome-associated encephalopathy in patients with COVID-19. *Eur. J. Neurol.* 28, 248–258. doi: 10.1111/ene.14491

Pilotto, A., Masciocchi, S., Volonghi, I., Crabbio, M., Magni, E., De Giuli, V., et al. (2021). Clinical presentation and outcomes of severe acute respiratory syndrome coronavirus 2-related encephalitis: the ENCOVID multicenter study. *J. Infect. Dis.* 223, 28–37. doi: 10.1093/infdis/jiaa609

Pilotto, A., Odolini, S., Masciocchi, S., Comelli, A., Volonghi, I., Gazzina, S., et al. (2020). Steroid-responsive encephalitis in coronavirus disease 2019. *Ann. Neurol.* 88, 423–427. doi: 10.1002/ana.25783

Plummer, A. M., Matos, Y. L., Lin, H. C., Ryman, S. G., Birg, A., Quinn, D. K., et al. (2023). Gut-brain pathogenesis of postacute COVID-19 neurocognitive symptoms. *Front. Neurosci.* 17:1232480. doi: 10.3389/fnins.2023.1232480

Pugin, D., Vargas, M. I., Thieffry, C., Schibler, M., Grosgurin, O., Pugin, J., et al. (2020). COVID-19-related encephalopathy responsive to high-dose glucocorticoids. *Neurology* 95, 543–546. doi: 10.1212/WNL.0000000000010354

Rachel, L. B., Laura, B., Michael, P. L., Tehmina, B., Michael, S. Z., Chandrashekar, H., et al. (2023). Pathophysiology, diagnosis, and management of neuroinflammation in covid-19. *BMJ* 382:e073923. doi: 10.1136/bmj-2022-073923

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71, 635–700. doi: 10.1146/annurev.biochem.71.110601.135414

Reinhold, D., Farztdinov, V., Yan, Y., Meisel, C., Sadlowski, H., Kühn, J., et al. (2023). The brain reacting to COVID-19: analysis of the cerebrospinal fluid proteome, RNA and inflammation. *J. Neuroinflammation* 20:30. doi: 10.1186/s12974-023-02711-2

Richwine, A. F., Parkin, A. O., Buchanan, J. B., Chen, J., Markham, J. A., Juraska, J. M., et al. (2008). Architectural changes to CA1 pyramidal neurons in adult and aged mice after peripheral immune stimulation. *Psychoneuroendocrinology* 33, 1369–1377. doi: 10.1016/j.psyneuen.2008.08.003

Robba, C., Ball, L., Battaglini, D., Cardim, D., Moncalvo, E., Brunetti, I., et al. (2021). Early effects of ventilatory rescue therapies on systemic and cerebral oxygenation in mechanically ventilated COVID-19 patients with acute respiratory distress syndrome: a prospective observational study. *Crit. Care* 25:111. doi: 10.1186/s13054-021-03537-1

Rock, R. B., Olin, M., Baker, C. A., Molitor, T. W., and Peterson, P. K. (2008). Central nervous system tuberculosis: pathogenesis and clinical aspects. *Clin. Microbiol. Rev.* 21, 243–61, table of contents, table of contents. doi: 10.1128/CMR.00042-07

Salama, C., Han, J., Yau, L., Reiss, W. G., Kramer, B., Neidhart, J. D., et al. (2021). Tocilizumab in patients hospitalized with Covid-19 pneumonia. *N. Engl. J. Med.* 384, 20–30. doi: 10.1056/NEJMoa2030340

Schmidt, S. K., Ebel, S., Keil, E., Woite, C., Ernst, J. F., Benzin, A. E., et al. (2013). Regulation of IDO activity by oxygen supply: inhibitory effects on antimicrobial and immunoregulatory functions. *PLoS One* 8:e63301. doi: 10.1371/journal.pone.0063301

Seifert, G., Schilling, K., and Steinhäuser, C. (2006). Astrocyte dysfunction in neurological disorders: a molecular perspective. *Nat. Rev. Neurosci.* 7, 194–206. doi: 10.1038/nrn1870

Shah, P., Patel, J., Soror, N. N., and Kartan, R. (2021). Encephalopathy in COVID-19 patients. *Cureus* 13:e16620. doi: 10.7759/cureus.16620

Silva, Y. P., Bernardi, A., and Frozza, R. L. (2020). The role of short-chain fatty acids from gut microbiota in gut-brain communication. *Front. Endocrinol.* 11:25. doi: 10.3389/fendo.2020.00025

Singh, B., Lant, S., Cividini, S., Cattrall, J. W. S., Goodwin, L. C., Benjamin, L., et al. (2022). Prognostic indicators and outcomes of hospitalized COVID-19 patients with neurological disease: an individual patient data meta-analysis. *PLoS One* 17:e0263595. doi: 10.1371/journal.pone.0263595

Solomon, I. H., Normandin, E., Bhattacharyya, S., Mukerji, S. S., Keller, K., Ali, A. S., et al. (2020). Neuropathological features of Covid-19. *N. Engl. J. Med.* 383, 989–992. doi: 10.1056/NEJMc2019373

Starkey, J., Kobayashi, N., Numaguchi, Y., and Moritani, T. (2017). Cytotoxic lesions of the Corpus callosum that show restricted diffusion: mechanisms, causes, and manifestations. *Radiographics* 37, 562–576. doi: 10.1148/rg.2017160085

Tapajós, R., Castro, D., Melo, G., Balogun, S., James, M., Pessoa, R., et al. (2019). Malaria impact on cognitive function of children in a peri-urban community in the Brazilian Amazon. *Malar. J.* 18:173. doi: 10.1186/s12936-019-2802-2

Taquet, M., Geddes, J. R., Husain, M., Luciano, S., and Harrison, P. J. (2021). 6-month neurological and psychiatric outcomes in 236 379 survivors of COVID-19: a retrospective cohort study using electronic health records. *Lancet Psychiatry* 8, 416–427. doi: 10.1016/S2215-0366(21)00084-5

Teixeira, P. C., Dorneles, G. P., Santana Filho, P. C., da Silva, I. M., Schipper, L. L., Postiga, I. A. L., et al. (2021). Increased LPS levels coexist with systemic inflammation and result in monocyte activation in patients with severe COVID-19. *Int. Immunopharmacol.* 100:108125. doi: 10.1016/j.intimp.2021.108125

Tong, L., Balazs, R., Soiampornkul, R., Thangnipon, W., and Cotman, C. W. (2008). Interleukin-1 beta impairs brain derived neurotrophic factor-induced signal transduction. *Neurobiol. Aging* 29, 1380–1393. doi: 10.1016/j.neurobiolaging.2007.02.027

Tsunooka, N., Maeyama, K., Hamada, Y., Imagawa, H., Takano, S., Watanabe, Y., et al. (2004). Bacterial translocation secondary to small intestinal mucosal ischemia during cardiopulmonary bypass. Measurement by diamine oxidase and peptidoglycan. European journal of cardio-thoracic surgery: official journal of the European Association for Cardio-thoracic. *Surgery* 25, 275–280. doi: 10.1016/j.ejcts.2003.11.008

Umapathi, T., Quek, W. M. J., Yen, J. M., Khin, H. S. W., Mah, Y. Y., Chan, C. Y. J., et al. (2020). Encephalopathy in COVID-19 patients; viral, parainfectious, or both? *eNS* 21:100275. doi: 10.1016/j.ensci.2020.100275

Vakhtin, A. A., Ryman, S. G., Flores, R. A., and Jung, R. E. (2014). Functional brain networks contributing to the Parieto-frontal integration theory of intelligence. *NeuroImage* 103, 349–354. doi: 10.1016/j.neuroimage.2014.09.055

Vargas-Caraveo, A., Sayd, A., Maus, S. R., Caso, J. R., Madrigal, J. L. M., García-Bueno, B., et al. (2017). Lipopolysaccharide enters the rat brain by a lipoprotein-mediated transport mechanism under physiological conditions. *Sci. Rep.* 7:13113. doi: 10.1038/s41598-017-13302-6

Vengalil, A., Nizamutdinov, D., Su, M., and Huang, J. H. (2023). Mechanisms of SARS-CoV-2-induced encephalopathy and encephalitis in COVID-19 cases. *Neurosci. Insights* 18:263310552311725. doi: 10.1177/26331055231172522

Vijay, N., and Morris, M. E. (2014). Role of monocarboxylate transporters in drug delivery to the brain. *Curr. Pharm. Des.* 20, 1487–1498. doi: 10.2174/13816128113199990462

Villapol, S. (2020). Gastrointestinal symptoms associated with COVID-19: impact on the gut microbiome. *Transl. Res.* 226, 57–69. doi: 10.1016/j.trsl.2020.08.004

Wiersinga, W. J., Rhodes, A., Cheng, A. C., Peacock, S. J., and Prescott, H. C. (2020). Pathophysiology, transmission, diagnosis, and treatment of coronavirus disease 2019 (COVID-19): a review. *JAMA* 324, 782–793. doi: 10.1001/jama.2020.12839

Wu, Y., Cheng, X., Jiang, G., Tang, H., Ming, S., Tang, L., et al. (2021). Altered oral and gut microbiota and its association with SARS-CoV-2 viral load in COVID-19 patients during hospitalization. *NPJ Biofilms Microbiomes* 7:90. doi: 10.1038/s41522-021-00232-5

Xu, E., Xie, Y., and Al-Aly, Z. (2022). Long-term neurologic outcomes of COVID-19. *Nat. Med.* 28, 2406–2415. doi: 10.1038/s41591-022-02001-z

Zhang, L., Han, H., Li, X., Chen, C., Xie, X., Su, G., et al. (2021). Probiotics use is associated with improved clinical outcomes among hospitalized patients with COVID-19. *Ther. Adv. Gastroenterol.* 14:175628482110356. doi: 10.1177/17562848211035670

Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273. doi: 10.1038/s41586-020-2012-7

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The investigation of the role of oral-originated *Prevotella*-induced inflammation in childhood asthma

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Background and objectives: The oral and gut microbiota play significant roles in childhood asthma pathogenesis. However, the communication dynamics and pathogenic mechanisms by which oral microbiota influence gut microbiota and disease development remain incompletely understood. This study investigated potential mechanisms by which oral-originated gut microbiota, specifically *Prevotella* genus, may contribute to childhood asthma etiology.

Methods: Oral swab and fecal samples from 30 asthmatic children and 30 healthy controls were collected. Microbiome composition was characterized using *16S rRNA* gene sequencing and metagenomics. Genetic distances identified potential oral-originated bacteria in asthmatic children. Functional validation assessed pro-inflammatory properties of *in silico* predicted microbial mimicry peptides from enriched asthma-associated species. Fecal metabolome profiling combined with metagenomic correlations explored links between gut microbiota and metabolism. HBE cells treated with *Prevotella bivia* culture supernatant were analyzed for lipid pathway impacts using UPLC-MS/MS.

Results: Children with asthma exhibited distinct oral and gut microbiota structures. *Prevotella bivia, P. disiens, P. oris* and *Bacteroides fragilis* were enriched orally and intestinally in asthmatics, while *Streptococcus thermophilus* decreased. *P. bivia, P. disiens* and *P. oris* in asthmatic gut likely originated orally. Microbial peptides induced inflammatory cytokines from immune cells. Aberrant lipid pathways characterized asthmatic children. *P. bivia* increased pro-inflammatory and decreased anti-inflammatory lipid metabolites in HBE cells.

Conclusion: This study provides evidence of *Prevotella* transfer from oral to gut microbiota in childhood asthma. *Prevotella*'s microbial mimicry peptides and effects on lipid metabolism contribute to disease pathogenesis by eliciting immune responses. Findings offer mechanistic insights into oral-gut connections in childhood asthma etiology.

KEYWORDS

asthma, oral microbiota, gut microbiota, migration, immune, lipid metabolome

Highlights

- *Prevotella* migrating from oral cavity to gut become the driver of childhood asthma.
- Microbial mimicry peptides secreted by *Prevotella* would trigger self-immune effect to induce asthma.
- *Prevotella* would exacerbate inflammation in childhood asthma by induing immune-related lipid metabolites in host.

1 Introduction

Oral microbiota plays a crucial role in the human microbial community, consisting of more than 700 species (Palmer, 2000). Due to its easy accessibility for sampling and its connection to overall health, the oral microbiome has become an integral part of the human microbiome program (HMP). The major phyla found in oral microbiota include Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria (Dewhirst et al., 2010). Research has demonstrated that the imbalance in the oral microbiota community is associated not only with local oral diseases but also with respiratory, cardiovascular and neoplastic conditions (Genco and Van Dyke, 2010; Le Bars et al., 2017). For example, lower level of *Lactobacillus* in oral microbiota during the first seven years after birth is closely linked to increased risk of developing of allergic conditions (Dzidic et al., 2018). Additionally, elevated levels of *Moraxella* and *Haemophilus* in sputum samples have been correlated with neutrophilic asthma (Durack et al., 2017). It is worth noting that oral microbiota is not solely colonized in the oral cavity; they can migrate to other body sites, leading to local inflammatory reaction and infection. Recent research has highlighted the correlation between the oral and gut microbiota. For example, oral administration of *Porphyromonas gingivalis* has been shown to disrupt the gut microbiota community, characterized by an increase in Bacteroidetes, a decrease in Firmicutes, and elevated serum endotoxin level, ultimately triggering gut inflammation (Kato et al., 2018). *Streptococcus salivarius*, an early colonizer in the oral cavity, is also capable of colonizing the gut mucosa. Notably, it down-regulated the expression of nuclear transcription factor NF-κB in human intestinal cells, which plays an important role in intestinal inflammatory response and homeostasis (Couvigny et al., 2015). Meanwhile, maintaining gut microbiota homeostasis is essential for lung health. Communication between the gut and lungs occurs via the gut-lung axis. For example, a decrease in *Bifidobacteria* and an increase in *Clostridium* in the gut have been associated with early-life asthma (Arrieta et al., 2015). Furthermore, animal experimental studies have demonstrated that germ-free mice are more susceptible to lung diseases and allergic inflammation (Simonyte Sjödin et al., 2016). The effect of gut microbiota on mucosal immunity is not only restricted to the gastrointestinal tract; it extends to the bronchial epithelium and lymphoid tissue as well, thereby affecting certain inflammatory diseases (Yuan et al., 2022). T and B cells induced by intestinal lamina propria can migrate through the lymphatic system, inducing immune responses (Enaud et al., 2020; Zhang et al., 2020). Probiotics, such as *Lactobacillus* and *Bifidobacteria*, which have been shown in mouse models to regulate T cell responses and suppress allergic reactions (Wang et al., 2017; Pyclik et al., 2021). The shared genera that coexist in both oral microbiota and gut microbiota have become a subject of interest in studying the potential role of oral-originated gut species in diseases. Hu et al. (2023) found that oral microbiota from alcohol dependence can colonize ectopically in the intestines. Recent investigations have indicated a translocation and migration of specific oral taxa into the gut of COVID-19 patients (Wu et al., 2021). Certain bacteria originated from the oral cavity may influence the development and health of the immune system, potentially contributing to the development or exacerbation of inflammatory diseases such as cardiovascular disease and rheumatoid arthritis (Hajishengallis, 2015; Graves et al., 2019). During this process, microbial-derived mimic antigen epitopes are activated, promoting the imprinting of specific T helper cells and the accumulation of inflammatory cells, thereby influencing systemic immune responses (Chen et al., 2021).

Additionally, metabolites derived from gut microbiota, such as short-chain fatty acids (SCFAs), are crucial signaling molecules for maintaining immune homeostasis. These SCFAs can enter the circulatory system via the lymphatic system and participate in regulating immune responses in the lungs, providing beneficial effects in alleviating asthma and chronic obstructive pulmonary diseases (Roduit et al., 2019). Bacterial-derived inflammatory mediators were associated with an increased risk of atopy and asthma. For example, an elevated concentration of the gut microbiota-derived metabolite 12,13-diHOME in infant feces was linked to an increased risk of atopy and asthma in childhood. This metabolite was produced by *Bifidobacterium* and *Faecalibacterium* which could promote allergic inflammation by reducing the frequency of lung Treg populations and *IL-10* production (Fujimura et al., 2016; Levan et al., 2019).

Based on these clues, we aimed to elucidate the communication between oral microbiota and gut microbiota and investigate the potential mechanism of the effects of oral-originated gut microbiota on childhood asthma in the present study. *16S rRNA* gene sequencing and metagenome sequencing were used to analyze the alterations in oral and gut microbiota in a case–control study. Genetics analysis was used to identify key oral-originated gut microbiota in the children with asthma. Metabolomic analysis and *in vitro* experiments was used to explore the potential mechanism.

2 Methods

2.1 Samples

In case–control study, 30 children with asthma and 30 controls without allergic diseases were recruited from Nanjing Children's hospital affiliated to Nanjing Medical University and Jiangsu Province Hospital of Chinese medicine. Covariates were considered in this study including age, sex (male vs. female), BMI (body mass index),

passive smoking (yes vs. no). Inclusion criteria: The children with asthma were diagnosed by physicians without taking antibiotics during the relief stage in recent three months. The control group did not have any respiratory system-related diseases or without allergic disease. Exclusion criteria: (1) subjects with recent surgical history were excluded; (2) subjects with recent Oral problems were excluded; (3) subjects with underlying diseases were excluded; (4) subjects with overweight or obesity were excluded. The demographic information of the children with asthma in the study was in Supplementary Table S1. Oral swabs and fecal samples were collected and stored at −80°C for further experiments. Blood samples were collected from control individuals. Peripheral blood mononuclear cells (PBMCs) were isolated using human lymphocyte separation medium (Fcmacs biotech co., ltd.). This study was approved by the ethics Committee of Nanjing Medical University [approval no. Nan med Univ ethical review (2019)02080-1]. Written informed consent was obtained from all guardians.

2.2 *16S rRNA* gene sequencing

Total genomic DNA was extracted from oral swabs and fecal samples according to the E.Z.N.A.® soil kit (Omega Biotek, Norcross, GA). The V3-V4 variable region of the bacterial *16S rRNA* gene was amplified by PCR, using the forward primer 338F (5'-ACTCCTAC GGGAGGCAGCAG-3′) and reverse primer 806R (5'-GGACTACHVG GGTWTCTAAT-3′). The PCR amplification system included 4 μL 5 × FastPfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL of each primer (0.5 μM), and 10 ng DNA template. PCR conditions were as following: 95°C denaturation for 3 min, 27 cycles (95°C for 30 s, 55°C for 30 s and 72°C for 45 s), finally 72°C for 10 min. The PCR products were purified and recovered by AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Sequencing was performed using the Miseq PE300 platform (Illumina, San Diego, USA). The raw sequences were quality controlled and assembled using Trimmomatic and FLASH software. The operational taxonomic units (OTUs) were clustered based on 97% similarity using UPARSE (version 7.1[1]) software, and the OTUs sequences were annotated using the RDP classifier[2] software. The free online platform of the Majorbio I-Sanger Cloud Platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd)[3] was used for data analysis.

2.3 Metagenomics sequencing

Metagenomic sequencing was performed on the Illumina NovaSeq platform (Illumina, USA). The raw reads from metagenome sequencing were used to generate clean reads using the fastp[4] on the free online platform of Majorbio Cloud Platform.[5] These high-quality reads were then assembled to contigs using MEGAHIT,[6] which makes use of succinct de Bruijn graphs. Contigs with the length being or over 300 bp were selected as the final assembling result. Open reading frames (ORFs) in contigs were identified using MetaGene.[7] Representative sequences of non-redundant gene catalog were annotated based on the NCBI NR database using blastp as implemented in DIAMOND v0.9.19 with e-value cutoff of 1e-5 using Diamond[8] for taxonomic annotations. Functional differences were found by LEfSe.

2.4 Strain-level analysis based on the genetic distance

The contigs, which have been assembled, were aligned with the reference gene sequences of NCBI strains using Blast software (version 2.9.0). Sequences with a similarity ≥80% and a length ≥ 200 bp were selected. The selected sequences were then subjected to gene annotation using Prokka software (version 1.13). The annotation results for the sequences identified as *16S rRNA* gene were further aligned using the MUSCLE method in MEGA software (version 11.0.13), and the genetic distance was calculated using the p-distance method.

2.5 Functional analysis of microbial mimicry peptides

Epitopes extracted from the Immune Epitope Database (IEDB)[9] were identified as triggers for immune reaction in asthma. These epitopes' amino acid sequences were then compared to the protein sequences of species. Only epitopes with a minimum identity and coverage of 80% were chosen for subsequent experimental investigation. These microbial mimicry peptides were synthesized by Nanjing Branch of GenScript Biotech Company. PBMCs were isolated from anticoagulated blood using human lymphocyte separation fluid and re-suspended in R1640 complete medium (Zhang et al., 2021). Peptide segments were added to the cells for stimulation at a final concentration of 40 μg/mL, and the cells were cultured at 37°C and 5% CO_2 for 48 h before collection. Total RNA was extracted from the cells using the TRIzol method (Tiangen, China), and the concentration was measured using a nucleic acid protein analyzer (Thermo NanoDrop 2000, US). Reverse transcription was performed using the HiScript II Q RT SuperMix kit (Vazyme Biotech) following the instructions provided. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to quantify asthma-related inflammatory factors *IL-4, IL-5, IL-6, IL-8, IL-13*, and *IL-17A*. The PCR system consisted of cDNA, primer (0.2 μmol/L) and ChamQ Universal SYBR qPCR Master Mix (5 μL), with a total system volume of 10 μL. The primers were synthesized by Nanjing Branch of GenScript Biotech Company, with the following sequences: For *IL-4*, the forward primer was 5'-CGGCAACTTTGTCCACGGA-3′, and the reverse primer was 5'-TCTGTTACGGTCAACTCGGTG-3′; for *IL-5*, the forward primer was 5'-GGAATAGGCACACTGGAGAGTC-3′,

1 http://drive5.com/uparse/
2 http://rdp.cme.msu.edu/
3 www.i-sanger.com
4 https://github.com/OpenGene/fastp, version 0.20.0.
5 cloud.majorbio.com
6 https://github.com/voutcn/megahit, version 1.1.2.
7 http://metagene.cb.k.u-tokyo.ac.jp/
8 http://www.diamondsearch.org/index.php, version 0.8.35.
9 http://www.iedb.org/

and the reverse primer was 5'-CTCTCCGTCTTTCTTCTCCACAC-3′; for *IL-6*, the forward primer was 5'-CCTTCGGTCCAGTTGCCTTCTC CCT-3′, and the reverse primer was 5'-GGGCTGAGATGCCGTCGA GGATGTA-3′; for *IL-8*, the forward primer was 5'-CCACCGGAAG GAACCATCTC-3′, and the reverse primer was 5'-TTCCTTGGG GTCCAGACAGA-3′; for *IL-13*, the forward primer was 5′-CCTCATGGCGCTTTTGTTGAC-3′, and the reverse primer was 5′- TCTGGTTCTGGGTGATGTTGA-3′; for *IL-17A*, the forward primer was 5'-AGATTACTACAACCGATCCACCT-3′, and the reverse primer was 5'-GGGGACAGAGTTCATGTGGTA-3′; for the reference gene *β-actin*, the forward primer was 5'-CATGTACGTTGC TATCCAGGC-3′, and the reverse primer was 5'-CTCCTTAATGTCA CGCACGAT-3′. According to the instructions of the kit, PCR reactions were performed in a LightCycler® 96 detection system (Roche Diagnostics (Shanghai) Ltd., Switzerland). The amplification parameters were as follows: an initial denaturation step at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s, with a final extension step at 60°C for 60 s. The melting curve was set according to the instrument's standard. For data analysis, *β-actin* was used as the reference gene for normalization, and triplicate measurements were performed. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6 Non-targeted metabolomic analysis of fecal samples

Fifty milligrams of feces were placed into a 1.5 mL Eppendorf tube, followed by the addition of 1 mL of pre-chilled ultrapure water and magnetic beads. The mixture was then ground at 30 Hz for 3 min at 4°C. The quality control sample was prepared by mixing 50 μL of each individual sample. The sample, along with the quality control mixture, was then centrifuged at 4°C and 12,000 rpm for 15 min, and the supernatant was collected. The remaining pellet was retreated according to the above steps. The above supernatant was mixed well and then centrifuged at 4°C and 12,000 rpm for 15 min. Finally, 200 μL of the supernatant was taken for further analysis.

For the metabolomic analysis, the method was well established in our laboratory. Briefly, a Dionex Ultimate 3,000 UPLC (Dionex, Germering, Germany) combined with a Q-Orbitrap-MS system equipped with a heated electrospray ionization source (Q-Exactive plus MS, Thermo Scientific, MA) was used. The column was a C_{18} column (100 mm × 2.1 mm, 1.9 μm), and the column temperature was set at 40°C. The mobile phase solvents, solvent A (0.1% formic acid-water) and solvent B (0.1% formic acid-acetonitrile), were used for sample elution. The column was washed with 100% solvent B and equilibrated with 100% solvent A. The flow rate was 0.2 mL/min, and the injection volume was 5 μL. The MS parameters were as follows: sheath gas flow rate of 10 arbitrary units, spray voltage of 3.0 kV, capillary temperature of 320°C, and auxiliary gas heater temperature of 30°C. A heated electrospray ionization source was used, and the samples were detected in full scan mode. Data for samples in the positive ion mode and negative ion mode were collected in the range of 30–800 m/z.

The raw data was processed using the MSConvert software to convert it to the RAW format. The R software was used for normalization, data transformation, centering, and standardization. Then, the data was imported into the online database XCMS Online Metabolomics (Scripps Research Institute, USA). After exporting the data, a pre-selection was performed based on the criteria of metabolite features >50% and relative standard deviation (RSD) ≤30%. Subsequently, the R software (version 3.5.1) was used for principal component analysis (PCA) to simplify and reduce the dimensionality of the data. The overall distribution between sample groups was analyzed using distance algorithms. The variable importance in the projection (VIP) was used as an important parameter to indicate disruptions in the body's metabolic network. Candidate variables were selected based on VIP ≥1.0 and statistical significance (adjusted *p*-value <0.05). Metabolites were compared with the HMDB Library[10] based on positive and negative ion modes (M + H and M-H).

2.7 HBE cells treated with the supernatant of *Prevotella bivia*

Human bronchial epithelial (HBE) cells were generously provided by Dr. Haiyan, Chu from the School of Public Health, Nanjing Medical University. HBE cells were cultured in DMEM (Gibco, USA) containing 10% FBS (TransGen Biotech Co., Ltd., Beijing, China) supplemented with 1% penicillin–streptomycin at 37°C with 5% CO_2. *Prevotella bivia* (ATCC 29303) and modified reinforced clostridial broth (ATCC Medium 2,107) were purchased from Mingzhou Biotechnology Co., LTD. The strain was cultured in Medium 2,107 at 37°C in the Bugbox anaerobic system (Bug Box, Ruskinn Technology, Leeds, UK) for 48 h. The cultures were centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was filtered to remove bacteria. HBE cells were treated for 24 h either 10% of bacterial supernatant or ATCC Medium 2,107.

2.8 Analysis of lipid metabolic profiles by UPLC-MS/MS

HBE cells were washed twice with PBS and resuspended in 100 μL ultrapure water extraction solution (containing protease inhibitors, PMSF, and EDTA). Five hundred microliter of lipid extraction solution and 50 μL cell suspension were mixed by vortexing for 2 min. The mixed samples were sonicated for 5 min, added with 100 μL water, vortexed for 1 min, and then centrifuged at 4°C and 12,000 rpm for 10 min. The 200 μL supernatant was collected and dried with nitrogen. The dried supernatant was re-dissolved in 200 μL of isopropanol/acetonitrile (50%/50%) for further analysis. Metabolic data were acquired by two data acquisition systems, i.e., ultra-performance liquid chromatography (UPLC, ExionLC™ AD) and tandem mass spectrometry (MS/MS, QTRAP® 6,500+). The samples were separated on a Thermo Accucore™ C30 column (2.6 μM, 2.1 mm × 100 mm i.d.) at a flow rate of 0.35 mL/min, and the temperature of 45°C. The solvent system was as follows: A: 0.1% formic acid and 10 mmol/L ammonium formate in acetonitrile/water (60%/40%); B: 0.1% formic acid and 10 mmol/L ammonium formate in acetonitrile/isopropanol (10%/90%). The samples were detected in full scan mode. The characteristic ions of each lipid metabolite were processed by multiple

10 http://www.hmdb.ca/

reaction monitoring. Lipid metabolites were identified by MWLDB (Metware lipidomics database v 3.0) provided by MetWare, Co., Ltd.[11] (Wuhan, China). The semiquantitative analysis of the metabolites were calculated based on the peak area ratio of the analyte to the internal standard (Supplementary Table S2).

2.9 Statistical analysis

Wilcoxon's rank sum tests and student's *t* tests were used to determine the significance of differences in alpha diversity, composition, genetic distance and inflammatory factor expression between two groups. The aforementioned tests were carried out by utilizing either R version 3.5.1 or GraphPad Prism version 8.0. A two-sided *p*-value of less than 0.05 was considered statistically significant.

3 Results

3.1 The alteration of the microbial diversity of oral and gut microbiota in the children with asthma

In this study, the mean (± SD) of age of children with asthma and the control was 6.52 ± 2.60 years and 6.06 ± 2.78 years, respectively; the number of males and females in the group with asthma was 14:16 and 18:12 in the control group. There was no significant difference in the distribution of age and sex between two groups. BMI of all subjects was all in the normal range. All of subjects reported without passive smoking.

The oral microbiota and gut microbiota of both the control and the children with asthma were analyzed through *16S rRNA* gene sequencing. Compared to the control group, the Chao, Sobs and Shannon indices of oral microbiota and gut microbiota in the children with asthma were significantly decreased, and the Simpson index increased (Figure 1A). Beta (β) diversity analysis using PCoA (Principal co-ordinates analysis) was used to visualize the differences among groups. Bray-Curtis distance demonstrated statistically significant differences in β diversity of oral microbiota and gut microbiota between two groups (Figure 1B).

3.2 The changes in the composition of oral and gut microbiota in the children with asthma

The dominant bacterial phyla in oral microbiota were Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria, while the top eight dominant bacterial genera were *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, *Haemophilus*, *Actinomyces*, *Leptotrichia* and *Granulicatella* (Figure 2A); the dominant phyla of gut microbiota were Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobiota; the top eight dominant genera were *Bacteroides*, *Blautia*, *Faecalibacterium*, *Bifidobacterium*, *Enterococcus*, *Allobaculum*, *Romboutsia*, and *Clostridium_sensu_stricto_1* (Figure 2B), which were consistent with previous studies (Hu et al., 2022; Zheng et al., 2022).

In oral microbiota, the relative abundance of Proteobacteria was significantly decreased, and although the relative abundance of Firmicutes and Bacteroidetes was increased, there was no statistical significance in the children with asthma. At the genus level (relative abundance >5%), the relative abundance of *Prevotella* and *Veillonella* was significantly increased, and *Streptococcus* showed a decreased trend in the children with asthma (Figure 2C). In gut microbiota, there was an increase in the relative abundance of Firmicutes and Bacteroidetes without significance, and the relative abundance of Proteobacteria was significantly increased and Actinobacterioat was significantly decreased in the children with asthma. At the genus level (relative abundance >5%), the relative abundance of *Enterococcus* and *Allobaculum* was significantly increased, while the relative abundance of *Blautia*, *Bifidobacterium*, *Faecalibacterium*, *Eubacterium_hallii_group*, and *Fusicatenibacter* was significantly decreased in the children with asthma (Figure 2D).

3.3 The enrichment of oral-originated microbiota in the gut of children with asthma

One hundred and twenty-one shared OTUs coexisted in oral and gut microbiota shown in the Venn diagram (Supplementary Figure S1). The relative abundance of the top 20 shared genera showed the relationship between oral and gut microbiota (high in oral and low in gut microbiota, vice versa) (Figure 3A). Furthermore, we conducted a co-occurrence network analysis on the distribution patterns between samples and species. In the control group, only *Streptococcus* was present in both the oral cavity and gut, while *Prevotella* and *Veillonella* were exclusively found in the oral cavity. In the group with asthma, *Prevotella*, *Veillonella*, and *Streptococcus* were observed in both oral cavity and gut (Figure 3B).

Further analysis of microbiota metagenomic sequencing results revealed a significant increase in the abundance of *P. bivia*, *P. disiens*, and *P. oris* in both oral cavity and gut. Additionally, there was a significant increase in the relative abundance of *Bacteroides fragilis* in the oral and gut microbiota of children with asthma, while the relative abundance of *Streptococcus thermophilus* was significantly decreased (Figures 3C,D). To confirm the oral-gut migration of these species in the children asthma, we conducted a strain-level analysis based on *16S rRNA* gene using oral and fecal metagenome of the control and fecal metagenome of the group with asthma. Genetic distance of *16S rRNA* gene was calculated by p-distance. The strains of *Prevotella bivia*, *P. disiens*, *P. oris* and *S. thermophilus* in the gut of the group with asthma had a closer relationship to those in the oral cavity of the control, indicating these species originated from the oral microbiota (Figures 4A–E).

3.4 Microbial mimicry peptides from the enriched microbiota in the gut induced the expression of inflammatory factors

Through aligning the amino acid sequences of *P. bivia*, *P. disiens*, *P. oris*, and *B. fragilis* with the IEDB database, microbial mimicry

11 http://www.metware.cn/

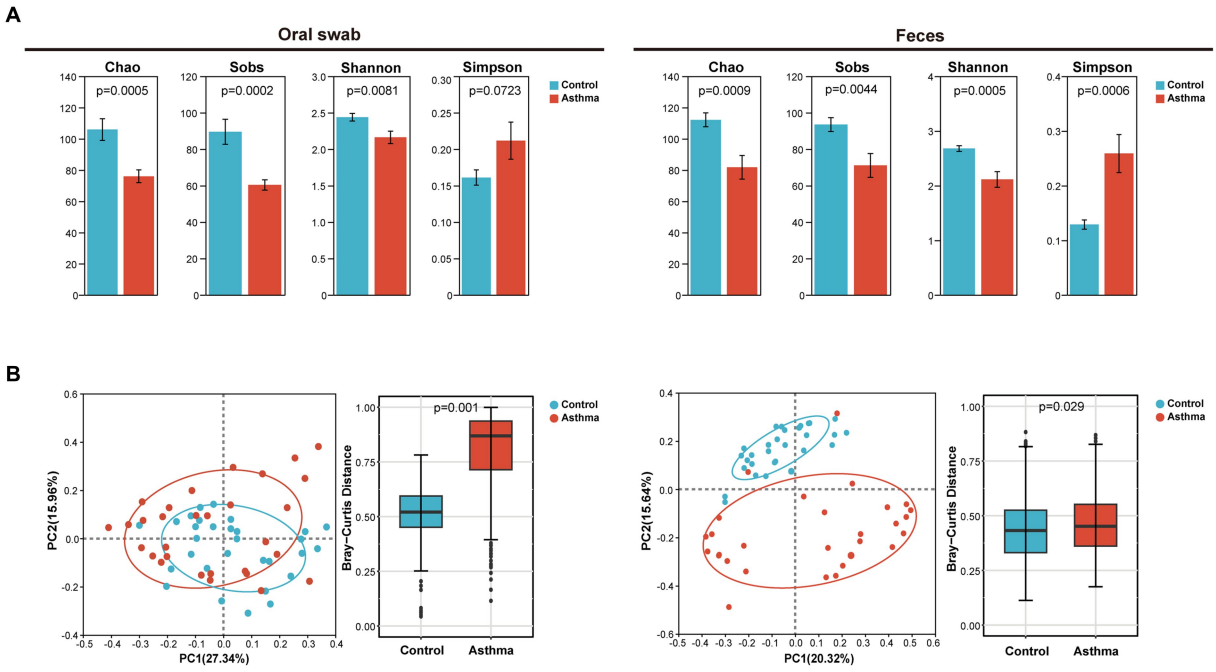


FIGURE 1
The changes in microbial diversity in oral and gut samples of the children with asthma. **(A)** Chao, sobs, shannon and simpson index of oral microbiota and gut microbiota in subjects with or without asthma. **(B)** PCoA and Bray-Curtis distance of oral and gut microbiota. All microbiota was analyzed using *16S rRNA* gene sequencing. $n = 30$ for oral swabs and 30 for feces. Student's *t*-test was used for statistical analysis in **(A)** and permutational multivariate analysis of variance in **(B)**.

peptides that exhibited more than 80% similarity to asthma antigenic epitopes were selected (Supplementary Table S3). These peptides were used to stimulate PBMCs, and the expression of relevant inflammatory factors was subsequently evaluated. The results showed that Ppb from *P. bivia* induced *IL-4, IL-5, IL-6,* and *IL-8* expression; Rpd from *P. disiens* induced *IL-6* and *IL-8* expression. Fbf was derived from *B. fragilis*, and induced *IL-4, IL-5, IL-6, IL-8*, and *IL-17A* expression (Figure 4F).

3.5 Gut microbiota interacted with fecal metabolome related to childhood asthma

Based on gut metagenomic data, we identified KEGG pathways with an LDA (Linear discriminant analysis) score greater than 2 by LEfSe analysis (Supplementary Figure S2). The asthma group was characterized by amino acid metabolism and lipid metabolism. Spearman correlation analysis was performed between the above 5 different species and selected pathways. *B. fragilis, P. bivia*, *P. disiens* and *P. oris* had associations with these two types of metabolic pathways (Figure 5A).

In order to elucidate the functional changes in gut microbiota and their interaction with the host, we conducted a comprehensive analysis of the metabolome in fecal samples using an untargeted ultrahigh-performance liquid chromatography-mass spectrometry (UPLC-MS) approach. The score plots generated from PCA depicted distinct separation between the control and the group with asthma (Figure 5B). The differential metabolism were determined based on a variable importance in projection (VIP) score greater than 1 and a false discovery rate lower than 0.05. The certain metabolism such as malic acid, SDA, ALA, and EPA (fatty acyl); DG and MG (glycerolipids); LPC, PC, and PA (glycerophospholipids) were decreased, while L-carnitine (fatty acyl) and Cer (sphingolipids) were increased compared to the control (Figure 5C; Supplementary Table S4).

Next, a redundancy analysis (RDA) using metagenomic and metabolomic data revealed a complex co-occurrence pattern and correlation between bacteria associated with childhood asthma and characterized metabolites from the essential metabolic pathways. In the gut of the children with asthma, *P. bivia*, *P. disiens,* and *P. oris* showed a negative correlation with glycerophospholipid (LPC, PC, and PA), and *B. fragilis* showed a positive correlation with L-carnitine and Cer. As a probiotic, *S. thermophilus* showed an opposite trend compared to *Prevotella* and *Bacteroides* (Figure 5D).

3.6 *Prevotella bivia* affected lipid metabolism in HBE cells

Human bronchial epithelial (HBE) cells were cultured with the supernatant of *P. bivia* and were detected with lipid metabolome based on UPLC-MS/MS. PCA performed on all the samples revealed that a clear clustering of QC (quality control) samples, and lipid components in the control group and treatment group were well separated (Supplementary Figure S3). A total of 1,221 lipid metabolites were detected in the samples, categorized into 45 lipid subclasses, and the types and quantities of lipid subclasses detected were presented in Supplementary Figure S4A. The distribution of various lipid subclasses between the two groups was shown in Supplementary Figure S4B. Based

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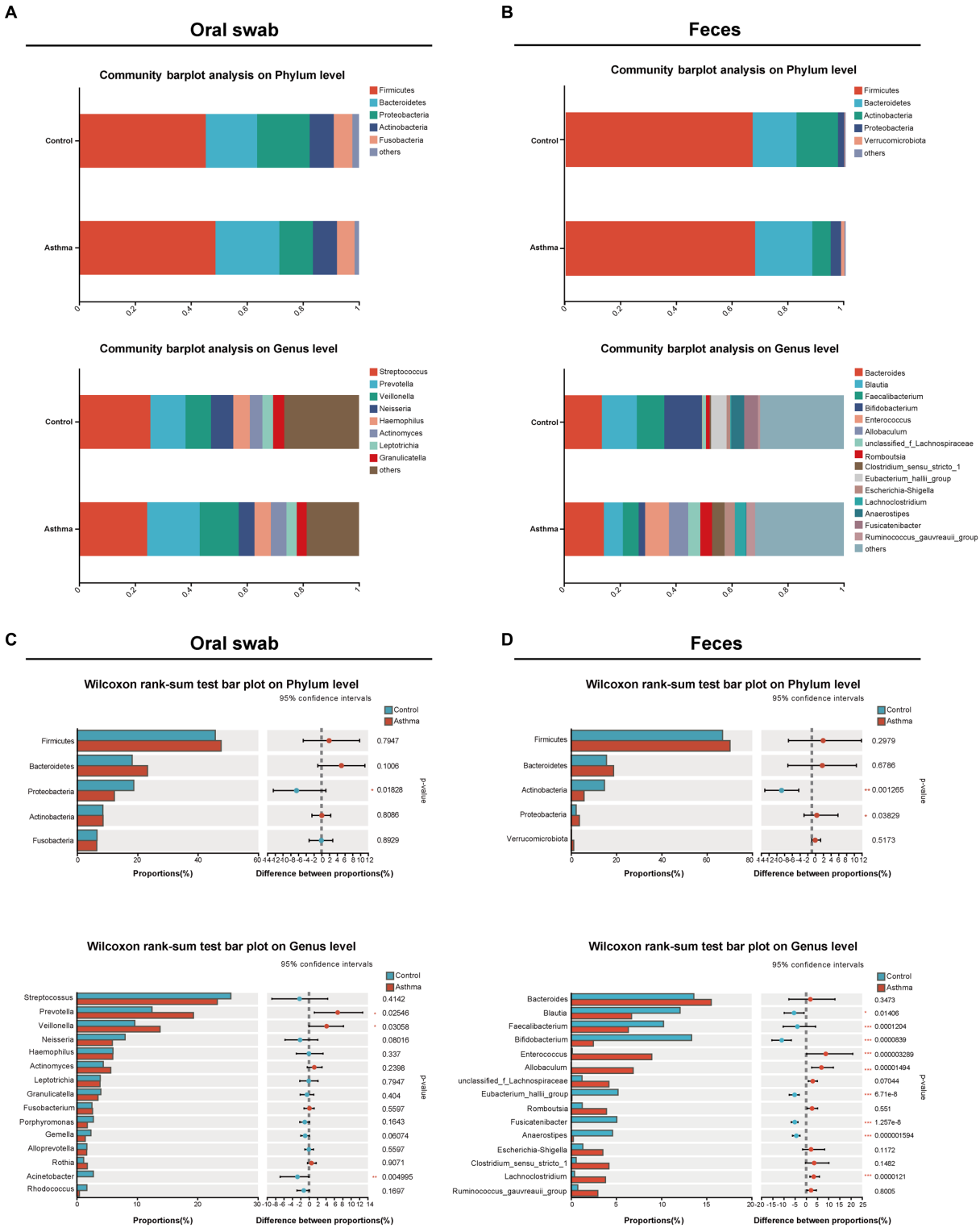


FIGURE 2
The alterations of oral and gut microbial composition in the children with asthma. Bar plots showing relative abundances of oral microbiota **(A)** and gut (fecal) microbiota **(B)** microbiota at phylum level and genus level in subjects with or without asthma. The distribution of microbial communities in each group was displayed using mean values. The comparison of the phyla and genera in oral microbiota **(C)** and gut (fecal) microbiota **(D)** between two groups. Wilcoxon rank-sum test was used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

on volcano plot analysis, 220 differential lipid metabolites were selected with VIP > 1 and adjusted *p*-value <0.05. Among them, 55 showed a significant increase, while 165 showed a significant decrease in the treatment group (Figure 6A). Through visualizing the distribution of differential lipids in different lipid subclasses (Supplementary Figure S5) and filtering the top 20 based on fold

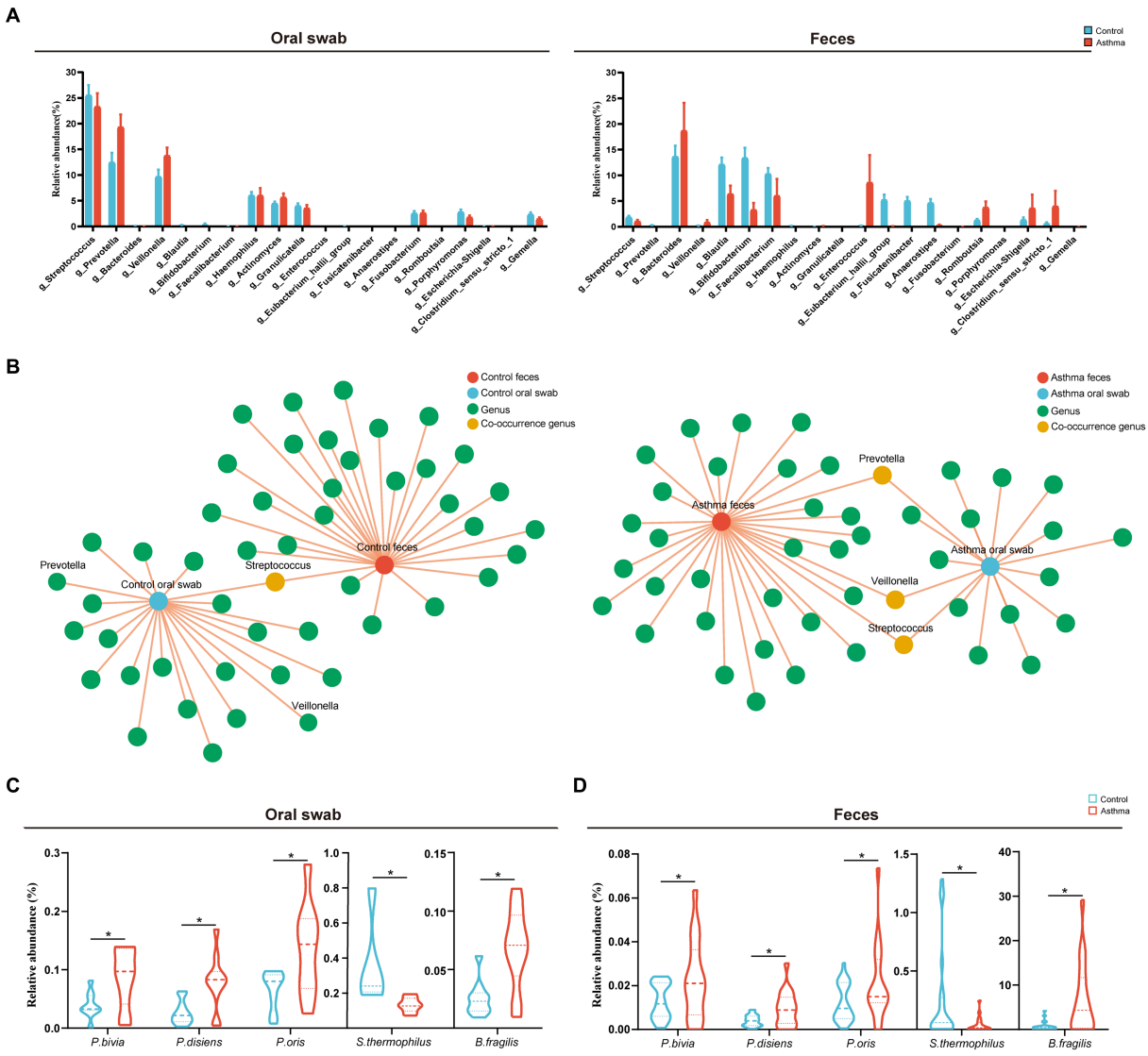


FIGURE 3
Shared microbiota in the oral cavity and gut of the children with asthma. **(A)** Relative abundances of the top 20 shared genera in subjects with or without asthma. **(B)** Co-occurrence network analysis of oral and gut microbial genera in patients with asthma and healthy controls. **(C,D)** The comparison of the relative abundance of *Prevotella bivia*, *Prevotella disiens*, *Prevotella oris*, *Streptococcus thermophilus* and *Bacteroides fragilis* in oral and gut microbiota between two groups. $n = 10$ in the control and the children with asthma, respectively, for microbiota metagenomic sequencing. *$p < 0.05$, Wilcoxon rank-sum test.

change, in the treatment group, it revealed that a significant decrease in LPC, LPG, PA, PC, PG, PI, and PS, and a significant increase in CE, Cer, and TG (Figures 6B,C). These results were partially consistent with findings from previous non-targeted metabolomics analysis. Most of lipid metabolites (87.72%) were enriched in metabolic pathways (Supplementary Figure S6). Based on the differential lipid metabolites, KEGG pathway enrichment analysis revealed pathway disruptions in the treated group, primarily enriched in glycerophospholipid metabolism ($p < 0.05$) (Figure 6D).

4 Discussion

Several studies have suggested a potential link between alterations in oral and gut microbial composition and an increased risk of childhood asthma (Fujimura et al., 2016; Durack et al., 2020). However, there is limited research focusing on direct evidence of migration between oral microbiota and gut microbiota in the children with asthma. In the present study, we found that *Prevotella* can migrate from the oral cavity to the gut and influence childhood asthma by secreting microbial mimicry peptides and regulating lipid metabolites associated with inflammation.

Consistent with previous results, *16S rRNA* sequencing data showed that a significant decrease in alpha diversity of both oral microbiota and gut microbiota in the children with asthma (Patrick et al., 2020; Hu et al., 2022). In the oral microbiota of the children with asthma, there was a significant increase in the abundance of *Prevotella* and *Veillonella*. They are common commensals in the oral cavity, the increase of which has been reported to lead to the recruitment of monocytes and T cells chemokines, consequently increasing the risk

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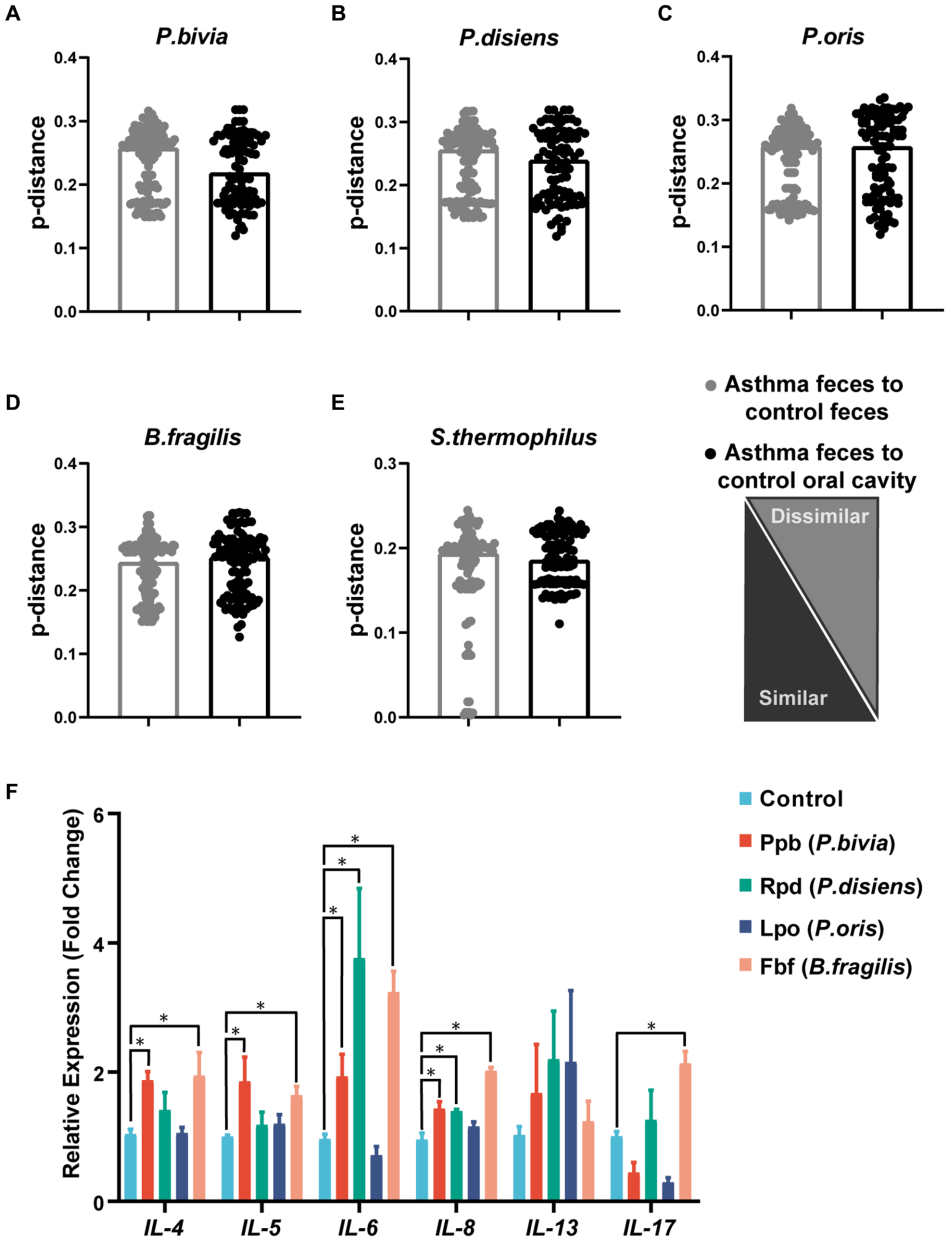


FIGURE 4
Genetic distance analysis of asthma-enriched species and functional validation of microbial peptide molecular mimicry. Indices of intrasubject similarity (p-distance) between the bacterial communities of the two source sites (oral cavity and gut) and the target site (gut) of that subject. As shown, smaller distances mean greater similarities between the microbial communities of the samples indicated **(A–E)**. After stimulation with different peptides, the mRNA transcription levels of *IL-4, IL-5, IL-6, IL-8, IL-13*, and *IL-17A* in PBMCs were measured ($n = 4$). *$p < 0.05$, student's *t* test was used for statistical analysis **(F)**.

of asthma (Thorsen et al., 2019). Metagenome sequencing revealed that *P. bivia, P. disiens, P. oris* and *B. fragilis* were increased and *Streptococcus thermophilu* was decreased in the gut of the children with asthma. *P. bivia, P. disiens,* and *P. oris* may be considered to originate from the oral cavity and transmit to the gut by genetic distance analysis. Some studies have demonstrated that *Prevotella* as key members of the oral microbiota were found in the gut of patients and associated with certain gastrointestinal disorders (Schmidt et al., 2019). *Prevotella* can mediate mucosal inflammation. *Prevotella* can active mucosal Th17 immune responses and neutrophil recruitment, further leading to systemic dissemination of inflammatory factors and bacterial products, and in turn may affect systemic disease outcomes, including asthma (Larsen, 2017). *B. fragilis* is a major colonizer of the human gut where *Bacteroides* is a predominant genus. *B. fragilis* colonized in feces at the age of 3 weeks increased the risk of developing asthma later on Vael et al. (2008). In mouse models, the colonization of *B. fragilis* in the gut led to impaired intestinal barrier function, increased secretion of IL-17 in the lungs during allergic airway inflammation, and oxidative stress (Wilson et al., 2023). Additionally, *S. thermophilus* in the gut of the children with asthma was more closely related to the oral cavity in genetics. What's more, the relative abundance of *S. thermophilus* was reduced in both locations in the

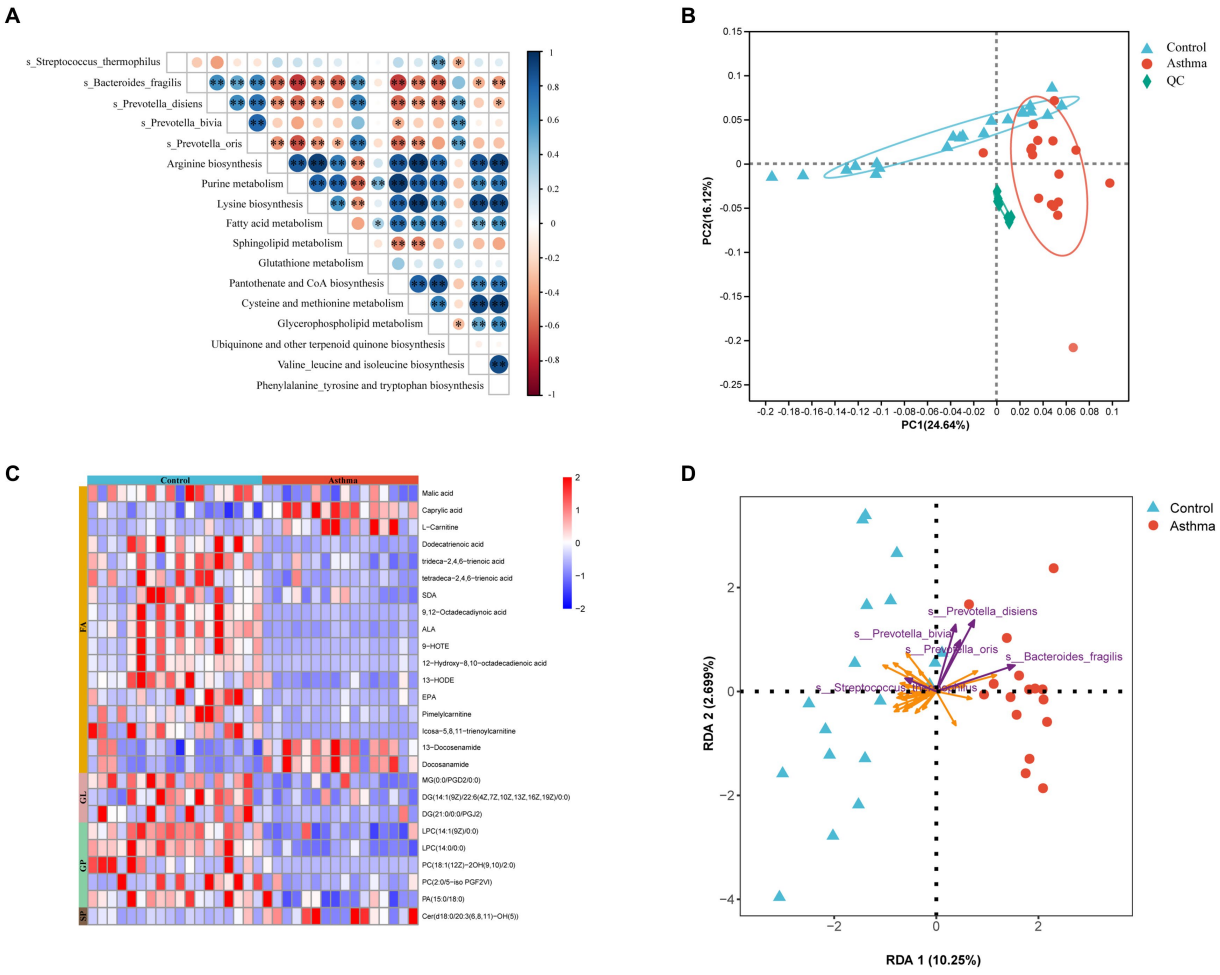


FIGURE 5
Correlations between asthma-related microbial species and metabolites in feces. **(A)** Heatmap representation of spearman correlation between microbial species and their corresponding KEGG pathways. *$p < 0.05$, **$p < 0.01$, Spearman. **(B)** PCA showed alterations of fecal metabolome in the children with asthma. **(C)** Heatmap analysis of differential lipid metabolites. Red color represents upregulated metabolites while blue color represents downregulated metabolites. **(D)** Redundancy analysis (RDA) of the variable relationships between differential microbiota and lipid metabolites. The purple arrows represent bacteria, and the orange arrows represent lipid metabolites. FA, Fatty Acyl; GL, Glycerolipids; GP, Glycerophospholipids; SP, Sphingolipids.

group with asthma. *S. thermophilus* is a probiotic that has anti-allergic effects and can reduce the production of allergen-specific IgE (Peng et al., 2007). The extracellular polysaccharides produced by *S. thermophilus* can protect the integrity of the intestinal barrier and reduce the expression of pro-inflammatory cytokines, playing an important role not only in gastrointestinal disease but also in other inflammatory disease prevention (Mizuno et al., 2020). For example, in the rat experiment of osteoarthritis, *S. thermophilus* can reduce synovial tissue inflammation (Lin et al., 2021).

Recently several studies have indicated that microbial mimicry peptides may trigger an immune response by activating autoimmune T cells and B cells as mimic autoantigens in some diseases (Chen et al., 2017). Five of microbial mimicry peptides derived from three *Prevotella* species and *B. fragilis* were capable of stimulating PBMCs to express *IL-4, IL-5, IL-6, IL-8,* and *IL-17A* genes. Rpd from *P. disiens* and Fbf from *B. fragilis* were similar to the *Blattella germanica* antigen which can be presented to T cells stimulating the secretion of IL-5 and inducing eosinophilic airway inflammation through HLA-DQ (Papouchado et al., 2001). The epitope from Ppb of *P. bivia* was similar to the dust mite allergen dermatophagoides farinae 2 (Der f 2). As a common allergen in asthma, Der f 2 can be presented to T cells through HLA-DQ or HLA-DR, stimulating the production of IL-4 (Inoue et al., 1997; Lee et al., 2018). These findings suggested that the enrichment of these four microbial species in the children with asthma may activate the immune response through microbial mimicry peptides, thus involved in childhood asthma.

Through non-targeted metabolomics analysis of fecal samples, we found that lipid metabolism was significantly disturbed in the children with asthma. Lipid metabolism is a key driver of inflammation, and recent studies suggested that disruptions in lipid metabolism play an important role in the pathogenesis of asthma (Monga et al., 2020; Jiang et al., 2021). Through RDA analysis of the relationship between gut microbiota and differential metabolites in feces, it was found that bacteria such as *Prevotella* and *Bacteroides*, well known for their association with inflammation, may activate

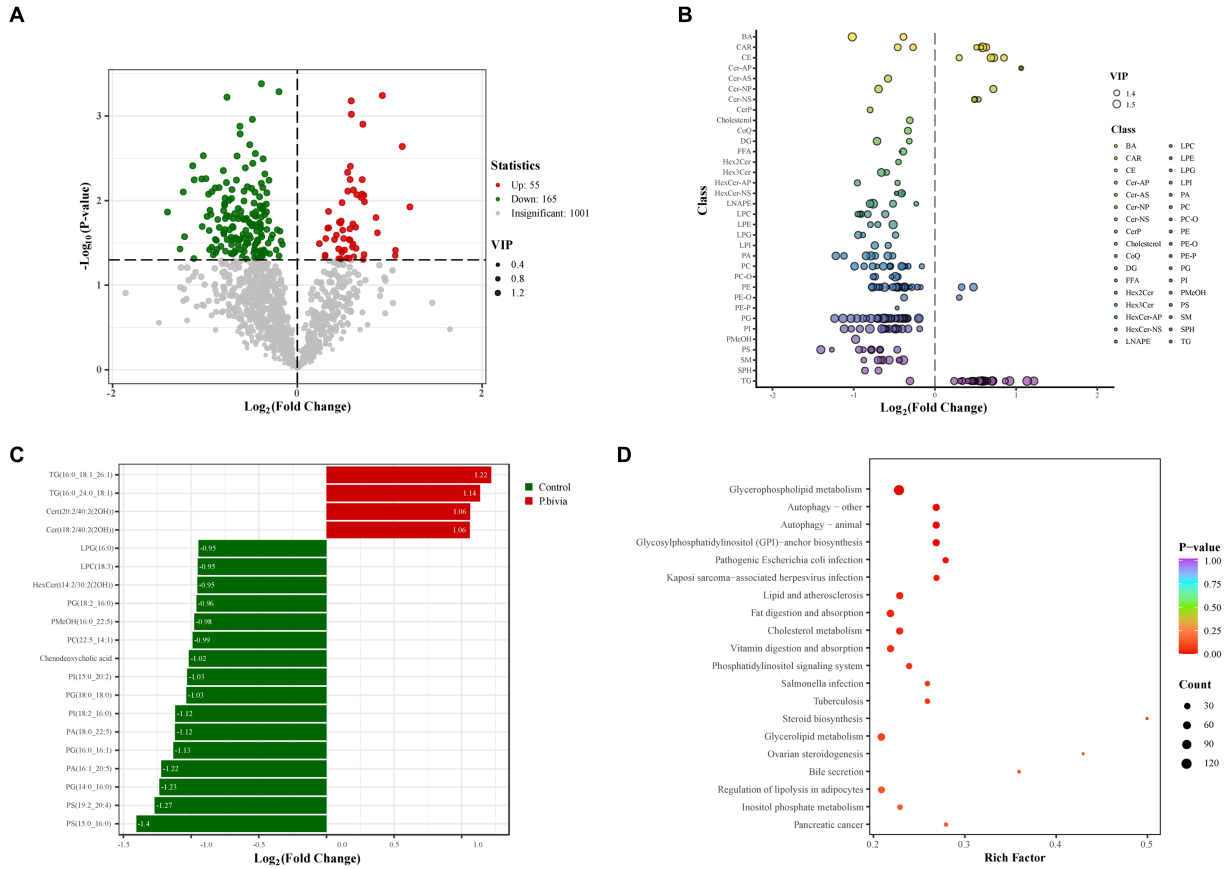


FIGURE 6
The disturbance of lipid metabolism in HBE cells treated with the supernatant of *Prevotella bivia*. **(A)** The volcano plot analysis. The criteria were VIP > 1 and *p*-value <0.5. The red (upregulated) and green (downregulated) dots represent lipid metabolites with significant differences, while gray dots indicate lipid metabolites with no significant differences. **(B)** The scatter plot illustrated the distribution of differential lipids in subclasses. The color of each circle represented different lipid subclasses, and the size of them indicated the VIP. **(C)** The bar chart showed the top twenty differential lipids in terms of fold change. **(D)** Enrichment of different lipid metabolites ($n = 3$).

immunity by influencing lipid metabolites. *In vitro* experiments, it was demonstrated that *Prevotella* can induce dyslipidemia in HBE cells.

Currently, some mechanism behind the association between the disturbance of lipid metabolome and asthma has been proposed. One could be inflammatory responses induced by pro-inflammatory lipid metabolites such as CE, Cer and TG. It is well known that asthma is a chronic inflammatory disease. Dyslipidemia may induce a shift in the Th1/Th2 balance with a predominance of Th2 cells, and lower Th1/Th2 ratio in both mouse and human lymphocytes (Zhou et al., 1998; Matia-Garcia et al., 2021). There is a significant increase in CE, Cer and TG levels in in *P. bivia-treated* HBE cells. Hypercholesterolemia was found to be associated with high prevalence of asthma (Ko et al., 2018). Moreover, research found that elevated CE levels triggered the toll-like receptor signaling pathway in macrophages, thereby enhancing the immune response (Fessler and Parks, 2011). Cer plays a central role in sphingolipid metabolism, and their role in airway damage is controversial due to the complexity of their metabolic pathways. Lu et al. (2023) found that *Prevotella* can affect the prognosis of COVID-19 by modulating the sphingolipid metabolism pathway. Izawa et al. (2012) demonstrated that extracellular Cer interacted with CD300f (an immunoinhibitory receptor) limiting ovalbumin (OVA)-induced chronic airway inflammation. Sakae et al. (2023) indicated that Cer inhibit goblet cell hyperplasia in the airways induced by OVA. These findings suggested that Cer may act as anti-asthmatic lipids. However, James et al. (2021) found that pulmonary Cer trigger the recruitment of neutrophils, exacerbating allergic asthma and the reduction of Cer synthesis resulted in a decrease in Th2 responses and relieving OVA-induced asthma (Shin et al., 2021). This may be attributed to different acyl chain lengths causing variations in the functionality of Cer (Grösch et al., 2012). TG was the most abundant lipid in the human body, and an increase in their levels can adversely affect overall health. A cross-sectional study involving Korean adolescents indicated that elevated TG levels in the blood could lead to chronic inflammation in children, resulting in airflow obstruction and an increased prevalence of asthma (Chanachon et al., 2022) and a significant correlation was observed between TG levels and FeNO (fractional exhaled nitric oxide) in the patients with asthma (Jiang et al., 2021). *In vitro* experiments have shown that TG induced proliferation of CD4+ T cells and significantly increased the gene and protein levels of IL-17 and IFN-γ, thereby promoting inflammatory responses (Tan et al., 2022).

Another mechanism may be glycerophospholipids-involved in signaling and immune responses, which are major components of cell

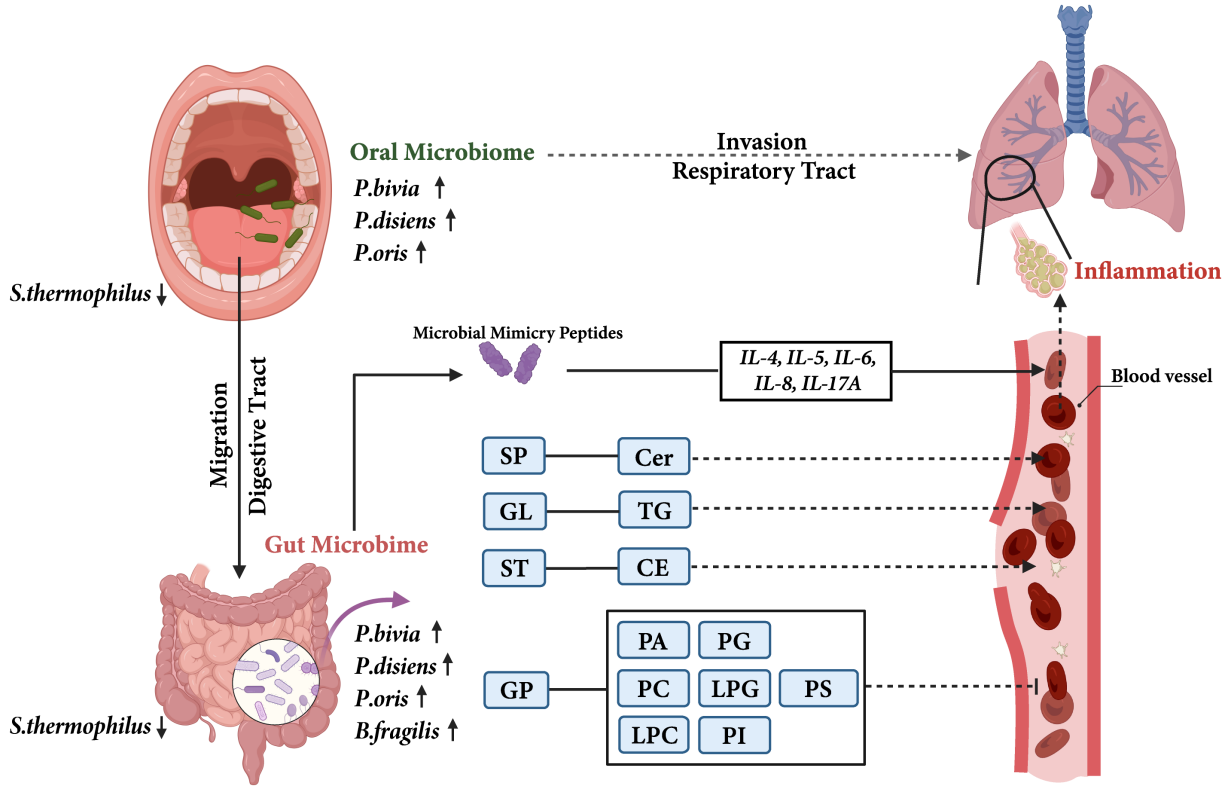


FIGURE 7
The potential mechanisms of oral-originated gut microbiota involvement in childhood asthma. The solid arrows indicated the results of this study, and the dashed arrows indicated previously reported results. SP, Sphingolipids; Cer, Ceramide; GL, Glycerolipids; TG, Triglyceride; ST, Sterol lipids; CE, Cholesterylesters; GP, Glycerophospholipids; PA, Phosphatidic acid; PC, Phosphatidylcholine; LPC, Lysophosphatidylcholine; PG, Phosphatidylglycerol; LPG, Lysophosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine.

membranes and precursors of signaling molecules. They are secreted into the alveoli by airway epithelial cells to reduce alveolar surface tension and block pathogen invasion. Studies have shown that the decreasing of glycerophospholipids are involved in the pathogenesis of lung infections, asthma, and COPD (Mander et al., 2002; Gai et al., 2019). What's more, we also observed a significant decrease in glycerophospholipid metabolites in the children with asthma. As a degradation product of PC, LPC can exert anti-inflammatory effects by reducing leukocyte extravasation, downregulating the formation of pro-inflammatory mediators IL-5 and IL-6, and upregulating the expression of anti-inflammatory mediators IL-4 and IL-10 (Hung et al., 2012; Monga et al., 2020). LPG is a precursor for *de novo* synthesis of PG which plays an important role, especially, in lung (Greenough, 1998). PG was a component of pulmonary surfactant that could reduce the surface tension at the alveolar air-liquid interface and exhibited immunomodulatory properties (Klein et al., 2020). PA can be degraded into LPA and pre-treatment with LPA has been shown to reduce the IL-13-induced STAT6 phosphorylation and cytokine release, accordingly exerting anti-inflammatory effects (Zhao and Natarajan, 2013). PI can increase airway hyperresponsiveness in asthma patients (Hallstrand et al., 2013). Stokes et al. (2016) found that liposomes containing PS could inhibit the inflammatory response induced by human rhinovirus, a major factor in acute exacerbations of chronic airway diseases. Therefore, based on these results, we postulated the potential mechanisms of oral-originated gut microbiota involved in childhood asthma (Figure 7).

This study has some limitations. First, the present study was a pilot study and had a small sample size. Second, due to the depth of metagenomic sequencing, homology analysis between oral microbiota and gut microbiota was only calculated by genetic distance and cannot fully represent the comprehensive relationship. Further research should be carried out to establish a direct causal relationship between oral-originated gut species and childhood asthma in multi-center samples by deep sequencing.

In conclusion, our study provided evidence that oral-originated microbiota in the gut, by secreting microbial mimicry peptides and altering in lipid metabolism, can influence childhood asthma. These findings highlighted the potential role of the oral-gut microbial axis in understanding the pathogenesis of asthma and metabolomic analysis of lipid profiles may give a new strategy for new biomarkers and new drug targets for asthma.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: https://www.ncbi.nlm.nih.gov/, PRJNA1086671.

Ethics statement

The studies involving humans were approved by Nanjing Medical University Clinical Research Ethics Committee under the reference number (2019)02080-1. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

TY: Data curation, Formal analysis, Writing – original draft, Visualization, Validation, Methodology, Investigation. YB: Data curation, Formal analysis, Methodology, Writing – original draft. SC: Formal analysis, Writing – original draft. PJ: Formal analysis, Writing – original draft. ZZ: Writing – original draft, Formal analysis. LL: Resources, Writing – original draft. YK: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. QW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1400079/full#supplementary-material

References

Arrieta, M.-C., Stiemsma, L. T., Dimitriu, P. A., Thorson, L., Russell, S., Yurist-Doutsch, S., et al. (2015). Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci. Transl. Med.* 7:307ra152. doi: 10.1126/scitranslmed.aab2271

Chanachon, P. N., Jotikasthira, W., Kiewngam, P., Sawatchai, A., Kanchongkittiphon, W., and Manuyakorn, W. (2022). TG/HDL-C ratio independent of obesity associates with airflow obstruction in children with asthma. *Indian J. Pediatr.* 89:92. doi: 10.1007/s12098-021-03942-y

Chen, B.-d., Jia, X.-M., Xu, J.-Y., Zhao, L.-D., Ji, J.-Y., Wu, B.-X., et al. (2021). An autoimmunogenic and Proinflammatory profile defined by the gut microbiota of patients with untreated systemic lupus erythematosus. Arthritis. *Rheumatol.* 73, 232–243. doi: 10.1002/art.41511

Chen, B., Sun, L., and Zhang, X. (2017). Integration of microbiome and epigenome to decipher the pathogenesis of autoimmune diseases. *J. Autoimmun.* 83, 31–42. doi: 10.1016/j.jaut.2017.03.009

Couvigny, B., de Wouters, T., Kaci, G., Jacouton, E., Delorme, C., Doré, J., et al. (2015). Commensal *Streptococcus salivarius* modulates PPARγ transcriptional activity in human intestinal epithelial cells. *PLoS One* 10:e0125371. doi: 10.1371/journal.pone.0125371

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R., Yu, W.-H., et al. (2010). The human oral microbiome. *J. Bacteriol.* 192, 5002–5017. doi: 10.1128/JB.00542-10

Durack, J., Christian, L. S., Nariya, S., Gonzalez, J., Bhakta, N. R., Ansel, K. M., et al. (2020). Distinct associations of sputum and oral microbiota with atopic, immunologic, and clinical features in mild asthma. *J. Allergy Clin. Immunol.* 146, 1016–1026. doi: 10.1016/j.jaci.2020.03.028

Durack, J., Lynch, S. V., Nariya, S., Bhakta, N. R., Beigelman, A., Castro, M., et al. (2017). Features of the bronchial bacterial microbiome associated with atopy, asthma, and responsiveness to inhaled corticosteroid treatment. *J. Allergy Clin. Immunol.* 140, 63–75. doi: 10.1016/j.jaci.2016.08.055

Dzidic, M., Abrahamsson, T. R., Artacho, A., Collado, M. C., Mira, A., and Jenmalm, M. C. (2018). Oral microbiota maturation during the first 7 years of life in relation to allergy development. *Allergy* 73, 2000–2011. doi: 10.1111/all.13449

Enaud, R., Prevel, R., Ciarlo, E., Beaufils, F., Wieërs, G., Guery, B., et al. (2020). The gut-lung Axis in health and respiratory diseases: a place for inter-organ and inter-kingdom Crosstalks. *Front. Cell. Infect. Microbiol.* 10:9. doi: 10.3389/fcimb.2020.00009

Fessler, M. B., and Parks, J. S. (2011). Intracellular lipid flux and membrane microdomains as organizing principles in inflammatory cell signaling. *J. Immunol.* 187, 1529–1535. doi: 10.4049/jimmunol.1100253

Fujimura, K. E., Sitarik, A. R., Havstad, S., Lin, D. L., Levan, S., Fadrosh, D., et al. (2016). Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. *Nat. Med.* 22, 1187–1191. doi: 10.1038/nm.4176

Gai, X. Y., Zhang, L. J., Chang, C., Guo, C. L., Abulikemu, M., Li, W. X., et al. (2019). Metabolomic analysis of serum Glycerophospholipid levels in eosinophilic and neutrophilic asthma. *Biomed. Environ. Sci.* 32, 96–106. doi: 10.3967/bes2019.013

Genco, R. J., and Van Dyke, T. E. (2010). Prevention: reducing the risk of CVD in patients with periodontitis. *Nat. Rev. Cardiol.* 7, 479–480. doi: 10.1038/nrcardio.2010.120

Graves, D. T., Corrêa, J. D., and Silva, T. A. (2019). The Oral microbiota is modified by systemic diseases. *J. Dent. Res.* 98, 148–156. doi: 10.1177/0022034518805739

Greenough, A. (1998). Neonatal chronic lung disease and exogenous surfactant therapy. *Eur. J. Pediatr.* 157, S16–S18. doi: 10.1007/PL00014283

Grösch, S., Schiffmann, S., and Geisslinger, G. (2012). Chain length-specific properties of ceramides. *Prog. Lipid Res.* 51, 50–62. doi: 10.1016/j.plipres.2011.11.001

Hajishengallis, G. (2015). Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44. doi: 10.1038/nri3785

Hallstrand, T. S., Lai, Y., Altemeier, W. A., Appel, C. L., Johnson, B., Frevert, C. W., et al. (2013). Regulation and function of epithelial secreted phospholipase A2 group X in asthma. *Am. J. Respir. Crit. Care Med.* 188, 42–50. doi: 10.1164/rccm.201301-0084OC

Hu, J., Bao, Y., Huang, H., Zhang, Z., Chen, F., Li, L., et al. (2022). The preliminary investigation of potential response biomarkers to PAHs exposure on childhood asthma. *J. Expo. Sci. Environ. Epidemiol.* 32, 82–93. doi: 10.1038/s41370-021-00334-4

Hu, L., Ni, Z., Zhao, K., Li, X., Gao, X., Kang, Y., et al. (2023). The association between oral and gut microbiota in male patients with alcohol dependence. *Front. Microbiol.* 14:1203678. doi: 10.3389/fmicb.2023.1203678

Hung, N. D., Sok, D.-E., and Kim, M. R. (2012). Prevention of 1-palmitoyl lysophosphatidylcholine-induced inflammation by polyunsaturated acyl lysophosphatidylcholine. *Inflamm. Res.* 61, 473–483. doi: 10.1007/s00011-012-0434-x

Inoue, R., Matsuoka, T., Kondo, N., Nishimura, Y., and Matsushita, S. (1997). Identification of Dermatophagoides farinae-2-derived peptides and class II HLA

molecules recognized by T cells from atopic individuals. *Int. Arch. Allergy Immunol.* 114, 354–360. doi: 10.1159/000237694

Izawa, K., Yamanishi, Y., Maehara, A., Takahashi, M., Isobe, M., Ito, S., et al. (2012). The receptor LMIR3 negatively regulates mast cell activation and allergic responses by binding to extracellular ceramide. *Immunity* 37, 827–839. doi: 10.1016/j.immuni.2012.08.018

James, B. N., Oyeniran, C., Sturgill, J. L., Newton, J., Martin, R. K., Bieberich, E., et al. (2021). Ceramide in apoptosis and oxidative stress in allergic inflammation and asthma. *J. Allergy Clin. Immunol.* 147, 1936–1948.e9. doi: 10.1016/j.jaci.2020.10.024

Jiang, T., Dai, L., Li, P., Zhao, J., Wang, X., An, L., et al. (2021). Lipid metabolism and identification of biomarkers in asthma by lipidomic analysis. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* 1866:158853. doi: 10.1016/j.bbalip.2020.158853

Kato, T., Yamazaki, K., Nakajima, M., Date, Y., Kikuchi, J., Hase, K., et al. (2018). Oral Administration of *Porphyromonas gingivalis* alters the gut microbiome and serum metabolome. *mSphere* 3:3. doi: 10.1128/mSphere.00460-18

Klein, M. E., Mauch, S., Rieckmann, M., Martínez, D. G., Hause, G., Noutsias, M., et al. (2020). Phosphatidylserine (PS) and phosphatidylglycerol (PG) nanodispersions as potential anti-inflammatory therapeutics: comparison of in vitro activity and impact of pegylation. *Nanomedicine* 23:102096. doi: 10.1016/j.nano.2019.102096

Ko, S.-H., Jeong, J., Baeg, M. K., Han, K.-D., Kim, H. S., Yoon, J.-S., et al. (2018). Lipid profiles in adolescents with and without asthma: Korea National Health and nutrition examination survey data. *Lipids Health Dis.* 17:158. doi: 10.1186/s12944-018-0807-4

Larsen, J. M. (2017). The immune response to Prevotella bacteria in chronic inflammatory disease. *Immunology* 151, 363–374. doi: 10.1111/imm.12760

Le Bars, P., Matamoros, S., Montassier, E., Le Vacon, F., Potel, G., Soueidan, A., et al. (2017). The oral cavity microbiota: between health, oral disease, and cancers of the aerodigestive tract. *Can. J. Microbiol.* 63, 475–492. doi: 10.1139/cjm-2016-0603

Lee, Y. Y., Lee, S. Y., Park, S.-Y., Choi, H.-J., Kim, E.-G., and Han, J.-S. (2018). Therapeutic potential of a phospholipase D1 inhibitory peptide fused with a cell-penetrating peptide as a novel anti-asthmatic drug in a Der f 2-induced airway inflammation model. *Exp. Mol. Med.* 50, 1–11. doi: 10.1038/s12276-018-0083-4

Levan, S. R., Stamnes, K. A., Lin, D. L., Panzer, A. R., Fukui, E., McCauley, K., et al. (2019). Elevated faecal 12,13-diHOME concentration in neonates at high risk for asthma is produced by gut bacteria and impedes immune tolerance. *Nat. Microbiol.* 4, 1851–1861. doi: 10.1038/s41564-019-0498-2

Lin, Y.-Y., Chen, N.-F., Yang, S.-N., Jean, Y.-H., Kuo, H.-M., Chen, P.-C., et al. (2021). Effects of *Streptococcus thermophilus* on anterior cruciate ligament transection-induced early osteoarthritis in rats. *Exp. Ther. Med.* 21:222. doi: 10.3892/etm.2021.9653

Lu, S., Zhou, Y., Hu, Y., Wang, J., Li, H., Lin, Y., et al. (2023). Metatranscriptomic analysis revealed Prevotella as a potential biomarker of oropharyngeal microbiomes in SARS-CoV-2 infection. *Front. Cell. Infect. Microbiol.* 13:1161763. doi: 10.3389/fcimb.2023.1161763

Mander, A., Langton-Hewer, S., Bernhard, W., Warner, J. O., and Postle, A. D. (2002). Altered phospholipid composition and aggregate structure of lung surfactant is associated with impaired lung function in young children with respiratory infections. *Am. J. Respir. Cell Mol. Biol.* 27, 714–721. doi: 10.1165/rcmb.4746

Matia-Garcia, I., Vadillo, E., Pelayo, R., Muñoz-Valle, J. F., García-Chagollán, M., Loaeza-Loaeza, J., et al. (2021). Th1/Th2 balance in young subjects: relationship with cytokine levels and metabolic profile. *J. Inflamm. Res.* 14, 6587–6600. doi: 10.2147/JIR.S342545

Mizuno, H., Tomotsune, K., Islam, M. A., Funabashi, R., Albarracin, L., Ikeda-Ohtsubo, W., et al. (2020). Exopolysaccharides from *Streptococcus thermophilus* ST538 modulate the antiviral innate immune response in porcine intestinal Epitheliocytes. *Front. Microbiol.* 11:894. doi: 10.3389/fmicb.2020.00894

Monga, N., Sethi, G. S., Kondepudi, K. K., and Naura, A. S. (2020). Lipid mediators and asthma: scope of therapeutics. *Biochem. Pharmacol.* 179:113925. doi: 10.1016/j.bcp.2020.113925

Palmer, R. J. (2000). Composition and development of oral bacterial communities. *Periodontol.* 64, 20–39. doi: 10.1111/j.1600-0757.2012.00453.x

Papouchado, B. G., Chapoval, S. P., Marietta, E. V., Weiler, C. R., and David, C. S. (2001). Cockroach allergen-induced eosinophilic airway inflammation in HLA-DQ/human CD4(+) transgenic mice. *J. Immunol.* 167, 4627–4634. doi: 10.4049/jimmunol.167.8.4627

Patrick, D. M., Sbihi, H., Dai, D. L. Y., Al Mamun, A., Rasali, D., Rose, C., et al. (2020). Decreasing antibiotic use, the gut microbiota, and asthma incidence in children: evidence from population-based and prospective cohort studies. *Lancet Respir. Med.* 8, 1094–1105. doi: 10.1016/S2213-2600(20)30052-7

Peng, S., Lin, J.-Y., and Lin, M.-Y. (2007). Antiallergic effect of milk fermented with lactic acid bacteria in a murine animal model. *J. Agric. Food Chem.* 55, 5092–5096. doi: 10.1021/jf062869s

Pyclik, M. J., Srutkova, D., Razim, A., Hermanova, P., Svabova, T., Pacyga, K., et al. (2021). Viability status-dependent effect of *Bifidobacterium longum* ssp. longum CCM 7952 on prevention of allergic inflammation in mouse model. *Front. Immunol.* 12:707728. doi: 10.3389/fimmu.2021.707728

Roduit, C., Frei, R., Ferstl, R., Loeliger, S., Westermann, P., Rhyner, C., et al. (2019). High levels of butyrate and propionate in early life are associated with protection against atopy. *Allergy* 74, 799–809. doi: 10.1111/all.13660

Sakae, H., Ogiso, Y., Matsuda, M., Shimora, H., Deering, T., Fox, T. E., et al. (2023). Ceramide Nanoliposomes as potential therapeutic reagents for asthma. *Cells* 12:12. doi: 10.3390/cells12040591

Schmidt, T. S., Hayward, M. R., Coelho, L. P., Li, S. S., Costea, P. I., Voigt, A. Y., et al. (2019). Extensive transmission of microbes along the gastrointestinal tract. *eLife* 8:8. doi: 10.7554/eLife.42693

Shin, S.-H., Cho, K.-A., Yoon, H.-S., Kim, S.-Y., Kim, H.-Y., Pewzner-Jung, Y., et al. (2021). Ceramide synthase 2 null mice are protected from ovalbumin-induced asthma with higher T cell receptor signal strength in CD4+ T cells. *Int. J. Mol. Sci.* 22:2713. doi: 10.3390/ijms22052713

Simonyte Sjödin, K., Vidman, L., Rydén, P., and West, C. E. (2016). Emerging evidence of the role of gut microbiota in the development of allergic diseases. *Curr. Opin. Allergy Clin. Immunol.* 16, 390–395. doi: 10.1097/ACI.0000000000000277

Stokes, C. A., Kaur, R., Edwards, M. R., Mondhe, M., Robinson, D., Prestwich, E. C., et al. (2016). Human rhinovirus-induced inflammatory responses are inhibited by phosphatidylserine containing liposomes. *Mucosal Immunol.* 9, 1303–1316. doi: 10.1038/mi.2015.137

Tan, S., Feng, X., Liu, Z., Wang, Q., Jiang, Q., Ye, X., et al. (2022). The pro-inflammatory effect of triglyceride on human CD4+ T cells and experimental autoimmune uveitis. *Clin. Immunol.* 240:109056. doi: 10.1016/j.clim.2022.109056

Thorsen, J., Rasmussen, M. A., Waage, J., Mortensen, M., Brejnrod, A., Bønnelykke, K., et al. (2019). Infant airway microbiota and topical immune perturbations in the origins of childhood asthma. *Nat. Commun.* 10:5001. doi: 10.1038/s41467-019-12989-7

Vael, C., Nelen, V., Verhulst, S. L., Goossens, H., and Desager, K. N. (2008). Early intestinal *Bacteroides fragilis* colonisation and development of asthma. *BMC Pulm. Med.* 8:19. doi: 10.1186/1471-2466-8-19

Wang, X., Hui, Y., Zhao, L., Hao, Y., Guo, H., and Ren, F. (2017). Oral administration of *Lactobacillus paracasei* L9 attenuates PM2.5-induced enhancement of airway hyperresponsiveness and allergic airway response in murine model of asthma. *PLoS One* 12:e0171721. doi: 10.1371/journal.pone.0171721

Wilson, N. G., Hernandez-Leyva, A., Rosen, A. L., Jaeger, N., McDonough, R. T., Santiago-Borges, J., et al. (2023). The gut microbiota of people with asthma influences lung inflammation in gnotobiotic mice. *iScience* 26:105991. doi: 10.1016/j.isci.2023.105991

Wu, Y., Cheng, X., Jiang, G., Tang, H., Ming, S., Tang, L., et al. (2021). Altered oral and gut microbiota and its association with SARS-CoV-2 viral load in COVID-19 patients during hospitalization. *NPJ Biofilms Microbiomes* 7:61. doi: 10.1038/s41522-021-00232-5

Yuan, X., Li, X., Kang, Y., Pang, L., Hei, G., Zhang, X., et al. (2022). Gut mycobiota dysbiosis in drug-naïve, first-episode schizophrenia. *Schizophr. Res.* 250, 76–86. doi: 10.1016/j.schres.2022.10.011

Zhang, D., Li, S., Wang, N., Tan, H.-Y., Zhang, Z., and Feng, Y. (2020). The cross-talk between gut microbiota and lungs in common lung diseases. *Front. Microbiol.* 11:301. doi: 10.3389/fmicb.2020.00301

Zhang, R., Lu, S., Yang, X., Li, M., Jia, H., Liao, J., et al. (2021). miR-19a-3p downregulates tissue factor and functions as a potential therapeutic target for sepsis-induced disseminated intravascular coagulation. *Biochem. Pharmacol.* 192:114671. doi: 10.1016/j.bcp.2021.114671

Zhao, Y., and Natarajan, V. (2013). Lysophosphatidic acid (LPA) and its receptors: role in airway inflammation and remodeling. *Biochim. Biophys. Acta* 1831, 86–92. doi: 10.1016/j.bbalip.2012.06.014

Zheng, P., Zhang, K., Lv, X., Liu, C., Wang, Q., and Bai, X. (2022). Gut microbiome and metabolomics profiles of allergic and non-allergic childhood asthma. *J Asthma Allergy.* 15, 419–435. doi: 10.2147/JAA.S354870

Zhou, X., Paulsson, G., Stemme, S., and Hansson, G. K. (1998). Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J. Clin. Invest.* 101, 1717–1725. doi: 10.1172/JCI1216

Glossary

HMP	Human microbiome program
SCFAs	Short-chain fatty acids
BMI	Body mass index
PBMCs	Peripheral blood mononuclear cells
OTUs	Operational taxonomic units
VIP	Variable importance in the projection
RSD	Relative standard deviation
PCoA	Principal co-ordinates analysis
QC	Quality control
PCA	Principal component analysis
LDA	Linear discriminant analysis
LEfSe	LDA effect size
HBE	Human bronchial epithelial
RDA	Redundancy analysis
Der f 2	Dermatophagoides farinae 2
FeNO	Fractional exhaled nitric oxide
CE	Cholesterylesters
Cer	Ceramide
TG	Triglyceride
LPC	Lysophosphatidylcholine
LPG	Lysophosphatidylglycerol
PA	Phosphatidic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SDA	Stearidonic acid
EPA	Eicosapentaenoic acid
DG	Diglyceride
MG	Monoglyceride
TC	Total cholesterol
FA	Fatty Acyl
GL	Glycerolipids
GP	Glycerophospholipids
SP	Sphingolipids

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Longitudinal microbiome investigation throughout prion disease course reveals pre- and symptomatic compositional perturbations linked to short-chain fatty acid metabolism and cognitive impairment in mice

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Commensal intestinal bacteria shape our microbiome and have decisive roles in preserving host metabolic and immune homeostasis. They conspicuously impact disease development and progression, including amyloid-beta (Aβ) and alpha (α)-synuclein pathology in neurodegenerative diseases, conveying the importance of the brain–gut–microbiome axis in such conditions. However, little is known about the longitudinal microbiome landscape and its potential clinical implications in other protein misfolding disorders, such as prion disease. We investigated the microbiome architecture throughout prion disease course in mice. Fecal specimens were assessed by 16S ribosomal RNA sequencing. We report a temporal microbiome signature in prion disease and uncovered alterations in Lachnospiraceae, Ruminococcaceae, Desulfovibrionaceae, and Muribaculaceae family members in this disease. Moreover, we determined the enrichment of Bilophila, a microorganism connected to cognitive impairment, long before the clinical manifestation of disease symptoms. Based on temporal microbial abundances, several associated metabolic pathways and resulting metabolites, including short-chain fatty acids, were linked to the disease. We propose that neuroinflammatory processes relate to perturbations of the intestinal microbiome and metabolic state by an interorgan brain–gut crosstalk. Furthermore, we describe biomarkers possibly suitable for early disease diagnostics and anti-prion therapy monitoring. While our study is confined to prion disease, our discoveries might be of equivalent relevance in other proteinopathies and central nervous system pathologies.

KEYWORDS

gut–brain–microbiome axis, microbiome, fecal 16S rRNA seq, metabolism, short-chain fatty acids, prion disease, neuroinflammation, intracerebral pathologies

Introduction

Microorganisms, especially host-resident bacteria that colonize mammalian intestines in large quantities, are collectively termed as gut microbiome. They are paramount players of the gut–brain–microbiome axis (Pickard et al., 2017). Their identified functions have vastly increased in the last decade and range from the metabolism of essential nutrients to regulation of cellular development and prevention of host dysbiosis and pathogen invasion (Bradford et al., 2017). Many reports describe that physiological processes of the central nervous system (CNS) rely on gut microbiota. There is a complex bidirectional and interorgan crosstalk between the brain and the intestine by a variety of different systems including endocrine, immune, autonomic, enteric nervous systems and microbiota-derived micro- and macromolecules (Lach et al., 2018; Lai et al., 2021). Microbiota exert their effects through short-chain fatty acids (SCFAs), branched-chain amino acids, peptidoglycans and peptides, indoxyl sulfate, trimethylamine-*N*-oxide, tryptophan metabolism, blood–brain barrier permeability modification, neurotransmitters modulation, accurate microglia function control, and vagus nerve excitation (Braniste et al., 2014; Erny et al., 2015; Strandwitz, 2018; Cryan et al., 2019; Colombo et al., 2021; Lai et al., 2021). Microbiota-produced SCFAs are believed to influence microglia activity via meningeal immune cells (Rothhammer et al., 2016; Martin-Pena and Tansey, 2023; Seo et al., 2023). In addition to peripheral immune homeostasis, the presence of a complex microbiome and SCFA receptors is crucial for proper function and maturation of microglia (Erny et al., 2015) and regulation of neuronal circuits (Yu and Hsiao, 2021). There is increasing evidence that the microbiome and dysbiosis largely influence several conditions such as autism, anxiety, schizophrenia, brain tumor pathogenesis (i.e., tumor growth), multiple sclerosis (MS), as well as protein misfolding disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD), and prion disease (PrD) via the gut–brain–microbiome axis (Minter et al., 2016; D'Argenio and Sarnataro, 2019; Mehrian-Shai et al., 2019; Montalban-Arques et al., 2021; Montella et al., 2021; Claudino Dos Santos et al., 2023; Heston et al., 2023; Mahbub et al., 2024). Furthermore, targeting of microbiota-derived SCFAs has been proven to effectively modulate and potentiate the immunotherapeutic response in colorectal cancer therapy *in vivo* (Montalban-Arques et al., 2021). Although there is a consensus that gut microbiome composition can be altered in patients with AD, PD, or ALS (Martin-Pena and Tansey, 2023), microbiome perturbations and the neuro-immunological crosstalk along the gut–brain–microbiome axis in prion disease are underreported and still poorly understood.

Prion diseases (PrDs) are incurable protein misfolding disorders characterized by a long incubation period (years to decades) and a rapid disease progression upon symptom onset (Brown et al., 1986). These diseases are caused by a single cellular membrane protein, the prion protein (PrP^{C}), that undergoes a pathological transformation into its aggregation-prone form (PrP^{Sc}). PrP^{Sc} self-assembles and propagates into neurotoxic extracellular PrP aggregates accommodated by microglia and astrocyte activation and neuroinflammation (Aguzzi and Weissmann, 1998; Scheckel and Aguzzi, 2018; Scheckel et al., 2020). Toxic PrP aggregates seed into most brain regions, including the brain stem, affecting neuronal circuits as well as efferent and afferent neuronal signaling thereof (Mirabile et al., 2015; Carta and Aguzzi, 2022). However, it is unclear whether brain (stem) pathology could relate to microbiome architectural changes, and if so, whether this even happens before the onset of disease symptoms. Recent promising human and rodent data report prion-specific preclinical hippocampal transcriptional (Sorce et al., 2020) and peripheral muscle tissue metabolism (Caredio et al., 2023) changes. This indicates that the discovery and clinical employment of early PrD biomarkers are feasible. Moreover, it may be that such biomarkers are also applicable in other neurodegenerative diseases sharing similar pathomechanisms (Barnham et al., 2006).

The question we therefore addressed was whether prion disease and neuroinflammation are associated with alterations in the microbiome composition in a temporal manner. We therefore investigated the gut microbiome of prion-infected animals throughout the entire prion disease course by fecal 16S ribosomal RNA sequencing.

Here, we report a temporal microbiome signature of prion disease. We detected pre- as well as post-symptomatic microbiome perturbations that might lead to the development of novel, readily accessible biomarkers and alternative therapeutic approaches. Our data provide novel insights into the brain–gut–microbiome axis and its dynamics in prion disease. Our study might therefore have broader clinical implications in a variety of proteinopathies and intracerebral pathologies.

Results

Temporal shift in microbiome composition along the prion disease course

Microbiota have been shown to influence Aβ aggregation and α-synuclein pathology in AD and PD via microglia modulations, respectively (Erny et al., 2015; D'Argenio and Sarnataro, 2019; Colombo et al., 2021; Claudino Dos Santos et al., 2023). As microglia activation (Aguzzi and Zhu, 2017) and brain stem pathology (Mirabile et al., 2015; Reis et al., 2015) are hallmarks in prion disease and correspond to the clinical phenotype, we hypothesized that the microbiome signature changes during the prion disease course. To this end, we first investigated whether prion-infected animals differ from control mice regarding microbiome composition throughout the course of prion disease. To do so, we employed a well-studied murine model of prion disease and intracerebrally injected Rocky Mountain Laboratory 6 (RML6) prion strain and non-infectious brain homogenate (NBH) into wild-type male mice of similar age (Supplementary Table S1; Sorce et al., 2020; Frontzek et al., 2022). Each experimental group consisted of $n = 21$ animals (total $n = 42$ mice) that were initially co-housed in five cages per experimental group (Supplementary Table S2). Pooled fecal specimen derived from each cage containing up to five mice were collected with a weekly interval from baseline (prior to any injections) to terminal stage, denominated as weeks post-inoculation (wpi; see Figure 1A). Consecutively, we processed the fecal specimen from RML6 and NBH-treated mice from $n = 15$ time points and performed 16S V4 ribosomal RNA sequencing and phylogenetic reconstruction (Figure 1B) to characterize the microbiome landscape and alterations between the two conditions over time. After data quality check and denoising, amplicon sequence variants (ASVs) were generated upon

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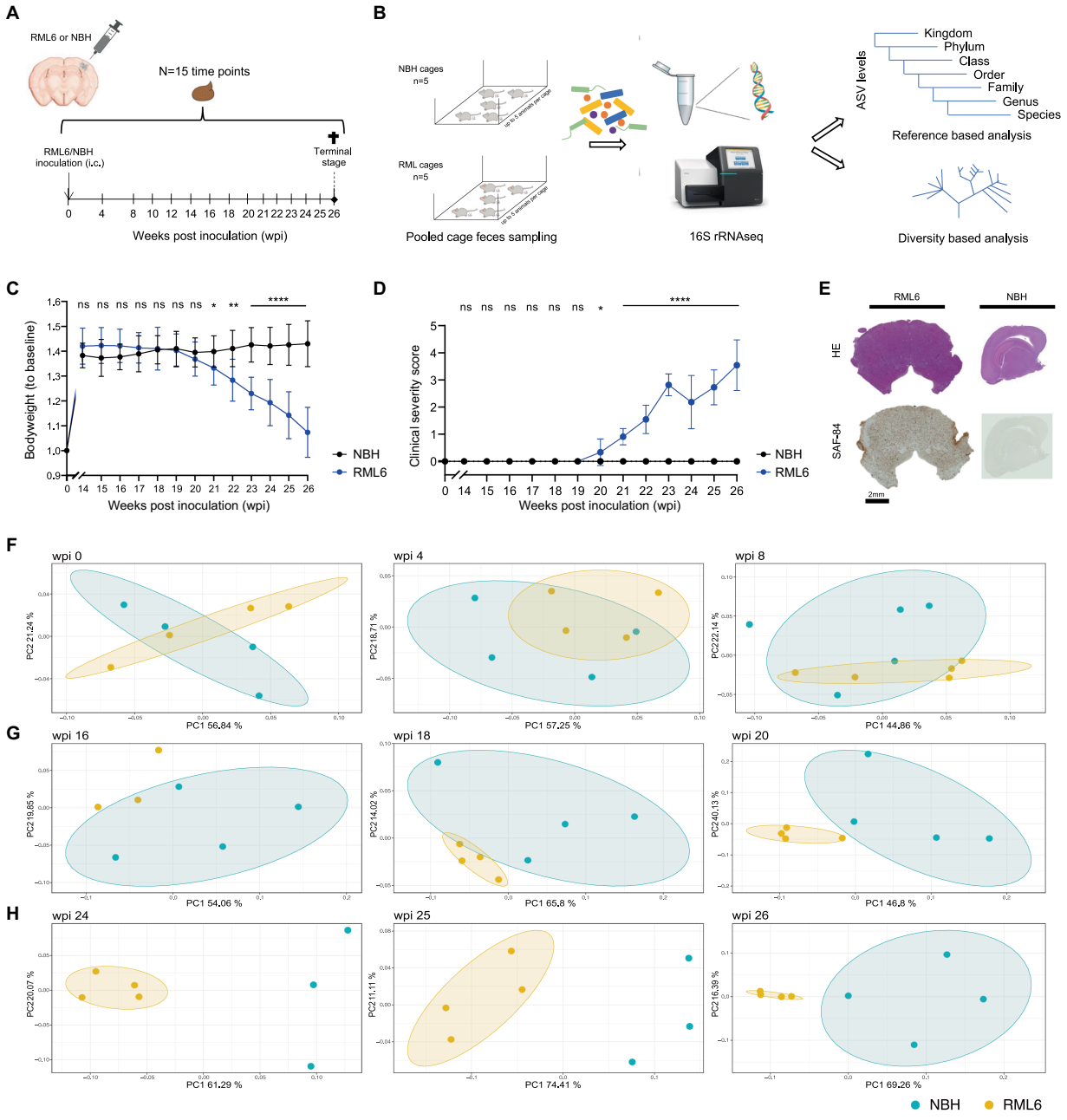


FIGURE 1
Identification of disease-specific microbiome alterations in prion disease. **(A)** Intracerebral inoculation and feces sampling scheme throughout the course of prion disease. **(B)** Overview of the experimental pipeline shows 16S rRNA sequencing and consecutive reference as well as diversity-based analysis. **(C)** Body weight development among the experimental animals. Of note: Prion-infected animals (RML6 inoculated) show bodyweight loss from 19 wpi. Two-tailed Student's *t*-test was performed for each time point. **(D)** Clinical severity score over time shows the clinical course of experimental animals. Of note: Clinical symptoms of experimental animals are congruent with the body weight loss displayed in **(C)**. Two-tailed Student's *t*-test was performed. **(E)** Representative HE-stained and SAF84 IHC brain sections (coronal for RML6 and sagittal for NBH) of experimental animals at the terminal stage show diffuse prion aggregation throughout the brain, including the brainstem (left panel). SAF84 positivity appears brown in IHC. No SAF84 positivity in NBH control mice. **(F–H)** Principal coordinate analysis on sequencing data showing weighted UniFrac beta distance matrix from three different time points **(F)** at the early stage (wpi 0, 4, 8), **(G)** at the middle symptomatic stage (wpi 16, 18, 20), and **(H)** at the end stage (wpi 24, 25, 26) of prion disease course. A difference between the experimental groups can be detected from wpi 16. A single data point represents pooled fecal sequencing data originating from one animal cage. By default, four cages per group were used. Of note, sequencing data sets from time points wpi16 (RML6), wpi24 (NBH), and wpi25 (NBH) did not pass quality control, resulting in the absence of respective vector illustrations. $*p < 0.05$; $**p < 0.01$; $****p < 0.0001$; ns: not significant.

paired read merging (Callahan et al., 2016). The sequencing data had sufficient depth to capture the most expected features and was consecutively analyzed using a diversity- as well as reference-based approach. Utilizing this murine model of prion disease leads to first clinical symptoms at approximately 20 weeks' post-inoculation (Sorce et al., 2020; Caredio et al., 2023). This was assessed by body weight loss and a clinical severity score (Figures 1C,D). To assess successful prion inoculation and propagation, we performed HE and SAF-84

immunohistochemistry on brain slices upon euthanasia and detected prion aggregates throughout the brain (Zhu et al., 2019; Frontzek et al., 2022; Figure 1E). We then went on and calculated both the microbiome diversity within (alpha diversity) and across (beta diversity) experimental groups. The former was calculated using the faith phylogenetic matrix to compute the richness and incorporate phylogenetic relations. The latter was calculated using the weighted UniFrac distance matrix to quantify the dissimilarity between communities (Diallo et al., 2007). We identified trends in the alpha diversity among microbiomes of experimental groups starting just before the onset of clinical symptoms (Supplementary Figure S1). By performing a principal coordinate analysis (PCoA) on all samples, divided into early, middle symptomatic, and end stages, we identified a progressive separation of the two experimental groups over time (Figures 1F–H and Supplementary Table S3). While in the early stage of disease no separation was observable (left to right, $p = 0.830$, 0.306, 0.298) (Figure 1F), the RML6 and NBH-treated groups gradually formed distinct clusters, starting from wpi 16–20 (left to right, $p = 0.215$, 0.033, 0.022) (Figure 1G), which was most pronounced toward the end stage of disease (left to right, $p = 0.029$, 0.029, 0.031) (Figure 1H). From these data, we conclude that the overall microbiome composition shifts in a temporal manner in mice inoculated with prions, starting around 12 weeks before symptom onset.

Compositional microbiome assessment identified enrichment of Lachnospiraceae and Ruminococcaceae in prion-diseased animals

The bacterial taxonomy based on genome phylogeny represents a hierarchical structure consisting of seven levels (Parks et al., 2018). Accordingly, we analyzed the data to investigate the abundance of different microbiota in depth. First, we plotted all the detected families over time and condition in a heat map (Figure 2A). Second, we extended the analyses to all detected genera throughout the experimental groups (Figure 2B), allowing to potentially identify a prion disease-specific microbiome signature that could be exploited to develop early preclinical disease biomarkers. Overall, we detected $n = 45$ family and $n = 106$ corresponding genera members among the analyzed samples. Neurodegenerative diseases result in a cognitive decline, are often associated with movement disorders, decreased activity, and altered metabolic serum profiles eventually associated with microbiome structural changes (Erkkinen et al., 2018; Wang et al., 2021). Apart from one report describing that mice and humans carrying the AD high-risk Apolipoprotein E4 allele show an enrichment in Ruminococcaceae (Tran et al., 2019), little is known about the role of Lachnospiraceae and Ruminococcaceae in neurodegenerative diseases. When focusing on Lachnospiraceae and Ruminococcaceae, we detected a significant increase in the abundance of these taxa at the time point of symptom onset at week 20 in prion-diseased mice as compared to control mice (Figure 3A, upper and middle panels). These differences persisted, even with a slight upward trend, until the terminal stage of the disease. From this analysis, we conclude that our data are in line with the findings reported by others and that neurodegenerative proteinopathies relate to changes in microbiome structure throughout the course of prion disease as compared to control animals.

Diminished Muribaculum signature after prion disease symptom onset

Muribaculum has been described to play a protective role in the development of cognitive impairment in APP/PS1 transgenic mice, a murine model of AD. For instance, mice that had high microbial diversity food showed an improvement in spatial learning, objective recognition, memory as well as hippocampal Aβ plaque pathology as compared to APP/PS1 transgenic animals that were fed with aseptic food over the same period. Fecal 16S ribosomal RNA sequencing and analysis of these transgenic experimental mice revealed that cognitive function correlated positively with the presence of Muribaculum (Liu et al., 2020). Another group studied hippocampal gene expression in association with changes in microbial species in rats that were fed with a high-fat and high-sugar diet as such a diet leads to cognitive impairment over time (Leigh et al., 2020). A multiple regression analysis identified Muribaculum as a significant predictor of performance in the novel place recognition task. Therefore, we assessed microbiota and their relative abundance that have been reported to be positively and negatively associated with cognitive impairment, with a special focus on microbiome strains such as Muribaculum. At baseline, there was no difference between the experimental groups. After prion inoculations, the relative abundance of Muribaculum decreased over time. At week 14, upon prion infection (the time point when pre-symptomatic hippocampal gene expression changes occur), Muribaculum abundance differed significantly between control and prion-diseased mice. At week 18–20 post-prion inoculation, when disease symptoms manifest, the abundance of Muribaculum dropped markedly. Moreover, the abundance of Muribaculum slightly increased over time in NBH-injected control animals (Figure 3A, lower panel). These findings suggest that (intracerebral) prion pathology is associated with microbiome alterations and compositional shifts that favor and/or reflect cognitive impairment of prion disease in an anterograde manner.

Early preclinical intestinal Bilophila expansion in prion disease

As previously described, first disease symptoms typically occur at approximately week 20 post-prion inoculation (Sorce et al., 2020; Caredio et al., 2023). As it has been shown that intracerebral transcriptional and muscle metabolism changes already occur before the onset of disease symptoms (Sorce et al., 2020; Caredio et al., 2023), we examined whether there are microbiota that are either depleted or enriched in prion disease prior to and after the onset of disease symptoms. Relative to NBH mice, RML6-injected prion mice had a significant enrichment in relative abundance for Lachnospiraceae and Ruminococccceae. Lachnospiraceae were enriched after 14 wpi in the RML6-inoculated group, whereas Ruminococcaceae were enriched in the terminal stage of disease. On the other hand, Muribaculaceae were depleted in RML6 as compared to NBH control mice (Figure 3A, lower panels). By looking at the genera level, we consecutively identified several microbiotas that were either significantly enriched or depleted in RML6-infected mice compared to NBH-injected mice. After the onset of first clinical symptoms, Xylanophilum and Oscillibacter were significantly enriched in RML6 mice compared to NBH mice, whereas the opposite was true for Muribaculum (Figure 3A, upper and middle panels). Already at 4 wpi, and thus 16 weeks prior to any disease symptoms, Bilophila were significantly

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FIGURE 2
Detected families in experimental groups across selected disease time points. **(A)** The heat map displays the detected microbiome families and their abundance among experimental groups from baseline to terminal stage of the disease. Every time point contains the analysis of 10 fecal specimen. **(B)** The heat map displays the detected microbiome genera and their abundance among experimental groups across selected time points from baseline to terminal stage of the disease. Every time point contains the analysis of 10 pooled fecal cage specimen.

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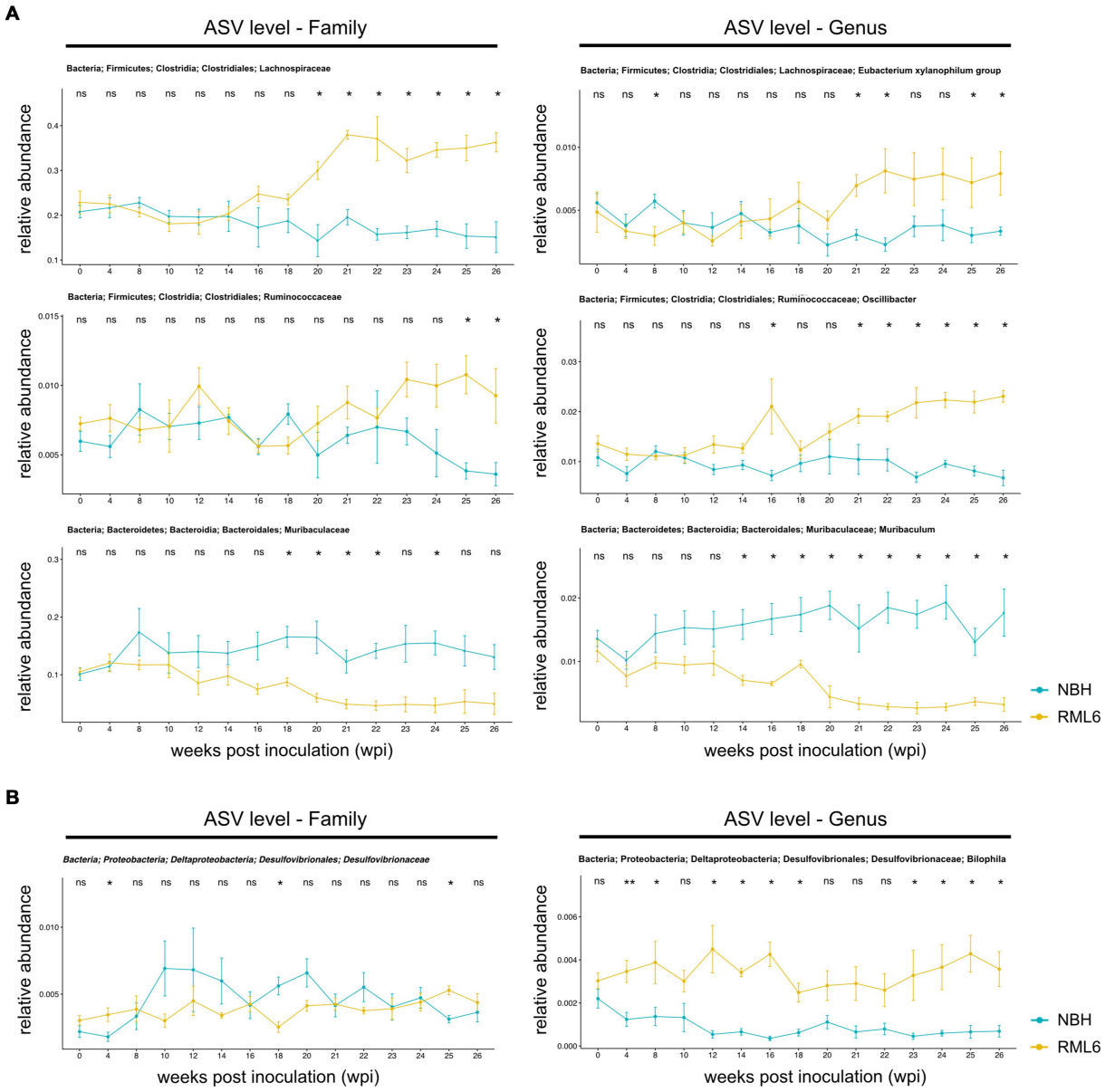


FIGURE 3
Relative abundance of significant gut microbes in prion disease. **(A)** Relative abundances of representative family (left panels) and associated genera (right panels) members. Within family, there are significant differences in clostridiales and bacteroidales between the control group (NBH) and prion (RML6)-infected animals. On the genera level, there are significant differences in abundance of Lachnospiraceae, Ruminococcaceae, and Muribaculaceae between control (NBH) and prion (RML6)-injected animals. Two-tailed Student's *t*-test was performed. **(B)** Relative abundances of Desulfovibrionaceae family (left panel) and Bilophila genera (right panel). Bilophila show relative pre-symptomatic expansion starting 4 weeks post-prion inoculation as compared to NBH-inoculated mice. Pairwise comparison of each group at each time point and Holm–Bonferroni corrected for multiple comparisons. *$p < 0.05$; **$p < 0.01$; ns: not significant.

enriched in prion-infected animals (Figure 3B). Throughout the prion disease course, Bilophila were largely absent and not detectable in control mice, suggesting an association of this genera with neurodegeneration and prion disease pathogenesis. From this, we conclude that certain bacteria are enriched prior to disease symptoms, whereas others are enriched toward the terminal stage of disease. Our findings suggest that microbiome alterations occur even earlier in the disease pathogenesis than the previously described transcriptional intrahippocampal changes in prion-diseased animals (Sorce et al., 2020).

Functional metabolic pathway prediction advocates significant roles for lipid metabolism and short-chain fatty acids in prion disease

Gut bacteria can affect the lipid metabolism of the host (Jian et al., 2022; Brown et al., 2023). Short-chain fatty acids (SCFAs) and their main members, acetate, propionate, and butyrate, are synthesized via the Embden–Meyerhof–Parnas (glycolysis, for six-carbon sugars) and the pentose phosphate pathway (oxidative and non-oxidative branches for five-carbon sugars). They convert monosaccharides into phosphoenolpyruvate (PEP), followed by fermentation into these organic

acids (Miller and Wolin, 1996; den Besten et al., 2013; Silva et al., 2020). SCFAs serve as energy sources, trophic intestinal factors, and regulators of regulatory T cells and exert crucial physiological effects on various organs, including the brain (Pascale et al., 2018; Dalile et al., 2019). Butyrate-producing bacteria include members of the families Ruminococcaceae and Lachnospiraceae (Louis and Flint, 2009; Louis et al., 2010; Louis and Flint, 2017; Parada Venegas et al., 2019). Both families were significantly more abundant in prion-infected animals (Figure 3). Moreover, it has been reported that butyrate promotes the fitness of bacteroidales, the most abundant human microbiome genus (Park et al., 2022). Therefore, we performed a functional metabolic prediction analysis based on relative bacterial abundances and 16S sequences to delineate pathways potentially relevant for prion disease pathogenesis. We first checked the main metabolic expressions being associated with genera and detected the absence of "fatty acid salvage" among bacteroides in RML6 in comparison with control mice, pointing to the importance of fatty acids in prion disease pathology (Figure 4). We then investigated the main pathways that yield SCFA precursors, namely pentose phosphate pathway, glycolysis, and fatty acid beta oxidation, as described above. All pathways were predicted to be enhanced in prion-diseased mice beyond 14 weeks of infection (Figure 5A). This prompted us to explicitly query for SCFA pathway expressions such as PEP and pyruvate (both derive from the above-mentioned pathways), which undergo fermentation into SCFAs and their precursors. Intriguingly, starting from week 14 upon prion infection, butanoate (butyrate), propanoate (propionate), acetate, lactate, and acetone fermentation (from pyruvate, L-lysine, and acetyl-CoA) were predicted to be upregulated in prion disease (Figure 5B). Moreover, methanogenesis (from acetate), coenzyme A biosynthesis, and acetyl coenzyme A pathways associated with SCFAs are predicted to be enriched in prion disease (Figure 5C). From these data, we learn that, in accordance with the associated microbiome landscape, the lipid metabolism and SCFA metabolic pathways are seemingly enhanced during prion disease course.

Discussion

Even after decades of research, a cure for neurodegenerative disorders is still missing (Prusiner, 1998; Checkoway et al., 2011; Erkkinen et al., 2018). Here, we aimed to pursue an alternative approach and assessed the microbiome in prion disease. A better understanding of the role of the intestinal microbiome in the pathogenesis of CNS diseases may allow the identification of biomarkers, novel drug targets, and important pathways. In this context, targeting the microbiome appears promising as fecal microbiota transplantation (FMT) has successfully been applied to restore microbiome dysbiosis and alleviate Alzheimer's disease-like pathogenesis *in vivo* (Sun et al., 2019).

To enhance the understanding of the interorgan crosstalk along the brain–gut–microbiome axis and its role in extra-intestinal CNS diseases such as prion disease, we investigated and characterized the temporal microbiome signature *in vivo*. It is now well recognized that a microbiome–brain crosstalk is essential in health and disease (Cryan et al., 2019; Nagpal and Cryan, 2021). The microbiome and its associated metabolites are not only crucial for peripheral immune homeostasis but are also paramount for immune cells, neuronal circuits, and brain function (Strandwitz, 2018; Lai et al., 2021; Yu and Hsiao, 2021). Moreover, it is recognized that microbiome alterations are associated with CNS and extra-axial pathologies such as autoimmune, neuroinflammatory, neuropsychiatric, and neurodegenerative diseases (Scher et al., 2013; Tremlett et al., 2016; Lach et al., 2018; Nagpal and Cryan, 2021). To determine the effects of altered cerebral homeostasis on microbiome composition, we analyzed fecal specimen on a weekly basis, assessed body weight changes, and performed a clinical severity scoring system throughout the disease course.

The brainstem harbors several critical neuronal structures, including motor and autonomic centers, the locus coeruleus, and the nucleus of the solitary tract, from where vagus nerve fibers originate. These structures were shown to be delicate areas in prion disease pathology (Mirabile et al., 2015). We, therefore, first confirmed the presence of prion aggregates throughout the brain, including the cortex and brainstem, upon euthanasia. Presumably, microglia serve as relays in the brain–gut axis as they carry out broad responsibilities in balancing and restoring host homeostasis, influencing synaptic remodeling, and communicating with afferent and efferent peripheral neurons (Abdel-Haq et al., 2019; Defaye et al., 2022). It is therefore conceivable that prion aggregate-induced microglia activation (Aguzzi and Zhu, 2017; Aguzzi, 2022) mediates an altered bidirectional neuronal (i.e., vagus nerve-mediated) crosstalk between anatomical regions of the brain(stem) and the intestine (Savchenko et al., 2000; Badoer, 2010; Hickman et al., 2018).

To determine whether microbiome perturbations occur in intracerebrally inoculated prion-diseased animals, we assessed microbiota diversity using alpha and beta diversity based on their relative abundance and observed diversity changes. Especially beta diversity was significantly changed in relation to disease state. It is likely that the large standard deviations are the main contributor to the overall non-significant change in alpha diversity. We then checked for differences in relative abundance among various taxonomy levels and found significant differences in preclinical, symptomatic, and terminal disease stages. In prion-diseased animals, we detected a significant enrichment in Lachnospiraceae, Desulfovibrionaceae, and Ruminococcaceae and a depletion of Muricaculaceae. Intriguingly, data from patients suffering from MS, an autoimmune neuroinflammation disorder, show comparable alterations among these taxa members (Tremlett et al., 2016). As described above, data suggest that Muribaculum correlates positively with cognitive function (Leigh et al., 2020; Liu et al., 2020). The identification of Muribaculum reduction in prion-infected animals at week 14 of disease course might indeed reflect a temporal association between hippocampal gene expression changes and microbiota colonization. The discovery of Bilophila enrichment 4 weeks after prion inoculation and thus long before the manifestation of any clinical symptoms was unexpected. It has been described that preclinical hippocampal transcriptional and muscle metabolism changes occur 8 weeks post-prion infection and therefore well before the onset of any clinical symptoms, which normally occur 20 weeks after prion inoculation (Sorce et al., 2020; Caredio et al., 2023). Our finding that Bilophila abundance changes 4 weeks post-prion inoculation suggests that peripheral and metabolic disease-associated alterations, including intestinal microbiome changes, occur even earlier than previously appreciated. To this end, we investigated the metabolic profile, calculated based on the apparent murine microbiome constituents with a focus on fatty acid metabolism, and identified enhanced phosphate pentose pathway, glycolysis, and beta oxidation metabolic processes in prion disease. In general, upregulation of these pathways

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FIGURE 4
Functional metabolic prediction analysis in prion disease. Left column of both experimental conditions (NBH and RML6) represents the genus, and right column represents the metabolic pathway. Link between the left and right columns represents correlated terms.

results in elevated levels of PEP and pyruvate molecules that can undergo fermentation into SCFAs (Fernie et al., 2004; den Besten et al., 2013). It might therefore be conceivable that upregulated bacterial metabolic pathways mirror additional energy needs in the setting of neuroinflammation or that upregulation of SCFA synthesis reflects an effort to maintain immune cell capacity to dampen pathogenic and inflammatory processes, with the aim to restore central immune homeostasis by influencing microglia activity via

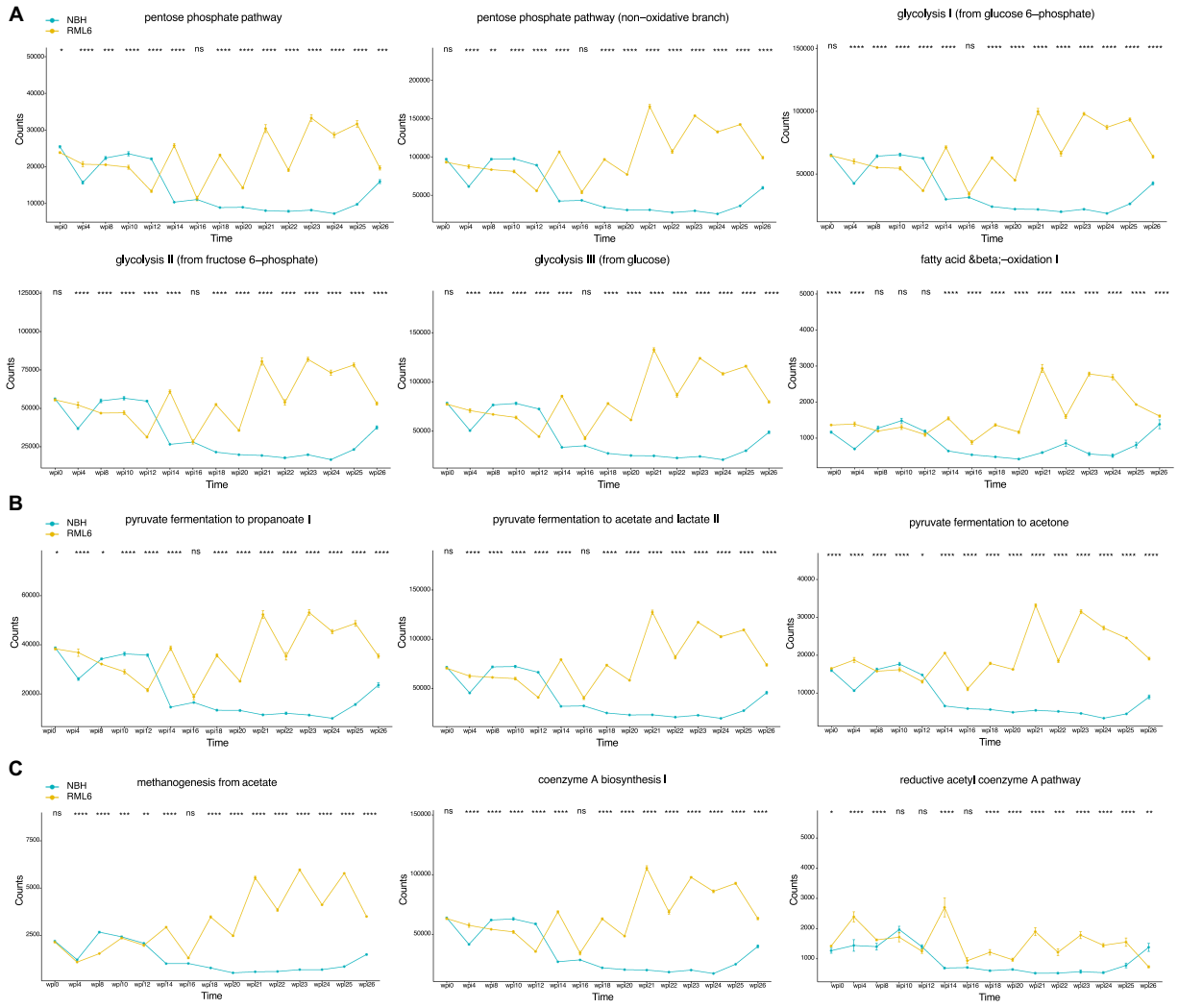


FIGURE 5
Temporal deconvolution of associated metabolic pathways based on relative microbiota abundance. **(A)** Pentose phosphate pathway, glycolysis, and fatty acid beta oxidation. **(B)** SCFAs, namely, acetate, propionate, and butyrate, are metabolized mainly from pyruvate, L-lysine, and acetyl-CoA fermentation. **(C)** Enrichment in methanogenesis (from acetate), coenzyme A biosynthesis, and acetyl coenzyme A pathway underlines the importance of SCFAs in prion disease. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

meningeal immune cell interaction (Rothhammer et al., 2016; Martin-Pena and Tansey, 2023; Seo et al., 2023). Butyrate-producing bacteria include Ruminococcaceae and Lachnospiraceae members (Louis and Flint, 2009; Louis et al., 2010; Louis and Flint, 2017; Parada Venegas et al., 2019), whereas it has been shown that the presence of Bilophila (*wadsworthia*) influences SCFA synthesis toward suppressed fecal SCFA concentrations (Natividad et al., 2018). Moreover, Bilophila monocolonization increases susceptibility to cognitive impairment by disrupting hippocampal synaptic plasticity and neurogenesis in mice (Olson et al., 2021). Therefore, our data suggest that the relative increase in Lachnospiraceae and Ruminococcaceae, but not Bilophila, might indeed be responsible for enhanced SCFA production in prion disease. As Lachnospiraceae have also been linked to other neurodegenerative diseases, including PD, it is possible that this family contributes to a variety of protein misfolding diseases in a more unspecific manner (Vascellari et al., 2020). Although it has recently been reported that prion disease pathogenesis was not worsened in germ-free mice (Bradford et al., 2017), we report significant dynamic microbiome compositional and metabolic perturbations in prion-diseased animals, supporting the relevance of a brain–gut–microbiome mechanism of unclear significance. The fact that antimicrobial agents such as doxycycline have been tested successfully in preclinical and clinical settings in prion disease therapy and prevention further supports the relevance and involvement of the microbiome in prion pathology (Stewart et al., 2008; Forloni et al., 2019). Mechanistically, it may be that prion-induced and microglia-derived molecules as well as altered neurotransmitter signaling result in a less stringent immune-controlled gut microbial environment, possibly mediated by an aberrant vagus nerve or circulatory signaling, in line with the fact that central neuronal loss and gliosis disturb the brain–gut axis (Abdel-Haq et al., 2019; Cryan et al., 2019). Another route of action might be that brainstem-mediated imbalances in vagus nerve function influence intestinal glial cells since this cell population has been shown to govern intestinal motility at the level of the myenteric plexus (Rao et al., 2017). Finally, it is also possible that altered

movement and eating behaviors, both occurring at different stages of neurodegenerative diseases (Guenther et al., 2001; Fostinelli et al., 2020), result in metabolic changes that favor perturbations in the microbiome.

Conclusion

We observed perturbations in the gut microbiome of mice throughout prion disease course in a temporal manner. These alterations were characterized by taxonomic depletions and enrichments in a limited number of microbial taxa rather than colossal compositional shifts as changes were only significantly evident at beta diversity level. This study suggests that neuroinflammatory processes are associated with an impaired brain–gut–microbiome axis and that microbiome and metabolic changes occur prior to clinical symptoms. Finally, we speculate that our findings might have diagnostic and therapeutic implications as microbiota and their metabolites could serve as early disease and therapy monitoring biomarkers or as targets for disease-modifying therapies that aim to ameliorate inflammation and disease progression in humans. It is conceivable that targeting the microbiome and related metabolites not only has implications on immunotherapy responses in malignancies but also in other conditions along the gut–brain axis.

Limitations and potential future directions

Despite the description of a dynamic temporal microbiome signature and underlying altered metabolic pathways in prion disease, additional work is required to resolve precise molecular mechanisms involved. Although the findings of this study hold promise for treating and anticipating development of prion disease, discoveries need to be corroborated and studied in human patients suffering from prion disease at present, and optimally also in individuals with a positive prion disease family history to assess translational perspectives. In addition to our intracerebral inoculation prion model that involves Brain stem pathology, future studies that may focus on the myenteric plexus–microbiome crosstalk may also consider harnessing the intraperitoneal route of prion infection. Whether the findings reported here indicate a specific microbiome signature of prion disease or of broader neuroinflammatory diseases and processes remains to be determined.

Materials and methods

Mice

Animal care and experimental protocols were in accordance with the Swiss Animal Protection Law. Mice were bred in a high hygienic-grade facility of the University Hospital of Zurich (BZL), housed in groups of 2–5, and monitored for pathogens such as bacteria, parasites, and viruses as described in the Federation of European Laboratory Animal Associations (FELASA). Individual housing was avoided in the best possible way. The light/dark cycle of mice consisted of 12/12 h with artificial light from 7 a.m. to 7 p.m. at 21 ± 1°C. Animals were fed with Kliba-Nafag (3242.PX.S12, vitamin-fortified, irradiated). C57BL/6/J male wild-type mice were obtained from Charles River (Germany). We performed the experiments with the highest possible age congruence among the experimental (RML and NBH) groups. Mice were randomly assigned to the experiments. Mice from these experimental cohorts were also used for a ribosomal profiling study in prion disease (Scheckel et al., 2020). Therefore, the decrease in animal numbers over time was intentional.

Prion inoculation, disease symptoms, and severity assessment

Six- to 10-week-old male mice were intracerebrally (right hemisphere between eye and ear) injected with 30 µL of RML6 prions (passage 6 of Rocky Mountain Laboratory strain mouse-adapted scrapie prions) containing 8.02 log LD_{50} of infectious units per ml. Control mice were inoculated with 30 µL of non-infectious brain homogenate (NBH) at the same dilution. Upon inoculation, mice were monitored three times per week up to the first clinical symptoms and scored with a clinical severity score. After the clinical onset, mice were monitored daily. Mice were sacrificed at respective time points corresponding to an age of 13–26 weeks. The scoring system consisted of two categories: (A) Grooming and (B) Activity/Ataxia/Posture from which each category (A and B) has a possible score of 0–5 points. The clinical severity score results from the sum of categories A and B. Category A—0: shiny, well-kept hair coat; 1: signs of reduced grooming; 2: dull coat; 3: ungroomed coat; 4: piloerection; 5: discharge from eyes. Category B—0: normal moving and no sign of kyphosis; 1: occasionally limp while walking and reduced nest-building activity; 2: limping and mild kyphosis but able to straighten its spine; 3: rarely tumbles, able to position itself correctly, shows mild but persistent kyphosis and inability to straighten its spine completely; 4: tumbling, able to position itself correctly, persistent kyphosis and leg stiffness while walking; 5: severe activity reduction. Mice are directly euthanized upon clinical symptoms of the terminal disease stage, such as persistent kyphosis, severe ataxia, hind-limb clasping, and piloerection.

Fecal specimen collection

Once a week, stool specimens were harvested from all experimental and control cages at the same time point. Upon collection, specimens were snap-frozen in liquid nitrogen and stored at −80°C until sequencing preparations. Mice were euthanized using carbon dioxide and decapitation upon anesthesia. For histology and immunohistochemistry, brains were immediately dissected and fixed in 4% formalin at 4°C overnight, followed by a prion decontamination step (1-h formic acid treatment at RT followed by a 2-h formalin post-fixation step) before paraffin embedding. Fecal specimens were sent for sequencing according to the guidelines of Microsynth (Switzerland).

16S ribosomal RNA sequencing and amplicon sequence variant analysis

Illumina Nextera barcoded, two-step PCR libraries (16S V4) were used to build up the library, and Illumina MiSeq, v2, 2x250bp was used for sequencing. We used the QIIME2 pipeline for the analysis of

16S rRNA (Bolyen et al., 2019). After checking data quality, denoising with DADA2 was performed to merge the paired reads, generating amplicon sequence variants (ASVs) (Callahan et al., 2016). Alpha rarefaction module was used to ensure that we had sufficient depth to capture most features. We then calculated both alpha and beta diversity using the core-metrics-phylogenetic module. For alpha analysis, we used the faith phylogenetic matrix to compute the richness and incorporate features of phylogenetic relations. In beta diversity, we used the weighted UniFrac distance matrix to quantify the dissimilarity between communities (Diallo et al., 2007). Principal coordinate analysis (PCoA) was used for better visualization of beta diversity. Taxonomy was assigned to ASVs using the q2-feature-classifier classify-sklearn naïve Bayes taxonomy classifier against the pre-trained Naïve Bayes silva-132-99-nb-classifier trained against Silva (release 132) full-length sequences (Bokulich et al., 2018). Taxa barplot module was used for the visualization of the different taxonomy composition.

Functional metabolic pathway prediction

To predict metagenome functions and pathway abundance based on 16S amplicon sequences, we used the PICRUSt2 algorithm (Douglas et al., 2020). In short, genus and pathway abundance were prepared upon sample aggregation (NBH and RML6) across different time points (wpi 0–26). Correlation was run between genus and pathways within each condition, followed by filtering the significant correlation with a value less than 0.001 and a correlation value more or less than 0.7. Since this might lead to multiple correlations (one genus could correlate with more than one pathway), Sankey plots were plotted for each experimental condition separately for data visualization and interpretation. Assessment of significant metabolic processes between experimental conditions (NBH vs. RML6) and their temporal dimension was performed.

Histology and immunohistochemistry

Formalin-fixed, formic acid-treated, and paraffin-embedded brain sections (2–4 µm) were deparaffinized with 3 cycles of xylene treatment followed by re-hydration with 100% EtOH, 96% EtOH, 70% EtOH, and water (each treatment 5 min), respectively. Hematoxylin and eosin (HE) stain and immunohistochemistry (IHC) for SAF84 were performed according to standard procedures at our institute. In short, deparaffinized sections were treated with formaldehyde for 30 min followed by 98% formic acid treatment for 6 min and then washed with distilled water for 30 min. After incubation with protease 1 (Ventana) for 16 min, sections were incubated with polyclonal anti-PrP antibody (SAF84, SPI Bio) at a dilution of 1:200. Sections were counterstained with hematoxylin. An Olympus BX61 system was used to acquire images.

Statistical analysis

A two-way analysis of variance (ANOVA) was conducted to compare the relative abundance of taxa between two groups (NBH and RML6) at multiple time points. The pairwise comparison of each group at each time point was also performed and corrected for multiple comparisons using the Holm–Bonferroni method. A p-value of less than 0.05 was considered significant. Additionally, a pairwise PERMANOVA of beta diversity was performed to assess differences in overall community composition between the two groups at each time point. The PERMANOVA was performed using the "adonis" function in the "vegan" package in R. All statistical analyses were performed using the R statistical software. The two-way ANOVA was used to determine the significance of the effect of both the treatment (NBH vs. RML6) and time point on the relative abundance of taxa, while the pairwise comparisons were used to identify which specific time points showed significant differences between the two groups. The PERMANOVA was used to assess the significance of differences in overall community composition between the two groups at each time point. The Holm-Bonferroni correction was applied to control the inflation of type I errors due to multiple comparisons.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: BioProject, accession number: PRJNA1114866.

Ethics statement

The animal study was approved by animal care and experimental protocols were in accordance with the Swiss Animal Protection Law and approved by the Veterinary office of the Canton of Zurich, Switzerland (permits ZH040-15, ZH243-18). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ML: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. YM: Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – review & editing. ME: Writing – review & editing. SM: Writing – review & editing. PS: Project administration, Resources, Writing – review & editing. AA: Funding acquisition, Resources, Writing – review & editing. MS: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1412765/full#supplementary-material

References

Abdel-Haq, R., Schlachetzki, J. C. M., Glass, C. K., and Mazmanian, S. K. (2019). Microbiome-microglia connections via the gut-brain axis. *J. Exp. Med.* 216, 41–59. doi: 10.1084/jem.20180794

Aguzzi, A. (2022). The protean roles of neuroinflammation in trauma, infections, autoimmunity and neurodegeneration. *Semin. Immunol.* 61-64:101655. doi: 10.1016/j.smim.2022.101655

Aguzzi, A., and Weissmann, C. (1998). Prion diseases. *Haemophilia* 4, 619–627. doi: 10.1046/j.1365-2516.1998.440619.x

Aguzzi, A., and Zhu, C. (2017). Microglia in prion diseases. *J. Clin. Invest.* 127, 3230–3239. doi: 10.1172/JCI90605

Badoer, E. (2010). Microglia: activation in acute and chronic inflammatory states and in response to cardiovascular dysfunction. *Int. J. Biochem. Cell Biol.* 42, 1580–1585. doi: 10.1016/j.biocel.2010.07.005

Barnham, K. J., Cappai, R., Beyreuther, K., Masters, C. L., and Hill, A. F. (2006). Delineating common molecular mechanisms in Alzheimer's and prion diseases. *Trends Biochem. Sci.* 31, 465–472. doi: 10.1016/j.tibs.2006.06.006

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., et al. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6:90. doi: 10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Bradford, B. M., Tetlow, L., and Mabbott, N. A. (2017). Prion disease pathogenesis in the absence of the commensal microbiota. *J. Gen. Virol.* 98, 1943–1952. doi: 10.1099/jgv.0.000860

Braniste, V., al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Tóth, M., et al. (2014). The gut microbiota influences blood-brain barrier permeability in mice. *Sci. Transl. Med.* 6:263ra158. doi: 10.1126/scitranslmed.3009759

Brown, P., Cathala, F., Castaigne, P., and Gajdusek, D. C. (1986). Creutzfeldt-Jakob disease: clinical analysis of a consecutive series of 230 neuropathologically verified cases. *Ann. Neurol.* 20, 597–602. doi: 10.1002/ana.410200507

Brown, E. M., Clardy, J., and Xavier, R. J. (2023). Gut microbiome lipid metabolism and its impact on host physiology. *Cell Host Microbe* 31, 173–186. doi: 10.1016/j.chom.2023.01.009

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caredio, D., Koderman, M., Frontzek, K., Sorce, S., Nuvolone, M., Bremer, J., et al. (2023). Skeletal-muscle glutamine synthase is upregulated in preclinical prion diseases. *bioRxiv*. doi: 10.1101/2023.10.31.564879

Carta, M., and Aguzzi, A. (2022). Molecular foundations of prion strain diversity. *Curr. Opin. Neurobiol.* 72, 22–31. doi: 10.1016/j.conb.2021.07.010

Checkoway, H., Lundin, J. I., and Kelada, S. N. (2011). Neurodegenerative diseases. *IARC Sci. Publ.* 163, 407–419.

Claudino Dos Santos, J. C., Oliveira, L. F., Noleto, F. M., Gusmao, C. T. P., Brito, G. A. C., and Viana, G. S. B. (2023). Gut-microbiome-brain axis: the crosstalk between the vagus nerve, alpha-synuclein and the brain in Parkinson's disease. *Neural Regen. Res.* 18, 2611–2614. doi: 10.4103/1673-5374.373673

Colombo, A. V., Sadler, R. K., Llovera, G., Singh, V., Roth, S., Heindl, S., et al. (2021). Microbiota-derived short chain fatty acids modulate microglia and promote Aβ plaque deposition. *Elife* 10:e59826. doi: 10.7554/eLife.59826

Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., et al. (2019). The microbiota-gut-brain axis. *Physiol. Rev.* 99, 1877–2013. doi: 10.1152/physrev.00018.2018

Dalile, B., Van Oudenhove, L., Vervliet, B., and Verbeke, K. (2019). The role of short-chain fatty acids in microbiota-gut-brain communication. *Nat. Rev. Gastroenterol. Hepatol.* 16, 461–478. doi: 10.1038/s41575-019-0157-3

D'Argenio, V., and Sarnataro, D. (2019). Microbiome influence in the pathogenesis of prion and Alzheimer's diseases. *Int. J. Mol. Sci.* 20:4704. doi: 10.3390/ijms20194704

Defaye, M., Abdullah, N. S., Iftinca, M., Hassan, A., Agosti, F., Zhang, Z., et al. (2022). Gut-innervating TRPV1+ neurons drive chronic visceral pain via microglial P2Y12 receptor. *Cell. Mol. Gastroenterol. Hepatol.* 13, 977–999. doi: 10.1016/j.jcmgh.2021.12.012

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., and Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* 54, 2325–2340. doi: 10.1194/jlr.R036012

Diallo, A. B., Lapointe, F. J., and Makarenkov, V. (2007). A new effective method for estimating missing values in the sequence data prior to phylogenetic analysis. *Evol. Bioinformatics* 2, 237–246

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Erkkinen, M. G., Kim, M. O., and Geschwind, M. D. (2018). Clinical neurology and epidemiology of the major neurodegenerative diseases. *Cold Spring Harb. Perspect. Biol.* 10:a033118. doi: 10.1101/cshperspect.a033118

Erny, D., Hrabe de Angelis, A. L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., et al. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* 18, 965–977. doi: 10.1038/nn.4030

Fernie, A. R., Carrari, F., and Sweetlove, L. J. (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Curr. Opin. Plant Biol.* 7, 254–261. doi: 10.1016/j.pbi.2004.03.007

Forloni, G., Roiter, I., and Tagliavini, F. (2019). Clinical trials of prion disease therapeutics. *Curr. Opin. Pharmacol.* 44, 53–60. doi: 10.1016/j.coph.2019.04.019

Fostinelli, S., De Amicis, R., Leone, A., Giustizieri, V., Binetti, G., Bertoli, S., et al. (2020). Eating behavior in aging and dementia: the need for a comprehensive assessment. *Front. Nutr.* 7:604488. doi: 10.3389/fnut.2020.604488

Frontzek, K., Bardelli, M., Senatore, A., Henzi, A., Reimann, R. R., Bedir, S., et al. (2022). A conformational switch controlling the toxicity of the prion protein. *Nat. Struct. Mol. Biol.* 29, 831–840. doi: 10.1038/s41594-022-00814-7

Guenther, K., Deacon, R. M., Perry, V. H., and Rawlins, J. N. (2001). Early behavioural changes in scrapie-affected mice and the influence of dapsone. *Eur. J. Neurosci.* 14, 401–409. doi: 10.1046/j.0953-816x.2001.01645.x

Heston, M. B., Hanslik, K. L., Zarbock, K. R., Harding, S. J., Davenport-Sis, N. J., Kerby, R. L., et al. (2023). Gut inflammation associated with age and Alzheimer's disease pathology: a human cohort study. *Sci. Rep.* 13:18924. doi: 10.1038/s41598-023-45929-z

Hickman, S., Izzy, S., Sen, P., Morsett, L., and El Khoury, J. (2018). Microglia in neurodegeneration. *Nat. Neurosci.* 21, 1359–1369. doi: 10.1038/s41593-018-0242-x

Jian, Z., Zeng, L., Xu, T., Sun, S., Yan, S., Zhao, S., et al. (2022). The intestinal microbiome associated with lipid metabolism and obesity in humans and animals. *J. Appl. Microbiol.* 133, 2915–2930. doi: 10.1111/jam.15740

Lach, G., Schellekens, H., Dinan, T. G., and Cryan, J. F. (2018). Anxiety, depression, and the microbiome: a role for gut peptides. *Neurotherapeutics* 15, 36–59. doi: 10.1007/s13311-017-0585-0

Lai, Y., Liu, C. W., Yang, Y., Hsiao, Y. C., Ru, H., and Lu, K. (2021). High-coverage metabolomics uncovers microbiota-driven biochemical landscape of interorgan transport and gut-brain communication in mice. *Nat. Commun.* 12:6000. doi: 10.1038/s41467-021-26209-8

Leigh, S. J., Kaakoush, N. O., Bertoldo, M. J., Westbrook, R. F., and Morris, M. J. (2020). Intermittent cafeteria diet identifies fecal microbiome changes as a predictor of spatial recognition memory impairment in female rats. *Transl. Psychiatry* 10:36. doi: 10.1038/s41398-020-0734-9

Liu, J., Yu, C., Li, R., Liu, K., Jin, G., Ge, R., et al. (2020). High-altitude Tibetan fermented milk ameliorated cognitive dysfunction by modified gut microbiota in Alzheimer's disease transgenic mice. *Food Funct.* 11, 5308–5319. doi: 10.1039/C9FO03007G

Louis, P., and Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* 294, 1–8. doi: 10.1111/j.1574-6968.2009.01514.x

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* 19, 29–41. doi: 10.1111/1462-2920.13589

Louis, P., Young, P., Holtrop, G., and Flint, H. J. (2010). Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. *Environ. Microbiol.* 12, 304–314. doi: 10.1111/j.1462-2920.2009.02066.x

Mahbub, N. U., Islam, M. M., Hong, S. T., and Chung, H. J. (2024). Dysbiosis of the gut microbiota and its effect on alpha-synuclein and prion protein misfolding: consequences for neurodegeneration. *Front. Cell. Infect. Microbiol.* 14:1348279. doi: 10.3389/fcimb.2024.1348279

Martin-Pena, A., and Tansey, M. G. (2023). The Alzheimer's risk gene APOE modulates the gut-brain axis. *Nature* 614, 629–630. doi: 10.1038/d41586-023-00261-4

Mehrian-Shai, R., Reichardt, J. K. V., Harris, C. C., and Toren, A. (2019). The gut-brain axis, paving the way to brain cancer. *Trends Cancer* 5, 200–207. doi: 10.1016/j.trecan.2019.02.008

Miller, T. L., and Wolin, M. J. (1996). Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Appl. Environ. Microbiol.* 62, 1589–1592. doi: 10.1128/aem.62.5.1589-1592.1996

Minter, M. R., Zhang, C., Leone, V., Ringus, D. L., Zhang, X., Oyler-Castrillo, P., et al. (2016). Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. *Sci. Rep.* 6:30028. doi: 10.1038/srep30028

Mirabile, I., Jat, P. S., Brandner, S., and Collinge, J. (2015). Identification of clinical target areas in the brainstem of prion-infected mice. *Neuropathol. Appl. Neurobiol.* 41, 613–630. doi: 10.1111/nan.12189

Montalban-Arques, A., Katkeviciute, E., Busenhart, P., Bircher, A., Wirbel, J., Zeller, G., et al. (2021). Commensal Clostridiales strains mediate effective anti-cancer immune response against solid tumors. *Cell Host Microbe* 29, 1573–1588.e7. doi: 10.1016/j.chom.2021.08.001

Montella, L., Sarno, F., Altucci, L., Cioffi, V., Sigona, L., Di Colandrea, S., et al. (2021). A root in synapsis and the other one in the gut microbiome-brain axis: are the two poles of ketogenic diet enough to challenge glioblastoma? *Front. Nutr.* 8:703392. doi: 10.3389/fnut.2021.703392

Nagpal, J., and Cryan, J. F. (2021). Microbiota-brain interactions: moving toward mechanisms in model organisms. *Neuron* 109, 3930–3953. doi: 10.1016/j.neuron.2021.09.036

Natividad, J. M., Lamas, B., Pham, H. P., Michel, M. L., Rainteau, D., Bridonneau, C., et al. (2018). *Bilophila wadsworthia* aggravates high fat diet induced metabolic dysfunctions in mice. *Nat. Commun.* 9:2802. doi: 10.1038/s41467-018-05249-7

Olson, C. A., Iniguez, A. J., Yang, G. E., Fang, P., Pronovost, G. N., Jameson, K. G., et al. (2021). Alterations in the gut microbiota contribute to cognitive impairment induced by the ketogenic diet and hypoxia. *Cell Host Microbe* 29, 1378–92 e6. doi: 10.1016/j.chom.2021.07.004

Parada Venegas, D., de la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., et al. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front. Immunol.* 10:277. doi: 10.3389/fimmu.2019.00277

Park, S. Y., Rao, C., Coyte, K. Z., Kuziel, G. A., Zhang, Y., Huang, W., et al. (2022). Strain-level fitness in the gut microbiome is an emergent property of glycans and a single metabolite. *Cell* 185, 513–29 e21. doi: 10.1016/j.cell.2022.01.002

Parks, D. H., Chuvochina, M., Waite, D. W., Rinke, C., Skarshewski, A., Chaumeil, P. A., et al. (2018). A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nat. Biotechnol.* 36, 996–1004. doi: 10.1038/nbt.4229

Pascale, A., Marchesi, N., Marelli, C., Coppola, A., Luzi, L., Govoni, S., et al. (2018). Microbiota and metabolic diseases. *Endocrine* 61, 357–371. doi: 10.1007/s12020-018-1605-5

Pickard, J. M., Zeng, M. Y., Caruso, R., and Nunez, G. (2017). Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunol. Rev.* 279, 70–89. doi: 10.1111/imr.12567

Prusiner, S. B. (1998). Prions. *Proc. Natl. Acad. Sci. U. S. A.* 95, 13363–13383. doi: 10.1073/pnas.95.23.13363

Rao, M., Rastelli, D., Dong, L., Chiu, S., Setlik, W., Gershon, M. D., et al. (2017). Enteric glia regulate gastrointestinal motility but are not required for maintenance of the epithelium in mice. *Gastroenterology* 153, 1068–1081.e7. doi: 10.1053/j.gastro.2017.07.002

Reis, R., Hennessy, E., Murray, C., Griffin, E. W., and Cunningham, C. (2015). At the centre of neuronal, synaptic and axonal pathology in murine prion disease: degeneration of neuroanatomically linked thalamic and brainstem nuclei. *Neuropathol. Appl. Neurobiol.* 41, 780–797. doi: 10.1111/nan.12232

Rothhammer, V., Mascanfroni, I. D., Bunse, L., Takenaka, M. C., Kenison, J. E., Mayo, L., et al. (2016). Type I interferons and microbial metabolites of tryptophan modulate astrocyte activity and central nervous system inflammation via the aryl hydrocarbon receptor. *Nat. Med.* 22, 586–597. doi: 10.1038/nm.4106

Savchenko, V. L., McKanna, J. A., Nikonenko, I. R., and Skibo, G. G. (2000). Microglia and astrocytes in the adult rat brain: comparative immunocytochemical analysis demonstrates the efficacy of lipocortin 1 immunoreactivity. *Neuroscience* 96, 195–203.

Scheckel, C., and Aguzzi, A. (2018). Prions, prionoids and protein misfolding disorders. *Nat. Rev. Genet.* 19, 405–418. doi: 10.1038/s41576-018-0011-4

Scheckel, C., Imeri, M., Schwarz, P., and Aguzzi, A. (2020). Ribosomal profiling during prion disease uncovers progressive translational derangement in glia but not in neurons. *Elife* 9:e62911. doi: 10.7554/eLife.62911

Scher, J. U., Sczesnak, A., Longman, R. S., Segata, N., Ubeda, C., Bielski, C., et al. (2013). Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *Elife* 2:e01202. doi: 10.7554/eLife.01202

Seo, D. O., O'Donnell, D., Jain, N., Ulrich, J. D., Herz, J., Li, Y., et al. (2023). ApoE isoform- and microbiota-dependent progression of neurodegeneration in a mouse model of tauopathy. *Science* 379:eadd1236. doi: 10.1126/science.add1236

Silva, Y. P., Bernardi, A., and Frozza, R. L. (2020). The role of short-chain fatty acids from gut microbiota in gut-brain communication. *Front. Endocrinol.* 11:25. doi: 10.3389/fendo.2020.00025

Sorce, S., Nuvolone, M., Russo, G., Chincisan, A., Heinzer, D., Avar, M., et al. (2020). Genome-wide transcriptomics identifies an early preclinical signature of prion infection. *PLoS Pathog.* 16:e1008653. doi: 10.1371/journal.ppat.1008653

Stewart, L. A., Rydzewska, L. H., Keogh, G. F., and Knight, R. S. (2008). Systematic review of therapeutic interventions in human prion disease. *Neurology* 70, 1272–1281. doi: 10.1212/01.wnl.0000308955.25760.c2

Strandwitz, P. (2018). Neurotransmitter modulation by the gut microbiota. *Brain Res.* 1693, 128–133. doi: 10.1016/j.brainres.2018.03.015

Sun, J., Xu, J., Ling, Y., Wang, F., Gong, T., Yang, C., et al. (2019). Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Transl. Psychiatry* 9:189. doi: 10.1038/s41398-019-0525-3

Tran, T. T. T., Corsini, S., Kellingray, L., Hegarty, C., Le Gall, G., Narbad, A., et al. (2019). APOE genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer's disease pathophysiology. *FASEB J.* 33, 8221–8231. doi: 10.1096/fj.201900071R

Tremlett, H., Fadrosh, D. W., Faruqi, A. A., Zhu, F., Hart, J., Roalstad, S., et al. (2016). Gut microbiota in early pediatric multiple sclerosis: a case-control study. *Eur. J. Neurol.* 23, 1308–1321. doi: 10.1111/ene.13026

Vascellari, S., Palmas, V., Melis, M., Pisanu, S., Cusano, R., Uva, P., et al. (2020). Gut microbiota and metabolome alterations associated with Parkinson's disease. *mSystems.* 5:e00561-20. doi: 10.1128/mSystems.00561-20

Wang, G., Zhou, H. H., Luo, L., Qin, L. Q., Yin, J., Yu, Z., et al. (2021). Voluntary wheel running is capable of improving cognitive function only in the young but not the middle-aged male APPSwe/PS1De9 mice. *Neurochem. Int.* 145:105010. doi: 10.1016/j.neuint.2021.105010

Yu, K. B., and Hsiao, E. Y. (2021). Roles for the gut microbiota in regulating neuronal feeding circuits. *J. Clin. Invest.* 131:e143772. doi: 10.1172/JCI143772

Zhu, C., Li, B., Frontzek, K., Liu, Y., and Aguzzi, A. (2019). SARM1 deficiency up-regulates XAF1, promotes neuronal apoptosis, and accelerates prion disease. *J. Exp. Med.* 216, 743–756. doi: 10.1084/jem.20171885

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Causal associations of gut microbiota and pulmonary tuberculosis: a two-sample Mendelian randomization study

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Background: The prevalence of pulmonary tuberculosis (PTB) as an infectious disease continues to contribute significantly to global mortality. According to recent studies, the gut microbiota of PTB patients and healthy controls (HCs) show significant disparities. However, the causal relationship between them has yet to be elucidated.

Methods: We conducted a study using Mendelian Randomization (MR) to explore the potential causal link between gut microbiota and pulmonary tuberculosis (PTB). The summary statistics of the gut microbiota were acquired from the MiBioGen consortium, while data on PTB were sourced from pheweb.jp. A range of statistical methodologies were employed to evaluate causality, encompassing inverse variance weighting (IVW), MR-Egger, weighted median (WM), weighted model, and simple model. We utilized instrumental variables (IVs) that have a direct causal relationship with PTB to annotate SNPs, aiming to discover the genes harboring these genetic variants and uncover potential associations between host genes and the microbiome in patients with PTB.

Results: Among the 196 bacterial traits in the gut microbiome, we have identified a total of three microbiomes that exhibit a significant association with PTB. The occurrence of Dorea (P = 0.0458, FDR-adjusted P = 0.0458) and Parasutterella (P = 0.0056, FDR-adjusted P = 0.0168) was linked to an elevated risk of PTB, while the presence of Lachnoclostridium (P = 0.0347, FDR-adjusted P = 0.0520) demonstrated a protective effect against PTB. Our reverse Two-Sample Mendelian Randomization (TSMR) analysis did not yield any evidence supporting the hypothesis of reverse causality from PTB to alterations in the intestinal flora.

Conclusion: We have established a connection between the gut microbiota and PTB through gene prediction analysis, supporting the use of gut microecological therapy in managing PTB and paving the way for further understanding of how gut microbiota contributes to PTB's development.

KEYWORDS

GM, PTB, MR, Dorea, *Parasutterella*, *Lachnoclostridium*

1 Introduction

The etiological agent of tuberculosis is Mycobacterium tuberculosis, a pathogenic bacterium primarily transmitted through airborne routes (Sia and Rengarajan, 2019). According to the 2022 report by the World Health Organization on tuberculosis (TB), there were a total of 10.6 million diagnosed TB cases, resulting in approximately 1.13 million fatalities in the same year. However, the net reduction from 2015 to 2022 fell short of the WHO End TB Strategy milestone, which aimed for a 50% decrease by 2025, achieving only an 8.7% decline. Furthermore, it is projected that around 410,000 individuals (95% UI:370,000–450,000) were impacted by multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB) in 2022 (Bagcchi, 2023). Moreover, drug-resistant Mtb and the increasing incidence of TB-HIV coinfection pose significant public health threats. The majority of TB cases in clinical practice are pulmonary tuberculosis (PTB), accounting for 80–90% (Dheda et al., 2017). PTB can lead to lung granuloma formation as well as tissue liquefaction and cavity development (Gupta et al., 2022).

The human gut microbiota consists of bacteria, archaea, viruses, and microbial eukaryotes, all of which play a pivotal role in maintaining human health (Cresci and Bawden, 2015). The gut microbiota can exert influence on the occurrence and progression of lung diseases through the modulation of circulating immune cells, inflammatory mediators, bacterial metabolites, and bacterial translocation, which is referred to as the gut-lung axis (Wang et al., 2023). Recent scientific studies indicate a possible link between the gut microbiome and the development of pulmonary tuberculosis (PTB) (Chhabra et al., 2021). Significant alterations have been observed in the gut microbiota composition of PTB patients compared to healthy individuals (Hu et al., 2019). In Burkina Faso, the growth of *M. tuberculosis* strains in vitro was found to be inhibited by two Firmicutes species, namely *E. casseliflavus* and *E. mundtii*, which were isolated from patients who tested negative for TB (Fellag et al., 2020). A study conducted in western China revealed a direct relationship between the gut microbiota and the number of peripheral $CD4^+$ T cells in individuals with tuberculosis (Luo et al., 2017). Conversely, a study conducted in India found that individuals with tuberculosis had an increased presence of bacteria that produce butyrate and propionate (Hajian et al., 2019). Furthermore, during anti-TB chemotherapy treatment, both intestinal flora diversity and fecal metabolite abundance were restored among PTB patients (Meng et al., 2022). Although several studies have described associations between bacterial function changes and PTB (Li et al., 2019; Wang et al., 2022), the specific mechanisms underlying these relationships remain unclear.

The majority of pertinent studies are of an observational nature, rendering the establishment of causal conclusions a challenging endeavor. While in vitro experiments can elucidate specific mechanisms by which certain bacteria respond to PTB; identifying truly causative bacteria from thousands remains difficult using this approach alone. Mendelian randomization (MR) establishes causal relationships between exposure and outcomes by using single nucleotide polymorphisms (SNPs) as instrumental variables (IVs), simulating a randomized controlled trial. The present study employed a two-sample Mendelian randomization approach to investigate the potential causal association between gut microbiota and PTB, with gut microbiota as the exposure factor and PTB as the outcome variable, aiming to explore their genetic relationship.

2 Materials and methods

2.1 Study design

The Two-Sample Mendelian Randomization (TSMR) approach was employed to investigate the causal relationship between intestinal bacterial taxa and PTB. To ensure robust findings, three assumptions must be met when conducting TSMR analysis: (1) there is a significant association between genetic variations and factors of exposure; (2) genetic variations do not show any correlation with confounding variables; and (3) the influence of genetic variants on the outcome is solely through exposure factors, excluding any alternative pathways like horizontal pleiotropy. The genetic variants that satisfy these three assumptions can serve as instrumental variables in TSMR analysis (Emdin et al., 2017).

2.2 Data sources

The statistical data on the gut microbiota were obtained from a comprehensive genome-wide association study (GWAS) conducted by the MiBioGen consortium,[1] which involved 18,340 participants across 24 cohorts (Kurilshikov et al., 2021). We eliminated 15 bacterial traits that lacked precise nomenclature, leading to a conclusive collection of 196 bacterial traits spanning across 9 Phyla, 16 Classes, 20 Orders, 32 Families, and 119 Genera. Data for PTB cases and controls were sourced from pheweb.jp,[2] comprising a total of 170871 controls, 7800 cases and 12,454,677 SNPs.

2.3 Instrumental variables

The instrumental variables were identified in a genome-wide association study (GWAS) of intestinal flora using the following criteria: (1) to increase the number of SNPs as instrumental variables, we selected SNPs that showed correlation with the risk factors at a relatively lenient statistical significance threshold ($P < 1 \times 10^{-5}$) (Sanna et al., 2019; Li et al., 2022; Liu et al., 2022; Huang et al., 2023), (2) to eliminate SNPs in high linkage disequilibrium ($R2 > 0.1$) based on reference data from individuals of European ancestry obtained from the 500 Genomes project, and (3) to assess the strength of each SNP by calculating its F statistic and discard weak instrumental SNPs ($F < 10$).

2.4 Mendelian randomization analysis

Five popular Mendelian randomization (MR) methods were employed for analyzing valid instrumental variables (IVs):

1 https://mibiogen.gcc.rug.nl

2 https://pheweb.jp/pheno/PTB

inverse variance-weighted (IVW) test, weighted median, MR-Egger regression, simple mode, and weighted mode. The IVW method was primarily used among various approaches due to its slightly higher statistical power in certain situations. It incorporated weights derived from the reciprocal of outcome variance for fitting purposes, regardless of whether an intercept term was included in the regression model. Complementary assessments were conducted using the remaining four methods, each relying on distinct assumptions regarding potential pleiotropy. Consistency between these complementary approaches and the IVW estimation results would strengthen the robustness of effect estimation.

The odds ratio (OR) and 95% confidence interval (CI) were computed as per standard procedures. A significance level of $P < 0.05$ was used to determine statistical significance. Additionally, we utilized the False Discovery Rate (FDR) correction method to assess the potential for multiple comparisons in our findings. A significant causal relationship was determined to be present when the adjusted P-value was less than 0.05. Conversely, if an unadjusted *p*-value was < 0.05 but the FDR-adjusted p-value was > 0.05, it was considered as suggestive evidence of a potential association (Zhang et al., 2024). The FDR method is detailed as follows: (1) Extract the p-values calculated using the same method. (2) List the p-values in ascending order. (3) Adjust the p-value according to the formula (adjusted *P*-value = *P*-value * (Total number of P-values / Ranking number of the current *P*-value)). The presence of heterogeneity among SNPs was evaluated using Cochran's Q statistic (Cohen et al., 2015). The absence of heterogeneity is suggested when the *P*-value exceeds 0.05. To evaluate horizontal pleiotropy, we employed the MR-PRESSO test in conjunction with the intercept derived from MR-Egger regression (Bowden et al., 2015). The absence of horizontal pleiotropy was indicated by a *P*-value greater than 0.05. To ensure the robustness of the findings, a sensitivity analysis was conducted using the leave-one-out method (Wu et al., 2020). All tests were conducted using the R software version 4.3 and employed the TwoSampleMR package in R, employing a two-sided approach.

3 Results

3.1 The selection of instrumental variables

We screened the instrumental variables of 196 bacteria, excluding 15 unidentified genera. A total of 13,659 instrumental variables achieved locus-wide significance ($p < 1 \times 10^{-5}$). After accounting for linkage disequilibrium effects specific to flora, we retained 2708 instrumental variables. Subsequently, we excluded weakly associated exposure factors ($F < 10$) and potential confounding factors from outcomes analysis, resulting in a final inclusion of 1708 instrumental variables representing the diverse flora.

3.2 Causal effects from gut microbiota on PTB risk

According to various Mendelian randomization (MR) analysis methods, we have obtained compelling evidence suggesting a potential causal relationship between the gut microbiota and the risk of PTB, with statistical significance set at 0.05 (**Figure 1**). The instrumental variable weighted (IVW) analysis revealed that Lachnoclostridium (OR = 0.82, 95%CI = 0.68 ~ 0.99, P = 0.0347) exhibited a protective effect against PTB. Furthermore, Parasutterella (OR = 1.23, 95%CI = 1.06 ~ 1.43, *P* = 0.0058) and Dorea (OR = 1.30, 95%CI = 1.00 ~ 1.68, *P* = 0 .0458) were found to be inversely associated with PTB (**Figure 2**). Based on the FDR correction results, it has been determined that there is a significant causal relationship between Parasutterella (adjusted *P* = 0.0168) and Dorea (adjusted *P* = 0.0458) with PTB. Additionally, it has been found that Lachnoclostridium (adjusted *P* = 0.0520) may have a potential causal relationship with PTB (**Table 1**).

3.3 Sensitivity analysis

The Cochran's Q test results indicated no evidence of heterogeneity among the independent variables (**Table 2**). The MR-Egger intercept test, excluding outlier variants, demonstrated the absence of horizontal pleiotropy (**Table 3**). Sensitivity analysis using the leave-one-out method confirmed the stability of our findings when systematically removing individual SNPs (**Figure 3**).

3.4 The result of reverse MR analysis

Subsequently, we conducted reverse Mendelian randomization (MR) analyses to assess the potential inverse associations between three bacterial traits and PTB. However, the IVW approach did not find any significant associations between PTB and the mentioned bacterial traits (OR: 0.94; 95% CI: 0.87 ~ 1.03; $p = 0.216$ for Dorea; OR: 0.98; 95% CI: 0.84 ~ 1.13; $p = 0.797$ for Parasutterella and OR: 1.05; 95% CI: 0.96 ~ 1.15; $p = 0.285$ for Lachnoclostridium) (**Table 4**).

4 Discussion

This study systematically investigates the causal relationship between gut microbiota and PTB using Mendelian randomization. Our findings suggest a significant association between Parasutterella and Dorea with an increased risk of PTB, while indicating that Lachnoclostridium may potentially exert a protective effect against PTB. The robustness of these associations is further confirmed through sensitivity analysis. Importantly, no evidence supporting causal effects of other intestinal flora on PTB was identified in our study.

The human gut microbiome (GM) plays a pivotal role in maintaining human health, including the regulation of

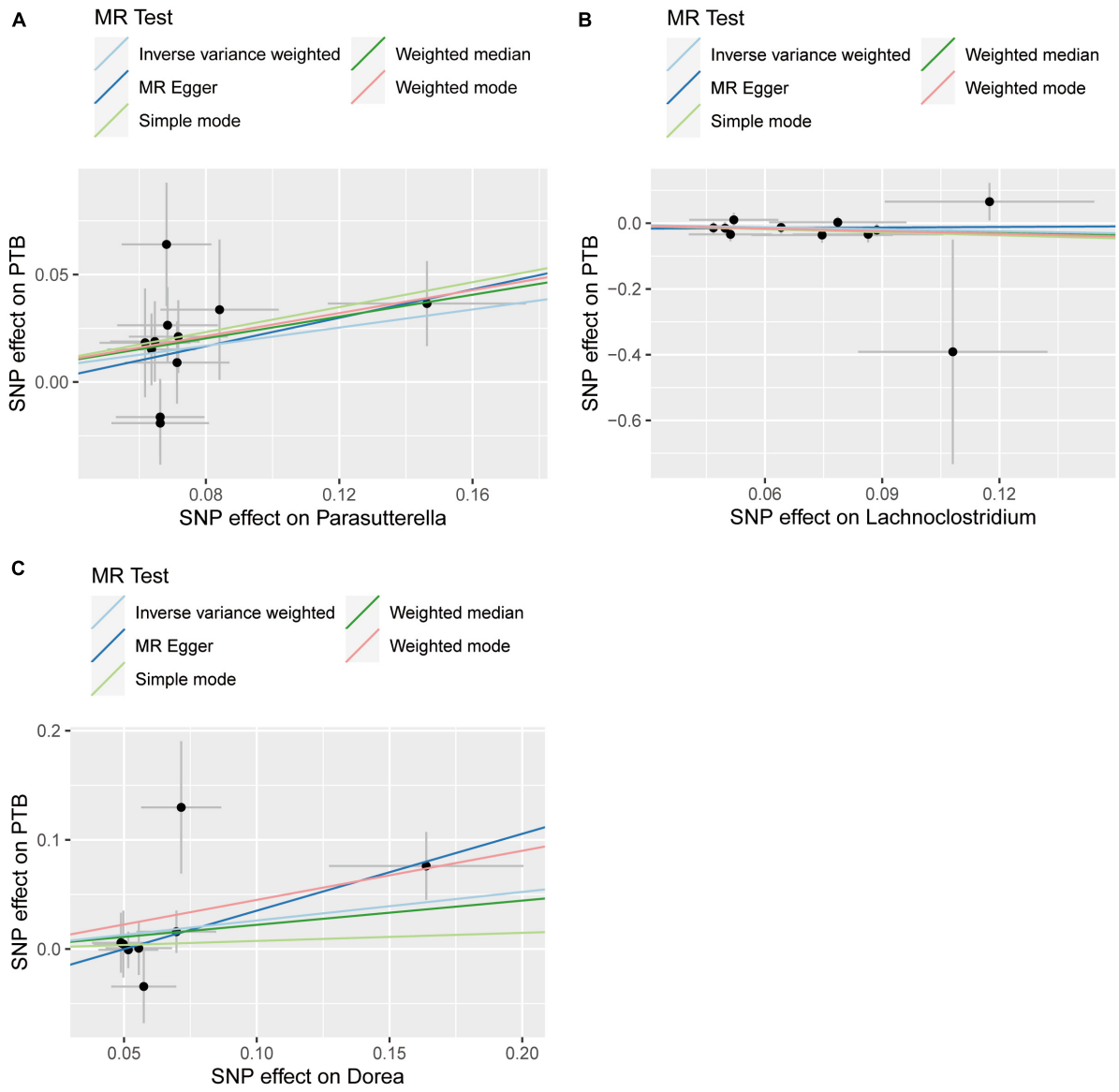


FIGURE 1
Summary of scatter plots of potential associations between gut microbiota and plumonary tuberculosis (PTB). **(A)** Potential positive associations between Parasutterella and PTB, **(B)** potential negative associations between Lachnoclostrdium and PTB, **(C)** potential positive associations between Dorea and PTB.

lung diseases. The gut microbiota and the lungs communicate through soluble microbial components and metabolites that are transported via circulation. Administering lipopolysaccharide (LPS) intrarectally to mice treated with antibiotics restored their capacity to mount efficient immune responses against influenza virus infection in the lungs (Ichinohe et al., 2011). Short-chain fatty acids (SCFAs), including butyrate, propionate, and acetate, are synthesized by the microbiota in the cecum and colon, exerting an influence on local intestinal immunity and immune cell development. Scientific evidence suggests that consumption of SCFAs can ameliorate pulmonary allergic reactions (Trompette et al., 2014; Cait et al., 2018) and confer protection against influenza virus infection (Steed et al., 2017). Additionally, immune cells possess the ability to migrate directly from the intestine to the respiratory tract through circulation, thereby shaping the immune microenvironment within the lungs. Consequently, the concept of a gut-lung axis was proposed.

The gut-lung axis may represent a potential mechanism underlying the occurrence and progression of pulmonary tuberculosis (PTB). Notably, studies have demonstrated significant differences in both diversity and taxonomic composition of the intestinal microbiota between PTB patients and healthy controls (HCs) (Ye et al., 2022). Furthermore, variations in gut microbiota profiles have been observed among different populations with PTB. A study conducted in western China revealed that Actinobacteria and Proteobacteria were significantly enriched in the feces of recurrent tuberculosis patients (RTB). Conversely, the phylum Bacteroidetes, which contains a variety of beneficial commensal organisms, was reduced (Luo et al., 2017). A study conducted in western China revealed that Actinobacteria and Proteobacteria

TABLE 1 False Discovery Rate (FDR) correction.

Outcome	Exposure	Method	*P*-value	FDR-adjusted *P*-value
PTB	*Parasutterella*	MR Egger	0.2514	0.3772
		Weighted median	0.0215	0.0645
		Inverse variance weighted	0.0056	0.0168
		Simple mode	0.0878	0.2635
		Weighted mode	0.0446	0.1337
	Lachnoclostridium	MR Egger	0.8897	0.8897
		Weighted median	0.0726	0.1089
		Inverse variance weighted	0.0347	0.0520
		Simple mode	0.1111	0.1667
		Weighted mode	0.1112	0.1112
	Dorea	MR Egger	0.0537	0.1612
		Weighted median	0.1912	0.1912
		Inverse variance weighted	0.0458	0.0458
		Simple mode	0.7737	0.7737
		Weighted mode	0.0478	0.0717

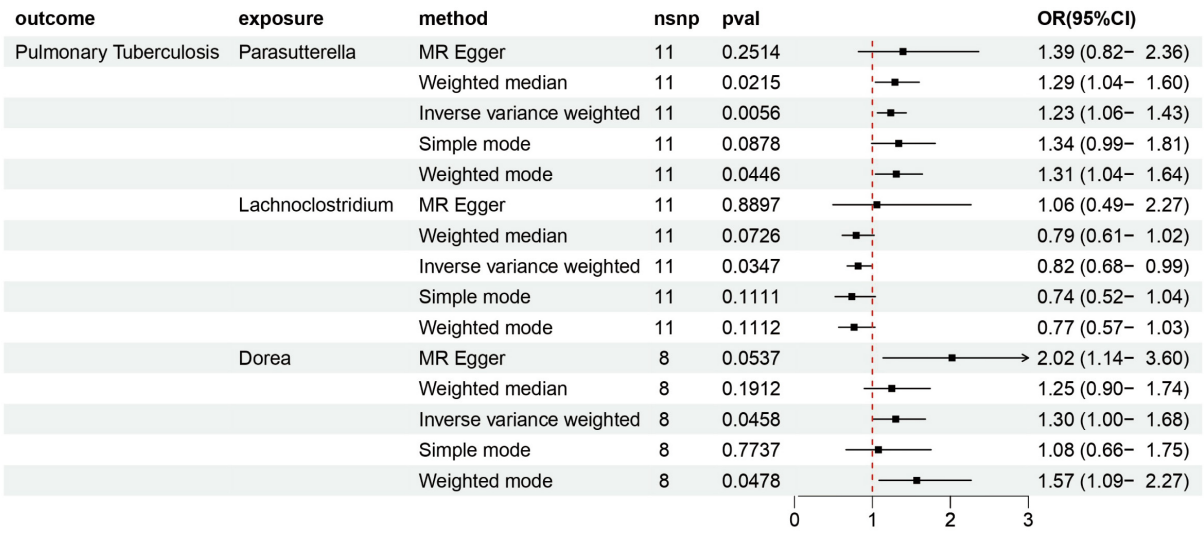


FIGURE 2
Forest plot of the causality between 3 bacterial taxa with the risks of PTB.

TABLE 2 Evaluation of heterogeneity using different methods.

Outcome	Exposure	Method	Cochran's Q	*P*-value
PTB	*Parasutterella*	MR Egger	9.75	0.37
		Inverse variance weighted	9.98	0.44
	Lachnoclostridium	MR Egger	7.90	0.54
		Inverse variance weighted	8.37	0.59
	Dorea	MR Egger	5.11	0.53
		Inverse variance weighted	7.86	0.34

were significantly enriched in the feces of recurrent tuberculosis patients (RTB). Conversely, the phylum Bacteroidetes, which contains a variety of beneficial commensal organisms, was reduced (Ye et al., 2022). At the same time, family *Veillonellacea* and *Bateroidaceae* and their genera *Veillonella* and *Bacteroides* significantly increased in the gut microbiota during anti-TB

TABLE 3 Evaluation of horizontal pleiotropy.

Outcome	Exposure	Egger-intercept	SE	*P*-value
PTB	*Parasutterella*	−0.01	0.02	0.65
	Lachnoclostridium	−0.02	0.03	0.51
	Dorea	−0.04	0.02	0.15

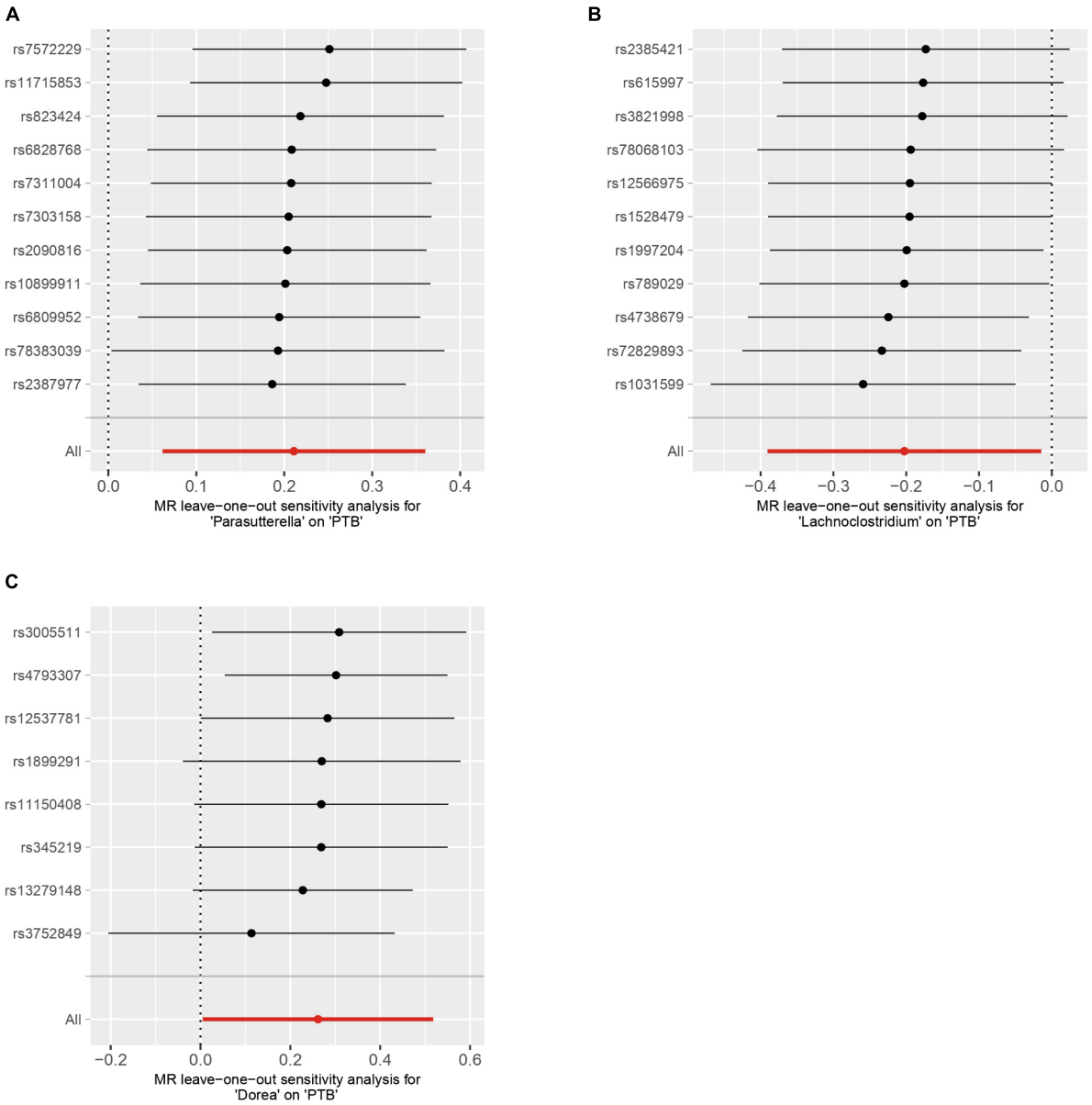


FIGURE 3
Sensitivity analysis by using the leave-one-out-method. **(A)** Parasutterella on PTB, **(B)** Lachnoclostridium on PTB **(C)** Dorea on PTB.

TABLE 4 Reverse MR analysis by using the IVW method.

Bacterium	Method	OR (95%CI)	*P*-value
Parasutterella	Inverse variance weighted	0.98(0.84 ~ 1.13)	0.797
Lachnoclostridium	Inverse variance weighted	1.05(0.96 ~ 1.15)	0.285
Dorea	Inverse variance weighted	0.94(0.87 ~ 1.03)	0.216

chemotherapy (Meng et al., 2022). However, it is important to note that these studies do not establish a causal relationship between gut microbiota and pulmonary tuberculosis. Although certain specific gut microbial species including E. casseliflavus and E. mundtii have shown inhibitory effects on Mycobacterium tuberculosis growth in vitro (Fellag et al., 2020), this does not necessarily imply a direct causal relationship in vivo.

Our study identified a causal association between three gut microbiota, namely Parasutterella, Dorea, and Lachnoclostridium, and PTB. Notably, Parasutterella has been found to have a causal relationship with various other conditions in several Mendelian randomization studies, including COVID-19 (Chen H. et al., 2023), intrahepatic cholangiocarcinoma (ICC) (Chen Z. et al., 2023), chronic kidney disease (CKD) (Ren et al., 2023), and prostatitis (Shen et al., 2024). Meanwhile, Dorea is associated with autism spectrum disorder (ASD) (Li Z. et al., 2023), inflammatory bowel disease (IBD) (Zhang et al., 2021), hypertensive disorders in pregnancy (HDP) (Wu et al., 2023a), polycystic ovary syndrome (PCOS) (Min et al., 2023), hemorrhagic stroke (Shen et al., 2023), age-related macular degeneration (AMD) (Li and Lu, 2023), nicotine dependence (Chen Y. et al., 2023), and insomnia (Li Z. et al., 2023). Similarly, Lachnochlostrium is linked to multiple diseases, including Alzheimer's disease (AD) (Ning et al., 2022), nonalcoholic fatty liver disease (NAFLD) (Zhang et al., 2023), myasthenia gravis (Su et al., 2023), type 2 diabetes (Li and Li, 2023), gestational diabetes mellitus (Wu et al., 2023b), renal cell cancer (Yin et al., 2023), insomnia (Li Z. et al., 2023), peripheral artery disease (Shi et al., 2024), peripheral atherosclerosis (Jiang et al., 2023), Primary open-angle glaucoma (POAG) (Zhou et al., 2024), and intervertebral disc degeneration (Zheng et al., 2023). These studies demonstrate a causal association between gut microbiota and diseases. Mechanistically, colonization of the gastrointestinal tract by Parasutterella, without altering bacterial composition, leads to modifications in microbial-derived metabolites such as aromatic amino acids, bilirubin, purine derivatives, and bile acid derivatives (Ju et al., 2019). Lachnoclostridium utilizes cutC/D to convert choline into TMA, thereby promoting the development of atherosclerosis (Cai et al., 2022). Dorea serves as an indicator of immune activation and is positively correlated with T-cell production of IFN-γ (Riazati et al., 2022). Collectively, these findings underscore the pivotal roles played by these gut microbiotas in disease progression.

In conclusion, our study establishes a causal relationship between three specific gut microbiota and pulmonary tuberculosis, thereby offering novel insights into the investigation of the association between gut microbiota and pulmonary tuberculosis. They may modulate pulmonary tuberculosis by facilitating the circulation of immune cells, cytokines, bacterial derivatives, and other factors. However, the underlying mechanism remains unclear. The elucidation of the pathway linking intestinal flora and pulmonary tuberculosis necessitates further in vivo and in vitro experiments. Additionally, it should be noted that these findings are derived from the Japanese population and may not necessarily be representative of other ethnicities such as those in Europe and Africa. Lastly, due to insufficient detailed clinical information, subgroup analyses based on age and gender were not feasible.

5 Conclusion

In contrast to other studies, our investigation specifically identified three gut microbiota species that are causally associated with tuberculosis using a two-sample Mendelian randomization approach. These include one protective bacterium and two bacteria associated with an increased risk of PTB. These findings support the hypothesis that the gut microbiota plays a role in the development of PTB. Furthermore, the findings offer valuable perspectives and guidance for future mechanistic investigations, including the utilization of animal models or human trials based on biomarkers, which can facilitate the advancement and clinical application of potential microbiota-centered strategies against tuberculosis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Nanjing Integrated Chinese and Western Medicine Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because The data in this study came from public database and did not involve patient samples. Therefore, written informed consent was not required for this study.

Author contributions

ZL: Writing—original draft. WX: Writing—original draft, Methodology. YG: Writing—original draft, Formal analysis. FH: Writing—original draft, Data curation. GZ: Writing—review and editing, Writing—original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Bagcchi, S. (2023). WHO's global tuberculosis report 2022. *Lancet Microbe* 4:e20. doi: 10.1016/s2666-5247(22)00359-7

Bowden, J., Davey Smith, G., and Burgess, S. (2015). Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int. J. Epidemiol.* 44, 512–525. doi: 10.1093/ije/dyv080

Cai, Y., Huang, F., Lao, X., Lu, Y., Gao, X., and Alolga, R. (2022). Integrated metagenomics identifies a crucial role for trimethylamine-producing Lachnoclostridium in promoting atherosclerosis. *NPJ Biofilms Microbiomes* 8:11. doi: 10.1038/s41522-022-00273-4

Cait, A., Hughes, M., Antignano, F., Cait, J., Dimitriu, P., Maas, K., et al. (2018). Microbiome-driven allergic lung inflammation is ameliorated by short-chain fatty acids. *Mucosal Immunol.* 11, 785–795. doi: 10.1038/mi.2017.75

Chen, H., Ye, B., Su, W., Song, Y., Sun, P., Zhou, X., et al. (2023). The causal role of gut microbiota in susceptibility and severity of COVID-19: a bidirectional Mendelian randomization study. *J. Med. Virol.* 95:e28880. doi: 10.1002/jmv.28880

Chen, Y., Zhao, M., Ji, K., Li, J., Wang, S., Lu, L., et al. (2023). Association of nicotine dependence and gut microbiota: a bidirectional two-sample Mendelian randomization study. *Front. Immunol.* 14:1244272. doi: 10.3389/fimmu.2023.1244272

Chen, Z., Shi, W., Chen, K., Lu, C., Li, X., and Li, Q. (2023). Elucidating the causal association between gut microbiota and intrahepatic cholangiocarcinoma through Mendelian randomization analysis. *Front. Microbiol.* 14:1288525. doi: 10.3389/fmicb.2023.1288525

Chhabra, S., Kashyap, A., Bhagat, M., Mahajan, R., and Sethi, S. (2021). Anemia and nutritional status in tuberculosis patients. *Int. J. Appl. Basic Med. Res.* 11, 226–230. doi: 10.4103/ijabmr.ijabmr_76_21

Cohen, J., Chalumeau, M., Cohen, R., Korevaar, D., Khoshnood, B., and Bossuyt, P. (2015). Cochran's Q test was useful to assess heterogeneity in likelihood ratios in studies of diagnostic accuracy. *J. Clin. Epidemiol.* 68, 299–306. doi: 10.1016/j.jclinepi.2014.09.005

Cresci, G., and Bawden, E. (2015). Gut microbiome: what we do and don't know. *Nutrition Clin. Practice* 30, 734–746. doi: 10.1177/0884533615609899

Dheda, K., Gumbo, T., Maartens, G., Dooley, K., McNerney, R., and Murray, M. (2017). The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *Lancet Respiratory Med.* doi: 10.1016/s2213-2600(17)30079-6 Online ahead of print.

Emdin, C., Khera, A., and Kathiresan, S. (2017). Mendelian randomization. *JAMA* 318, 1925–1926. doi: 10.1001/jama.2017.17219

Fellag, M., Gouba, N., Bedotto, M., Sakana, M., Zingué, D., Tarnagda, Z., et al. (2020). Culturomics discloses anti-tubercular enterococci exclusive of pulmonary tuberculosis: a preliminary report. *Microorganisms* 8:1544. doi: 10.3390/microorganisms8101544

Gupta, M., Bagri, S., Garg, A., Singh, D., Choudhary, P., and Sahni, S. (2022). Pulmonary function in cured pulmonary tuberculosis cases. *Indian J. Tuberculosis* 69, 535–538. doi: 10.1016/j.ijtb.2021.08.024

Hajian, B., Scocchera, E., Shoen, C., Krucinska, J., Viswanathan, K., and Erlandsen, H. (2019). Drugging the folate pathway in mycobacterium tuberculosis: the role of multi-targeting agents. *Cell Chem. Biol.* 26, 781–791.e6. doi: 10.1016/j.chembiol.2019.02.013.

Hu, Y., Feng, Y., Wu, J., Liu, F., Zhang, Z., Hao, Y., et al. (2019). The gut microbiome signatures discriminate healthy from pulmonary tuberculosis patients. *Front. Cell Infect. Microbiol.* 9:90. doi: 10.3389/fcimb.2019.00090

Huang, Y., Chen, X., Ye, J., Yi, H., and Zheng, X. (2023). Causal effect of gut microbiota on DNA methylation phenotypic age acceleration: a two-sample Mendelian randomization study. *Sci. Rep.* 13:18830. doi: 10.1038/s41598-023-46308-4

Ichinohe, T., Pang, I., Kumamoto, Y., Peaper, D., Ho, J., Murray, T., et al. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc. Natl. Acad. Sci. U S A.* 108, 5354–5359. doi: 10.1073/pnas.1019378108

Jiang, S., Yu, C., Lv, B., He, S., Zheng, Y., Yang, W., et al. (2023). Two-sample Mendelian randomization to study the causal association between gut microbiota and atherosclerosis. *Front. Immunol.* 14:1282072. doi: 10.3389/fimmu.2023.1282072

Ju, T., Kong, J., Stothard, P., and Willing, B. (2019). Defining the role of Parasutterella, a previously uncharacterized member of the core gut microbiota. *ISME J.* 13, 1520–1534. doi: 10.1038/s41396-019-0364-5

Kurilshikov, A., Medina-Gomez, C., Bacigalupe, R., Radjabzadeh, D., Wang, J., and Demirkan, A. (2021). Large-scale association analyses identify host factors influencing human gut microbiome composition. *Nat. Genet.* 53, 156–165. doi: 10.1038/s41588-020-00763-1

Li, C., and Lu, P. (2023). Association of gut microbiota with age-related macular degeneration and glaucoma: a bidirectional mendelian randomization study. *Nutrients* 15:4646. doi: 10.3390/nu15214646

Li, H., and Li, C. (2023). Causal relationship between gut microbiota and type 2 diabetes: a two-sample Mendelian randomization study. *Front. Microbiol.* 14:1184734. doi: 10.3389/fmicb.2023.1184734

Li, P., Wang, H., Guo, L., Gou, X., Chen, G., Lin, D., et al. (2022). Association between gut microbiota and preeclampsia-eclampsia: a two-sample Mendelian randomization study. *BMC Med.* 20:443. doi: 10.1186/s12916-022-02657-x

Li, W., Zhu, Y., Liao, Q., Wang, Z., and Wan, C. (2019). Characterization of gut microbiota in children with pulmonary tuberculosis. *BMC Pediatrics* 19:445. doi: 10.1186/s12887-019-1782-2

Li, Y., Deng, Q., and Liu, Z. (2023). The relationship between gut microbiota and insomnia: a bi-directional two-sample Mendelian randomization research. *Front. Cell. Infect. Microbiol.* 13:1296417. doi: 10.3389/fcimb.2023.1296417

Li, Z., Liu, S., Liu, F., Dai, N., Liang, R., Lv, S., et al. (2023). Gut microbiota and autism spectrum disorders: a bidirectional Mendelian randomization study. *Front. Cell. Infect. Microbiol.* 13:1267721. doi: 10.3389/fcimb.2023.1267721

Liu, K., Zou, J., Fan, H., Hu, H., and You, Z. (2022). Causal effects of gut microbiota on diabetic retinopathy: a mendelian randomization study. *Front. Immunol.* 13:930318. doi: 10.3389/fimmu.2022.930318

Luo, M., Liu, Y., Wu, P., Luo, D., Sun, Q., and Zheng, H. (2017). Alternation of gut microbiota in patients with pulmonary tuberculosis. *Front. Physiol.* 8:822. doi: 10.3389/fphys.2017.00822

Meng, R., Dong, W., Gao, J., Lu, C., Zhang, C., Liao, Q., et al. (2022). Clostridium, *Bacteroides* and Prevotella associates with increased fecal metabolites Trans-4-Hydroxy-L-proline and Genistein in active pulmonary tuberculosis patients during anti-tuberculosis chemotherapy with isoniazid-rifampin-pyrazinamide-ethambutol (HRZE). *Indian J. Microbiol.* 62, 374–383. doi: 10.1007/s12088-022-01003-2

Min, Q., Geng, H., Gao, Q., and Xu, M. (2023). The association between gut microbiome and PCOS: evidence from meta-analysis and two-sample mendelian randomization. *Front. Microbiol.* 14:1203902. doi: 10.3389/fmicb.2023.1203902

Ning, J., Huang, S., Chen, S., Zhang, Y., Huang, Y., and Yu, J. (2022). Investigating casual associations among gut microbiota, metabolites, and neurodegenerative diseases: a mendelian randomization study. *J. Alzheimer's Dis.* 87, 211–222. doi: 10.3233/jad-215411

Ren, F., Jin, Q., Jin, Q., Qian, Y., Ren, X., Liu, T., et al. (2023). Genetic evidence supporting the causal role of gut microbiota in chronic kidney disease and chronic systemic inflammation in CKD: a bilateral two-sample Mendelian randomization study. *Front. Immunol.* 14:1287698. doi: 10.3389/fimmu.2023.1287698

Riazati, N., Kable, M., Newman, J., Adkins, Y., Freytag, T., Jiang, X., et al. (2022). Associations of microbial and indoleamine-2,3-dioxygenase-derived tryptophan metabolites with immune activation in healthy adults. *Front. Immunol.* 13:917966. doi: 10.3389/fimmu.2022.917966

Sanna, S., van Zuydam, N., Mahajan, A., Kurilshikov, A., Vich Vila, A., and Võsa, U. (2019). Causal relationships among the gut microbiome, short-chain fatty acids and metabolic diseases. *Nat. Genet.* 51, 600–605. doi: 10.1038/s41588-019-0350-x

Shen, C., Chen, Z., Zhang, W., Chen, X., Zheng, B., and Shi, C. (2024). Preliminary study of the effect of gut microbiota on the development of prostatitis. *BMC Med. Genomics* 17:35. doi: 10.1186/s12920-024-01812-y

Shen, Y., Liu, H., Meng, X., Gao, A., Liu, Y., Ma, W., et al. (2023). The causal effects between gut microbiota and hemorrhagic stroke: a bidirectional two-sample Mendelian randomization study. *Front. Microbiol.* 14:1290909. doi: 10.3389/fmicb.2023.1290909

Shi, H., Yuan, X., Wu, F., Li, X., Fan, W., Yang, X., et al. (2024). Genetic support of the causal association between gut microbiota and peripheral artery disease: a bidirectional Mendelian randomization study. *Aging* 16, 762–778. doi: 10.18632/aging.205417

Sia, J., and Rengarajan, J. (2019). Immunology of Mycobacterium tuberculosis Infections. *Microbiol Spectr.* 7, 1–57. doi: 10.1128/microbiolspec.GPP3-0022-2018

Steed, A., Christophi, G., Kaiko, G., Sun, L., Goodwin, V., and Jain, U. (2017). The microbial metabolite desaminotyrosine protects from influenza through type I interferon. *Science* 357, 498–502. doi: 10.1126/science.aam5336

Su, T., Yin, X., Ren, J., Lang, Y., Zhang, W., and Cui, L. (2023). Causal relationship between gut microbiota and myasthenia gravis: a bidirectional mendelian randomization study. *Cell Biosci.* 13:204. doi: 10.1186/s13578-023-01163-8

Trompette, A., Gollwitzer, E., Yadava, K., Sichelstiel, A., Sprenger, N., Ngom-Bru, C., et al. (2014). Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat. Med.* 20, 159–166. doi: 10.1038/nm.3444

Wang, L., Cai, Y., Garssen, J., Henricks, P., Folkerts, G., and Braber, S. (2023). The bidirectional gut-lung axis in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 207, 1145–1160. doi: 10.1164/rccm.202206-1066TR

Wang, S., Yang, L., Hu, H., Lv, L., Ji, Z., Zhao, Y., et al. (2022). Characteristic gut microbiota and metabolic changes in patients with pulmonary tuberculosis. *Microb. Biotechnol.* 15, 262–275. doi: 10.1111/1751-7915.13761

Wu, F., Huang, Y., Hu, J., and Shao, Z. (2020). Mendelian randomization study of inflammatory bowel disease and bone mineral density. *BMC Med.* 18:312. doi: 10.1186/s12916-020-01778-5

Wu, X., Li, Q., Lin, D., Cai, J., Huang, H., and Tan, H. (2023a). Gut microbiota and hypertensive disorders in pregnancy: evidence from the Mendelian randomization study. *Aging* 15, 9105–9127. doi: 10.18632/aging.205019

Wu, X., Lin, D., Li, Q., Cai, J., Huang, H., Xiang, T., et al. (2023b). Investigating causal associations among gut microbiota, gut microbiota-derived metabolites, and gestational diabetes mellitus: a bidirectional Mendelian randomization study. *Aging* 15, 8345–8366. doi: 10.18632/aging.204973

Ye, S., Wang, L., Li, S., Ding, Q., Wang, Y., Wan, X., et al. (2022). The correlation between dysfunctional intestinal flora and pathology feature of patients with pulmonary tuberculosis. *Front. Cell Infect. Microbiol.* 12:1090889. doi: 10.3389/fcimb.2022.1090889

Yin, Z., Liu, B., Feng, S., He, Y., Tang, C., Chen, P., et al. (2023). Large genetic causal analysis of the gut microbiota and urological cancers: a bidirectional mendelian randomization study. *Nutrients* 15:4086. doi: 10.3390/nu15184086

Zhang, L., Zi, L., Kuang, T., Wang, K., Qiu, Z., Wu, Z., et al. (2023). Investigating causal associations among gut microbiota, metabolites, and liver diseases: a Mendelian randomization study. *Front. Endocrinol.* 14:1159148. doi: 10.3389/fendo.2023.1159148

Zhang, Y., Ren, E., Zhang, C., Wang, Y., Chen, X., and Li, L. (2024). The protective role of oily fish intake against type 2 diabetes: insights from a genetic correlation and Mendelian randomization study. *Front. Nutrition* 11:1288886. doi: 10.3389/fnut.2024.1288886

Zhang, Z., Qu, H., Zhao, N., Wang, J., Wang, X., Hai, R., et al. (2021). Assessment of causal direction between gut microbiota and inflammatory bowel disease: a mendelian randomization analysis. *Front. Genet.* 12:631061. doi: 10.3389/fgene.2021.631061

Zheng, D., Wu, Z., Li, L., Cheng, S., and Chang, J. (2023). Genetic analysis of the causal relationship between gut microbiota and intervertebral disc degeneration: a two-sample Mendelian randomized study. *Eur. Spine J.* doi: 10.1007/s00586-023-08059-8 Online ahead of print.

Zhou, X., Xu, J., Zhang, X., Zhao, Y., and Duan, X. (2024). Causal relationships between gut microbiota and primary open-angle Glaucoma: a mendelian randomization and mediation analysis of Glaucoma endophenotypes. *Exp. Eye Res.* 240:109788. doi: 10.1016/j.exer.2024.109788

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Association of symptomatic upper respiratory tract infections with the alteration of the oropharyngeal microbiome in a cohort of school children in Côte d'Ivoire

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Introduction: The oropharyngeal microbiome plays an important role in protection against infectious agents when in balance. Despite use of vaccines and antibiotic therapy to prevent respiratory tract infections, they remain one of the major causes of mortality and morbidity in Low- and middle-income countries. Hence the need to explore other approaches to prevention by identifying microbial biomarkers that could be leveraged to modify the microbiota in order to enhance protection against pathogenic bacteria. The aim of this study was to analyze the oropharyngeal microbiome (OPM) of schoolchildren in Côte d'Ivoire presenting symptoms of upper respiratory tract infections (URTI) for better prevention strategy.

Methods: Primary schools' children in Korhogo (n = 37) and Abidjan (n = 39) were followed for six months with monthly oropharyngeal sampling. Clinical diagnostic of URT infection was performed and nucleic acid extracted from oropharyngeal swabs were used for 16S rRNA metagenomic analysis and RT-PCR.

Results: The clinical examination of children's throat in Abidjan and Korhogo identified respectively 17 (43.59%) and 15 (40.54%) participants with visible symptoms of URTIs, with 26 episodes of infection in Abidjan and 24 in Korhogo. Carriage of *Haemophilus influenzae* (12%), *Streptococcus pneumoniae* (6%) and SARS-CoV-2 (6%) was confirmed by PCR. A significant difference in

alpha diversity was found between children colonized by *S. pneumoniae* and those that were not (p = 0.022). There was also a significant difference in alpha diversity between children colonised with *H. influenzae* and those who were not (p = 0.017). No significant difference was found for SARS-CoV-2. *Sphingomonas*, *Ralstonia* and Rothia were significantly enriched in non-carriers of *S. pneumoniae*; *Actinobacillus* was significantly enriched in non-carriers of *H. influenzae*; *Actinobacillus* and *Porphyromonas* were significantly enriched in non-carriers of SARS-CoV-2 (p < 0.001).

Discussion: Nearly 40% of children showed clinical symptoms of infection not related to geographical location. The OPM showed an imbalance during *H. influenzae* and *S. pneumoniae* carriage. This study provides a baseline understanding of microbiome markers in URTIs in children for future research, to develop targeted interventions aimed at restoring the microbial balance and reducing the symptoms associated with RTIs.

KEYWORDS

oropharyngeal microbiome, upper respiratory tract infections, SARS-CoV-2, 16S rRNA metagenomic, microbial markers, Côte d'Ivoire

1 Introduction

The human microbiome, represented by 10 to 100 trillion symbiotic microbial cells, is considered beneficial to the human host; it is involved in the differentiation of mucosal structure and function, and stimulates innate and adaptive immune systems (Ursell et al., 2012). The composition of the pharyngeal microbiota and the collection of its genetic information, "microbiome", plays a crucial role in maintenance of respiratory health by providing "colonization resistance" against pathogen invasion (Blaser and Falkow, 2009). However, an imbalance in its composition, for example through acquisition of new pathogens, viral co-infection, or other host or environmental factors, has been associated with increased risk of infections such as pneumonia, cystic fibrosis, colds, Covid-19, and meningitis (de Koff et al., 2016; Piters et al., 2016; Xiang et al., 2020; Diallo et al., 2023).

Respiratory tract infections (RTIs) are a major cause of morbidity and mortality worldwide, despite effective treatments with antibiotics and availability of certain vaccines. According to a recent World Health Organization (WHO) report, they are the leading cause of death among children and adults worldwide, and are responsible for around 4.25 million deaths worldwide every year (Mayor, 2010; Bogaert et al., 2011; World Health Organization, 2020).

In the inter-tropical zone of Côte d'Ivoire these RTIs are one of the ten leading causes of death among children and represent the second cause of morbidity after malaria according to the annual Health report of 2020, with a morbidity rate of 54.77‰ (Centers for Disease Control and Prevention, 2023). Although little studied, restoring the airway microbiome is a possible route to prevent RTIs and could become an additional intervention tool alongside antibiotics and vaccines (Cho and Blaser, 2012). In the airways of children, viruses such as rhinoviruses, influenza viruses, respiratory syncytial virus, adenoviruses and coronaviruses are the main causes of viral respiratory infections (Mahony, 2008). Bacteria such as *Haemophilus influenzae, Corynebacteria* species, *Streptococcus pneumoniae, Staphylococcus aureus, Moraxella catarrhalis and Prevotella melanino-genica* frequently cause bacterial respiratory infections (Brook et al., 1981; Karaman et al., 2009; Maleki et al., 2020).

Some of these potential pathogens are also commonly found as members of the normal flora of the human oral cavity and can be detected in nasal or respiratory secretions. The asymptomatic or symptomatic presence of these microorganisms, combined with a general lower application of hygiene measures, make children more likely to transmit these pathogens to others. The likelihood of transmission is further enhanced when large numbers of children are gathered, such as in day care centres and schools (Msd Manual Consumer Version, 2023). It is therefore important to characterize the composition of the OPM of schoolchildren in order to better prepare public health interventions. The concept of using microbial markers as a non-invasive diagnostic tool for diseases has been gradually developed due to new sequencing technologies (Gao et al., 2021). The presence of microorganisms, and their associated metabolites, can provide signatures specific to an infection (Times, 2020). Microbial signatures are used, not for the diagnosis of infections as such, but to predict certain aspects of classical infectious diseases, such as disease severity and progression (Khanna et al., 2016). Microbiome can be affected by multiple factors, such as life style, diet, environment and therefore studies in a particular setting may not be representative of the microbiome elsewhere. However, in Côte d'Ivoire, to our knowledge, there are no studies of the OPM in children and therefore no baseline data to build on.

A better understanding of the interactions between micro-organisms in the oropharynx will contribute to the development of new therapeutic agents or preventive measures likely to improve respiratory health outcomes and help stratify at-risk populations to better target current interventional approaches. This study aims to characterize the variations in the OPM of school children from

northern and southern Côte d'Ivoire with visible clinical upper respiratory tract infections (URTI) in order to determine potential biomarkers that could be used for surveillance and responses.

2 Materials and methods

2.1 Study sites and participants

Two sites were selected for this study conducted in Côte d'Ivoire, West Africa: one in the northern town of Korhogo and one in the southern city of Abidjan. A public school was targeted in both cities. Children between 8 and 12 years old were invited to participate in the study that was conducted from November 2020 to April 2021. A cohort of schoolchildren was selected and followed in two primary school in Korhogo (n = 37) and Abidjan (n = 39). These numbers derived from published studies of the nasopharyngeal microbiome that used a similar sample size and were able to identify a distinct microbial profile and relevant changes (Biesbroek et al., 2014; Borges et al., 2018). The inclusion factors were school enrolment, age between 08 to 12 years and consent from parents. Children outside this age range were excluded.

That cohort was skewed toward girls, with a total of 29 girls (76.3) and 9 boys (23.7) in Abidjan. Korhogo had a more balanced male-female ratio, with 19 girls (51.4%) and 18 boys (48.6%).

Prior to inclusion in the study, informed consent was obtained from parents or legal guardian and written assent from the participant older than 10 years. Ethical approval from the National Ethical Committee was also obtained (IRB000111917). A questionnaire on infection risk factors and oral health was also administered to participants (Supplementary Tables 1, 2) by the team medical doctor.

Oral health questionnaire was completed by the team doctor every month prior to sampling. The criteria for consideration as symptomatic URTIs were the presence of visible signs such as nasal discharge, swelling, redness, irritation of the throat.

The risk factor questionnaire was completed by children's parents or legal guardians after obtention of the signed informed consent. Nutritional status was defined in terms of the child's body mass index. The modalities considered for the analysis were: Mild malnutrition, Moderate malnutrition, Severe malnutrition, normal, obesity and excess weight. A nurse from the school's associated clinic, part of the health system in Côte d'Ivoire, was also present at each visit to attend to the children care and refer them, to the clinic if deemed necessary. Children aged from 8 to 12 years were invited to participate based on the school administration list, using a random selection to obtain the total number of participants. One participant dropped out of the study in Abidjan (S2), the other variation in number of participants were due to children absence from school on respective sampling days.

2.2 Sample collection

Oropharyngeal swab samples were collected monthly from each participant using sterile swabs. Swabs were stored in 1 ml of RNAprotect to protect RNA stability. RNAprotect samples were aliquoted into two different tubes and stored at −80°C. The first sample was used for 16S rRNA sequencing and RT-PCR detection of *S. pneumoniae* and *H. influenzae*. The second one was used for detection of SARS-CoV-2.

All swab samples were collected by qualified medical personnel trained in the study's sampling procedures under the supervision of school medical officers. The physician performed a clinical examination of the children's oral health prior to any sampling to determine the presence of infections or visible irritations in the children's throats. This made it possible to identify a number of children with visible sign of URTIs throughout the cohort (nasal discharge, swelling, redness and irritation of the throat); as well as children with no clinical signs of infection.

2.3 DNA extraction

DNA samples were extracted from the oropharyngeal samples using a Qiagen DNeasy PowerSoil kit (Qiagen, Germany) following the manufacturer's instructions. DNA samples were quantified by a Qubit 4 fluorometer (Invitrogen, Carlsbad, CA, USA), and molecular size was estimated by 1% agarose gel electrophoresis.

2.4 PCR amplification of the V3-V4 region

The V3-V4 hypervariable region of the 16S rRNA gene was targeted for sequencing. Forward and reverse primers targeting this region were generated with an Illumina adapter overhang sequence appended to the primer pair for compatibility with Illumina index and sequencing adapters (Klindworth et al., 2013). Amplifications were done in 25 µl reactions with 12.5 µl Q5® Hot Start High-Fidelity 2X Master Mix (NEB), 5 µl of 1 µM forward and reverse 16S primer and 2.5 µl of template. Reactions were carried out on ABI Veriti thermocyclers (Applied Biosytems) under the following conditions: 95°C for 3 min, 25 cycles of; 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by 72°C for 5 min and a final hold at 4°C. The amplified products were then verified on 1.5 % agarose gel with a product of ∼550 bp expected.

2.5 Library preparation and sequencing

Amplified products were further purified using Agencourt AMPure XP beads (BeckmanCoulter) for library preparation. Libraries were then prepared by ligating Illumina dual indices and Illumina sequencing adapters to the purified amplicons using the NexteraXT index kit. Attachment of the indices was performed using 5 µL of the 16S amplicon DNA, 5 µL of Illumina Nextera XT Index Primer 1 (N7xx), 5 µL of Nextera XT Index Primer 2 (S5xx), 25 µL of Q5® Hot Start High-Fidelity 2X Master Mix (NEB), and 10 µL of PCR-grade water (Ambion). The reactions were carried out on ABI Veriti thermocyclers (Applied Biosytems) under the following conditions 95 °C for3 min, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, a final extension at 72°C for 5 min and a final hold at 4°C. The libraries were then purified using Agencourt AMPure XP beads

(BeckmanCoulter) and thereafter size distribution and library quality control performed using the Agilent 2100 Bioanalyzer (Agilent) to confirm the expected size distribution and the quality. The libraries were finally quantified using the Qubit dsDNA HS kit on the Qubit 4.0 flourometer (Life Technologies) normalized and pooled at equimolar concentration based on the Qubit results. A total of 10pM of the pooled library was then spiked with 8% Phix (v3) for sequencing. Sequencing was done on the Illumina MiSeq system using 2 x 300 bp PE sequencing with the MiSeq® Reagent Kit v3 (600 cycle).

2.6 Identification of pathogens by RT-PCR

All the RT-PCR tests were done retrospectively on stored samples multiple months after the end of the sample collection.

- Bacterial RT-PCR

Detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* was done using a multiplex assay targeting *S. pneumoniae*, *H. influenzae* and *Neisseria meningitidis*. A mastermix of 15 μL was prepared as follow: 7.5 μL of 2x Master Mix, 0.5 μL of each primer and probe, and 1 μL of MgCl2 along with 2 μL of DNA previously extracted for the 16SrNA sequencing. Primers and probes used are in Table 1.

2.7 RNA extraction

Ribonucleic acid (RNA) extraction was performed on oropharyngeal samples using the QIAamp viral RNA kit for viral pathogens (Qiagen, Germany) according to the manufacturer's recommendations. RNA quality was checked using the Qubit RNA HS kit.

- **RT-qPCR for SARS-CoV-2**

RT-qPCR of SARS-CoV-2 was performed in 20 μL of reaction medium containing 10 μL of Luna Universal Probe One-Step Reaction Mix (2X), 1 μL of Luna WarmStart RT Enzyme Mix (20X), 0.8 μL of each E Sarbeco Forward and reverse primer (10 μM), 0.4 μL of E Serbeco Probe, 5 μL of Nuclease-free Water, and 2 μL of RNA. Primers and probe used have shown in Table 2 (Corman et al., 2020).

2.8 16S data processing

The raw 16S rRNA sequences were analyzed using R-4.2.1 software. They were quality filtered and denoised using the filterAndTrim function of DADA2 (Divisive Amplicon Denoising Algorithm) (Callahan et al., 2016). Poor quality ends were truncated, with a truncation length of 270 nts and 260 nts for forward and reverse reads respectively. The resulting sequences were then dereplicated, denoised and merged, and the removeBimeraDenovo function was used to remove chimeras in these sequences. The resulting amplicon sequence variants (ASVs) were classified by taxonomy and mapped to a reference set of operational taxonomic units (OTUs) at 99% sequence similarity using the Silva database (Silva_nr_v138) (Quast et al., 2013) with the assign-Taxonomy function. The data set was decontaminated using the "decontam" package (Nm et al., 2018) and then combined into a phyloseq object.

2.9 Statistical analysis

Shannon index, alpha-diversity and relative abundances (at phylum and genus level) were performed using the phyloseq package (McMurdie and Holmes, 2013). Prior to statistical analysis, the data were rarefied with a depth of 30000 reads per sample. Shapiro-Wilk test were used to test data normality for quantitative variables before comparing microbial diversity between the groups.

Wilcoxon rank sum tests and Kruskal-Wallis were used to compare microbial diversity in participant groups. Fisher or Chi-square tests were used for categorical data from the risk factors questionnaire, as well as the relationship between clinical symptom of infections and geographical location. Values of $p < 0.05$ were considered significant. All data were plotted using the ggplot2 package.

Principal coordinate analysis (PCoA) was performed according to the Bray-Curtis dissimilarity matrix for beta diversity. The association between oropharyngeal microbiome and symptomatic URTIs was also tested by permutational multivariate analysis of variance using the vegan package.

Bacteria genus differentially enriched according to status (COVID-19 and not; *H. influenzae* and not; *S. pneumoniae* and not) were analyzed at OTUs level using the DESeq2 method (McMurdie and Holmes, 2013). The package DESeq2 provides methods to test bacteria differentially enriched by use of negative binomial generalized linear models.

3 Results

3.1 Clinical and RT-PCR diagnosis of upper respiratory tract infections

During the study, 17 (43.59%) participants in Abidjan and 15 (40.54%) in Korhogo had visible signs of URTI following the clinical examination, with 26 episodes of infection in Abidjan and 24 in Korhogo. Eleven children had repeated episodes of infection.

The prevalence of these symptoms by site, visit and sex is presented in Table 3. These symptoms of infection (runny nose, coughing, redness and throat irritation) were not differently associated with geographical location ($p > 0.05$; Fisher test). The change in prevalence of clinical symptoms of respiratory infections was significant throughout the different visits in the north ($p = 0.026$, Fisher test), but not in the south ($p > 0.05$), with a high prevalence in February and April. There was no significant difference between the application of hand hygiene measures, the nutritional status of participants (in terms of the child's body mass index), promiscuity or contact with animals in the two study sites

TABLE 1 List of primers and probes used for bacterial RT-PCR.

Organisms	Primers and probes	Sequences (5′-3′)	5′ dye	3′ quencher	References
S. pneumoniae	*lytA*- CDC-F	ACGCAATCTAGCAGATGAAGCA			Carvalho et al., 2007
	lytA-CDC-R	TCGTGCGTTTTAATTCCAGCT			
	lytA- CDC-Probe	TGCCGAAAACGCTTGATACAGGGAG	ROX	BHQ2	
	*SP_2020*_F	TAAACAGTTTGCCTGTAGTCG			Tavares et al., 2019
	*SP_2020*_R	CCCGGATATCTCTTTCTGGA			
	*SP_2020*_Probe	AACCTTTGTTCTCTCTCGTGGCAGCTCAA	Cy5	BHQ2	
H. influenzae	*HelS*-F	CCGGGTGCGGTAGAATTTAATAA			Arjarquah et al., 2022
	HelA-R	CTGATTTTTCAGTGCTGTCTTTGC			
	Hel- Probe	ACAGCCACAACGGTAAAGTGTTCTACG	FAM	BHQ1	

TABLE 2 Primers and probe used to target the SARS-CoV-2 E gene in oropharyngeal samples.

Primer/probe	Sequences (5′-3′)
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA
E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ

($p > 0.5$). We also found no link between socio-economic factors and clinical symptoms of URT infections ($p > 0.5$).

In addition to the clinical signs of infection identified, the proportions of micro-organisms targeted per site, visit and gender are shown in Table 4. *Haemophilus influenzae* (12%), *Streptococcus pneumoniae* (6%) and SARS-CoV-2 (6%) were identified in participants presenting symptoms of URTIs (Table 5). Co-infection between *H. influenzae* and *S. pneumoniae* (2%) was also observed in some children with clinical suspicion of URTI. The prevalence of *H. influenzae* and *S. pneumoniae* was associated with geographical location ($p < 0.001$; Chi-square tests) with a higher prevalence in Korhogo than in Abidjan.

3.2 Characterization of the structure of the oropharyngeal microbiome based on 16S rRNA analysis

Of the 434 samples received after sequencing, 427 passed the filter for 16S rRNA analysis. Of these sequences, 46 were from participants with clinical symptoms of infection out of 50. Analysis of alpha diversity showed that there was no significant difference between the microbial profile of children with clinical suspicion of upper respiratory infection and that of uninfected children using Shannon's diversity index ($p = 0.25$) (**Figure 1**). A geographical difference was however observed in the microbial profile of children from the two study sites (Abidjan and Korhogo) showing a greater abundance and microbial diversity in individuals from Abidjan than in those from Korhogo using a Shannon diversity index ($p < 0.001$, Wilcoxon rank-sum test) (**Figure 2**). No significant difference was observed in the microbial profile of participants over time and according to gender ($p > 0.5$).

Microbial diversity of each individual who had at least one URTI symptom during the cohort was plotted over time (per visit). This showed a generally high microbial abundance in the presence of infection. More people were infected when Shannon diversity was high in Abidjan, with values between 2.4 and 4 (**Figure 3**).

Analysis of alpha diversity showed a significant difference between symptomatic carriers and non-carriers of *S. Pneumoniae* and *H. influenzae* ($p < 0.05$). Microbial diversity was lower in participants carrying both micro-organisms. Alpha diversity showed no association between symptomatic carriers and non-carriers of SARS-CoV-2 ($p = 0.94$) (Figure 4). Beta diversity results (Supplementary Figure 1) also revealed that symptomatic carriers and non-carriers of *S. Pneumoniae* and *H. influenzae* have distinct OPM compositions ($p = 0.001$).

The most represented phyla in the samples were Proteobacteria, Bacteroidota, Firmicutes and Fusobacteriota with proportions higher than 15%. At the genus level, 18 different genera were detected in the children's oropharynx, with an abundance greater than 1%. Taxonomic classification also showed that the relative abundances of the different bacterial genera in the participants' upper respiratory tracts differed from one infection to another. We found that the genus *Streptococcus* were significantly more abundant in children who had no clinical symptoms of infection than in those who did (17.37% VS 11.92%, $p = 0.001$) (Figure 5).

When zooming only on children that had clinically diagnosed URTI, *Leptotrichia* was dominant in carriers of *S. pneumoniae* compared to non-carriers (4.27%; 1.45%; $p = 0.028$). The abundance of *Actinobacillus* was also very low in all three types of carriage (Figure 6). The proportions of these different genus in terms of abundance are shown in Table 6. Identification to species level was not possible for all ASVs.

3.3 Determination of microbial markers involved in symptomatic upper respiratory tract infections

Microbial markers were identified as predominant in carriage of each microorganism. The results revealed that the genera

TABLE 3 Prevalence of suspected clinical infections in both study sites.

Site	Visits						Gender		Total
	S1	S2	S3	S4	S5	S6	Boys	Girls	Episodes of infection
Abidjan total (n)	**38**	**38**	**38**	**38**	**37**	**36**	**53**	**172**	
Infection Yes n (%)	3 (7.9)	10 (26.3)	3 (7.9)	2 (5.26)	4 (10.81)	4 (11.11)	1 (1.89)	25 (14.5)	26
Infection No n (%)	35 (92.1)	28 (73.7)	35 (92.1)	36 (94.7)	33 (89.2)	32 (88.9)	52 (98.1)	147 (85.5)	199
Korhogo total (n)	**37**	**37**	**37**	**37**	**36**	**36**	**107**	**113**	
Infection Yes n (%)	1 (2.7)	3 (8.11)	1 (2.7)	8 (21.62)	4 (11.11)	7 (19.44)	7 (6.6)	17 (15)	24
Infection No n (%)	36 (97.3)	34 (91.9)	36 (97.3)	29 (78.4)	32 (88.9)	29 (80.6)	100 (93.4)	96 (85)	196

For visits, S represents the different surveys (S1=Survey 1, S6=Survey 6).

TABLE 4 RT-PCR identification of *H. influenzae*, *S. pneumoniae*, and SARS-CoV-2 by site, visit and gender.

Site	Visits						*p*-value	Gender		Total episodes of infection	*p*-value
	S1	S2	S3	S4	S5	S6		Boys	Girls		
Total Abidjan (n)	**38**	**38**	**38**	**38**	**37**	**36**		**53**	**172**		
H. influenzae Yes n (%)	4 (10.5)	5 (13.16)	5 (13.16)	3 (7.89)	7 (18.92)	2 (5.56)	0.57	8 (15.09%)	18 (10.47%)	26	0.5
H. influenzae No n (%)	34 (89.5)	33 (86.84)	33 (86.84)	35 (92.1)	30 (81.08)	34 (94.44)		45 (84.91%)	154 (89.53%)	199	
S. pneumoniae Yes n (%)	0	3 (7.89)	1 (2.63)	0	1 (2.70)	1 (2.78)	0.32	3 (5.66%)	3 (1.74%)	6	0.14
S. pneumoniae No n (%)	38 (100)	35 (92.11)	37 (97.37)	38 (100)	36 (97.30)	35 (97.22)		50 (94.34%)	169 (98.26%)	219	
SARS-CoV-2 Yes n (%)	0	0	0	1 (2.63)	1 (2.70)	3 (8.33)	0.06	1 (1.89%)	4 (2.33%)	5	1
SARS-CoV-2 No n (%)	38 (100)	38 (100)	38 (100)	37 (97.37)	36 (97.30)	33 (91.67)		52 (98.11%)	168 (97.67%)	220	
Total Korhogo (n)	**37**	**37**	**37**	**37**	**36**	**36**		**107**	**113**		
H. influenzae Yes n (%)	5 (13.51)	7 (18.92)	9 (24.32)	12 (32.43)	11 (30.56)	12 (33.33)	0.28	31 (28.97%)	25 (22.12%)	56	0.31
H. influenzae No n (%)	32 (86.49)	30 (81.08)	28 (75.68)	25 (67.57)	25 (69.44)	24 (66.67)		76 (71.03%)	88 (77.88%)	164	
S. pneumoniae Yes n (%)	6 (16.2)	2 (5.41)	4 (10.81)	13 (35.14)	3 (8.33)	8 (22.22)	0.006	22 (20.56%)	14 (12.39%)	36	0.15
S. pneumoniae No n (%)	31 (83.8)	35 (94.59)	33 (89.19)	24 (64.86)	33 (91.67)	28 (77.78)		85 (79.44%)	99 (87.61%)	184	
SARS-CoV-2 Yes n (%)	0	0	0	7 (18.92)	3 (8.33)	0	<0.001	5 (4.7%)	5 (4.42%)	10	1
SARS-CoV-2 No n (%)	37 (100)	37 (100)	37 (100)	30 (81.08)	33 (91.67)	36 (100)		102 (95.3%)	108 (95.58%)	210	

"Yes" and "No" indicate the presence or absence of microorganisms. For visits, S represents the different surveys (S1 = Survey 1, S6 = Survey 6).

Sphingomonas, Ralstonia and *Rothia* were significantly enriched in the absence of *S. pneumoniae* (5.71%; 4.23%; 2.08%) when compared to carriers (0%) ($p < 0.001$; log2 fold-change: −26.1; −24.8; −9.9). *Actinobacillus* was significantly enriched in the absence of *Haemophilus influenzae* (2.73% VS 0%; $p < 0.001$); *Actinobacillus* and *Porphyromonas* were microbial markers of presence of SARS-CoV-2 (0%; 0.39%) when compared to non-carriers (2.54%, 7.57%; with $p < 0.001$; log2 fold-change: −25.8 and −5.1) (Table 7).

4 Discussion

The importance of respiratory infections in children, particularly in low- and middle-income countries, is very evident as it has been shown that 30% of the annual mortality rate in children is associated with acute respiratory infections (Liu et al., 2018). This study is the first to report on the oropharyngeal microbiome in children with suspicions of URTIs in Côte d'Ivoire using 16S rRNA sequencing.

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TABLE 5 Prevalence of microorganisms identified by RT-PCR.

		H. influenzae		*p*-value	SARS-CoV-2		*p*-value	*S. pneumoniae*		*p*-value	*H. influenzae* and *S. pneumoniae*		*P*-value
		Yes	No		Yes	No		Yes	No		Yes	No	
Clinical infections	Yes n (%)	**6 (12%)**	44 (88%)	0.25	**3 (6%)**	47 (94%)	0.23	**3 (6%)**	47 (94%)	0.6	**1 (2%)**	49 (98%)	0.34
	No n (%)	76 (19.24%)	319 (80.76%)		12 (3%)	383 (97%)		39 (9.9%)	356 (90.1%)		26 (6.58%)	369 (93.42%)	

Values in bold represent the prevalence of participants with symptoms of clinical infection and carrying microorganisms detected by RT-PCR.

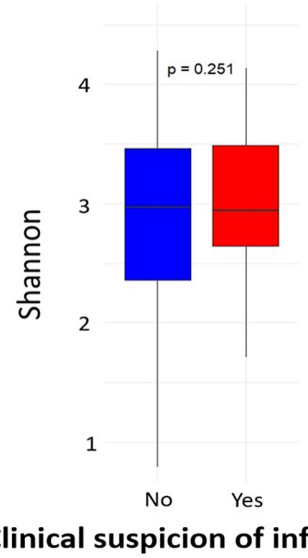


FIGURE 1
Alpha diversity (Shannon index) of the oropharyngeal microbiome in participants with clinical symptom of infections and healthy children.

Several participants were attending school despite harboring symptoms of respiratory tract infections such as nasal discharge, swelling, redness and irritation of the throat. This suggests that common URTIs symptoms (runny nose, coughing, etc.) are not considered alarming enough for some parents to keep children at home. Previous research has shown that infectious disease management in schools is essential to minimize the spread of respiratory infections (Ridenhour et al., 2011). In addition, health campaigns promoting hand hygiene and use of hand sanitiser have been shown to be effective in reducing illness and absenteeism (White et al., 2005; Azor-Martínez et al., 2014).

Variation in clinical symptoms of respiratory infections was significant throughout the year in participants in Korhogo (north) but not in Abidjan (south), with a high prevalence in February and April. This means that seasonality could be a factor to be taken into account in the occurrence of these infections in the northern part of the country, given that the different sampling waves covered both the dry season and the approach of the rainy season. Previous studies have shown a link between the seasonality of URTIs and environmental factors. In fact, certain viruses such as rhinovirus, adenovirus and influenza viruses A and B have been more active during certain seasons. Temperature and humidity also played a role in their transmission. (Price et al., 2019).

Paynter et al. (2015) have also shown that human immunity varies according to seasons, which could contribute to the seasonal nature of respiratory infections. This study also confirmed the presence of *Haemophilus influenzae, Streptococcus pneumoniae* and SARS-CoV-2 in the participants' oropharynx. Despite the fact that these three types of infections were more strongly detected in children in the north than in those in the south, *Haemophilus influenzae* remained the most common species carried in the oropharynx.

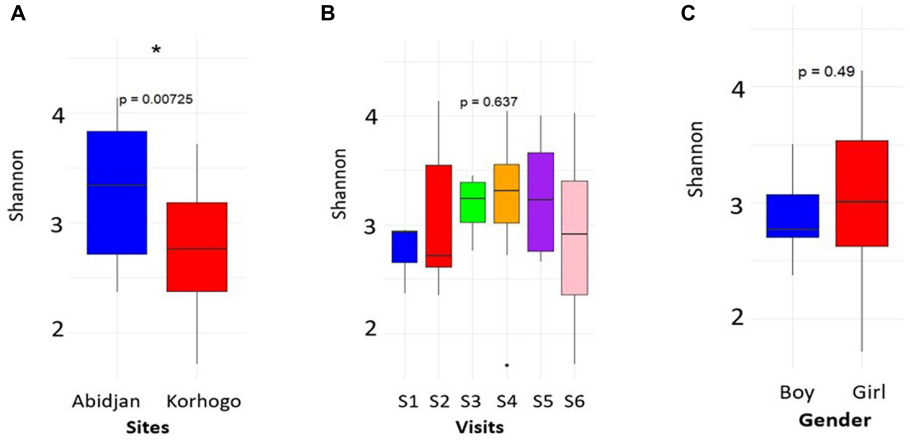


FIGURE 2
Plots showing the alpha diversity (Shannon index) of the oropharyngeal microbiome in participants by site **(A)**, visit **(B)** and gender **(C)**. The asterisk shows a geographical difference in the participants' microbiome. For visits, S represents the different surveys (S1 = Survey 1).

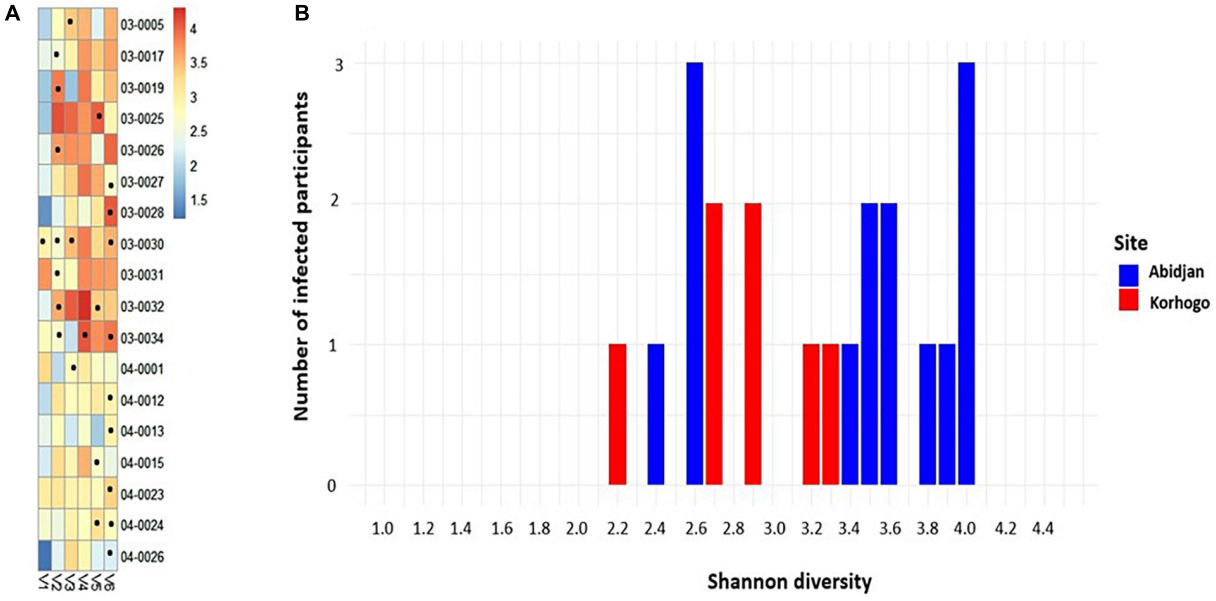


FIGURE 3
Heat map of the relative abundances of differential OTUs in the group of positive samples whose sequences were received. Each point represents the visit during which the participant had a symptom of irritation, redness or inflammation in the throat **(A)**. Bar plot showing the number of people with clinical infections as a function of Shannon diversity at each study site (blue in Abidjan and red in Korhogo) **(B)**. **(B)** Produced taking into account the Shannon diversity of the first figure.

These results are in line with a previous study showing the persistence of *Haemophilus influenzae* as a major cause of acute respiratory infections in sub-Saharan Africa (Lagare et al., 2015).

The fact that the prevalence of carriage of these micro-organisms is considerably different from one area to another may be due to a number of factors, such as seasonal difference between the two sites and promiscuity (Wimalasena et al., 2021). The link between seasonality and respiratory infections has also been demonstrated in another study in Kenya that showed an association between the rainy season and nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae* independent of the effect of age (Abdullahi et al., 2008).

The role of children in the transmission of COVID-19 has been a subject of debate worldwide (Dattner et al., 2021). A study by Jiehao et al. (2020) of children diagnosed with COVID-19 indicates that the potential risk of transmission from infected children to adult contacts should not be overlooked. Our study retrospectively identified COVID-19 patients with what can be considered benign to mild symptoms. SARS-CoV-2 was identified in this study in 15 participants between February and March 2021 (05 in Abidjan and 10 in Korhogo), coinciding with a period of high virus spread in Côte d'Ivoire according to WHO (World Health Organization, 2024). This results reinforce the idea that children were mostly asymptomatic carriers during the

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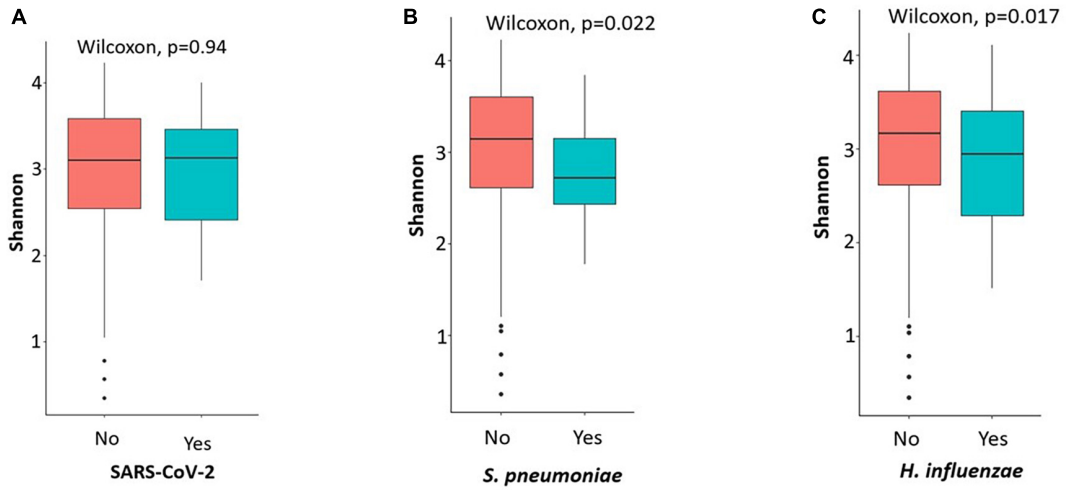


FIGURE 4
Plots showing the alpha diversity (Shannon index) of the oropharyngeal microbiome in participants carriers of SARS-CoV-2 and not **(A)**, *S. pneumoniae* **(B)** and *H. influenzae* **(C)**. Microbial diversity was low when participants were carriers of *H. influenzae* and *S. pneumoniae*.

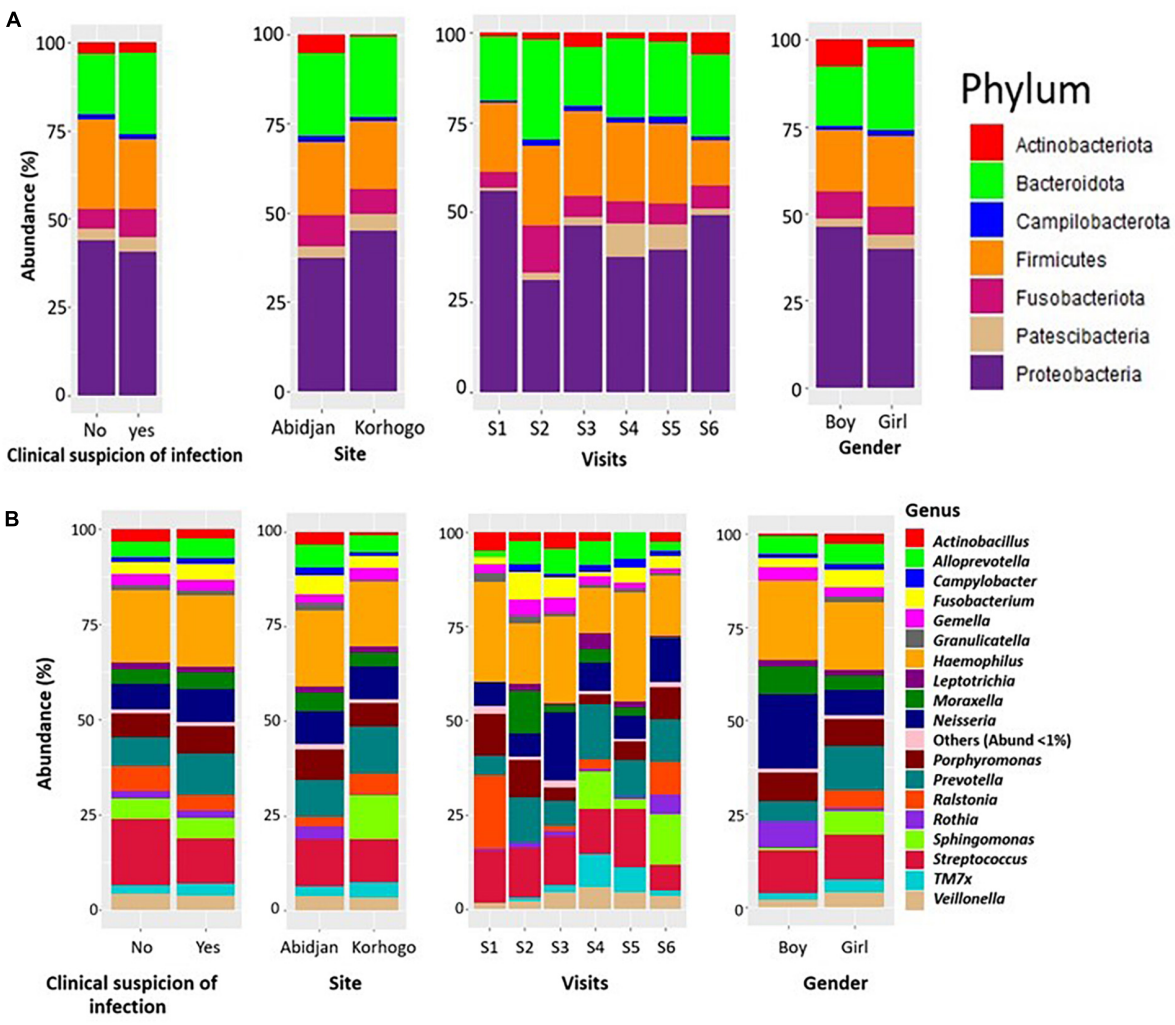


FIGURE 5
Bar charts illustrating the relative abundance of bacterial phylum **(A)** and genus **(B)** in participants with clinically suspected throat infection throughout the cohort. The representations were made generally, according to site, visit and gender.

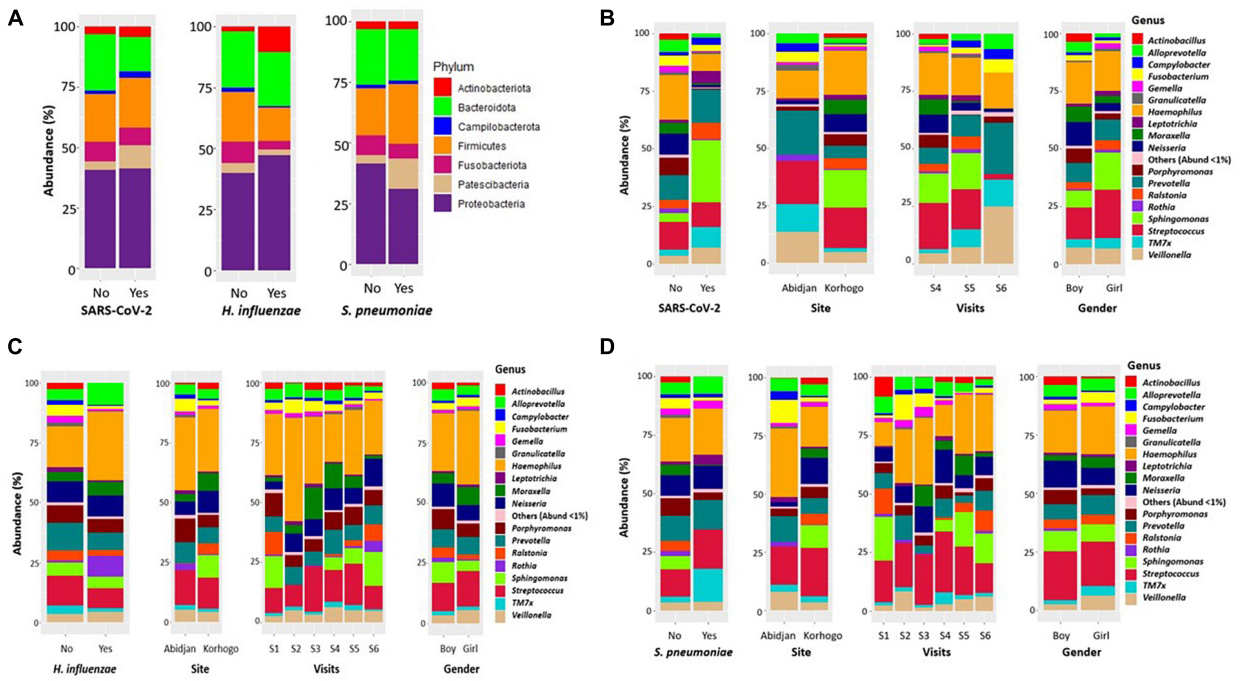


FIGURE 6
Bar charts illustrating the relative abundance of bacterial phylum **(A)** and genera in the oropharynx of participants carrying SARS-Cov-2 **(B)**, *H. influenzae* **(C)** and *S. pneumoniae* **(D)** All bacterial genera whose abundance was less than 1% were represented by "other.".

pandemic and could have played a role in the transmission of the virus to their household members (Zhu et al., 2020). More carriage studies in African children could have helped better study the diversity of the virus.

16S rRNA sequencing analysis showed that the oropharyngeal microbial diversity was significantly reduced in children who were symptomatic carriers of *H. influenzae* and *S. pneumoniae* infection, indicating the presence of oropharyngeal microbial dysbiosis in these children. Recent data using whole genome sequencing have also shown a reduction in the diversity of the oropharyngeal microbiome in elderly and adult patients with *Streptococcus pneumoniae* associated pneumonia (Piters et al., 2016). Laufer et al. (2011) also observed lower microbial diversity in the presence of *S. pneumoniae* in the URT of children with otitis media. These results suggest that dysbiosis of the URT microbiome may be associated with various respiratory infections, underlining the importance of maintaining a healthy microbial balance in this region.

16S rRNA analysis also showed that the dynamics of the microbiome between children in the north and south differed significantly ($p < 0.001$); with greater bacterial diversity in children from Abidjan than those from Korhogo. This could be explained by different lifestyles, including diet, genetics and host physiology as demonstrated in a previous study conducted on the salivary microbiome of populations living in different geographical and climatic environments (Li et al., 2014). It shows that there is considerable geographic variation in the microbiota of selected individuals. This diversity should be taken into account in future control strategies based on microbiota modulation.

The phyla that were highly represented in both groups of participants (children with clinical suspicion of infections and not) are Firmicutes, Proteobacteria and Bacteroidota with relative abundance above 15%. These are part of the five major bacterial phyla identified by the Human Microbiome Project (ScienceDirect Topics, 2007; Gao et al., 2014). Other phyla such as Fusobacteriota and Actinobacteriota were also represented to a lesser extent in the microbiome of children in this cohort. The identification of most of these phyla was also done in the study by Xia et al. (2022) on the pulmonary microbiome. This could be explained by the fact that the lungs microbiota represents an extension of the upper respiratory tract. The oropharynx is the junction between the mouth, nasopharynx, larynx, lower respiratory tract and gastrointestinal tract. It is also exposed to exogenous and endogenous microorganisms (Chen et al., 2021). Therefore, the set of species in the oropharyngeal microbiota may generally be larger than in other niches.

Of the microbial genera most present in terms of proportion (*Haemophilus, Streptococcus, Prevotella, Neisseria and Porphyromonas*), only *Streptococcus* showed a statistically significant difference in abundance between the groups of symptomatic and non-symptomatic children ($p = 0.001$). The prevalence of these bacteria in the oropharyngeal microbiome has been demonstrated in previous studies in healthy and asthmatic children, those suffering from Cystic Fibrosis, and in studies focusing on COVID-19 (Charlson et al., 2011; Boutin et al., 2015; Depner et al., 2017).

The genus *Sphingomonas, Ralstonia* and *Rothia* were identified as microbial markers of symptomatic carriage of *S. pneumoniae*. This suggests that their low abundance may also play a role in the modification of the microbiome and the onset of infection, as these three bacterial genera were not present in infected children. These results differ from those of Piters et al. (2016) who showed a

TABLE 6 Relative abundance of the 18 most abundant bacterial genera in the microbiome of individuals according to whether or not they are symptomatic carriers.

Genus	Clinical infections		P	Sars-Cov-2			*H. influenzae*			*S. pneumoniae*		
	No	Yes		No	Yes	p	No	Yes	p	No	Yes	p
Actinobacillus	3.35	2.38	0.499	2.54	0	0.397	2.73	0	0.205	2.54	0	0.769
Alloprevotella	3.89	5.17	0.792	5.4	1.77	0.4	4.57	9.15	0.187	5.01	7.43	0.165
Campylobacter	1.42	1.67	0.202	1.58	3.07	0.509	1.86	0.45	0.064	1.69	1.42	0.539
Fusobacterium	3.24	4.25	0.03	4.36	2.64	0.339	4.67	1.47	0.074	4.45	1.36	0.211
Gemella	2.77	2.63	0.218	2.78	0.49	0.123	2.91	0.78	0.082	2.58	3.38	0.982
Granulicatella	1.26	1.27	0.425	1.3	0.88	0.391	1.42	0.26	0.24	1.36	0	0.297
Haemophilus	19.12	18.69	0.852	19.49	7.34	0.065	17.2	28.67	0.08	18.62	19.79	0.767
Leptotrichia	1.72	1.63	0.542	1.37	5.29	0.111	1.77	0.68	0.36	1.45	4.27	**0.028**
Moraxella	3.79	4.28	0.831	4.53	0.7	0.604	4.08	5.57	0.225	4.55	0.41	1
Neisseria	6.72	8.74	0.82	9.27	1.08	0.127	8.74	8.71	0.473	8.65	10.04	0.838
Porphyromonas	6.06	7.1	0.465	7.57	0.39	**0.01**	7.3	5.77	0.894	7.38	3.09	0.306
Prevotella	7.73	11	0.019	10.76	14.57	0.265	11.59	7.09	0.433	10.89	12.63	0.802
Ralstonia	6.47	3.96	0.196	3.74	7.06	0.191	4.19	2.41	0.559	4.23	0	0.28
Rothia	1.96	1.95	0.296	2.05	0.49	0.453	0.91	8.85	0.749	2.08	0	0.1
Sphingomonas	5.42	5.34	0.636	3.84	26.83	0.095	5.44	4.69	0.985	5.71	0	0.192
Streptococcus	17.37	11.92	**0.001**	12.01	10.71	0.847	12.47	8.28	0.286	11.59	16.77	0.09
TM7x	2.32	3.2	0.555	2.79	8.97	0.057	3.44	1.59	0.534	2.44	14.05	0.371
Veillonella	4.14	3.65	0.167	3.41	7.08	0.4	3.53	4.44	0.432	3.64	3.85	0.601
Others	1.24	1.17		1.21	0.64		1.18	1.14		1.15	1.53	

The different p-values were calculated using the Wilcoxon rank sum test.

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TABLE 7 Table showing the results for the most significant genera and phyla in the three types of infections using the DESeq2 differential abundance analysis.

baseMean	log2Fold Change	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus
H. influenzae carriage											
449.478154	−26.08070999	3.73333343	−6.9859043	2.83027E−12	5.09449E−11	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Actinobacillus*
S. pneumoniae carriage											
880.016161	−26.12988877	4.16702087	−6.2706402	3.59567E−10	6.4722E−09	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
966.564475	−24.75841785	4.52357889	−5.4731925	4.42E−08	3.978E−07	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia*
181.960938	−9.963195029	2.71187828	−3.6739094	0.000238867	0.001433205	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	*Rothia*
Sars-Cov-2 carriage											
449.478154	−25.78980787	5.3122002	−4.854826	1.20492E−06	1.92788E−05	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	*Actinobacillus*
1721.68874	−5.128442329	1.11016396	−4.619536	3.84599E−06	3.07679E−05	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Porphyromonadaceae	*Porphyromonas*

significantly higher relative abundance of *Rothia* in oropharyngeal microbiota of elderly pneumonia patients, suggesting that *Rothia* may play a role in the pathogenesis of the infection. *Rothia* has also been implicated in T helper 17 (Th17)-induced lung inflammation and pneumonia in immunocompromised patients (Segal et al., 2016). Furthermore, abundance of this microorganism in the upper respiratory tract has also been associated with an increased risk of otitis media in children (Laufer et al., 2011), suggesting a potential role for this bacterium in the pathogenesis of respiratory infections generally. Thus, variations in microbial composition between different age groups could contribute to differences in susceptibility to infection (Belibasakis, 2018). *Ralstonia*, in particular the species *insidiosa*, has been isolated from the respiratory tract of cystic fibrosis patients (Lin et al., 2023). This bacterium has low toxicity and is a conditional pathogen. However, contraction of bacterial or viral pathogens, environmental factors or immunological disturbances can potentially lead to dysbiosis and proliferation of pathogens, which could result in symptomatic infections due to this microorganism.

The genus *Actinobacilus* and *Porphyromonas* were identified as microbial markers in symptomatic SARS-CoV-2 carriage with very low abundance in carriers' individuals ($p < 0.001$). This decrease in *Actinobacillus* genus and *Porphyromonas* was observed in several previous studies in patients with COVID-19 (Ren et al., 2021; Soffritti et al., 2021; Wu et al., 2021; Cui et al., 2022; Gupta et al., 2022; Rafiqul Islam et al., 2022). These results suggest a potential role for these bacteria in the pathogenesis of COVID-19. However, further research is needed to fully understand the impact of these microbial markers in the context of COVID-19. *Actinobacilus* has also been identified as a microbial marker in symptomatic *Haemophilus influenzae* carriers.

These results demonstrate the importance of conducting regular surveillance of the carriage of upper respiratory tract pathogens in order to identify bacteria or viruses that are most prevalent and to prepare for the deployment of appropriate public health measures in the event of an epidemic. The microbiome results presented could be used as baseline data for the identification of biomarkers involved in URTIs in children despite the limited number of individuals.

The human upper respiratory tract microbiome is being studied more and more thanks to the development of new sequencing tools. However, the oropharynx has received less attention and, to our knowledge, few studies have been performed on the microbiome in Africa, especially on diseases of public health importance (Allali et al., 2021; Diallo et al., 2023). Additional studies including larger numbers of participants with a stratified spectrum of respiratory infections severity, will be useful to better understand the changes of the microbiota based on the progression of the disease.

The integration of other omics technologies such as RNA sequencing or metatranscriptomics will provide more specific gene expression information to better understand the metabolic characteristics of the children respiratory microbiome (Shakya et al., 2019). This approach could lead to a better understanding of the disease and more targeted approaches for treatment and prevention.

This study has certain limitations. Firstly, it used a cohort and therefore generalization of the results must be done with caution. Secondly, we were unable to identify causal relationships between symptomatic carriage of microorganisms and microbial

markers in the oropharynx using either functional or mechanistic data as this would require whole genome metagenomic. Finally, the monthly sampling may have prevented the identification of rapidly evolving changes in the microbiome focusing on rather long-lasting changes.

5 Conclusion

In our study cohort 40% of children showed clinical symptoms of infection without geographical location variation.

OPM of children is highly diverse and varies considerably between both sites with greater microbiota diversity in Abidjan (stable seasonality humid) than in Korogho (variable seasonality dry/rainy) in children presenting clinical signs of URT infection. A significant difference in the microbiota diversity was also found in children that carry *S. Pneumoniae* and *H. influenzae* ($P < 0.05$) with lower microbial diversity in patients with both infections. However, the OPM was not significantly different when comparing children with or without SARS-CoV-2 carriage. 16S rRNA analysis showed that *Sphingomonas, Ralstonia and Rothia* were significantly enriched by non-carriers of *S. pneumoniae; Actinobacillus* was significantly enriched by non-carriers of *H. influenzae; Actinobacillus* and *Porphyromonas* were significantly enriched by non-carriers of SARS-CoV-2 ($p < 0.001$). Our study has shown that changes in the microbiome composition may favor predisposition for certain microorganisms to cause symptomatic infections. Further characterization of the response of the microbiota to airway infections down to the species level as well as gene expression using novel sequencing technologies such as shotgun sequencing or metatranscriptomics is needed to better understand the transcriptional changes of the host and microbiota during infection. This will provide a better understanding of pathogenesis of respiratory tract infections in children over the long term, and will help understand the effects of current and future preventive measures.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra/PRJNA1064912, from SRX23222127 to SRX23221695.

Ethics statement

This project was approved by the Ethics Committee for Life Sciences and Health (CNESVS) of the Ministry of Health and Public Hygiene of the Republic of Côte d'Ivoire before the work was carried out; (Ethical approval number: IRB000111917). The National Programme for School and University Health (PNSSU) was associated to the study from the onset (selection of schools) and throughout the process to ensure the safety of the interventions. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

KM: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Conceptualization, Validation. KD: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review and editing, Formal analysis, Supervision, Validation. KB: Supervision, Validation, Writing – review and editing. KT: Investigation, Methodology, Writing – original draft, Validation. KG: Investigation, Methodology, Writing – review and editing. L-ST: Formal analysis, Methodology, Visualization, Writing – review and editing, Validation. AO: Formal analysis, Validation, Writing – review and editing, Visualization. BG: Supervision, Writing – review and editing. JN: Data curation, Methodology, Writing – review and editing, Validation. RW: Funding acquisition, Supervision, Writing – review and editing, Validation, Project administration. GA: Project administration, Supervision, Writing – review and editing, Resources, Validation. BB: Investigation, Project administration, Resources, Supervision, Validation, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Abdullahi, O., Nyiro, J., Lewa, P., Slack, M., and Scott, J. A. G. (2008). The descriptive epidemiology of *Streptococcus pneumoniae* and *Haemophilus influenzae* nasopharyngeal carriage in children and adults in Kilifi District, Kenya. *Pediatr. Infect. Dis. J.* 27, 59–64. doi: 10.1097/INF.0b013e31814da70c

Allali, I., Abotsi, R. E., Tow, L. A., Thabane, L., Zar, H. J., Mulder, N. M., et al. (2021). Human microbiota research in Africa: A systematic review reveals gaps and priorities for future research. *Microbiome* 9:241. doi: 10.1186/s40168-021-01195-7

Arjarquah, A. K., Obodai, E., Anie, H. A., Osei, M. A., Odoom, J. K., Bonney, J. H. K., et al. (2022). Occurrence of influenza and bacterial infections in cancer patients receiving radiotherapy in Ghana. *PLoS One* 17:e0271877. doi: 10.1371/journal.pone.0271877

Azor-Martínez, E., Gonzalez-Jimenez, Y., Seijas-Vazquez, M. L., Cobos-Carrascosa, E., Santisteban-Martínez, J., Martínez-López, J. M., et al. (2014). The impact of common infections on school absenteeism during an academic year. *Am. J. Infect. Control* 42, 632–637. doi: 10.1016/j.ajic.2014.02.017

Belibasakis, G. N. (2018). Microbiological changes of the ageing oral cavity. *Arch. Oral. Biol.* 96, 230–232. doi: 10.1016/j.archoralbio.2018.10.001

Biesbroek, G., Tsivtsivadze, E., Sanders, E. A. M., Montijn, R., Veenhoven, R. H., Keijser, B. J. F., et al. (2014). Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am. J. Respir. Crit. Care Med.* 190, 1283–1292. doi: 10.1164/rccm.201407-1240OC

Blaser, M. J., and Falkow, S. (2009). What are the consequences of the disappearing human microbiota? *Nat. Rev. Microbiol.* 7, 887–894. doi: 10.1038/nrmicro2245

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., van Gils, E., et al. (2011). Variability and diversity of nasopharyngeal microbiota in children: A metagenomic analysis. *PLoS One* 6:e17035. doi: 10.1371/journal.pone.0017035

Borges, L. G. D. A., Giongo, A., Pereira, L., Trindade, F. J., Gregianini, T. S., Campos, F. S., et al. (2018). Comparison of the nasopharynx microbiome between influenza and non-influenza cases of severe acute respiratory infections: A pilot study. *Health Sci. Rep.* 1:e47. doi: 10.1002/hsr2.47

Boutin, S., Graeber, S. Y., Weitnauer, M., Panitz, J., Stahl, M., Clausznitzer, D., et al. (2015). Comparison of microbiomes from different niches of upper and lower airways in children and adolescents with cystic fibrosis. *PLoS One* 10:e0116029. doi: 10.1371/journal.pone.0116029

Brook, I., Yocum, P., and Friedman, E. M. (1981). Aerobic and anaerobic bacteria in tonsils of children with recurrent tonsillitis. *Ann. Otol. Rhinol. Laryngol.* 90, 261–263. doi: 10.1177/000348948109000313

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High resolution sample inference from illumina amplicon data. *Nat. Methods* 13:581. doi: 10.1038/nmeth.3869

Carvalho, M., Tondella, M., McCaustland, K., Weidlich, L., McGee, L., Mayer, L., et al. (2007). Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. *J. Clin. Microbiol.* 45, 2460–2466. doi: 10.1128/JCM.02498-06

Centers for Disease Control and Prevention (2023). *CDC in Côte d'Ivoire: Global Health*. Atlanta, GA: Centers for Disease Control and Prevention.

Charlson, E. S., Bittinger, K., Haas, A. R., Fitzgerald, A. S., Frank, I., Yadav, A., et al. (2011). Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am. J. Respir. Crit. Care Med.* 184, 957–963. doi: 10.1164/rccm.201104-0655OC

Chen, Y., Xu, C., Zhong, C., Lyu, Z., Liu, J., Chen, Z., et al. (2021). Temporal characteristics of the oropharyngeal and nasal microbiota structure in crewmembers stayed 180 days in the controlled ecological life support system. *Front. Microbiol.* 11:617696. doi: 10.3389/fmicb.2020.617696

Cho, I., and Blaser, M. J. (2012). The human microbiome: At the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/nrg3182

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., et al. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eur. Surveill.* 25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045

Cui, G.-Y., Rao, B.-C., Zeng, Z.-H., Wang, X.-M., Ren, T., Wang, H.-Y., et al. (2022). Characterization of oral and gut microbiome and plasma metabolomics in COVID-19 patients after 1-year follow-up. *Mil. Med. Res.* 9:32. doi: 10.1186/s40779-022-00387-y

Dattner, I., Goldberg, Y., Katriel, G., Yaari, R., Gal, N., Miron, Y., et al. (2021). The role of children in the spread of COVID-19: Using household data from Bnei Brak, Israel, to estimate the relative susceptibility and infectivity of children. *PLoS Comput. Biol.* 17:e1008559. doi: 10.1371/journal.pcbi.1008559

de Koff, E. M., Groot, K. M., and Bogaert, D. (2016). Development of the respiratory tract microbiota in cystic fibrosis. *Curr. Opin. Pulm. Med.* 22, 623–628. doi: 10.1097/MCP.0000000000000316

Depner, M., Ege, M. J., Cox, M. J., Dwyer, S., Walker, A. W., Birzele, L. T., et al. (2017). Bacterial microbiota of the upper respiratory tract and childhood asthma. *J. Allergy Clin. Immunol.* 139:826–834.e13. doi: 10.1016/j.jaci.2016.05.050

Diallo, K., Missa, K. F., Tuo, J. K., Amoikon, T. L. S., Bla, B. K., and Bonfoh, B. (2023). Narrative review of application of metagenomic approaches to study the link between oropharyngeal microbiome and infectious diseases. *Front. Microbiol.* 14:1292526. doi: 10.3389/fmicb.2023.1292526

Gao, M., Wang, H., Luo, H., Sun, Y., Wang, L., Ding, S., et al. (2021). Characterization of the human oropharyngeal microbiomes in SARS-CoV-2 infection and recovery patients. *Adv. Sci.* 8:2102785. doi: 10.1002/advs.202102785

Gao, Z., Kang, Y., Yu, J., and Ren, L. (2014). Human pharyngeal microbiome may play a protective role in respiratory tract infections. *Genom. Proteomics Bioinform.* 12, 144–150. doi: 10.1016/j.gpb.2014.06.001

Gupta, A., Bhanushali, S., Sanap, A., Shekatkar, M., Kharat, A., Raut, C., et al. (2022). Oral dysbiosis and its linkage with SARS-CoV-2 infection. *Microbiol. Res.* 261:127055. doi: 10.1016/j.micres.2022.127055

Jiehao, C., Jin, X., Daojiong, L., Zhi, Y., Lei, X., Zhenghai, Q., et al. (2020). A case series of children with 2019 novel coronavirus infection: Clinical and epidemiological features. *Clin. Infect. Dis.* 71, 1547–1551. doi: 10.1093/cid/ciaa198

Karaman, E., Enver, O., Alimoglu, Y., Gonullu, N., Bahar, H., Mamal Torun, M., et al. (2009). Oropharyngeal flora changes after tonsillectomy. *Otolaryngol. Head Neck Surg.* 141, 609–613. doi: 10.1016/j.otohns.2009.07.010

Khanna, S., Montassier, E., Schmidt, B., Patel, R., Knights, D., Pardi, D. S., et al. (2016). Gut microbiome predictors of treatment response and recurrence in primary *Clostridium difficile* infection. *Aliment. Pharmacol. Ther.* 44, 715–727. doi: 10.1111/apt.13750

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Lagare, A., Maïnassara, H. B., Issaka, B., Sidiki, A., and Tempia, S. (2015). Viral and bacterial etiology of severe acute respiratory illness among children < 5 years of age without influenza in Niger. *BMC Infect. Dis.* 15:515. doi: 10.1186/s12879-015-1251-y

Laufer, A. S., Metlay, J. P., Gent, J. F., Fennie, K. P., Kong, Y., and Pettigrew, M. M. (2011). Microbial communities of the upper respiratory tract and otitis media in children. *mBio* 2, e245–e210. doi: 10.1128/mBio.00245-10

Li, J., Quinque, D., Horz, H.-P., Li, M., Rzhetskaya, M., Raff, J. A., et al. (2014). Comparative analysis of the human saliva microbiome from different climate zones: Alaska, Germany, and Africa. *BMC Microbiol.* 14:316. doi: 10.1186/s12866-014-0316-1

Lin, S.-Z., Qian, M.-J., Wang, Y.-W., Chen, Q.-D., Wang, W.-Q., Li, J.-Y., et al. (2023). Children with infectious pneumonia caused by *Ralstonia insidiosa*: A case report. *World J. Clin. Cases* 11, 2002–2008. doi: 10.12998/wjcc.v11.i9.2002

Liu, P., Xu, M., He, L., Su, L., Wang, A., Fu, P., et al. (2018). Epidemiology of respiratory pathogens in children with lower respiratory tract infections in Shanghai, China, from 2013 to 2015. *Jpn. J. Infect. Dis.* 71, 39–44. doi: 10.7883/yoken.JJID.2017.323

Mahony, J. B. (2008). Detection of respiratory viruses by molecular methods. *Clin. Microbiol. Rev.* 21, 716–747. doi: 10.1128/CMR.00037-07

Maleki, A., Zamirnasta, M., Taherikalani, M., Pakzad, I., Mohammadi, J., Krutova, M., et al. (2020). The characterization of bacterial communities of oropharynx microbiota in healthy children by combining culture techniques and sequencing of the 16S rRNA gene. *Microb. Pathog.* 143:104115. doi: 10.1016/j.micpath.2020.104115

Mayor, S. (2010). Acute respiratory infections are world's third leading cause of death. *BMJ* 341:c6360. doi: 10.1136/bmj.c6360

McMurdie, P. J., and Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Msd Manual Consumer Version (2023). *Rotavirus infection – children's health issues*. Available online at: https://www.msdmanuals.com/home/children-s-health-issues/common-viral-infections-in-infants-and-children/rotavirus-infection (accessed August 09, 2023).

Nm, D., Dm, P., Sp, H., Da, R., and Bj, C. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6, D590–D596. doi: 10.1186/s40168-018-0605-2

Paynter, S., Ware, R. S., Sly, P. D., Williams, G., and Weinstein, P. (2015). Seasonal immune modulation in humans: Observed patterns and potential environmental drivers. *J. Infect.* 70, 1–10. doi: 10.1016/j.jinf.2014.09.006

Piters, W. A. A., Huijskens, E. G. W., Wyllie, A. L., Biesbroek, G., van den Bergh, M. R., Veenhoven, R. H., et al. (2016). Dysbiosis of upper respiratory tract microbiota in elderly pneumonia patients. *ISME J.* 10:97. doi: 10.1038/ismej.2015.99

Price, R. H. M., Graham, C., and Ramalingam, S. (2019). Association between viral seasonality and meteorological factors. *Sci. Rep.* 9:929. doi: 10.1038/s41598-018-37481-y

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Rafiqul Islam, S. M., Foysal, M. J., Hoque, M. N., Mehedi, H. M. H., Rob, M. A., Salauddin, A., et al. (2022). Dysbiosis of oral and gut microbiomes in SARS-CoV-2 infected patients in bangladesh: Elucidating the role of opportunistic gut microbes. *Front. Med.* 9:821777. doi: 10.3389/fmed.2022.821777

Ren, Z., Wang, H., Cui, G., Lu, H., Wang, L., Luo, H., et al. (2021). Alterations in the human oral and gut microbiomes and lipidomics in COVID-19. *Gut* 70, 1253–1265. doi: 10.1136/gutjnl-2020-323826

Ridenhour, B. J., Braun, A., Teyrasse, T., and Goldsman, D. (2011). Controlling the spread of disease in schools. *PLoS One* 6:e29640. doi: 10.1371/journal.pone.0029640

ScienceDirect Topics (2007). *Human microbiome project – an overview*. Available online at: https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/human-microbiome-project (accessed February 20, 2023).

Segal, L. N., Clemente, J. C., Tsay, J.-C. J., Koralov, S. B., Keller, B. C., Wu, B. G., et al. (2016). Enrichment of the lung microbiome with oral taxa is associated with lung inflammation of a Th17 phenotype. *Nat. Microbiol.* 1:16031. doi: 10.1038/nmicrobiol.2016.31

Shakya, M., Lo, C.-C., and Chain, P. S. G. (2019). Advances and challenges in metatranscriptomic analysis. *Front. Genet.* 10:904. doi: 10.3389/fgene.2019.00904

Soffritti, I., D'Accolti, M., Fabbri, C., Passaro, A., Manfredini, R., Zuliani, G., et al. (2021). Oral microbiome dysbiosis is associated with symptoms severity and local immune/inflammatory response in COVID-19 patients: A cross-sectional study. *Front. Microbiol.* 12:687513. doi: 10.3389/fmicb.2021.687513

Tavares, D. A., Handem, S., Carvalho, R. J., Paulo, A. C., de Lencastre, H., Hinds, J., et al. (2019). Identification of Streptococcus pneumoniae by a real-time PCR assay targeting SP2020. *Sci. Rep.* 9:3285. doi: 10.1038/s41598-019-39791-1

Times, M. (2020). *Microbiome-based diagnostics and biomarkers: Changing the paradigm in microbiology and medicine – Microbiome times magazine*. Available online at: https://www.microbiometimes.com/microbiome-based-diagnostics-and-biomarkers-changing-the-paradigm-in-microbiology-and-medicine/ (accessed March 20, 2023).

Ursell, L. K., Metcalf, J. L., Parfrey, L. W., and Knight, R. (2012). Defining the human microbiome. *Nutr. Rev.* 70:S38. doi: 10.1111/j.1753-4887.2012.00493.x

White, C., Kolble, R., Carlson, R., and Lipson, N. (2005). The impact of a health campaign on hand hygiene and upper respiratory illness among college students living in residence halls. *J. Am. Coll. Health* 53, 175–181. doi: 10.3200/JACH.53.4.175-181

Wimalasena, N. N., Chang-Richards, A., Wang, K. I.-K., and Dirks, K. N. (2021). Housing risk factors associated with respiratory disease: A systematic review. *Int. J. Environ. Res. Public Health* 18:2815. doi: 10.3390/ijerph18062815

World Health Organization *Côte d'Ivoire: WHO coronavirus disease (COVID-19) dashboard with vaccination data 2024*. Geneva: World Health Organization.

World Health Organization *Respiratory infections 2020*. Geneva: World Health Organization.

Wu, Y., Cheng, X., Jiang, G., Tang, H., Ming, S., Tang, L., et al. (2021). Altered oral and gut microbiota and its association with SARS-CoV-2 viral load in COVID-19 patients during hospitalization. *NPJ Biofilms Microb.* 7, 1–9. doi: 10.1038/s41522-021-00232-5

Xia, X., Chen, J., Cheng, Y., Chen, F., Lu, H., Liu, J., et al. (2022). Comparative analysis of the lung microbiota in patients with respiratory infections, tuberculosis, and lung cancer: A preliminary study. *Front. Cell. Infect. Microbiol.* 12:1024867. doi: 10.3389/fcimb.2022.1024867

Xiang, Z., Koo, H., Chen, Q., Zhou, X., Liu, Y., and Simon-Soro, A. (2020). Potential implications of SARS-CoV-2 oral infection in the host microbiota. *J. Oral Microbiol.* 13:1853451. doi: 10.1080/20002297.2020.1853451

Zhu, Y., Bloxham, C. J., Hulme, K. D., Sinclair, J. E., Tong, Z. W. M., Steele, L. E., et al. (2020). A meta-analysis on the role of children in SARS-CoV-2 in household transmission clusters. *Clin. Infect. Dis.* 72:ciaa1825. doi: 10.1093/cid/ciaa1825

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The microbiota: a crucial mediator in gut homeostasis and colonization resistance

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The gut microbiota is a complex and diverse community of microorganisms that colonizes the human gastrointestinal tract and influences various aspects of human health. These microbes are closely related to enteric infections. As a foreign entity for the host, commensal microbiota is restricted and regulated by the barrier and immune system in the gut and contributes to gut homeostasis. Commensals also effectively resist the colonization of pathogens and the overgrowth of indigenous pathobionts by utilizing a variety of mechanisms, while pathogens have developed strategies to subvert colonization resistance. Dysbiosis of the microbial community can lead to enteric infections. The microbiota acts as a pivotal mediator in establishing a harmonious mutualistic symbiosis with the host and shielding the host against pathogens. This review aims to provide a comprehensive overview of the mechanisms underlying host-microbiome and microbiome-pathogen interactions, highlighting the multi-faceted roles of the gut microbiota in preventing enteric infections. We also discuss the applications of manipulating the microbiota to treat infectious diseases in the gut.

KEYWORDS

microbiota, gut homeostasis, enteric infections, mucosal immunity, colonization resistance

1 Introduction

An enormous number of microbes, collectively termed gut microbiota, colonize the human gastrointestinal (GI) tract, including bacteria, viruses, fungi, archaea, and protozoa. Bacteria achieve the highest density, with an estimated more than 10^{14} bacteria in the gut (de Vos et al., 2022). The negative impact of microorganisms has long been the primary focus, but there is a growing interest in exploring their potential benefits for human health. These beneficial roles include supporting the maturation of the immune system, producing beneficial metabolites, promoting food digestion and vitamin production, enhancing gut barrier function, and resisting the colonization of pathogens (Dey et al., 2021).

Bacterial gastroenteritis poses a significant threat to global public health, and the microbiota is tightly linked to the infection in the gut (GBD, 2019 Antimicrobial Resistance Collaborators, 2022). Infectious enteritis occurs when pathogens invade the intestine and elicit a response from the immune system (Sarkar et al., 2024). The microbiota is recognized by the host as a foreign entity, making it a potential source of infection. In a healthy state, a harmonious interaction is maintained between the gut and the microbiota. The commensal microbiota, which dominates and occupies nearly all areas of the gut, is restricted and

regulated by the host and serves as an inevitable symbiont for the host (Kim et al., 2017). There exists another interaction between microbiota and pathogens in the gut. The microbiota efficiently impedes pathogen colonization by deploying multiple mechanisms to prevent infection. However, during dysbiosis, some beneficial commensals can exert adverse effects, and pathogenic microbes have counterstrategies to break the defense from the microbiota and host (Ducarmon et al., 2019; Caballero-Flores et al., 2023). Therefore, gut microbiota acts as a significant mediator in maintaining gut homeostasis and protecting against pathogen invasion. The current advancement of research and technologies supply mechanistic insights into host-microbiome and microbiome-pathogen interactions, enhancing our understanding of these dynamic interactions and promoting the development of innovative strategies to reduce the incidence and severity of enteric infections.

This review systematically elucidates the multi-faceted roles of microbiota as both a foreign entity and a symbiont in the gut. The commensal microbiota must be modulated by the host and maintain a balanced gut environment. It also aids the host in effectively resisting the invasion and colonization of pathogens (Figure 1). By delving into the intricate roles of gut microbiota, we aim to facilitate the comprehension and promotion of the mutually beneficial relationship between the host and microbiota to prevent and manage infectious diseases in the gut.

2 Composition of microbiota

The normal gut microbial community, known as the commensal microbiota, typically consists of six significant phyla, including *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*, among which the first two phyla make up over 90% of the bacterial population in the colon while the others are present in lower abundance (Hou et al., 2022). The total density of bacteria is higher in the colon than in the small intestine. Members of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* are the prominent residents of the small intestine (Ruigrok et al., 2023).

The *Bacteroidetes* phylum includes both anaerobic and aerobic, non-spore-forming, Gram-negative, rod-shaped bacteria that colonize the intestinal tract. The *Bacteroides* genus is one of the most predominant groups in the intestine. It has essential metabolic functions and maintains a beneficial relationship in the intestinal lumen, but some members can become pathogenic if they disseminate (Rajilić-Stojanović and de Vos, 2014). For example, *Bacteroides fragilis* is typically found in the lower GI tract and has beneficial effects. However, it is also commonly isolated from abdominal abscesses and bloodstream infections if the intestinal mucosa is perforated and traversed (Mazmanian and Kasper, 2006; Wexler and Wexler, 2007).

The *Firmicutes* phylum is composed of anaerobic bacteria that are predominantly Gram-positive and can produce endospores. These endospores are durable, dormant structures that allow the bacteria to remain viable in challenging environments and reactivate when conditions become more favorable. The class *Clostridia* encompasses a wide range of bacteria with different clusters. While some clusters, such as *XIVa* and *IV*, exert beneficial effects by producing butyrate and supporting intestinal health and immune balance, others, like cluster *I*, *Clostridium perfringens*, *Clostridium tetani*, and *Clostridium difficile*, are potential pathogens responsible for enteric infection (Kim et al., 2017). The *Firmicutes* phylum also involves the *Bacilli* class, which includes clinically significant oxygen-tolerant pathobionts such as *Enterococcus* species and *Streptococcus* species (Taur et al., 2012, 2013).

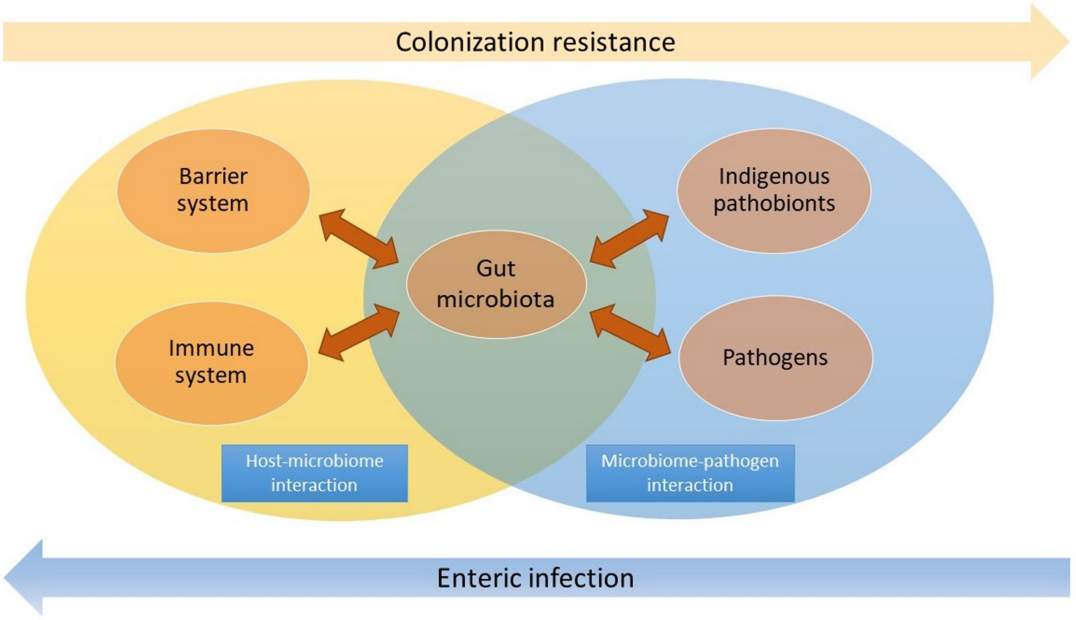


FIGURE 1
The microbiota acts as a vital mediator in the host-microbiome and microbiome-pathogen interactions. Commensal microbiota interacts with the barrier and immune system in the gut, while it also effectively resists pathogen colonization and overgrowth of indigenous pathobionts. The dysbiosis may lead to enteric infection.

These bacteria are generally present in low abundance but can become harmful during intestinal dysbiosis.

The *Actinobacteria* phylum comprises aerobic and anaerobic bacteria (Rajilić-Stojanović and de Vos, 2014). *Bifidobacteria* species are among the predominant genera in this phylum. They are recognized for their probiotic properties, including mechanisms such as competitive exclusion, bile salt hydrolase activity, immune modulation, and adherence to the mucus or intestinal epithelium (Kim et al., 2017), thus protecting the gut against potential infections caused by harmful bacteria.

The *Proteobacteria* phylum encompasses a wide array of Gram-negative bacteria, many of which are facultative anaerobes. An elevated presence of *Proteobacteria* in the gut microbiota may signify an imbalance and a heightened risk of illness (Shin et al., 2015). The family *Enterobacteriaceae* within the *Gammaproteobacteria* class includes pathogens such as *Escherichia coli* and *Klebsiella* species (Taur et al., 2012, 2013). These pathogens are typically in low abundance but can become dominant in the intestinal environment during dysbiosis.

The *Fusobacteria* phylum is a type of Gram-negative anaerobic bacilli that is recognized as an opportunistic pathogen due to its frequent isolation from anaerobic samples in various infections; its role as a cancer-causing member of the microbiota is still being uncovered (Brennan and Garrett, 2019; Harrandah, 2023). The *Verrucomicrobiota*, a phylum of Gram-negative bacteria, are mucin-degrading bacteria found in the intestinal mucosa. This phylum of bacteria can help maintain intestinal health and glucose homeostasis (Oren and Garrity, 2021; Jian et al., 2023).

Additionally, fungi are crucial gut microbiota components, influencing immune responses and local inflammation. The most extensively researched fungi in the gut microbiota include *Candida*, *Saccharomyces*, *Malassezia*, and *Cladosporium*. The gut microbiota also comprises viruses, phages, and archaea, with *Methanobrevibacter smithii* being the predominant archaea species (Hou et al., 2022).

In general, the gut microbiota is a complex and dynamic community of microorganisms colonizing the GI tract. The diversity and balance of the gut microbiota are critical for maintaining intestinal health and preventing diseases. The altered composition or distribution of gut microbiota could create opportunities for pathobionts to thrive and colonize in the gut, thus ultimately leading to infections (Sheppard, 2022). Notably, there is no strict boundary between beneficial and harmful microbes. The beneficial microbiota present in most situations has the potential to transform into harmful pathogens during dysbiosis quickly.

3 Crosstalk between gut and microbiota

While the gut microbiota has been shown to benefit the host, it is important to note that the host recognizes the microbiota as a foreign entity. To prevent excessive defensive responses, the barrier system and mucosal immune system in the gut contribute to maintaining the balance between the host and commensal microbes (Figure 2).

3.1 Barrier-microbiome interaction

Intestinal epithelial cells (IECs) are crucial in establishing physical and chemical barriers to maintain the separation of microbiota and immune cells (Maynard et al., 2012). Specifically, the barrier mechanisms vary between the small intestine and the colon.

3.1.1 Chemical barriers

The small intestine possesses fewer goblet cells than the colon, resulting in reduced mucus production. However, Paneth cells are present and produce chemical barrier molecules that help to maintain a separation between intestinal microbes and IECs.

Antimicrobial peptides (AMPs), such as defensins (including α-, β- and θ-defensins) and cathelicidins, are small cationic proteins that disrupt microbial cell membranes. α-defensin (cryptdin) is expressed in Paneth cells in the small intestine and helps protect against pathogenic bacteria (Bosch and Zasloff, 2021; Gong et al., 2021). Meanwhile, cathelicidin-related antimicrobial peptide (CRAMP) is expressed in the colonic epithelia and defends colorectal pathogens (Bosch and Zasloff, 2021). RegIII family proteins are antimicrobial C-type lectins mainly produced by Paneth cells, and they have bactericidal activity against Gram-positive bacteria (Mukherjee et al., 2014). The decrease of this AMP was unveiled to induce peripheral translocation of *Alcanligenes xylosoxidans*, a lymphoid-resident commensal bacteria (Sonnenberg et al., 2012). The expression of RegIII family proteins is controlled by the Toll-like receptor (TLR)/myeloid differentiation primary response 88 protein (MyD88) pathway and interleukin (IL)-22 stimulation, both of which are activated by the gut microbiota (Frantz et al., 2012; Bhinder et al., 2014). It was observed that the administration of bacterial-associated molecules such as lipopolysaccharide (LPS) or bacterial flagellin could increase the expression of RegIIIγ and enhance the eradication of vancomycin-resistant enterococci (VRE) species (Brandl et al., 2008; Kinnebrew et al., 2010), showing the microbiota can strengthen the gut barrier.

Frantz et al. (2012) showed that mice lacking MyD88 in IECs experienced a decrease in the production of AMPs and mucus, leading to a higher risk of colitis and bacterial infection in the gut. Mice without IL-22 signaling also exhibited increased sensitivity to dextran sulfate sodium (DSS)-induced colitis (Zenewicz et al., 2008), indicating that the compromised chemical barriers in the intestines heighten the susceptibility to inflammation. IL-22 was also revealed to inhibit the growth of segmented filamentous bacteria (SFB), which attached to IECs in the ileum and stimulated the production of serum amyloid A, promoting T helper 17 (Th17) differentiation in the lamina propria (LP) (Terao et al., 2014; Yang et al., 2022). Therefore, IL-22 is capable of preventing Th17-mediated intestinal inflammation.

In addition, Lipocalin 2 (Lcn2), released by immune cells like neutrophils, is a critical antimicrobial protein that sequesters bacterial iron-scavenging siderophores to prevent pathogen iron acquisition (Mayneris-Perxachs et al., 2022). Its expression is induced by inflammation and is dependent on the presence of the gut microbiota (Klüber et al., 2021). Calprotectin, a protein released by neutrophils and IECs during inflammation, also exerts activity against pathogens by sequestering essential divalent metals such as iron, zinc, calcium, and manganese (Zygiel and Nolan, 2018). These are significant for regulating gut microbiota composition and abundance.

3.1.2 Physical barriers

The large intestine, with a higher concentration of goblet cells, has a distinct mucosal barrier system that separates microbiota from the epithelial layer. The mucous layer comprises a firm inner layer and a

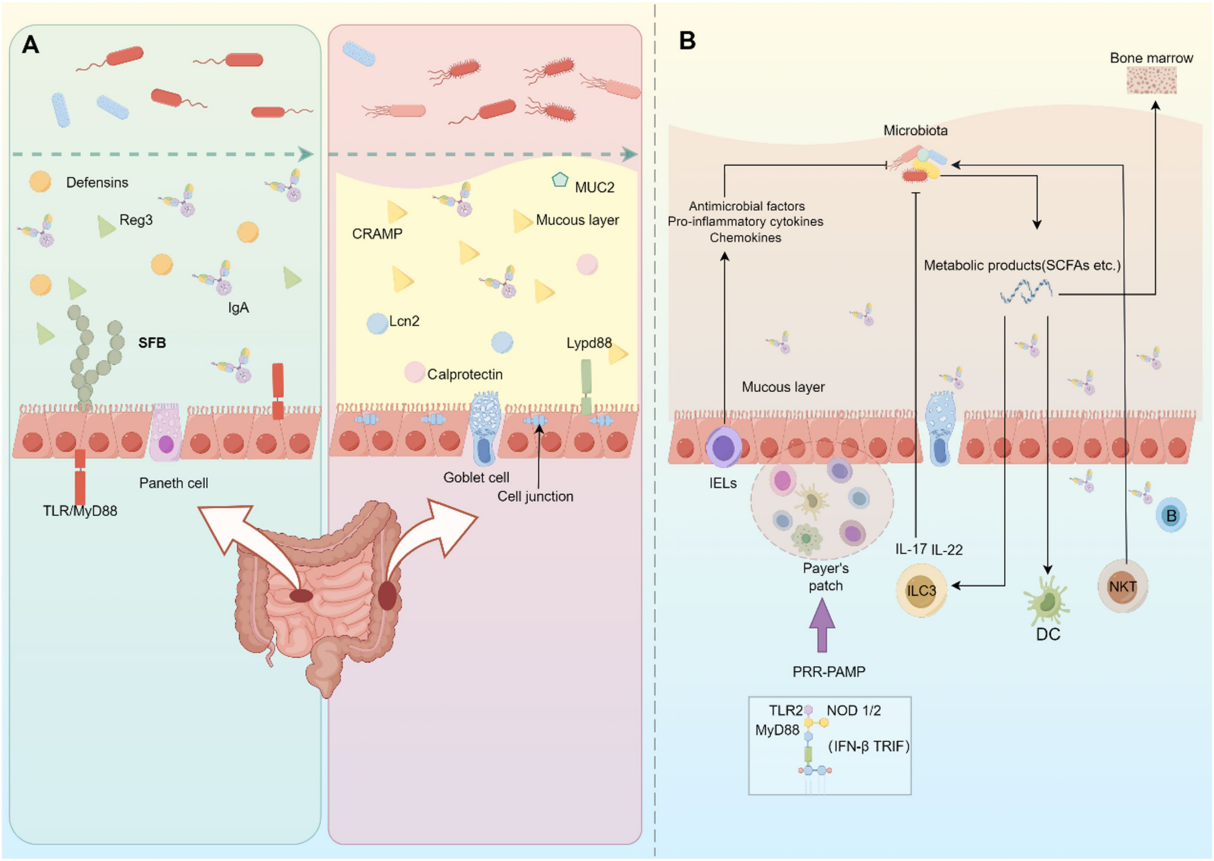


FIGURE 2
Host-microbiome interaction in the gut. **(A)** In the small intestine, chemical barriers mainly contribute to separating the gut microbiota and intestinal epithelial cells. Immune cells in the lamina propria regulate the generation of these chemical barriers. In the large intestine, the mucous layer and cell junctions hamper pathogen invasion in the gut. **(B)** The host immunity can recognize and respond to the presence of gut microbiota. This interaction involves various immune cells and cytokines, and the commensal microorganisms and their metabolites can impact the development and function of mucosal immunity.

loose outer layer. The inner layer, consisting of polymerized mucin 2 (MUC2), effectively prevents microorganisms from penetrating the colonic epithelium (Zhang et al., 2021). Transmembrane mucins such as MUC1, MUC13, and MUC17 also protect the intestine from enteric pathogens. Their reduction or deficiency resulted in an increased susceptibility to bacterial infections (van Putten et al., 2017; Sheng and Hasnain, 2022). Furthermore, The *Fut2* gene, responsible for transferring fucose to the end of glycans on cell surface glycoproteins, is linked to an increased susceptibility to Crohn's disease, a form of inflammatory bowel disease (McGovern et al., 2010; Li L. et al., 2024). A study showed that mice deficient in *Fut2* were significantly more vulnerable to bacterial infections (McGovern et al., 2010), highlighting the key role of *Fut2* in defending against infections.

Various antimicrobial molecules maintain the inner mucous layer in a nearly sterile state, including immunoglobulin A (IgA) and the defensin family of proteins (Palm et al., 2014), while the expression of these antimicrobial molecules in the colon is not as high as in the small intestine due to the absence of Paneth cells and lower IgA^+ plasma cells (Yanagibashi et al., 2013; Gesualdo et al., 2021). Nonetheless, Ly6/Plaur-domain-containing 8 (Lypd8), a highly N-glycosylated GPI-anchored protein, has been found to efficiently separate intestinal microbes and the epithelium in the colon (Okumura et al., 2016). This suggests that an alternative mechanism is involved in the large intestine to prevent pathogen invasion.

Another physical barrier against the gut microbiota is the cell junctions, specifically tight and adhesion junctions, which connect epithelial cells and control epithelial polarity paralleling the movement of solutes and fluids (Furuse, 2010). These cell junctions build a physical barrier to prevent microbial invasion through the paracellular pathway. Tight junctions comprise claudins, occludins, and intracellular zonula occludens proteins (García-Hernández et al., 2017), which are significant in preventing intestinal inflammation.

Collectively, IECs are essential for the formation and maintenance of the mucosal barrier. They produce barrier components that separate the microbiota from the intestinal immune cells.

3.2 Immune-microbiome interaction

Besides the physical separation between the microbiota and the gut, the balanced crosstalk between mucosal immunity and the microbiota is inevitable for sustaining homeostasis in the gut.

3.2.1 Gut-associated lymphoid tissues (GALTs)

GALTs are part of the mucosa-associated lymphoid tissues (MALTs) lining the host and environment. Their primary function is recognizing pathogens, initiating immune response, and maintaining immune tolerance to commensal flora (Mörbe et al., 2021). The histological components of GALTs include Peyer's patches (PPs), crypt patches, isolated lymphoid follicles (ILFs), appendix, and mesenteric lymph nodes (MLNs) (Mörbe et al., 2021). The formation of GALTs greatly depends on lymphoid tissue inducer (LTi) cells and their interaction with gut microbiota (Jiao et al., 2020). The molecular process involved in this mechanism centers around recognizing pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which then trigger the activation of downstream signaling pathways. Several key PRR-related molecules have been identified to play roles in this process, including TLR2, nucleotide-binding oligomerization domain 1/2 (NOD 1/2), MyD88, and TIR domain-containing adaptor protein-inducing interferon (IFN)-β (TRIF) (Clarke et al., 2010; Round et al., 2011; Li J.-H. et al., 2024). PRR-PAMP recognition drives the structural development of GALTs and contributes to conditioning the host defense function. Furtherly, metabolic byproducts from symbiotic bacteria, such as short-chain fatty acids (SCFAs), exert effects in modulating the immune response of GALTs through epigenetic mechanisms (Jiao et al., 2020). This helps to maintain immune tolerance towards beneficial microbes.

3.2.2 Innate lymphoid cells (ILCs)

ILCs are vital members of the innate compartment of mucosal immunity (Vivier et al., 2018). ILCs share specific immunological characteristics with Th cells. They are categorized into three significant subpopulations based on their determinant transcription factors and signature cytokines: Group 1 ILCs, like Th1 cells, are T-bet-dependent and secrete interferon gamma (IFN-γ) (Cherrier et al., 2018). Group 2 ILCs, like Th2 cells, are GATA3-dependent and secrete IL-5 and IL-13 (Mjösberg et al., 2012; Roediger et al., 2013). Group 3 ILCs, like Th17 and Th22, are retinoic acid-related orphan receptor (ROR) γt-dependent and secrete IL-17 and IL-22 (Cording et al., 2014; Vivier et al., 2018). Group 3 ILCs consist of the $CD4^+CD3^-CCR6^+$ subset (LTi cells) and ILC3 subpopulations without expression of the tissue homing factor C-C chemokine receptor 6 (CCR6) (Vivier et al., 2018).

While the ILC1 subset is detectable in germ-free (GF) mice, its number is significantly lower in the fetal intestine, where the gut microbiota is not yet established. This finding may suggest a critical regulatory influence by commensal microorganisms on the maturation and functional manifestation of ILC1 (Krabbendam et al., 2015). ILC1 also contribute to restoring the balance of the intestinal microbiome by inhibiting the growth of opportunistic pathogens like *Clostridium difficile* (Abt et al., 2015). ILC2 are stimulated by cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin that are generated in reaction to signals from commensal bacteria (Peterson and Artis, 2014). It was reported that butyrate, a member of SCFAs, could suppress the production of IL-5 and IL-13 by ILC2 through histone deacetylases (HDAC) inhibition (Thio et al., 2018). ILC3 participate in producing the cytokine IL-22 via the IL-23/IL-23R pathway, promoting the production of antimicrobial compounds like RegIII family proteins by epithelial cells (Dudakov et al., 2015). Meanwhile, ILC3-derived IL-22 and lymphotoxin α (LTα) promote commensal bacteria-dependent expression of *Fut2*, leading to the upregulation of epithelial fucosylation. Fucose generated by commensal bacteria acts as a host defense mechanism against pathogens (Pickard et al., 2014). The interplay between ILC3 and gut microbes is controlled by transcription factors such as inhibitors of DNA binding (ID) 2 and cytokines like IL-22 (Guo et al., 2015; Thaiss et al., 2016). Commensals also modulate the antigen presentation by ILC3, thus shaping the adaptive immune response (Hepworth et al., 2013, 2015). As a result, commensal microbiota is crucial in inducing the host defense function of ILC3.

LTi cells are a unique subset of $ROR\gamma t^+$ group 3 ILCs that express CCR6 (Huang et al., 2017). The activation of LTi cells is initiated by peptidoglycan derived from enteric Gram-negative commensals, which in turn triggers the activation of stromal cells and leads to the formation of ILFs. This process involves the recruitment of B cells and dendritic cells (DCs) (Bouskra et al., 2008). The microbiota also promotes the production of chemokine (C-C motif) ligand 20 (CCL20) and β-defensin 3, which further activate LTi cells through binding to CCR6 (Bouskra et al., 2008), creating a positive feedback loop for continuous activation and ILFs development.

3.2.3 Intraepithelial lymphocytes (IELs)

IELs are divided into two subsets: natural IELs (nIELs), which migrate directly from the thymus to the intestine, and peripherally induced IELs (pIELs), which differentiate from peripherally activated conventional T cells within the intestinal epithelium (Lockhart et al., 2024). The accumulation of these cell subsets in the intestinal epithelium is closely associated with the diversity of the gut microbiota. nIELs are primarily responsible for tolerance to the indigenous gut microbiota and immune responses against pathogens, whereas pIELs are more involved in immune responses to foreign pathogens. For example, commensal bacteria promote the survival of nIELs through IL-15 produced by IECs, support the cytotoxic potential of $TCR\alpha\beta^+$ nIELs, and support $TCR\gamma\delta^+$ nIELs in producing the antimicrobial peptide REGIIIγ (Lockhart et al., 2024). The gut microbiota can regulate pIELs through metabolic products and pattern recognition pathways. *Lactobacillus reuteri*, for instance, produces tryptophan metabolites that induce the expansion of $CD4^+CD8\alpha\alpha^+$ pIELs via the aryl hydrocarbon receptor (AhR) signaling pathway (Cervantes-Barragan et al., 2017). Additionally, *Bifidobacterium* species enhance the accumulation of $CD8\alpha\beta^+$ pIELs that produce antimicrobial peptides in a TLR-dependent manner (Chen et al., 2018). Members of the *Bacteroidetes* phylum may also promote the development and accumulation of $CD4^+CD8\alpha\alpha^+$ pIELs with anti-inflammatory properties (Bousbaine et al., 2022).

3.2.4 Natural killer T (NKT) cells

NKT cells are a specific T cell subset that share properties of both T cells and NK cells. NKT cells can identify glycolipid antigens presented by CD1d, which is expressed by DCs and IECs (Kayama et al., 2020). It was found that Paneth cells in *Cd1d*-deficient GF mice mono-colonized with *E. coli* showed reduced production of AMPs, leading to their dissemination to MLNs. The fecal microbial community of *Cd1d*-deficient mice was also altered, with increased *Bacteroides* and a lower frequency of *Firmicutes* (Nieuwenhuis et al., 2009). These demonstrate that CD1d-mediated activation of NKT cells is crucial for modulating the colonization, composition, and translocation of commensal bacteria (Nieuwenhuis et al., 2009).

3.2.5 Mucosal-associated invariant T (MAIT) cells

MAIT cells are a specialized subset of T lymphocytes characterized by their semi-invariant T-cell receptors (TCRs), which interact with antigens presented by the major histocompatibility complex (MHC) class I-related molecule 1 (MR1). These cells play a crucial role in the dynamic interaction between the host and the gut microbiota, serving a regulatory function in intestinal immunity by recognizing antigens presented by MR1. MAIT cells are particularly important in defending against bacterial infections; they produce cytokines such as IL-17 and IFN-γ, which enhance mucosal immune responses and contribute to tissue repair (Corbett et al., 2014; Fadlallah et al., 2018). They also recognize acute viral infections by sensing IL-18, IL-15, and type I interferons, and contribute to enhancing the adaptive immune response (Provine et al., 2021). In chronic viral infections like human immunodeficiency virus (HIV) and hepatitis C virus (HCV), a reduction in the number and function of MAIT cells is associated with gut microbiota dysbiosis, which may impair the host's antimicrobial defenses and the immune system's recovery (Hengst et al., 2016; Merlini et al., 2019). *Bacteroidetes*, abundant in the gut, are significant stimulants for MAIT cells and may further influence their phenotype in the intestinal mucosa (Tastan et al., 2018). The influence of the microbiota on MAIT cells thereafter highlighted by the fact that certain bacterial metabolites, such as those derived from the riboflavin synthesis pathway by bacteria like *E. coli* and *Proteus mirabilis*, are essential for the development of MAIT cells (Constantinides et al., 2019).

3.2.6 IgA-producing plasm cells

The bacterial symbionts can stimulate the production of IgA in plasm cells (Bunker et al., 2017). PPs are crucial for immune monitoring in the gut, consisting of B cell follicles and T cell zones (Da Silva et al., 2017). DCs are essential for the transportation of antigens from the microbiota to the PPs (Kogut et al., 2020). Within the follicle-associated epithelium of the PPs, specialized epithelial cells called microfold (M) cells contribute to antigen presentation with specific carbohydrates and receptors as binding sites for pathogens (Mabbott et al., 2013; Ohno, 2016). This facilitates the transport of antigens to the subepithelial dome or basolateral pockets where phagocytes, T cells, and B cells reside. These immune cells further stimulate the differentiation of IgA-producing plasma B cells, leading to the production of IgA (Rios et al., 2016; Nagashima et al., 2017). In PPs, follicular helper T (Tfh) cells play a crucial role in orchestrating robust IgA responses by their high expression of the inhibitory coreceptor programmed cell death 1 (PD-1) (Kawamoto et al., 2012). Furthermore, IgA-producing cells are concurrently generated in cecal patches, which are specialized lymphoid tissues found within the appendix. DCs residing within these cecal patches have been shown to potently augment the expression of CCR10 on B cells, thereby ensuring the effective migration of IgA-producing cells to the gut (Masahata et al., 2014; Kayama et al., 2020). IgA is transported through the polymeric immunoglobulin receptor on epithelial cells and released into the intestinal lumen as secretory IgA (SIgA) (Bunker et al., 2015, 2017). SIgA can bind to specific microbial antigens and confine the activity of pathogens (Fagarasan et al., 2010). SIgA is also able to support the survival of the commensal microbes. The absence of IgA results in a reduction in beneficial symbionts and excessive growth of pathogenic bacteria in the intestine (Donaldson et al., 2018; Fadlallah et al., 2018), ultimately leading to intestinal inflammation.

3.2.7 Phagocytes

Intestinal phagocytes mainly include macrophages, DCs, and other non-immune cells. Gut macrophages (Tim-4$^-$ macrophages) are derived from circulating monocytes (Shaw et al., 2018). These macrophages are directed to the intestine by the chemokine receptor CCR2, induced by commensal bacteria. The absence of gut microbiota was found to result in a lack of chemotaxis signals for local macrophage replenishment (Lavin et al., 2015). Additionally, a study revealed a positive correlation between myelopoiesis in the bone marrow, the diversity of gut microbiota, and serum TLR levels, suggesting that the resident flora might impact the spatially distant hematopoiesis process through PRR-PAMP pathways (Schürch et al., 2014). Therefore, the absence of commensals can increase susceptibility to bacterial infections due to a compromised immune response mediated by myeloid cells.

DCs in the gut are categorized into two subsets based on the expression of CD103 (αE integrin), a chemokine receptor CX3CR1, and CD11b (Cerovic et al., 2014). In the case of dysbiosis, such as in the *Salmonella* infection model, CD103$^+$ DCs were found to accumulate in the enteric epithelium layer and phagocytosed pathogenic bacteria (Farache et al., 2013). Lactic acid bacteria (LAB) were also discovered to stimulate immature DCs within the gut, inducing them to produce cytokines like IL-12 and IL-15 (Rizzello et al., 2011). In the steady state, commensal flora can block the migration of CX3CR1$^+$ DCs to MLNs to present both commensal and pathogenic antigens (Diehl et al., 2013), but dysbiosis may disturb this balance and cause inappropriate antigen presentation.

The metabolic products from gut microbiota also impact the local homeostasis of phagocytes and myelopoiesis in the bone marrow (Khosravi et al., 2014). SCFAs can modulate epigenetic processes by directly inhibiting HDACs and activating G-protein coupled receptors (GPCRs) (Woo et al., 2017). Exposure of macrophages to microbiota was reported to result in an elevation of HDAC3, subsequently leading to an increased production of the anti-inflammatory cytokine IL-10. This might be due to the enhanced deacetylation of IL-10 promoters by HDAC3 (Kobayashi et al., 2012). Another study found that SCFAs could affect HDACs levels in gut macrophages, inducing a downregulation of pro-inflammatory cytokines such as IL-6 and IL-12 (Chang et al., 2014). SCFAs were also revealed to support the transformation of macrophages into M2 macrophages, which possess anti-inflammatory properties (Ji et al., 2016). Additionally, Ganal et al. (2012) reported that DCs were unable to produce specific pro-inflammatory cytokines when stimulated by pathogens in GF mice. There was a significant decrease in the level of trimethylated H3K4 in DCs from GF mice (Ganal et al., 2012), suggesting the core role of microbial symbionts or their products in modifying the function of immune cells through epigenetic mechanisms.

In summary, the microbiota is regulated by the barrier system and immune system in the gut. A sustainable mutualism between the host and commensals is necessary for gut homeostasis. The dysfunction of the gut barrier and immune system potentially contributes to the development of infection through the induction of dysbiosis.

4 Colonization resistance and invasion

Colonization resistance refers to the ability of the commensal microbiota to prevent the invasion of pathogens and the overgrowth

of indigenous but potentially pathobionts (Caballero-Flores et al., 2023). The microbiota has developed various mechanisms to protect against infection in the gut. However, pathogens evolve strategies to overcome these defenses and thrive (Figure 3).

4.1 The competition for resources in the gut

4.1.1 Resource-based colonization resistance

In certain circumstances, the preferential consumption of nutrients in the gut by commensals can outcompete pathogenic microbes. Fabich et al. (2008) showed that *E. coli* competed for nutrients with EHEC, causing starvation of the pathogenic bacteria and thus restricting them. The reduction of dietary amino acids by the microbiota was found to enhance resistance to colonization by the pathogen *Citrobacter rodentium* (Caballero-Flores et al., 2020). Furthermore, more diverse microbiomes increase the probability of protection against pathogens. The overlap in nutrient-utilization profiles between the microbiota community and the pathogen was identified as the critical factor contributing to the benefits of increased microbiome diversity (Spragge et al., 2023). Nutrient utilization assays revealed that beneficial *Klebsiella oxytoca* could utilize 100 different carbon sources, whereas pathogenic *K. pneumoniae* could only utilize 56 carbon sources (Osbelt et al., 2021). Competition for vital metal cofactors such as iron, zinc, and manganese also impacts resistance to pathogenic bateria (Behnsen et al., 2021). These findings emphasize the colonization resistance against pathogens through scrambling for resources in the gut.

4.1.2 Resource-based invasion

Pathogens have numerous strategies to exploit resources in the gut, offering them a competitive edge over commensal microorganisms. They can utilize the molecules that commensal organisms use and alternative nutrient sources. For example, EHEC is able to utilize alternative sources of sugar, such as hexuronate, glucuronate, galacturonate, and sucrose, which are not typically used by commensal *E. coli* (Kamada et al., 2012; Maltby et al., 2013). Moreover, EHEC can use ethanolamine, a carbon and nitrogen source released into the intestine lumen during intestinal cell turnover, as a nutrient based on the *eut* operon in their genome (Garsin, 2010; Girardeau et al., 2011), while commensal *E. coli* cannot utilize ethanolamine as a nutrient. Other pathogenic bacteria, particularly those of food origin, such as *Salmonella Typhimurium* and *Listeria monocytogenes*, have a competitive advantage in the intestines over commensals due to their ability to utilize ethanolamine as an energy

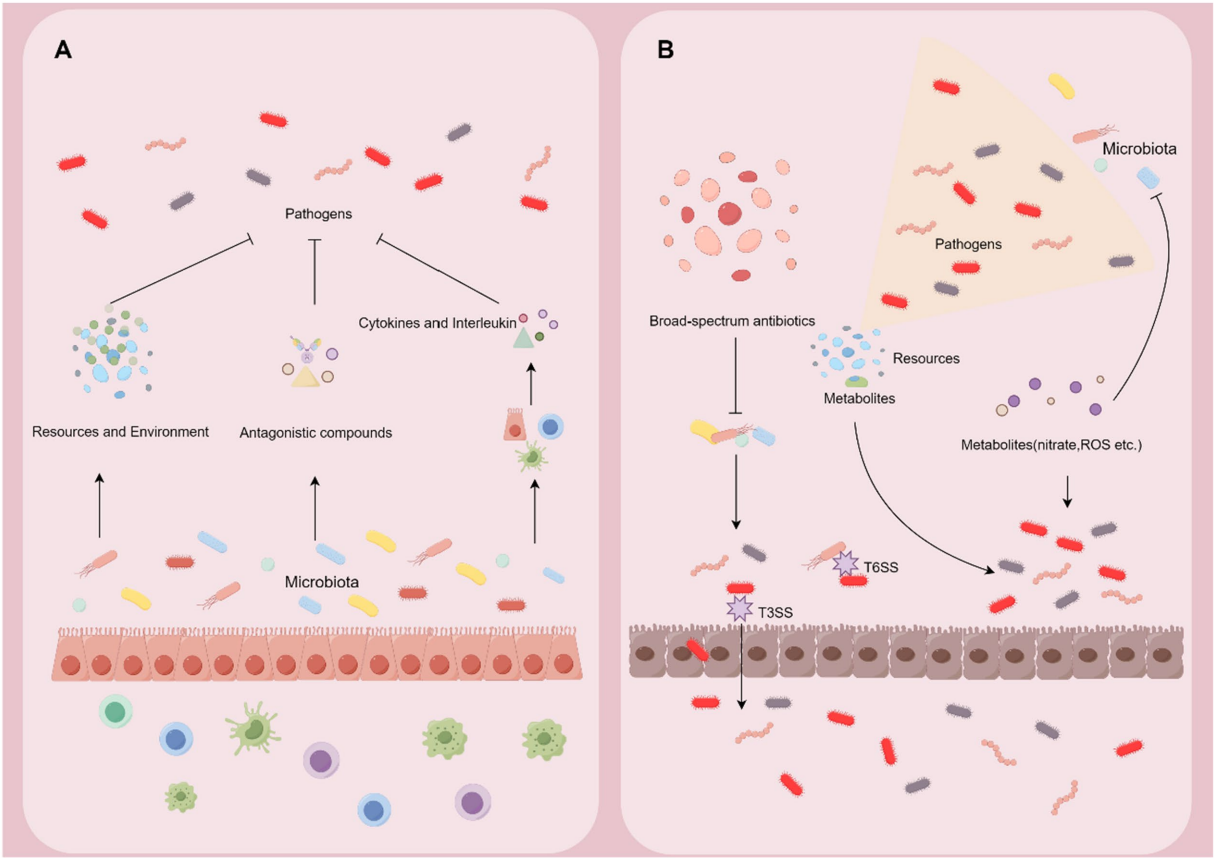


FIGURE 3
Microbiome-pathogen interaction in the gut. **(A)** Symbiotic microbiota can exert the effect of colonization resistance through altering the resource and environment in the gut, producing antagonistic compounds, and reinforcing the barrier and immune system. **(B)** Pathogens develop counterstrategies including disrupting the microbiota, exploiting nutrients and metabolites, and harnessing intestinal inflammation to break the defense from the microbiota and host.

source (Girardeau et al., 2011; Thiennimitr et al., 2011). In the case of *Citrobacter rodentium*, it stimulates the expression of genes involved in amino acid biosynthesis to produce its own amino acids, which are crucial for colonizing the gut to overcome the restriction on amino acids imposed by the commensals (Caballero-Flores et al., 2020). This pathogen also shows the ability to utilize alternative carbon sources for its initial proliferation in the gut (Jimenez et al., 2020), efficiently avoiding the limitation of indigenous bacteria. In addition, bacteria in the gut need to acquire iron, which is significant for their growth, relying on the 2,3-dihydroxy benzoate-based siderophore enterobactin (Ent), while innate immune cells produce Lcn2 to inhibit the iron acquisition and growth of commensal bacteria (Khan et al., 2021; Mayneris-Perxachs et al., 2022). However, pathogenic bacteria such as pathogenic *E. coli*, *S. typhimurium*, and *K. pneumoniae* possess a variant of Ent that allows them to evade this inhibition (Fischbach et al., 2006), indicating that some pathogens use nutrients more efficiently than commensals.

Notably, most of the symbiotic bacteria in the intestine are obligate anaerobes. Facultative anaerobe pathogens like *C. rodentium* can take advantage of oxygen availability by attaching to the epithelium and injecting effectors into host cells through Type III secretion systems (T3SS). They change the epithelial metabolism, decrease oxygen consumption, and respire excess oxygen (Lopez et al., 2016; Miller et al., 2020), ultimately enhancing their survival and growth. On the other hand, *C. rodentium* can occupy a unique niche on the intestinal epithelium surface, where commensal bacteria cannot reside, due to its expression of the distinct adhesion molecule intimin (Kamada et al., 2012), enabling *C. rodentium* to avoid competition for nutrients with commensal bacteria.

In general, the nutrient-rich intestine becomes a battleground where commensal and pathogenic microbes compete for dominance by applying various strategies for resources.

4.2 The effects of metabolites

4.2.1 Metabolite-based colonization resistance

Commensal microbiota and their metabolic byproducts can create an inhospitable environment for pathogens. The commensals are able to metabolize complex indigestible carbohydrates and mucin into SCFAs, including acetate, propionate, butyrate, valerate, and isovalerate (Nhu and Young, 2023). SCFAs are the primary energy source for colonic enterocytes and support the function of the intestinal barrier system (Fachi et al., 2019; Dang et al., 2023), but they also help resist colonization by pathogens (Osbelt et al., 2020; Pensinger et al., 2023). It was revealed that the administration of *Lactobacillus* to antibiotic-treated mice could result in an increase in fecal butyrate levels and a decrease in intestinal colonization by *K. pneumoniae*, highlighting the defensive function of commensals that produce butyrate (Djukovic et al., 2022). Butyrate was found to reduce the expression of virulence genes in *Salmonella* species, while fucose impacted the expression of virulence factors in enterohaemorrhagic *Escherichia coli* (EHEC) (Gantois et al., 2006; Curtis et al., 2012). Furthermore, the degradation of the primary bile acids in distal intestine by bacteria such as *Clostridium scindens* appeared to inhibit the growth of *C. difficile* and the virulence of *Vibrio cholerae* (Alavi et al., 2020; Foley et al., 2023). The optimum pH is essential for the development of enteropathogenic bacterial species such as *Bacillus cereus*, *E. coli*, and enterotoxigenic bacteria (Ceuppens et al., 2012; Hammami et al., 2013). *Bacteroides* were reported to resist colonization by changing the gut pH through the production of propionate (Brinkman et al., 2013), indicating that the SCFAs from commensals might help influence the pH of the environment, thus altering the physiological conditions required for pathogen virulence and survival.

The immune system can also be regulated by the metabolites from gut microbiota to resist pathogens. Pedicord et al. (2016) discovered that the commensal bacterium *Enterococcus faecium* could protect against *S. typhimurium* infection by producing secreted antigen A (SagA), a distinct peptidoglycan hydrolase. SagA can interact with PRRs in the IECs, leading to a strong innate immune response against pathogens (Pedicord et al., 2016). In a mice model of sepsis, fecal microbiota transplant (FMT) was found to improve pathogen clearance and restore host systemic immunity. This beneficial effect of FMT was associated with an increase in butyrate-producing *Bacteroidetes*, which enhanced the expression of interferon regulatory factor (IRF) 3, ultimately alleviating inflammation (Kim et al., 2020). The establishment of a specific *Bacteroidota* consortium in GF and antibiotic-treated mice could confer protection against *K. pneumoniae* by activating IL-36 signaling pathways (Sequeira et al., 2020), resulting in increased resistance to enteric pathogens. Furthermore, colonization of GF mice with commensal bacteria has been discovered to induce the presence of Th17 in the intestinal tissue (Ivanov et al., 2009), suggesting the modulation of microbiota on immune cells.

In addition, specific microbes can produce compounds that have antagonistic effects on pathogens. For example, substances with inhibitory effects on VRE include a lantibiotic produced by *Blautia producta* and bacteriocin produced by *Enterococcus faecalis* (Kommineni et al., 2015; Kim et al., 2019). *Lactobacillus lactis* and *Streptococcus* can produce Nisin-A, which is widely utilized as a food preservative due to its inhibiting the growth of gram-positive bacteria (Nhu and Young, 2023).

Commensal bacteria also demonstrate the ability to limit the colonization of pathogenic microorganisms other than bacteria by producing specific metabolites. *C. scindens* and *Clostridium orbiscindens* were discovered to exert protective effects against the Chikungunya and influenza viruses in mice, respectively. The mechanism of action for *C. scindens* involves the enhancement of the antiviral immune response through the production of deoxycholic acid. On the other hand, *C. orbiscindens* and its metabolite desaminotyrosine were reported to modulate type I IFN signaling, efficiently restraining influenza virus (Steed et al., 2017; Winkler et al., 2020). Fan et al. (2015) illustrated the critical role of commensal anaerobic bacteria, specifically clostridial *Firmicutes* (clusters *IV* and *XIVa*) and *Bacteroidetes*, in maintaining resistance to *Candida albicans* colonization in mice. This finding emphasized the significance of hypoxia-inducible factor-1α (HIF-1α) and the antimicrobial peptide LL-37 in determining the effect to resist *C. albicans* colonization (Fan et al., 2015). Another study using a mice model of parasitic protozoa revealed that *C. scindens* served a protective role against *Entamoeba histolytica* infection (Burgess et al., 2020). This protective mechanism relied on the metabolism of bile salts, as the bile salt-derived metabolite deoxycholate was found to activate the host bone marrow through epigenetic modifications, thereby boosting the immune response (Burgess et al., 2020). It is also evident that the commensal *E.coli* express anti-α-gal antibodies, which confer protection against

Plasmodium species infection (Yilmaz et al., 2014; Mandal and Schmidt, 2023), thus restricting the transmission of malaria parasites. These findings underscore the potential of gut microbiota and their metabolites to resist viral, fungal, and parasitic invasion, providing novel therapeutic options for treating these infections.

4.2.2 Metabolite-based invasion

The pathogen can take advantage of the metabolites produced by beneficial bacteria. The gut is rich in sugar molecules broken down by saccharolytic bacteria (Horrocks et al., 2023). It was found that *Bacteroides thetaiotaomicron* could break down sugar components, such as sialic acid and fucose, from the gut epithelial mucins. These sugar components could then be utilized by pathogenic bacteria such as *C. difficile* and *S. typhimurium* (Wang et al., 2013). In addition, *S. typhimurium* was exhibited to utilize gut microbiota-derived hydrogen to fuel its early-stage growth and expansion during infection (Maier et al., 2013). Taurocholate and cholate, generated by commensals, were reported to be potent triggers for the germination of *C. difficile* spores in the gut (Aguirre et al., 2021; Foley et al., 2023). A recent study revealed that the presence of the specific gut microbiota was able to resist *Campylobacter jejuni*, mainly because the microbiota rarely produced organic acids and amino acids, which were vital for *C. jejuni* growth (Shayya et al., 2023). These findings show that pathogens can effectively utilize the metabolites of resident bacteria as a source of energy to promote their growth.

Some pathogenic microbes rely on the molecules produced by microbiota as metabolic signals to promote their activity. For example, EHEC uses fucose as a signaling molecule to modify their metabolism and gene expression related to virulence and metabolic stimulus. This is achieved through a fucose-sensing signaling transduction system composed of FusK and FusR components, helping EHEC adapt to the intestinal lumen environment (Curtis et al., 2012). Similarly, ethanolamine acts as a signal molecule for EHEC and *S. typhimurium*, triggering the expression of their virulent genes (Kendall et al., 2012; Anderson et al., 2015). *C. difficile* can utilize microbiota-produced succinate to gain a growth advantage in the gut (Ferreyra et al., 2014). Regarding SCFAs, the variation of their distribution, concentration, and composition in the intestine results in the creation of distinct physiological environments sensed by pathogenic bacteria. It was observed that the concentration of acetate in the ileum region of the intestine enhanced the expression of pathogenicity island 1 (SPI-1)-encoded T3SS of *S. typhimurium*, promoting bacterium invasion (Khan et al., 2021). In contrast, propionate and butyrate in the colon were found to repress T3SS-1-related genes (Maurer et al., 2002). In the case of EHEC, T3SS expression was elevated by butyrate concentration in the colon via post-transcriptional activation of Lrp, a transcriptional regulator in EHEC (Takao et al., 2014). However, exposure to acetate and propionate in the small intestine was not evident to affect gene expression related to EHEC virulence or T3SS (Takao et al., 2014), showing that the susceptibility to infection may influenced by the metabolites of commensals.

The pathogens are also able to utilize the host immune response to gain a growth advantage over symbiotic microbes in the gut through their metabolism. Inflammation can lead to changes in the intestinal environment, creating an environment more conducive to the growth of pathogens (Fattinger et al., 2021). For example, pathogenic bacteria such as EHEC are facultative anaerobes and can use nitrate as an energy source (Lee et al., 2022). During intestinal inflammation, nitrate ($NO3^-$) production is increased in the intestine due to the migrated neutrophils and macrophages with inducible nitric oxide synthetase (iNOS). Pathogenic bacteria can apply the nitrate as an energy source through nitrate respiration, while commensal bacteria lack this ability (Winter et al., 2010). The cytokine IFN-γ also stimulates the production of hydrogen peroxide and nitric oxide in the gut, which can be converted into nitrates (Khan et al., 2021). Similarly, the influx of neutrophils during inflammation produces reactive oxygen species (ROS), which are responsible for converting $S_2O_2^{-3}$ generated by commensal bacteria into $S_4O_2^{-6}$. This form of sulfur cannot be utilized by symbionts but by pathogens like *S. typhimurium* through its specific operon *ttrSR ttrBCA* (Furne et al., 2001; Winter et al., 2010).

Furthermore, intestinal inflammation serves as a signal that triggers and amplifies the expression of virulence factors in the pathogens. A study (Licheng et al., 2005) presented that *Pseudomonas aeruginosa*, a bacterium that frequently caused infections in hospital settings, led to nosocomial infection by using its surface protein OprF to bind to the host immune factor IFN-γ. This interaction further activated a quorum sensing-dependent virulence factor PA-I lectin (Licheng et al., 2005). Additionally, IL-22 was exhibited to be abundant during *S. typhimurium* infection and correlated with high levels of galactoside 2-α-l-fucosyltransferase 2. This enzyme could promote the α(1,2)-fucosylation of mucus carbohydrates, activating fucose-related genes in other pathogenic members such as EHEC (Tu et al., 2014). The increase in mucus-derived carbohydrates in the gut lumen establishes a physiological niche enriched with pathogen-specific nutrients, altering the composition of the gut microbiota and potentially contributing to infection (Sonnenburg et al., 2005; Wang et al., 2013). Consequently, the ability to subvert colonization resistance and thrive in an inflammatory environment is a critical factor in the pathogenicity of many infectious agents.

4.3 Contact-dependent interactions

Some competitions between commensal microbes and pathogens rely on direct cell–cell contact. For indigenous microbiota, contact-dependent inhibition requires a specific receptor protein on the target cell and the encoding of various toxic effector domains with inhibitory mechanisms (Hayes et al., 2014; Wang et al., 2024). Commensal species like *E. coli* are equipped with type VI secretion systems (T6SS), a contractile nanomachine widely distributed among Gram-negative bacteria, which allow them to directly inject antimicrobial substances into their competitors like *C. rodentium*, efficiently neutralizing them and preventing the colonization (Flaugnatti et al., 2021; Serapio-Palacios et al., 2022). Furthermore, the probiotic *Bacillus* demonstrates the effect of influencing the quorum-quenching fengycins, previously known for their anti-fungal properties, effectively eradicating *Staphylococcus aureus* infection by interfering with its signaling mechanism (Piewngam et al., 2018; Su and Ding, 2023). These advanced defense strategies testify to the adaptability and complexity of bacterial survival mechanisms. However, the T6SS is also used by pathogens to compete with the native microbiota by injecting toxic effectors into nearby cells. Pathogens like *V. cholerae*, *S. typhimurium*, *Shigella sonnei*, and *C. rodentium* can apply their T6SS to kill competing beneficial microbes (Sana et al., 2016; Anderson et al.,

2017; Zhao et al., 2018; Serapio-Palacios et al., 2022), thereby promoting their colonization in the gut.

4.4 Disruption of microbiota

The dysbiosis can lead to enteric infections originating from the expansion of indigenous but potentially pathogenic microbes. Numerous factors, including host genetics, diet, and the use of antibiotics, can influence the composition and functionality of the gut microbiota (Pickard and Núñez, 2019; Qin et al., 2022). The disruption of the gut microbiota community reduces beneficial bacteria diversity or leads to an outgrowth of pathobionts, making the host more vulnerable to opportunistic infections caused by potential pathobionts (Gupta and Dey, 2023). *C. difficile* infection is a leading opportunistic infectious disease associated with diarrhea and colitis (de Nies et al., 2023). Under homeostatic conditions, the growth of *C. difficile* is controlled by commensals; however, broad-spectrum antibiotic treatment is able to induce *C. difficile* infection with the production of toxins TcdA and TcdB (Vuotto et al., 2024). Similarly, *S. typhimurium* and *E. coli* are bacterial enteric pathogens commonly linked to the consumption of contaminated food. The disruption of commensal microbiota promotes the growth of these pathogens and triggers inflammation (de Nies et al., 2023). The administration of broad-spectrum antibiotics can also create opportunities for the overgrowth of resistant pathogens (Diaz Caballero et al., 2023; Yip et al., 2023), leading to the development of difficult superinfections to treat.

Noteworthy, it was presented that gut fungal dysbiosis reduced the efficacy of FMT for treating recurrent *C. difficile* infections (Zuo et al., 2018), highlighting the interdependencies between bacteria and fungi in the GI tract. Antibiotic treatments can result in fungal overgrowth by reducing competing bacteria, while antifungal treatments can alter bacterial communities (Wheeler et al., 2016; Sovran et al., 2018). Fungi can act as a commensal that protects the host from bacterial pathogens by modulating the immunological responses of the host (van Tilburg Bernardes et al., 2020; Pérez, 2021). For example, *Candida albicans* influence host immune responses, especially Th17, which are crucial for protective immunity at barrier sites (Bacher et al., 2019). Conversely, indigenous bacteria can effectively limit the growth and invasion of fungal pathogens by targeting specific virulence factors, disrupting quorum sensing systems, secreting active metabolites, or triggering the immune response (Cong et al., 2023). Therefore, the interplay between bacteria and fungi within the gut microbiota substantially influences its balance.

Collectively, gut commensal microbes effectively exert robust resistance against pathogen colonization through diverse mechanisms. These mechanisms mainly include competition for resources, utilization of metabolic by-products, and direct contact suppression. In response, pathogens have evolved strategies to evade defenses, resulting in dysbiosis and infection. To further enhance colonization resistance, advanced therapies based on microbiota have been introduced to prevent and treat infections in the GI tract.

5 Microbiota-targeted therapy

For years, microbiota-targeted therapy has been employed to prevent and eradicate enteric infections (Sorbara and Pamer, 2022; Fekete et al., 2023). This specialized therapy involves a variety of approaches.

One approach is with FMT. The transplantation of beneficial microbiota helps restore the diversity and stability of the GI microbial community, protecting against the overgrowth of pathogenic microbes. FMT efficiently treats recurrent *C. difficile* infection (CDI), which is often refractory to conventional antibiotic therapy (Gupta et al., 2022; Gonzales-Luna et al., 2023). A study demonstrated that the administration of a consortium of five special strains effectively limited VRE gut colonization in antibiotic-treated mice by depleting nutrients, particularly fructose (Isaac et al., 2022). This provides a new strategy for applying FMT to treating enteric infections caused by various pathogens. FMT can be performed through multiple routes, such as oral capsules, enemas, or colonoscopy, depending on the severity of the infection and the patient's preference (Liwinski and Elinav, 2020). Nonetheless, further research is necessary to determine suitable donors for FMT, the most effective administration methods, and the pivotal factors influencing therapeutic effectiveness. Personalized approaches that consider the unique composition of gut microbiota and immune system in each individual are crucial for maximizing the efficacy of FMT.

Another approach is the administration of probiotics. Probiotics are live microorganisms that provide benefits to the host. These microorganisms can act by directly inhibiting the growth of pathogens, enhancing the immune response, and producing antimicrobial substances (Zhong et al., 2022). Furthermore, prebiotic supplementations are dietary compounds that selectively promote the growth and activity of beneficial microorganisms in the gut. They serve as a source of nutrients for these microbes, thus indirectly enhancing the colonization resistance. Prebiotics can be found in various foods such as fruits, vegetables, whole grains, and legumes (Sanders et al., 2019). The application of prebiotics and probiotics in treating enteric infections requires careful consideration of the specific strains and doses to ensure their efficacy. Understanding the metabolic pathways commensal microbiota uses for infection prevention will aid in developing next-generation prebiotics and probiotics with enhanced anti-pathogenic capacity. However, in some instances, it may be more beneficial to restrict bacterial nutrients rather than adding a prebiotic. This can be achieved through dietary changes, such as following a low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) diet (McCuaig and Goto, 2023). By removing the resource in the gut, the microbial community is altered, highlighting the impact of nutrient restriction on microbial composition.

Additionally, there has been a remarkable advancement in the application of small microbial molecules and engineered bacteriophages for the targeted eradication of specific pathogens. Small microbial molecules, such as butyrate, produced by beneficial microorganisms, act as supplements to overcome the challenges associated with engrafting live microorganisms (Chen et al., 2019). Engineered bacteriophages, which are viruses that specifically target and kill bacteria, can selectively eliminate pathogenic bacteria while sparing the beneficial ones (Divya Ganeshan and Hosseinidoust, 2019; Jault et al., 2019). The potential of harnessing bacteria that produce AMPs to enhance colonization resistance is also worth investigating further.

The integration of emerging metagenomics, novel imaging technologies, and mathematical modeling potentially contributes to

precision microbiome reconstitution therapy in the gut (Bilotta and Cong, 2019; Chetty and Blekhman, 2024). Further investigation is essential to uncover novel and unique therapeutic targets within the microbiota which can be utilized for the precise prevention and management of enteric infections.

6 Conclusion

This review mainly discusses about two crucial interactions involving microbiota in the gut. The first is the crosstalk between the host and microbial symbionts, while the second is the constant battle between the commensal microbiota and pathogens. The microbiota serves as an indispensable mediator in these interactions, orchestrating the gut ecosystem and the resistance to pathogen colonization, whereas dysbiosis can lead to enteric infection. Therapies focusing on the gut microbiota primarily aim to restore balance in the indigenous symbionts and enhance their protective functions. A deeper understanding of the mediating roles of microbiota is vital to providing effective and personalized treatments for enteric infections.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Abt, M. C., Lewis, B. B., Caballero, S., Xiong, H., Carter, R. A., Sušac, B., et al. (2015). Innate immune defenses mediated by two ILC subsets are critical for protection against acute *Clostridium difficile* infection. *Cell Host Microbe* 18, 27–37. doi: 10.1016/j.chom.2015.06.011

Aguirre, A. M., Yalcinkaya, N., Wu, Q., Swennes, A., Tessier, M. E., Roberts, P., et al. (2021). Bile acid-independent protection against Clostridioides difficile infection. *PLoS Pathog.* 17:e1010015. doi: 10.1371/journal.ppat.1010015

Alavi, S., Mitchell, J. D., Cho, J. Y., Liu, R., Macbeth, J. C., and Hsiao, A. (2020). Interpersonal gut microbiome variation drives susceptibility and resistance to cholera infection. *Cell* 181, 1533–1546.e13. doi: 10.1016/j.cell.2020.05.036

Anderson, C. J., Clark, D. E., Adli, M., and Kendall, M. M. (2015). Ethanolamine signaling promotes Salmonella niche recognition and adaptation during infection. *PLoS Pathog.* 11:e1005278. doi: 10.1371/journal.ppat.1005278

Anderson, M. C., Vonaesch, P., Saffarian, A., Marteyn, B. S., and Sansonetti, P. J. (2017). *Shigella sonnei* encodes a functional T6SS used for Interbacterial competition and niche occupancy. *Cell Host Microbe* 21, 769–776.e3. doi: 10.1016/j.chom.2017.05.004

Bacher, P., Hohnstein, T., Beerbaum, E., Röcker, M., Blango, M. G., Kaufmann, S., et al. (2019). Human anti-fungal Th17 immunity and pathology rely on Cross-reactivity against *Candida albicans*. *Cell* 176, 1340–1355.e15. doi: 10.1016/j.cell.2019.01.041

Behnsen, J., Zhi, H., Aron, A. T., Subramanian, V., Santus, W., Lee, M. H., et al. (2021). Siderophore-mediated zinc acquisition enhances enterobacterial colonization of the inflamed gut. *Nat. Commun.* 12:7016. doi: 10.1038/s41467-021-27297-2

Bhinder, G., Stahl, M., Sham, H. P., Crowley, S. M., Morampudi, V., Dalwadi, U., et al. (2014). Intestinal epithelium-specific MyD88 signaling impacts host susceptibility to infectious colitis by promoting protective goblet cell and antimicrobial responses. *Infect. Immun.* 82, 3753–3763. doi: 10.1128/IAI.02045-14

Bilotta, A. J., and Cong, Y. (2019). Gut microbiota metabolite regulation of host defenses at mucosal surfaces: implication in precision medicine. *Precis. Clin. Med.* 2, 110–119. doi: 10.1093/pcmedi/pbz008

Bosch, T. C. G., and Zasloff, M. (2021). Antimicrobial peptides-or how our ancestors learned to control the microbiome. *MBio* 12:e0184721. doi: 10.1128/mBio.01847-21

Bousbaine, D., Fisch, L. I., London, M., Bhagchandani, P., Rezende de Castro, T. B., Mimee, M., et al. (2022). A conserved Bacteroidetes antigen induces anti-inflammatory intestinal T lymphocytes. *Science* 377, 660–666. doi: 10.1126/science.abg5645

Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I. G., et al. (2008). Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* 456, 507–510. doi: 10.1038/nature07450

Brandl, K., Brandl, K., Plitas, G., Mihu, C. N., Ubeda, C., Jia, T., et al. (2008). Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* 455, 804–807. doi: 10.1038/nature07250

Brennan, C. A., and Garrett, W. S. (2019). *Fusobacterium nucleatum* - symbiont, opportunist and oncobacterium. *Nat. Rev. Microbiol.* 17, 156–166. doi: 10.1038/s41579-018-0129-6

Brinkman, B. M. N., Brinkman, B. M., Becker, A., Ayiseh, R. B., Hildebrand, F., Raes, J., et al. (2013). Gut microbiota affects sensitivity to acute DSS-induced colitis independently of host genotype. *Inflamm. Bowel Dis.* 19, 2560–2567. doi: 10.1097/mib.0b013e3182a8759a

Bunker, J. J., Erickson, S. A., Flynn, T. M., Henry, C., Koval, J. C., Meisel, M., et al. (2017). Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science* 358:eaan6619. doi: 10.1126/science.aan6619

Bunker, J. J., Flynn, T. M., Koval, J. C., Shaw, D. G., Meisel, M., McDonald, B. D., et al. (2015). Innate and adaptive humoral responses coat distinct commensal Bacteria with immunoglobulin a. *Immunity* 43, 541–553. doi: 10.1016/j.immuni.2015.08.007

Burgess, S. L., Leslie, J. L., Uddin, J., Oakland, D. N., Gilchrist, C., Moreau, G. B., et al. (2020). Gut microbiome communication with bone marrow regulates susceptibility to amebiasis. *J Clin Invest.* 130, 4019–4024. doi: 10.1172/JCI133605

Caballero-Flores, G., Pickard, J. M., Fukuda, S., Inohara, N., and Núñez, G. (2020). An enteric pathogen subverts colonization resistance by evading competition for amino acids in the gut. *Cell Host Microbe* 28, 526–533.e5. doi: 10.1016/j.chom.2020.06.018

Caballero-Flores, G., Pickard, J. M., and Núñez, G. (2023). Microbiota-mediated colonization resistance: mechanisms and regulation. *Nat. Rev. Microbiol.* 21, 347–360. doi: 10.1038/s41579-022-00833-7

Cerovic, V., Mowat, A. M. I., Bain, C. C., Mowat, A. M. I., and Milling, S. (2014). Intestinal macrophages and dendritic cells: what's the difference? *Trends Immunol.* 35, 270–277. doi: 10.1016/j.it.2014.04.003

Cervantes-Barragan, L., Chai, J. N., Tianero, M. D., Di Luccia, B., Ahern, P. P., Merriman, J., et al. (2017). *Lactobacillus reuteri* induces gut intraepithelial CD4+CD8αα+ T cells. *Science* 357, 806–810. doi: 10.1126/science.aah5825

Ceuppens, S., Rajkovic, A., Hamelink, S., Hamelink, S., Van de Wiele, T., Boon, N., et al. (2012). Enterotoxin production by *Bacillus cereus* under gastrointestinal conditions and their immunological detection by commercially available kits. *Foodborne Pathog. Dis.* 9, 1130–1136. doi: 10.1089/fpd.2012.1230

Chang, P. V., Chang, P. V., Hao, L., Offermanns, S., and Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. USA* 111, 2247–2252. doi: 10.1073/pnas.1322269111

Chen, B., Ni, X., Sun, R., Zeng, B., Wei, H., Tian, Z., et al. (2018). Commensal Bacteria-dependent CD8αβ+ T cells in the intestinal epithelium produce antimicrobial peptides. *Front. Immunol.* 9:1065. doi: 10.3389/fimmu.2018.01065

Chen, H., Nwe, P.-K., Yang, Y., Rosen, C. E., Bielecka, A. A., Kuchroo, M., et al. (2019). A forward chemical genetic screen reveals gut microbiota metabolites that modulate host physiology. *Cell* 177, 1217–1231.e18. doi: 10.1016/j.cell.2019.03.036

Cherrier, D. E., Serafini, N., and Di Santo, J. P. (2018). Innate lymphoid cell development: a T cell perspective. *Immunity* 48, 1091–1103. doi: 10.1016/j.immuni.2018.05.010

Chetty, A., and Blekhman, R. (2024). Multi-omic approaches for host-microbiome data integration. *Gut Microbes* 16:2297860. doi: 10.1080/19490976.2023.2297860

Clarke, T. B., Davis, K. M., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Zhou, A., et al. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16, 228–231. doi: 10.1038/nm.2087

Cong, L., Chen, C., Mao, S., Han, Z., Zhu, Z., and Li, Y. (2023). Intestinal bacteria-a powerful weapon for fungal infections treatment. *Front. Cell. Infect. Microbiol.* 13:1187831. doi: 10.3389/fcimb.2023.1187831

Constantinides, M. G., Link, V. M., Tamoutounour, S., Wong, A. C., Perez-Chaparro, P. J., Han, S.-J., et al. (2019). MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science* 366:eaax6624. doi: 10.1126/science.aax6624

Corbett, A. J., Eckle, S. B. G., Birkinshaw, R. W., Liu, L., Patel, O., Patel, O., et al. (2014). T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* 509, 361–365. doi: 10.1038/nature13160

Cording, S., Medvedovic, J., Eberl, G., Cherrier, M., and Eberl, G. (2014). Development and regulation of RORγt+ innate lymphoid cells. *FEBS Lett.* 588, 4176–4181. doi: 10.1016/j.febslet.2014.03.034

Curtis, M. M., Pacheco, A. R., Ritchie, J. M., Curtis, M. M., Ritchie, J. M., Munera, D., et al. (2012). Fucose sensing regulates bacterial intestinal colonization. *Nature* 492, 113–117. doi: 10.1038/nature11623

Da Silva, C., Wagner, C., Bonnardel, J., Gorvel, J.-P., and Lelouard, H. (2017). The Peyer's patch mononuclear phagocyte system at steady state and during infection. *Front. Immunol.* 8:1254. doi: 10.3389/fimmu.2017.01254

Dang, Y., Ma, C., Chen, K., Chen, Y., Jiang, M., Hu, K., et al. (2023). The effects of a high-fat diet on inflammatory bowel disease. *Biomol. Ther.* 13:905. doi: 10.3390/biom13060905

de Nies, L., Kobras, C. M., and Stracy, M. (2023). Antibiotic-induced collateral damage to the microbiota and associated infections. *Nat. Rev. Microbiol.* 21, 789–804. doi: 10.1038/s41579-023-00936-9

de Vos, W. M., Tilg, H., Van Hul, M., and Cani, P. D. (2022). Gut microbiome and health: mechanistic insights. *Gut* 71, 1020–1032. doi: 10.1136/gutjnl-2021-326789

Dey, P., Chaudhuri, S. R., Efferth, T., and Pal, S. (2021). The intestinal 3M (microbiota, metabolism, metabolome) zeitgeist - from fundamentals to future challenges. *Free Radic. Biol. Med.* 176, 265–285. doi: 10.1016/j.freeradbiomed.2021.09.026

Diaz Caballero, J., Wheatley, R. M., Kapel, N., López-Causapé, C., Van der Schalk, T., Quinn, A., et al. (2023). Mixed strain pathogen populations accelerate the evolution of antibiotic resistance in patients. *Nat. Commun.* 14:4083. doi: 10.1038/s41467-023-39416-2

Diehl, G. E., Longman, R. S., Zhang, J.-X., Breart, B., Galan, C., Bréart, B., et al. (2013). Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX 3 CR1 hi cells. *Nature* 494, 116–120. doi: 10.1038/nature11809

Divya Ganeshan, S., and Hosseinidoust, Z. (2019). Phage therapy with a focus on the human microbiota. *Antibiotics* 8:131. doi: 10.3390/antibiotics8030131

Djukovic, A., Garzón, M. J., Canlet, C., Cabral, V., Lalaoui, R., García-Garcerá, M., et al. (2022). Lactobacillus supports Clostridiales to restrict gut colonization by multidrug-resistant Enterobacteriaceae. *Nat. Commun.* 13:5617. doi: 10.1038/s41467-022-33313-w

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W.-C., et al. (2018). Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science* 360, 795–800. doi: 10.1126/science.aaq0926

Ducarmon, Q. R., Zwittink, R. D., Hornung, B. V. H., van Schaik, W., Young, V. B., and Kuijper, E. J. (2019). Gut microbiota and colonization resistance against bacterial enteric infection. *Microbiol. Mol. Biol. Rev.* 83, e00007–e00019. doi: 10.1128/MMBR.00007-19

Dudakov, J. A., Hanash, A. M., and van den Brink, M. R. M. (2015). Interleukin-22: Immunobiology and pathology. *Annu. Rev. Immunol.* 33, 747–785. doi: 10.1146/annurev-immunol-032414-112123

Fabich, A. J., Fabich, A. J., Jones, S. A., Jones, S. A., Chowdhury, F. Z., Cernosek, A., et al. (2008). Comparison of carbon nutrition for pathogenic and commensal *Escherichia coli* strains in the mouse intestine. *Infect. Immun.* 76, 1143–1152. doi: 10.1128/iai.01386-07

Fachi, J. L., Felipe, J. D. S., Pral, L. P., Da Silva, B. K., Corrêa, R. O., De Andrade, M. C. P., et al. (2019). Butyrate protects mice from *Clostridium difficile*-induced colitis through an HIF-1-dependent mechanism. *Cell Rep.* 27, 750–761.e7. doi: 10.1016/j.celrep.2019.03.054

Fadlallah, J., El Kafsi, H., Sterlin, D., Juste, C., Parizot, C., Dorgham, K., et al. (2018). Microbial ecology perturbation in human IgA deficiency. *Sci. Transl. Med.* 10:eaan1217. doi: 10.1126/scitranslmed.aan1217

Fagarasan, S., Fagarasan, S., Kawamoto, S., Kanagawa, O., and Suzuki, K. (2010). Adaptive immune regulation in the gut: T cell–dependent and T cell–independent IgA synthesis. *Annu. Rev. Immunol.* doi: 10.1146/annurev-immunol-030409-101314

Fan, D., Coughlin, L., Fan, D., Neubauer, M. M., Coughlin, L. A., Neubauer, M. M., et al. (2015). Activation of HIF-1α and LL-37 by commensal bacteria inhibits *Candida albicans* colonization. *Nat. Med.* 21, 808–814. doi: 10.1038/nm.3871

Farache, J., Koren, I., Milo, I., Gurevich, I., Kim, K.-W., Zigmond, E., et al. (2013). Luminal Bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* 38, 581–595. doi: 10.1016/j.immuni.2013.01.009

Fattinger, S. A., Sellin, M. E., and Hardt, W.-D. (2021). Salmonella effector driven invasion of the gut epithelium: breaking in and setting the house on fire. *Curr. Opin. Microbiol.* 64, 9–18. doi: 10.1016/j.mib.2021.08.007

Fekete, E. E., Figeys, D., and Zhang, X. (2023). Microbiota-directed biotherapeutics: considerations for quality and functional assessment. *Gut Microbes* 15:2186671. doi: 10.1080/19490976.2023.2186671

Ferreyra, J. A., Katherine, W., Katherine, W., Katherine, W., Bouley, D. M., Hryckowian, A. J., et al. (2014). Gut microbiota-produced succinate promotes *C. difficile* infection after antibiotic treatment or motility disturbance. *Cell Host Microbe* 16, 770–777. doi: 10.1016/j.chom.2014.11.003

Fischbach, M. A., Lin, H., Walsh, C. T., Liu, D. R., and Walsh, C. T. (2006). How pathogenic bacteria evade mammalian sabotage in the battle for iron. *Nat. Chem. Biol.* 2, 132–138. doi: 10.1038/nchembio771

Flaugnatti, N., Isaac, S., Lemos Rocha, L. F., Stutzmann, S., Rendueles, O., Stoudmann, C., et al. (2021). Human commensal gut Proteobacteria withstand type VI secretion attacks through immunity protein-independent mechanisms. *Nat. Commun.* 12:5751. doi: 10.1038/s41467-021-26041-0

Foley, M. H., Walker, M. E., Stewart, A. K., O'Flaherty, S., Gentry, E. C., Patel, S., et al. (2023). Bile salt hydrolases shape the bile acid landscape and restrict Clostridioides difficile growth in the murine gut. *Nat. Microbiol.* 8, 611–628. doi: 10.1038/s41564-023-01337-7

Frantz, A. L., Rogier, E. W., Weber, C. R., Shen, L., Cohen, D. A., Fenton, L. A., et al. (2012). Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides. *Mucosal Immunol.* 5, 501–512. doi: 10.1038/mi.2012.23

Furne, J. K., Springfield, J., Koenig, T., Koenig, T., DeMaster, E. G., DeMaster, E. G., et al. (2001). Oxidation of hydrogen sulfide and methanethiol to thiosulfate by rat tissues: a specialized function of the colonic mucosa. *Biochem. Pharmacol.* 62, 255–259. doi: 10.1016/s0006-2952(01)00657-8

Furuse, M. (2010). Molecular basis of the Core structure of tight junctions. *Cold Spring Harb. Perspect. Biol.* 2:2907. doi: 10.1101/cshperspect.a002907

Ganal, S., Ganal, S. C., Sanos, S. L., Kallfass, C., Oberle, K., Johner, C., et al. (2012). Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity* 37, 171–186. doi: 10.1016/j.immuni.2012.05.020

Gantois, I., Ducatelle, R., Pasmans, F., Hautefort, I., Haesebrouck, F., Haesebrouck, F., et al. (2006). Butyrate specifically Down-regulates Salmonella Pathogenicity Island 1 gene expression. *Appl. Environ. Microbiol.* 72, 946–949. doi: 10.1128/aem.72.1.946-949.2006

García-Hernández, V., Garcia-Hernandez, V., Quiros, M., and Nusrat, A. (2017). Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. *Ann. N. Y. Acad. Sci.* 1397, 66–79. doi: 10.1111/nyas.13360

Garsin, D. A. (2010). Ethanolamine utilization in bacterial pathogens: roles and regulation. *Nat. Rev. Microbiol.* 8, 290–295. doi: 10.1038/nrmicro2334

GBD 2019 Antimicrobial Resistance Collaborators (2022). Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the global burden of disease study 2019. *Lancet* 400, 2221–2248. doi: 10.1016/S0140-6736(22)02185-7

Gesualdo, L., Di Leo, V., and Coppo, R. (2021). The mucosal immune system and IgA nephropathy. *Semin. Immunopathol.* 43, 657–668. doi: 10.1007/s00281-021-00871-y

Girardeau, J. P., Bertin, Y., Girardeau, J.-P., Chaucheyras-Durand, F., Chaucheyras-Durand, F., Lyan, B., et al. (2011). Enterohaemorrhagic *Escherichia coli* gains a competitive advantage by using ethanolamine as a nitrogen source in the bovine intestinal content. *Environ. Microbiol.* 13, 365–377. doi: 10.1111/j.1462-2920.2010.02334.x

Gong, T., Fu, J., Shi, L., Chen, X., and Zong, X. (2021). Antimicrobial peptides in gut health: a review. *Front. Nutr.* 8:751010. doi: 10.3389/fnut.2021.751010

Gonzales-Luna, A. J., Carlson, T. J., and Garey, K. W. (2023). Gut microbiota changes associated with Clostridioides difficile infection and its various treatment strategies. *Gut Microbes* 15:2223345. doi: 10.1080/19490976.2023.2223345

Guo, X., Liang, Y., Liang, Y., Zhang, Y., Lasorella, A., Lasorella, A., et al. (2015). Innate lymphoid cells control early colonization resistance against intestinal pathogens through ID2-dependent regulation of the microbiota. *Immunity* 42, 731–743. doi: 10.1016/j.immuni.2015.03.012

Gupta, U., and Dey, P. (2023). Rise of the guardians: gut microbial maneuvers in bacterial infections. *Life Sci.* 330:121993. doi: 10.1016/j.lfs.2023.121993

Gupta, K., Tappiti, M., Nazir, A. M., Koganti, B., Memon, M. S., Aslam Zahid, M. B., et al. (2022). Fecal microbiota transplant in recurrent *Clostridium Difficile* infections: a systematic review. *Cureus* 14:e24754. doi: 10.7759/cureus.24754

Hammami, R., Lacroix, C., Fernandez, B., Lacroix, C., and Fliss, I. (2013). Anti-infective properties of bacteriocins: an update. *Cell. Mol. Life Sci.* 70, 2947–2967. doi: 10.1007/s00018-012-1202-3

Harrandah, A. M. (2023). The role of Fusobacteria in oral cancer and immune evasion. *Curr. Opin. Oncol.* 35, 125–131. doi: 10.1097/CCO.0000000000000927

Hayes, C. S., Koskiniemi, S., Ruhe, Z. C., Poole, S. J., and Low, D. A. (2014). Mechanisms and biological roles of contact-dependent growth inhibition systems. *Cold Spring Harb. Perspect. Med.* 4:a010025. doi: 10.1101/cshperspect.a010025

Hengst, J., Strunz, B., Deterding, K., Ljunggren, H.-G., Leeansyah, E., Manns, M. P., et al. (2016). Nonreversible MAIT cell-dysfunction in chronic hepatitis C virus infection despite successful interferon-free therapy. *Eur. J. Immunol.* 46, 2204–2210. doi: 10.1002/eji.201646447

Hepworth, M. R., Fung, T. C., Masur, S. H., Fung, T. C., Masur, S. H., Kelsen, J. R., et al. (2015). Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria–specific CD4+ T cells. *Science* 348, 1031–1035. doi: 10.1126/science.aaa4812

Hepworth, M. R., Monticelli, L. A., Fung, T. C., Ziegler, C. G. K., Sinha, R., Grunberg, S., et al. (2013). Innate lymphoid cells regulate CD4 + T-cell responses to intestinal commensal bacteria. *Nature* 498, 113–117. doi: 10.1038/nature12240

Horrocks, V., King, O. G., Yip, A. Y. G., Marques, I. M., and McDonald, J. A. K. (2023). Role of the gut microbiota in nutrient competition and protection against intestinal pathogen colonization. *Microbiology* 169:001377. doi: 10.1099/mic.0.001377

Hou, K., Wu, Z.-X., Chen, X.-Y., Wang, J.-Q., Zhang, D., Xiao, C., et al. (2022). Microbiota in health and diseases. *Signal Transduct. Target. Ther.* 7:135. doi: 10.1038/s41392-022-00974-4

Huang, Q., Seillet, C., and Belz, G. T. (2017). Shaping innate lymphoid cell diversity. *Front. Immunol.* 8:1569. doi: 10.3389/fimmu.2017.01569

Isaac, S., Flor-Duro, A., Carruana, G., Puchades-Carrasco, L., Quirant, A., Lopez-Nogueroles, M., et al. (2022). Microbiome-mediated fructose depletion restricts murine gut colonization by vancomycin-resistant Enterococcus. *Nat. Commun.* 13:7718. doi: 10.1038/s41467-022-35380-5

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous Bacteria. *Cell* 139, 485–498. doi: 10.1016/j.cell.2009.09.033

Jault, P., Leclerc, T., Jennes, S., Pirnay, J. P., Que, Y.-A., Resch, G., et al. (2019). Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect. Dis.* 19, 35–45. doi: 10.1016/S1473-3099(18)30482-1

Ji, J., Ji, J., Shu, D., Shu, D., Zheng, M., Wang, J., et al. (2016). Microbial metabolite butyrate facilitates M2 macrophage polarization and function. *Sci. Rep.* 6:24838. doi: 10.1038/srep24838

Jian, H., Liu, Y., Wang, X., Dong, X., and Zou, X. (2023). *Akkermansia muciniphila* as a next-generation probiotic in modulating human metabolic homeostasis and disease progression: a role mediated by gut-liver-brain axes? *Int. J. Mol. Sci.* 24:3900. doi: 10.3390/ijms24043900

Jiao, Y., Wu, L., Huntington, N. D., and Zhang, X. (2020). Crosstalk between gut microbiota and innate immunity and its implication in autoimmune diseases. *Front. Immunol.* 11:282. doi: 10.3389/fimmu.2020.00282

Jimenez, A. G., Ellermann, M., Abbott, W., and Sperandio, V. (2020). Diet-derived galacturonic acid regulates virulence and intestinal colonization in enterohaemorrhagic Escherichia coli and *Citrobacter rodentium*. *Nat. Microbiol.* 5, 368–378. doi: 10.1038/s41564-019-0641-0

Kamada, N., Kim, Y. G., Sham, H. P., Vallance, B. A., Vallance, B. A., Puente, J. L., et al. (2012). Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science* 336, 1325–1329. doi: 10.1126/science.1222195

Kawamoto, S., Tran, T. H., Maruya, M., Suzuki, K., Doi, Y., Tsutsui, Y., et al. (2012). The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science* 336, 485–489. doi: 10.1126/science.1217718

Kayama, H., Okumura, R., and Takeda, K. (2020). Interaction between the microbiota, epithelia, and immune cells in the intestine. *Annu. Rev. Immunol.* 38, 23–48. doi: 10.1146/annurev-immunol-070119-115104

Kendall, M. M., Gruber, C. C., Parker, C., and Sperandio, V. (2012). Ethanolamine controls expression of genes encoding components involved in Interkingdom signaling and virulence in Enterohemorrhagic *Escherichia coli* O157:H7. *MBio* 3, e00050–e00012. doi: 10.1128/mbio.00050-12

Khan, I., Bai, Y., Zha, L., Ullah, N., Ullah, H., Shah, S. R. H., et al. (2021). Mechanism of the gut microbiota colonization resistance and enteric pathogen infection. *Front. Cell. Infect. Microbiol.* 11:716299. doi: 10.3389/fcimb.2021.716299

Khosravi, A., Yáñez, A., Price, J., Mérad, M., Chow, A., Merad, M., et al. (2014). Gut microbiota promote hematopoiesis to control bacterial infection. *Cell Host Microbe* 15, 374–381. doi: 10.1016/j.chom.2014.02.006

Kim, S. G., Becattini, S., Moody, T. U., Shliaha, P. V., Littmann, E. R., Seok, R., et al. (2019). Microbiota-derived lantibiotic restores resistance against vancomycin-resistant Enterococcus. *Nature* 572, 665–669. doi: 10.1038/s41586-019-1501-z

Kim, S., Covington, A., and Pamer, E. G. (2017). The intestinal microbiota: antibiotics, colonization resistance, and enteric pathogens. *Immunol. Rev.* 279, 90–105. doi: 10.1111/imr.12563

Kim, S. M., DeFazio, J. R., DeFazio, J. R., Hyoju, S., Sangani, K., Keskey, R., et al. (2020). Fecal microbiota transplant rescues mice from human pathogen mediated sepsis by restoring systemic immunity. *Nat. Commun.* 11:2354. doi: 10.1038/s41467-020-15545-w

Kinnebrew, M. A., Ubeda, C., Zenewicz, L. A., Smith, N., Flavell, R. A., and Pamer, E. G. (2010). Bacterial flagellin stimulates toll-like receptor 5—dependent defense against vancomycin-resistant Enterococcus infection. *J. Infect. Dis.* 201, 534–543. doi: 10.1086/650203

Klüber, P., Meurer, S. K., Lambertz, J., Schwarz, R., Zechel-Gran, S., Braunschweig, T., et al. (2021). Depletion of Lipocalin 2 (LCN2) in mice leads to Dysbiosis and persistent colonization with segmented filamentous Bacteria. *Int. J. Mol. Sci.* 22:13156. doi: 10.3390/ijms222313156

Kobayashi, T., Matsuoka, K., Matsuoka, K., Sheikh, S. Z., Russo, S. M., Mishima, Y., et al. (2012). IL-10 regulates Il12b expression via histone deacetylation: implications for intestinal macrophage homeostasis. *J. Immunol.* 189, 1792–1799. doi: 10.4049/jimmunol.1200042

Kogut, M. H., Lee, A., and Santin, E. (2020). Microbiome and pathogen interaction with the immune system. *Poult. Sci.* 99, 1906–1913. doi: 10.1016/j.psj.2019.12.011

Kommineni, S., Bretl, D. J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., et al. (2015). Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature* 526, 719–722. doi: 10.1038/nature15524

Krabbendam, L., Bernink, J. H., Germar, K., Krabbendam, L., Germar, K., de Jong, E. C., et al. (2015). Interleukin-12 and -23 control plasticity of CD127(+) group 1 and group 3 innate lymphoid cells in the intestinal Lamina Propria. *Immunity* 43, 146–160. doi: 10.1016/j.immuni.2015.06.019

Lavin, Y., Lavin, Y., Rahman, A., Mortha, A., Rahman, A., Mérad, M., et al. (2015). Regulation of macrophage development and function in peripheral tissues. *Nat. Rev. Immunol.* 15, 731–744. doi: 10.1038/nri3920

Lee, J.-Y., Tsolis, R. M., and Bäumler, A. J. (2022). The microbiome and gut homeostasis. *Science* 377:eabp9960. doi: 10.1126/science.abp9960

Li, J.-H., Chen, Y., Ye, Z.-H., Chen, L.-P., Xu, J.-X., Han, J., et al. (2024). Suppression of MyD88 disturbs gut microbiota and activates the NLR pathway and hence fails to ameliorate DSS-induced colitis. *Precis. Clin. Med.* 7:pbae013. doi: 10.1093/pcmedi/pbae013

Li, L., Cheng, R., Wu, Y., Lin, H., Gan, H., and Zhang, H. (2024). Diagnosis and management of inflammatory bowel disease. *J. Evid. Based Med.* 17, 409–433. doi: 10.1111/jebm.12626

Licheng, W., Estrada, O., Zaborina, O., Bains, M., Shen, L., Kohler, J. E., et al. (2005). Recognition of host immune activation by *Pseudomonas aeruginosa*. *Science* 309, 774–777. doi: 10.1126/science.1112422

Liwinski, T., and Elinav, E. (2020). Harnessing the microbiota for therapeutic purposes. *Am. J. Transplant.* 20, 1482–1488. doi: 10.1111/ajt.15753

Lockhart, A., Mucida, D., and Bilate, A. M. (2024). Intraepithelial lymphocytes of the intestine. *Annu. Rev. Immunol.* 42, 289–316. doi: 10.1146/annurev-immunol-090222-100246

Lopez, C. A., Miller, B. M., Rivera-Chávez, F., Velazquez, E. M., Byndloss, M. X., Chávez-Arroyo, A., et al. (2016). Virulence factors enhance *Citrobacter rodentium* expansion through aerobic respiration. *Science* 353, 1249–1253. doi: 10.1126/science.aag3042

Mabbott, N. A., Donaldson, D. S., Ohno, H., Williams, I. R., and Mahajan, A. (2013). Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol.* 6, 666–677. doi: 10.1038/mi.2013.30

Maier, L. A., Vyas, R., Cordova, C. D., Lindsay, H., Schmidt, T., Schmidt, T. S. B., et al. (2013). Microbiota-derived hydrogen fuels *Salmonella Typhimurium* invasion of the gut ecosystem. *Cell Host Microbe* 14, 641–651. doi: 10.1016/j.chom.2013.11.002

Maltby, R., Leatham-Jensen, M. P., Gibson, T., Cohen, P. S., and Conway, T. (2013). Nutritional basis for colonization resistance by human commensal *Escherichia coli* strains HS and Nissle 1917 against *E. coli* O157:H7 in the mouse intestine. *PLoS One* 8:53957. doi: 10.1371/journal.pone.0053957

Mandal, R. K., and Schmidt, N. W. (2023). Mechanistic insights into the interaction between the host gut microbiome and malaria. *PLoS Pathog.* 19:e1011665. doi: 10.1371/journal.ppat.1011665

Masahata, K., Umemoto, E., Kayama, H., Kayama, H., Kotani, M., Nakamura, S., et al. (2014). Generation of colonic IgA-secreting cells in the caecal patch. *Nat. Commun.* 5:3704. doi: 10.1038/ncomms4704

Maurer, R., Lawhon, S. D., Russell Maurer, M., Suyemoto, M., and Altier, C. (2002). Intestinal short-chain fatty acids alter *Salmonella typhimurium* invasion gene expression and virulence through BarA/SirA. *Mol. Microbiol.* 46, 1451–1464. doi: 10.1046/j.1365-2958.2002.03268.x

Maynard, C. L., Elson, C. O., Hatton, R. D., and Weaver, C. T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489, 231–241. doi: 10.1038/nature11551

Mayneris-Perxachs, J., Moreno-Navarrete, J. M., and Fernández-Real, J. M. (2022). The role of iron in host-microbiota crosstalk and its effects on systemic glucose metabolism. *Nat. Rev. Endocrinol.* 18, 683–698. doi: 10.1038/s41574-022-00721-3

Mazmanian, S. K., and Kasper, D. L. (2006). The love-hate relationship between bacterial polysaccharides and the host immune system. *Nat. Rev. Immunol.* 6, 849–858. doi: 10.1038/nri1956

McCuaig, B., and Goto, Y. (2023). Immunostimulating commensal Bacteria and their potential use as therapeutics. *Int. J. Mol. Sci.* 24:15644. doi: 10.3390/ijms242115644

McGovern, D., Jones, M., Jones, M., Jones, M. R., Jones, M., Jones, M., et al. (2010). Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. *Hum. Mol. Genet.* 19, 3468–3476. doi: 10.1093/hmg/ddq248

Merlini, E., Cerrone, M., van Wilgenburg, B., Swadling, L., Cannizzo, E. S., d'Arminio Monforte, A., et al. (2019). Association between impaired Vα7.2+CD161++CD8+ (MAIT) and Vα7.2+CD161-CD8+ T-cell populations and gut Dysbiosis in chronically HIV- and/or HCV-infected patients. *Front. Microbiol.* 10:1972. doi: 10.3389/fmicb.2019.01972

Miller, B. M., Liou, M. J., Zhang, L. F., Nguyen, H., Litvak, Y., Schorr, E.-M., et al. (2020). Anaerobic respiration of NOX1-derived hydrogen peroxide licenses bacterial growth at the colonic surface. *Cell Host Microbe* 28, 789–797.e5. doi: 10.1016/j.chom.2020.10.009

Mjösberg, J., Bernink, J. H., Golebski, K., Karrich, J. J., Karrich, J. J., Peters, C. P., et al. (2012). The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity* 37, 649–659. doi: 10.1016/j.immuni.2012.08.015

Mörbe, U. M., Jørgensen, P. B., Fenton, T. M., von Burg, N., Riis, L. B., Spencer, J., et al. (2021). Human gut-associated lymphoid tissues (GALT); diversity, structure, and function. *Mucosal Immunol.* 14, 793–802. doi: 10.1038/s41385-021-00389-4

Mukherjee, S., Zheng, H., Derebe, M. G., Callenberg, K. M., Partch, C. L., Rollins, D., et al. (2014). Antibacterial membrane attack by a pore-forming intestinal C-type lectin. *Nature* 505, 103–107. doi: 10.1038/nature12729

Nagashima, K., Sawa, S., Nitta, T., Tsutsumi, M., Okamura, T., Penninger, J. M., et al. (2017). Identification of subepithelial mesenchymal cells that induce IgA and diversify gut microbiota. *Nat. Immunol.* 18, 675–682. doi: 10.1038/ni.3732

Nhu, N. T. Q., and Young, V. B. (2023). The relationship between the microbiome and antimicrobial resistance. *Clin. Infect. Dis.* 77, S479–S486. doi: 10.1093/cid/ciad641

Nieuwenhuis, E. E. S., Nieuwenhuis, E. E. S., Nieuwenhuis, E. E. S., Lindenbergh, D., Matsumoto, T., Lindenbergh, D., et al. (2009). Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J. Clin. Invest.* 119, 1241–1250. doi: 10.1172/jci36509

Ohno, H. (2016). Intestinal M cells. *J. Biochem.* 159, 151–160. doi: 10.1093/jb/mvv121

Okumura, R., Kurakawa, T., Kurakawa, T., Nakano, T., Nakano, T., Kayama, H., et al. (2016). Lypd8 promotes the segregation of flagellated microbiota and colonic epithelia. *Nature* 532, 117–121. doi: 10.1038/nature17406

Oren, A., and Garrity, G. M. (2021). Valid publication of the names of forty-two phyla of prokaryotes. *Int. J. Syst. Evol. Microbiol.* 71:5056. doi: 10.1099/ijsem.0.005056

Osbelt, L., Thiemann, S., Smit, N., Lesker, T. R., Schröter, M., Gálvez, E. J. C., et al. (2020). Variations in microbiota composition of laboratory mice influence *Citrobacter rodentium* infection via variable short-chain fatty acid production. *PLoS Pathog.* 16:e1008448. doi: 10.1371/journal.ppat.1008448

Osbelt, L., Wende, M., Almási, É., Derksen, E., Muthukumarasamy, U., Lesker, T. R., et al. (2021). *Klebsiella oxytoca* causes colonization resistance against multidrug-resistant *K. pneumoniae* in the gut via cooperative carbohydrate competition. *Cell Host Microbe* 29, 1663–1679.e7. doi: 10.1016/j.chom.2021.09.003

Palm, N. W., de Zoete, M. R., Cullen, T. W., Barry, N. A., Stefanowski, J., Hao, L., et al. (2014). Immunoglobulin a coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* 158, 1000–1010. doi: 10.1016/j.cell.2014.08.006

Pedicord, V. A., Lockhart, A., Lockhart, A., Rangan, K. J., Craig, J. W., Craig, J. W., et al. (2016). Exploiting a host-commensal interaction to promote intestinal barrier function and enteric pathogen tolerance. *Sci. Immunol.* 1:eaai7732. doi: 10.1126/sciimmunol.aai7732

Pensinger, D. A., Fisher, A. T., Dobrila, H. A., Van Treuren, W., Gardner, J. O., Higginbottom, S. K., et al. (2023). Butyrate differentiates permissiveness to Clostridioides difficile infection and influences growth of Diverse *C. difficile* isolates. *Infect. Immun.* 91:e0057022. doi: 10.1128/iai.00570-22

Pérez, J. C. (2021). Fungi of the human gut microbiota: roles and significance. *Int. J. Med. Microbiol.* 311:151490. doi: 10.1016/j.ijmm.2021.151490

Peterson, L. W., and Artis, D. (2014). Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat. Rev. Immunol.* 14, 141–153. doi: 10.1038/nri3608

Pickard, J. M., Maurice, C. F., Kinnebrew, M. A., Abt, M. C., Schenten, D., Golovkina, T. V., et al. (2014). Rapid fucosylation of intestinal epithelium sustains host–commensal symbiosis in sickness. *Nature* 514, 638–641. doi: 10.1038/nature13823

Pickard, J. M., and Núñez, G. (2019). Pathogen colonization resistance in the gut and its manipulation for improved health. *Am. J. Pathol.* 189, 1300–1310. doi: 10.1016/j.ajpath.2019.03.003

Piewngam, P., Piewngam, P., Piewngam, P., Zheng, Y., Zheng, Y., Nguyen, T. H., et al. (2018). Pathogen elimination by probiotic Bacillus via signalling interference. *Nature* 562, 532–537. doi: 10.1038/s41586-018-0616-y

Provine, N. M., Amini, A., Garner, L. C., Spencer, A. J., Dold, C., Hutchings, C., et al. (2021). MAIT cell activation augments adenovirus vector vaccine immunogenicity. *Science* 371, 521–526. doi: 10.1126/science.aax8819

Qin, Y., Havulinna, A. S., Liu, Y., Jousilahti, P., Ritchie, S. C., Tokolyi, A., et al. (2022). Combined effects of host genetics and diet on human gut microbiota and incident disease in a single population cohort. *Nat. Genet.* 54, 134–142. doi: 10.1038/s41588-021-00991-z

Rajilić-Stojanović, M., and de Vos, W. M. (2014). The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiol. Rev.* 38, 996–1047. doi: 10.1111/1574-6976.12075

Rios, D., Wood, M. B., Li, J., Chassaing, B., Gewirtz, A. T., and Williams, I. R. (2016). Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria. *Mucosal Immunol.* 9, 907–916. doi: 10.1038/mi.2015.121

Rizzello, V., Rizzello, V., Bonaccorsi, I., Bonaccorsi, I., Dongarrà, M. L., Fink, L. N., et al. (2011). Role of natural killer and dendritic cell crosstalk in immunomodulation by commensal Bacteria probiotics. *Biomed. Res. Int.* 2011:473097. doi: 10.1155/2011/473097

Roediger, B., Kyle, R., Yip, K. H., Sumaria, N., Guy, T. V., Kim, B. S., et al. (2013). Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat. Immunol.* 14, 564–573. doi: 10.1038/ni.2584

Round, J. L., Melanie Lee, S., Li, J., Li, J. S., Li, J., Tran, G., et al. (2011). The toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 332, 974–977. doi: 10.1126/science.1206095

Ruigrok, R. A. A. A., Weersma, R. K., and Vich Vila, A. (2023). The emerging role of the small intestinal microbiota in human health and disease. *Gut Microbes* 15:2201155. doi: 10.1080/19490976.2023.2201155

Sana, T. G., Flaugnatti, N., Lugo, K. A., Lam, L. H., Jacobson, A., Baylot, V., et al. (2016). *Salmonella Typhimurium* utilizes a T6SS-mediated antibacterial weapon to establish in the host gut. *Proc. Natl. Acad. Sci. USA* 113, E5044–E5051. doi: 10.1073/pnas.1608858113

Sanders, M. E., Merenstein, D. J., Reid, G., Gibson, G. R., and Rastall, R. A. (2019). Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nat. Rev. Gastroenterol. Hepatol.* 16, 605–616. doi: 10.1038/s41575-019-0173-3

Sarkar, A., McInroy, C. J. A., Harty, S., Raulo, A., Ibata, N. G. O., Valles-Colomer, M., et al. (2024). Microbial transmission in the social microbiome and host health and disease. *Cell* 187, 17–43. doi: 10.1016/j.cell.2023.12.014

Schürch, C. M., Balmer, M. L., Schürch, C. M., Geuking, M. B., Saito, Y., Geuking, M. B., et al. (2014). Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. *J. Immunol.* 193, 5273–5283. doi: 10.4049/jimmunol.1400762

Sequeira, R. P., McDonald, J. A. K., Marchesi, J. R., and Clarke, T. B. (2020). Commensal Bacteroidetes protect against *Klebsiella pneumoniae* colonization and transmission through IL-36 signalling. *Nat. Microbiol.* 5, 304–313. doi: 10.1038/s41564-019-0640-1

Serapio-Palacios, A., Woodward, S. E., Vogt, S. L., Deng, W., Creus-Cuadros, A., Huus, K. E., et al. (2022). Type VI secretion systems of pathogenic and commensal bacteria mediate niche occupancy in the gut. *Cell Rep.* 39:110731. doi: 10.1016/j.celrep.2022.110731

Shaw, T. N., Shaw, T. N., Houston, S., Wemyss, K., Bridgeman, H. M., Barbera, T. A., et al. (2018). Tissue-resident macrophages in the intestine are long lived and defined by Tim-4 and CD4 expression. *J. Exp. Med.* 215, 1507–1518. doi: 10.1084/jem.20180019

Shayya, N. W., Bandick, R., Busmann, L. V., Mousavi, S., Bereswill, S., and Heimesaat, M. M. (2023). Metabolomic signatures of intestinal colonization resistance against *Campylobacter jejuni* in mice. *Front. Microbiol.* 14:1331114. doi: 10.3389/fmicb.2023.1331114

Sheng, Y. H., and Hasnain, S. Z. (2022). Mucus and mucins: the underappreciated host Defence system. *Front. Cell. Infect. Microbiol.* 12:856962. doi: 10.3389/fcimb.2022.856962

Sheppard, S. K. (2022). Strain wars and the evolution of opportunistic pathogens. *Curr. Opin. Microbiol.* 67:102138. doi: 10.1016/j.mib.2022.01.009

Shin, N.-R., Whon, T. W., and Bae, J.-W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Sonnenberg, G. F., Monticelli, L. A., Alenghat, T., Hutnick, N. A., Fung, T. C., Kunisawa, J., et al. (2012). Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal Bacteria. *Science* 336, 1321–1325. doi: 10.1126/science.1222551

Sonnenburg, J. L., Leip, D. D., Jian, X., Jian, X., Jian, X., Leip, D. D., et al. (2005). Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science* 307, 1955–1959. doi: 10.1126/science.1109051

Sorbara, M. T., and Pamer, E. G. (2022). Microbiome-based therapeutics. *Nat. Rev. Microbiol.* 20, 365–380. doi: 10.1038/s41579-021-00667-9

Sovran, B., Planchais, J., Jegou, S., Straube, M., Lamas, B., Natividad, J. M., et al. (2018). Enterobacteriaceae are essential for the modulation of colitis severity by fungi. *Microbiome* 6:152. doi: 10.1186/s40168-018-0538-9

Spragge, F., Bakkeren, E., Jahn, M. T., Araujo, E., Pearson, C. F., Wang, X., et al. (2023). Microbiome diversity protects against pathogens by nutrient blocking. *Science* 382:eadj3502. doi: 10.1126/science.adj3502

Steed, A. L., Steed, A., Christophi, G. P., Kaiko, G. E., Sun, L., Goodwin, V. M., et al. (2017). The microbial metabolite desaminotyrosine protects from influenza through type I interferon. *Science* 357, 498–502. doi: 10.1126/science.aam5336

Su, Y., and Ding, T. (2023). Targeting microbial quorum sensing: the next frontier to hinder bacterial driven gastrointestinal infections. *Gut Microbes* 15:2252780. doi: 10.1080/19490976.2023.2252780

Takao, M., Yen, H., and Tobe, T. (2014). LeuO enhances butyrate-induced virulence expression through a positive regulatory loop in enterohaemorrhagic *Escherichia coli*. *Mol. Microbiol.* 93, 1302–1313. doi: 10.1111/mmi.12737

Tastan, C., Karhan, E., Zhou, W., Fleming, E., Voigt, A. Y., Yao, X., et al. (2018). Tuning of human MAIT cell activation by commensal bacteria species and MR1-dependent T-cell presentation. *Mucosal Immunol.* 11, 1591–1605. doi: 10.1038/s41385-018-0072-x

Taur, Y., Taur, Y., and Pamer, E. G. (2013). The intestinal microbiota and susceptibility to infection in immunocompromised patients. *Curr. Opin. Infect. Dis.* 26, 332–337. doi: 10.1097/qco.0b013e3283630dd3

Taur, Y., Taur, Y., Xavier, J. B., Lipuma, L., Ubeda, C., Goldberg, J. D., et al. (2012). Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. *Clin. Infect. Dis.* 55, 905–914. doi: 10.1093/cid/cis580

Terao, Y., Goto, Y., Panea, C., Nakato, G., Cebula, A., Nakato, G., et al. (2014). Segmented filamentous Bacteria antigens presented by intestinal dendritic cells drive mucosal Th17 cell differentiation. *Immunity* 40, 594–607. doi: 10.1016/j.immuni.2014.03.005

Thaiss, C. A., Zmora, N., Levy, M., and Elinav, E. (2016). The microbiome and innate immunity. *Nature* 535, 65–74. doi: 10.1038/nature18847

Thiennimitr, P., Winter, S. E., Winter, M. G., Xavier, M. N., Tolstikov, V., Huseby, D. L., et al. (2011). Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. *Proc. Natl. Acad. Sci. USA* 108, 17480–17485. doi: 10.1073/pnas.1107857108

Thio, C. L.-P., Thio, C. L.-P., Chi, P.-Y., Chi, P.-Y., Lai, A. C.-Y., Lai, A. C.-Y., et al. (2018). Regulation of type 2 innate lymphoid cell-dependent airway hyperreactivity by butyrate. *J. Allergy Clin. Immunol.* 142, 1867–1883.e12. doi: 10.1016/j.jaci.2018.02.032

Tu, A. N., Pham, S. C., Clare, S., Goulding, D., Goulding, D., Arasteh, J. M., et al. (2014). Epithelial IL-22RA1-mediated Fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. *Cell Host Microbe* 16, 504–516. doi: 10.1016/j.chom.2014.08.017

van Putten, J. P. M., van Putten, J. P. M., and Strijbis, K. (2017). Transmembrane mucins: signaling receptors at the intersection of inflammation and Cancer. *J. Innate Immun.* 9, 281–299. doi: 10.1159/000453594

van Tilburg Bernardes, E., Pettersen, V. K., Gutierrez, M. W., Laforest-Lapointe, I., Jendzjowsky, N. G., Cavin, J.-B., et al. (2020). Intestinal fungi are causally implicated in microbiome assembly and immune development in mice. *Nat. Commun.* 11:2577. doi: 10.1038/s41467-020-16431-1

Vivier, E., Artis, D., Colonna, M., Diefenbach, A., di Santo, J. P., Eberl, G., et al. (2018). Innate lymphoid cells: 10 years on. *Cell* 174, 1054–1066. doi: 10.1016/j.cell.2018.07.017

Vuotto, C., Donelli, G., Buckley, A., and Chilton, C. (2024). Clostridioides difficile Biofilm. *Adv. Exp. Med. Biol.* 1435, 249–272. doi: 10.1007/978-3-031-42108-2_12

Wang, S., Mu, L., Yu, C., He, Y., Hu, X., Jiao, Y., et al. (2024). Microbial collaborations and conflicts: unraveling interactions in the gut ecosystem. *Gut Microbes* 16:2296603. doi: 10.1080/19490976.2023.2296603

Wang, B., Ng, K. M., Ferreyra, J. A., Higginbottom, S. K., Lynch, J. B., Kashyap, P. C., et al. (2013). Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature* 502, 96–99. doi: 10.1038/nature12503

Wexler, H. M., and Wexler, H. M. (2007). Bacteroides: the good, the bad, and the nitty-gritty. *Clin. Microbiol. Rev.* 20, 593–621. doi: 10.1128/cmr.00008-07

Wheeler, M. L., Limon, J. J., Bar, A. S., Leal, C. A., Gargus, M., Tang, J., et al. (2016). Immunological consequences of intestinal fungal Dysbiosis. *Cell Host Microbe* 19, 865–873. doi: 10.1016/j.chom.2016.05.003

Winkler, E. S., Shrihari, S., Hykes, B. L., Handley, S. A., Handley, S. A., Andhey, P. S., et al. (2020). The intestinal microbiome restricts alphavirus infection and dissemination through a bile acid-type I IFN signaling Axis. *Cell* 182, 901–918.e18. doi: 10.1016/j.cell.2020.06.029

Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Huseby, D. L., et al. (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. *Nature* 467, 426–429. doi: 10.1038/nature09415

Woo, V., Woo, V., and Alenghat, T. (2017). Host-microbiota interactions: epigenomic regulation. *Curr. Opin. Immunol.* 44, 52–60. doi: 10.1016/j.coi.2016.12.001

Yanagibashi, T., Hosono, A., Hosono, A., Oyama, A., Tsuda, M., Suzuki, A., et al. (2013). IgA production in the large intestine is modulated by a different mechanism than in the small intestine: *Bacteroides acidifaciens* promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ B cells. *Immunobiology* 218, 645–651. doi: 10.1016/j.imbio.2012.07.033

Yang, W., Yu, T., and Cong, Y. (2022). CD4+ T cell metabolism, gut microbiota, and autoimmune diseases: implication in precision medicine of autoimmune diseases. *Precis. Clin. Med.* 5:pbac018. doi: 10.1093/pcmedi/pbac018

Yilmaz, B., Portugal, S., Tran, T. M., Gozzelino, R., Gozzelino, R., Ramos, S., et al. (2014). Gut microbiota elicits a protective immune response against malaria transmission. *Cell* 159, 1277–1289. doi: 10.1016/j.cell.2014.10.053

Yip, A. Y. G., King, O. G., Omelchenko, O., Kurkimat, S., Horrocks, V., Mostyn, P., et al. (2023). Antibiotics promote intestinal growth of carbapenem-resistant Enterobacteriaceae by enriching nutrients and depleting microbial metabolites. *Nat. Commun.* 14:5094. doi: 10.1038/s41467-023-40872-z

Zenewicz, L. A., Yancopoulos, G. D., Valenzuela, D. M., Murphy, A. J., Stevens, S., and Flavell, R. A. (2008). Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* 29, 947–957. doi: 10.1016/j.immuni.2008.11.003

Zhang, T., Sasabe, J., Hullahalli, K., Sit, B., and Waldor, M. K. (2021). Increased *Listeria monocytogenes* dissemination and altered population dynamics in Muc2-deficient mice. *Infect. Immun.* 89, e00667–e00620. doi: 10.1128/IAI.00667-20

Zhao, W., Caro, F., Robins, W., and Mekalanos, J. J. (2018). Antagonism toward the intestinal microbiota and its effect on *Vibrio cholerae* virulence. *Science* 359, 210–213. doi: 10.1126/science.aap8775

Zhong, Y., Wang, S., Di, H., Deng, Z., Liu, J., and Wang, H. (2022). Gut health benefit and application of postbiotics in animal production. *J. Anim. Sci. Biotechnol.* 13:38. doi: 10.1186/s40104-022-00688-1

Zuo, T., Wong, S. H., Cheung, C. P., Lam, K., Lui, R., Cheung, K., et al. (2018). Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in *Clostridium difficile* infection. *Nat. Commun.* 9:3663. doi: 10.1038/s41467-018-06103-6

Zygiel, E. M., and Nolan, E. M. (2018). Transition metal sequestration by the host-defense protein calprotectin. *Annu. Rev. Biochem.* 87, 621–643. doi: 10.1146/annurev-biochem-062917-012312

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Oral microbiota and metabolites: key players in oral health and disorder, and microbiota-based therapies

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The review aimed to investigate the diversity of oral microbiota and its influencing factors, as well as the association of oral microbiota with oral health and the possible effects of dysbiosis and oral disorder. The oral cavity harbors a substantial microbial burden, which is particularly notable compared to other organs within the human body. In usual situations, the microbiota exists in a state of equilibrium; however, when this balance is disturbed, a multitude of complications arise. Dental caries, a prevalent issue in the oral cavity, is primarily caused by the colonization and activity of bacteria, particularly streptococci. Furthermore, this environment also houses other pathogenic bacteria that are associated with the onset of gingival, periapical, and periodontal diseases, as well as oral cancer. Various strategies have been employed to prevent, control, and treat these disorders. Recently, techniques utilizing microbiota, like probiotics, microbiota transplantation, and the replacement of oral pathogens, have caught the eye. This extensive examination seeks to offer a general view of the oral microbiota and their metabolites concerning oral health and disease, and also the resilience of the microbiota, and the techniques used for the prevention, control, and treatment of disorders in this specific area.

KEYWORDS

oral microbiota, bacteria, periodontal disease, oral cavity, microbiota metabolites

1 Introduction

In the 1700s, Antonie van Leeuwenhoek made a groundbreaking discovery while studying dental plaque under the microscope. The discovery of bacteria in dental plaque is one of the first areas of study in traditional microbiology (Mark Welch et al., 2016; Bowen et al., 2018; Lamont et al., 2018). The oral cavity is home to a rich population of microorganisms, many of which are unique and evolved specifically for oral colonization (Mark Welch et al., 2016; Lamont et al., 2018). The oral microbiota is the second most important and the richest microbial community after the gut and is one of the five research priorities of the Human Microbiome Project. Over 1,100 different taxonomic groups are recorded in the Human Oral Microbiome Database, and the genera *Streptococcus*, *Neisseria*, *Veillonella*, and *Actinomyces*

are associated with the core microbiome (Bäckhed et al., 2012; Wade, 2013; Franzosa et al., 2015; Bowen et al., 2018; Deo and Deshmukh, 2019; Herremans et al., 2022). Among them, facultative anaerobic bacteria such as *Streptococcus* and *Actinomyces* predominate in the oral cavity. The low oxygen tension in the subgingival area creates favorable conditions for an increasing number of strict anaerobes such as *Bacteroidaceae* spp. and *Spirochaetes*. Identification of microbial communities has a major role in physical and metabolic exchanges between species, which can be cooperative or antagonistic (Mark Welch et al., 2016; Bowen et al., 2018; Lamont et al., 2018). Since 2000, with the introduction of cost-effective genetic sequencing, scientists have focused on the diversity of oral microbial communities and understanding their impact on systemic diseases (Bäckhed et al., 2012). Using modern sequencing technology and next-generation sequencing (NGS) systems, the barriers of traditional culture-based techniques can now be overcome (Franzosa et al., 2015). The microbial community in the body has various functions and is crucial for host health. However, we only become aware of them when the microbiota is out of balance and disease occurs (Zaura et al., 2009; Deo and Deshmukh, 2019). Moreover, recent evidence supports a link between systemic disease and the oral microbiome. The potential for many oral microbes to interplay with an inflammatory microenvironment may be related to this phenomenon. In addition, poor oral hygiene is closely associated with adverse systemic health, and research on oral health and related factors has become a subject of attention (Caselli et al., 2020; Lippi and Mattiuzzi, 2020). Therefore, in the present review, we investigated the diversity of oral microbiota and its influencing factors, and also investigated the association of oral microbiota with oral health and the possible effects of dysbiosis and oral disorder (Figure 1).

2 Composition and diversity of the oral microbiota

The oral cavity is a complex habitat that allows microorganisms to interact with the host, and is continuously subjected to both inhaled and ingested microorganisms. Of the more than 700 species of viruses, bacteria, fungi, and protozoa in this microbial community, only 54% have been identified and can be cultured. Another 14% of these species are cultivable but have not so far been recognized, and the other 32% have not been cultivated at all (Bäckhed et al., 2012; Wade, 2013; Herremans et al., 2022). It is essential to notice that everyone has their own unique "microbial identity" consisting of their microbial community. The diversity of the human oral microbiome is influenced by the microhabitat. However, in general terms, the human oral microbiome can be defined as having two major but variable components (Franzosa et al., 2015; Deo and Deshmukh, 2019). The core microbiome is similar among healthy individuals (Zaura et al., 2009), while environmental factors and physiological changes specifically influence the diversity of the microbiome (Lippi and Mattiuzzi, 2020). As a result of both genetic and environmental factors, the composition of the oral microbial community can change over time (Caselli et al., 2020; Herremans et al., 2022). Based on the results of NGS, it has been shown that most of the bacteria in the mouth belong to the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Oriarchyota*, *Fusobacteria*, *Tenricotes*, *Actinobacteria*, and *Spirochaetes* (Dewhirst et al., 2010). *Chloroflexi*, TM7, GN02, *Synergistetes*, *Chlorobi*, SR1, and WPS-2 are among the lesser known potential phyla in the mouth (Camanocha and Dewhirst, 2014). The mouth is a complex environment that contains diverse microbial populations and undergoes significant changes during life stages (Sedghi et al., 2021). During the menstrual cycle, physiological hormones cause changes in bacteria such as *Oribacterium* spp., *Campylobacter* spp., *Haemophilus* spp., and *Prevotella* spp. (Bostanci et al., 2021). Another study found that the prevalence of oral *Neisseria* spp., *Porphyromonas* spp., and *Treponema* spp. was higher in pregnant women than in non-pregnant women, but *Streptococcus* spp. and *Veillonella* spp. were more common in non-pregnant women than in pregnant women (Saadaoui et al., 2021). These findings assume that the oral microbial ecosystem should be analyzed with respect to the age of the host and its particular biological niche within the oral cavity, as it is continuously exposed to external chemicals (Jiang et al., 2015; Baker and Edlund, 2018; Oliveira et al., 2021). Oral archaea in the oral microbiome are less diverse and less abundant than bacteria. Initially, it was assumed that they were only methanogenic, but several studies have also found non-methanogenic bacteria in the oral cavity (Li et al., 2009; Wade, 2013; Dame-Teixeira et al., 2020). Although not pathogenic, these microorganisms have been identified in inflamed pulp tissue, subgingival biofilms, and caries biofilm samples. Further research is required to determine their potential pathogenicity (Efenberger et al., 2015; Aleksandrowicz et al., 2020; Dame-Teixeira et al., 2020).

There has been very little research on the mycobiome, which refers to the fungal and other microbes present in the oral cavity. However, up to 101 species of fungi have been identified as part of the healthy oral microbiota, including *Cryptococcus* spp., *Aureobasidium* spp., *Aspergillus* spp., *Cladosporium* spp., *Saccharomyces* spp., *Candida* spp., *Fusarium* spp., and Aspergillus spp. (Arboleya et al., 2015; Fujiwara et al., 2017; Godlewska et al., 2017; Gomez-Arango et al., 2017; Grant et al., 2019). *Anelloviridae*, *Herpesviridae*, and *Papillomaviridae* are the most common oral virome (Holmes et al., 1998; Welch et al., 2020).

3 Factors influencing composition and diversity of oral microbiota

Various internal and external factors, as well as lifespan, can affect oral health. The concept of resilience refers to a system's capability to quickly recover its equilibrium state after a perturbation (Rosier et al., 2020). Thus, in the interplay between host and oral microbiota, resilience plays a vital contribution to the maintenance of health. Similarly, microbiota resilience could minimize the impact of perturbation while maintaining the symbiotic state, thereby inhibiting dysbiosis and preventing bacterial disease (Liu et al., 2012; Nascimento, 2018). Conversely, Bacteria maintain their balance to maintain oral health. The adaptability of the human oral cavity is affected by host conditions such as genetics, age, immune system, lifestyle and oral environment such as diet, pH, gingival crevicular fluid, and saliva. These factors can induce alterations in the diversity of the community of oral microbes (Chattopadhyay et al., 2019; Nascimento et al., 2019).

3.1 Internal factors

3.1.1 Host genetics

Modern genomic techniques have demonstrated the impact of host genetic factors on the diversity of the oral microbiota community

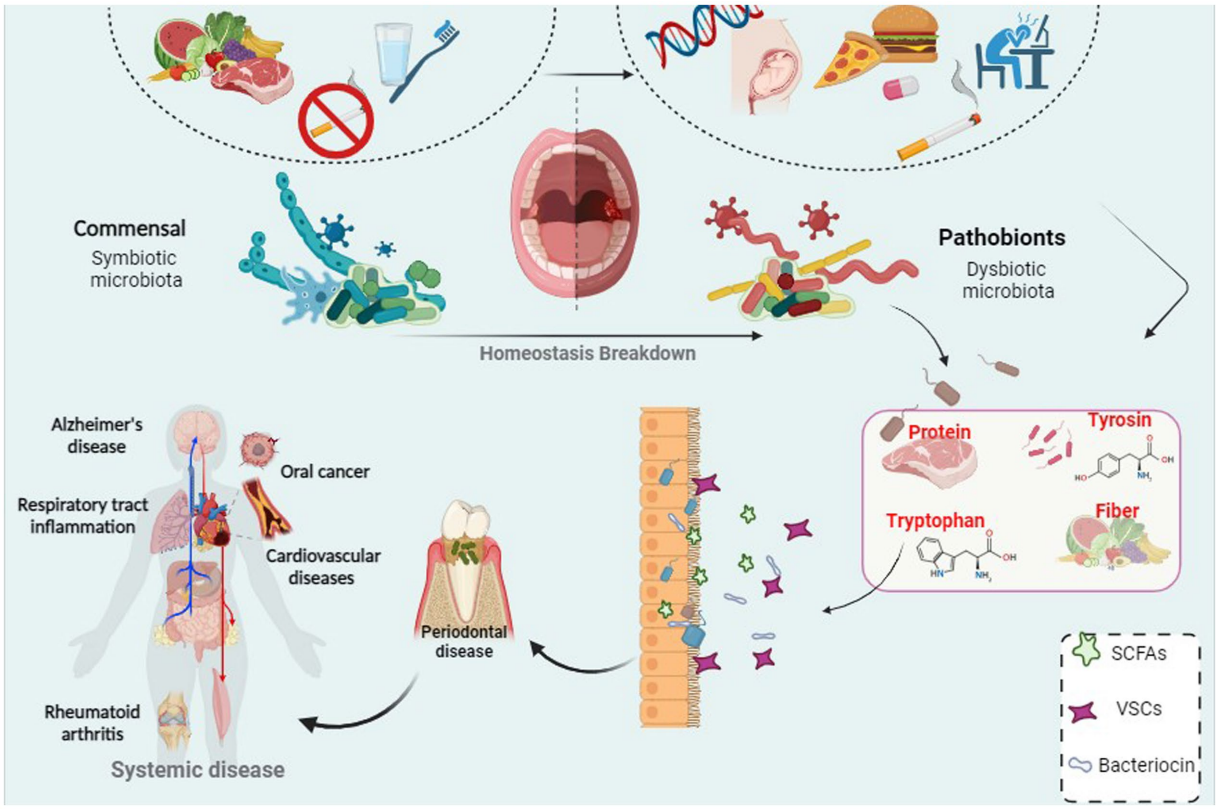


FIGURE 1
A correlation between bacterial infection and periodontal disease which can led to systemic disease and cancer.

(Heilbronner et al., 2021). However, reports on the heritability of the oral microbiota are conflicting (Wescombe et al., 2012; Bushin et al., 2020).

For example, Esberg et al. highlighted the effect of genetic factors on the composition of the oral microbiota through various factors such as immune pathways in 836 Swedish twins. By studying separate groups of adolescents and adults using 16S rRNA gene sequencing, this research indicated that genetic factors affect the host's immunity, the composition of oral microbiota species, and susceptibility to tooth disorders such as periodontitis and caries (Ferreira et al., 2014). Studies conducted on twins have proven that hereditary traits have a significant contribution in the composition of oral microbiota. Monozygotic twins had a much more similar oral microbial profile than dizygotic twins, highlighting the importance of host genetics (Lin et al., 2021; Zhang J. S. et al., 2022). In addition, research has shown that the abundance of cariogenic species in plaque and saliva is highly heritable trait (Zhang et al., 2018).

In a study of oral metagenomic analysis of over 1,915 cases, human genetics is responsible for a minimum 10% of the oral microbiome composition in all people. These findings suggest that the oral microbiome persists or recurs in people primarily because of changes in their genetics. It also appears that certain host miRNAs may regulate the growth of specific oral bacteria, which may help predict the risk of dental problems such as gum bleeding and plaque (Duran-Pinedo, 2021). A study conducted in 2021 analyzed the oral microbiota of adoptive mother–child pairs and concluded that acquired factors, such as contact and shared environment, are instrumental in altering and transferring oral microbiota from host genetics. However, because of inconsistent findings, more research is necessary to provide a better insight into the relationship between host genetics and the oral microbiome (Nowicki et al., 2018).

3.1.2 Saliva

The first stage of food digestion occurs in the oral cavity, where chewing, facilitated by the teeth, and subsequent swallowing occurs. Saliva contains peptides, vitamins, amino acids, glycoproteins, and proteins. As a result, the oral microbiota is enriched with saliva-derived nutrients for growth and development. In the context of cleaned teeth, salivary biomolecules form an "acquired pellicle" on the tooth surface by depositing a thin layer containing proline-rich proteins, statherin and histatins, which play a crucial role in oral health (Hannig et al., 2017; Hu et al., 2023). In addition, gingival crevicular fluid (GCF) provides a source of nutrition for the microbiota, containing glycoprotein, heme and albumin (Cornejo Ulloa et al., 2019; Wade, 2021). In the absence of compounds and immune properties of saliva, non-oral bacteria and fungi can colonize and multiply in a dry oral cavity (Dabdoub et al., 2016; Tuominen and Rautava, 2021). The buffering system of saliva contains bicarbonate and phosphate, which are essential for the maintenance of the pH value of the mouth at a neutral level, which is optimal for bacterial growth. The sugars in saliva are rapidly metabolized by the oral microbiota, resulting in the production of acidic components that significantly lower the pH of saliva. This acidic environment, facilitates irreversible tooth decay by activating the saccharolytic activity of

bacteria such as *Streptoccucs mutans*, *Actinomyces* spp., *Bifidobacteria* spp., *Propionibacterium acidifaciens* and *Lactobacilli*, which convert sugars into acidic products. Under these circumstances, microbial diversity is reduced, leading to the formation of cariogenic microbiota, including acid-tolerant and saccharolytic bacteria (Rosier et al., 2018; Cornejo Ulloa et al., 2019).

3.2 External factors

3.2.1 Indoor environment

The indoor microbiome and its metabolites, such as flavonoids and mycotoxins, play a significant role in shaping the oral microbiome's composition and function. The indoor environment, where individuals spend a substantial amount of time, hosts diverse microbial communities that can influence the human microbiota, including the oral microbiome. Studies suggest that the diversity and composition of indoor microbiomes can impact the microbial populations in the human body. A healthy indoor microbiome can introduce beneficial bacteria into the oral cavity to support oral health. Conversely, an indoor environment with a high prevalence of pathogenic microbes can disrupt the oral microbial balance, leading to conditions such as periodontal disease and dental caries. Effective management of the indoor environment through proper ventilation and regular cleaning is crucial to mitigate the negative impact on the oral microbiome (Adams et al., 2015; Zhang et al., 2023).

Flavonoids are plant polyphenols found in various foods and beverages, known for their antimicrobial and anti-inflammatory properties. These compounds significantly influence the oral microbiome by inhibiting the growth of harmful bacteria and promoting beneficial species. For instance, catechins in green tea exhibit antibacterial activity against *Streptococcus mutans*, a major contributor to dental caries (Ferrazzano et al., 2009). Flavonoids also reduce oxidative stress and inflammation in the oral cavity, thereby supporting a healthy and balanced oral microbiome (Meena et al., 2020). Mycotoxins are toxic metabolites produced by fungi and are commonly found in contaminated food and indoor environments. Exposure to mycotoxins, such as aflatoxins and ochratoxins, can adversely affect the oral microbiome. Mycotoxins disrupt the balance of oral microbial communities by selectively inhibiting beneficial bacteria and allowing pathogenic fungi and bacteria to proliferate. This disruption increases the risk of oral infections and inflammation, which can lead to systemic health problems. In addition, mycotoxins can impair the immune response, further compromising the resilience of the oral microbiome to pathogens (Bryden, 2012; Bai et al., 2021).

3.2.2 Diet and nutrition

Understanding the impact of diet on oral microbes is essential to developing successful oral disease prevention strategies (Yang et al., 2018). Since the 1960s, there has been a shift in dietary patterns toward a Westernized style. This change is characterized by the consumption of certain foods such as meat from farmed animals, high-sugar dairy products, refined vegetable oils, and processed grains. These diets have been in associated with pathological modifications in the oral microbiota, including an increase in the proportion of acid-producing and acid-tolerant organisms (Karpiński, 2019; Sun et al., 2020). Dietary variables significantly influence the oral microbiota biofilm, which may disrupt the homeostasis of specific bacterial species (Yang et al., 2018). Evidence suggests that macro- and micronutrients can modify pro- and anti-inflammatory cascades and influence the inflammatory state of an individual at rest. The oral microbiota is nourished by the diet, which also causes selective pressures that favor the growth and proliferation of organisms that are best suited to utilize certain food resources provided by the host (Lee et al., 2017).

An oral health study evaluated the effect of traditional diets on the dental health of "bush dwellers" and "village dwellers" who were consuming an increasingly externalized diet. The results supported the idea that hunter-gatherer societies have better dental health than agricultural societies. Women who resided in villages and ate a diet rich in agricultural products had a higher prevalence of tooth decay and periodontal disease than those who lived in the bush and consumed a diet rich in wild foods such as legumes. However, cultural factors such as excessive honey consumption and smoking affect the dental health of bush dwelling men, who do not maintain the same level of dental health as their female counterparts (Zhang L. et al., 2020b). In addition to the well-established association between simple carbohydrate consumption and dental caries, several dietary factors may help prevent dental caries. The results of an *in vivo* study revealed that frequent usage of hard candy increased the rate of oral streptococci such as *Streptococcus parasanguinis*, *Streptococcus gordonii*, and *Streptococcus sanguinis* while generally decreasing some species of the oral microbiota such as *Haemophilus* spp. and members of the *Proteobacteria* phylum (Abranches et al., 2018; Hampelska et al., 2020).

Recent research has shown that the entero-salivary nitrate-nitrite-nitric oxide pathway, which promotes nitric oxide (NO) maintenance, is a favorable symbiotic connection between oral microbiota and the human host. Here, nitrate concentrated in the salivary glands is converted to nitrite by facultative anaerobic oral bacteria, which is subsequently ingested and absorbed into the circulation before being further converted to NO. Moreover, the daily use of antiseptic mouthwash for up to 7 days can disrupt the oral microbiome of healthy non-athletes, resulting in a decrease in plasma and oral nitrite levels and elevated blood pressure without dietary intervention. This is an indication of the essential contribution of the oral microbiome to this condition. These findings suggest which nutrition can alter the oral microbiome that affects the entero-salivary pathway and NO homeostasis, but further research is needed to explore this possibility in depth (Vinasco et al., 2019; Wu et al., 2022).

3.2.3 Smoking

The oral area represents one of the first sites on the body to be subjected to cigarette smoke, leading to increased carcinogenesis, reduced mucosal immunity, and altered oral microbiota (Al Bataineh et al., 2020). Numerous toxicants from cigarette smoke can disrupt the oral cavity's microbial diversity by antibiotic effects, oxygen reduction, or another way (Wu et al., 2016). Smoking with alternation of oxygen levels can favor the survival of microaerophilic bacteria over commensal beneficial species (Jaspers, 2014). It also promotes the development of biofilm and increases the adherence of pathogens, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *S. mutans*, to the epithelial layer. According to some evidence, former smokers' oral microbiota was more similar to that of current smokers than non-smokers, suggesting smoking effects on oral microbiota may last for years. The oral cavity of smokers tends to be more colonized

with disease-associated bacteria such as *Actinomyces*, which have been associated with the occurrence of dental diseases such as periodontitis and caries. Additionally, *Actinomyces* spp. in the salivary microbiota have also been linked to the development of liver cancer (Li et al., 2020; Jia et al., 2021). Wu et al. found that current smokers had a reduced frequency of aerobic metabolic pathways, such as tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and higher frequencies of glycolysis and other oxygen-independent carbohydrate metabolism pathways. Non-smokers had more Proteobacteria than current smokers, while the difference between former smokers and never smokers was not significant. *Atopobium* spp. and *Streptococcus* spp. were enriched relative to never smokers, but the genera *Capnocytophaga* spp., *Peptostreptococcus* spp. and *Leptotrichia* spp. were reduced. The study also found that non-proteobacterial taxa was associated with smoking. Overall, evidence shows that cigarette smoke creates a setting that is more conducive to facultative aerobics than robust aerobics (Jia et al., 2021). On the other hand, cigarette smoking affects the respiratory immune system at several levels (Tuominen and Rautava, 2021), which resulted in the induction of inflammatory cytokines such as IL-8, TNF-α, and MCP-1, due to the stimulation of innate immune and inflammatory cells such as neutrophils and macrophages (Jaspers, 2014).

3.2.4 Pregnancy

It is vital to maintain good oral hygiene during pregnancy because the mother's oral hygiene has a direct effect on the child's oral hygiene. Hence, the elimination of maternal oral infections during pregnancy is critical, as it can prevent or delay colonization of the infant's oral cavity with oral pathogens (Jang et al., 2021). Altered oral microbiota during pregnancy may have implications for maternal oral health, birth outcomes, and infant oral health because a stable microbiota helps maintain stable oral and general health. Therefore, it is critical to understand changes in the oral microbiota during pregnancy, their relationship to maternal health, and their impact on birth outcomes (Jang et al., 2021). Current studies have revealed that the diversity of oral microbiota in pregnant women remains stable throughout pregnancy. Although, the diversity of the oral microbiota changes in a pathogenic manner and then returns to a normal or healthy microbiome after delivery. It is believed that these changes are caused by female hormones such as progesterone and estrogen (Ye and Kapila, 2021). Previous reports have shown a high frequency of *Prevotella intermedia sensu lato* over the second trimester. Subsequent studies have found that the growth of certain bacterial species, such as *Porphyromonas gingivalis* has been found to have elevated levels of progesterone and estrogen, which are part of the fumarate reductase system of the pathogen (Lin W. et al., 2018). A 2015 study by Fujiwara et al. found that the number of pathogenic bacteria, such as *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, in gingival crevices increased in pregnant women during the early and middle stages of pregnancy compared to non-pregnant women (Fujiwara et al., 2017).

4 Role of the oral microbiota in oral health

The oral cavity is a warm, moist, and nutrient-rich environment that provides a suitable niche for the colonization of microbiota. Early colonization is exhibited by Gram-positive bacteria, such as *Streptococci* (*S. sanguinis*, *S. mutans*, *S. sobrinus*, *S. gordonii*, and *S. oralis*), as well as *Actinomyces*, through adhesion to various surfaces, including gingival surfaces and teeth covered by pellicles (O'Mullane et al., 2016; Thurnheer and Belibasakis, 2018).

The oral microbiota strives to retain a healthy dynamic state and a symbiotic relationship in the complex oral ecosystem, which is referred to as eubiosis. In addition, these bacteria can self-aggregate and co-aggregate with other bacterial species, including some Gram-negative bacteria, through various bacterial glycoproteins and polysaccharides. This process serves as the basis for biofilm formation (Holmes et al., 1998; Bürgers et al., 2010). However, this state of eubiosis can be interrupted and transformed into a state of dysbiosis (Radaic and Kapila, 2021). Dysbiosis is characterized by the involvement of bacterial metabolites in periodontal disease, as well as a marked difference in the metabolic profiles of the oral microbiota when compared with healthy conditions (Heilbronner et al., 2021). In this situation, oral microbiota may be associated with the pathological processes of several diseases, such as inflammatory bowel disease (IBD), arthritis, colorectal and pancreatic cancer, and Alzheimer's disease, by serving as a reservoir for opportunistic pathogens.

5 Role of oral microbiota metabolites in health

The oral bacterial population produces a significant source of metabolites in the mouth which are associated with critical processes in human health or disease. Bacterial metabolites include by-products of carbohydrate, lipid, and protein metabolism such as short chain fatty acids (SCFAs), amines, and gasses (Mai et al., 2017) (Table 1). These metabolites are used by the medical and biotechnology industries as antimicrobial, antifungal, and anticancer agents. Saliva contains glycoproteins and amino acids that are broken down into their subunits by oral microbiota and human enzymes. Under anaerobic conditions, acid-producing bacteria including, *Actinomyces* spp., *Streptococcus* spp., and *Lactobacillus* spp. metabolize deuterated sugars to ethanol, lactic acid, formic acid, and acetic acid via the Embden-Meyerhof-Parnas pathway (Takahashi, 2015; Rosier et al., 2020). The nitrate-reducing bacteria use the acids that are produced as a source of carbon. Then, they raise the pH with ammonia from arginine and urea metabolism, neutralizing the acid and creating a balanced oral ecosystem. In this mechanism, nitrate can be reduced to ammonia (NH3) by the bacterial nitrate-reducing ammonium (DNRA) pathway, raising the pH (Efenberger et al., 2015). As a result, the simultaneous presence of acid-producing and nitrate-reducing bacteria helps maintain a neutral oral cavity (Kistler et al., 2013; Abusleme et al., 2021).

Lugdunin is an example of an oral microbiota metabolite. It is synthesized by *Staphylococcus lugdunensis*, a commensal bacterium found in the mouth. It belongs to thiazolidine-containing cyclic peptides and has been identified as a potent inhibitor of methicillin-resistant *S. aureus*, a superbug that poses a significant threat to human health. *S. gordonii* and *Actinomyces* spp. can metabolize basic amino acids such as arginine to produce ornithine, and citrulline and release other by-products including ammonia, ATP and CO_2 by a number of reactions referred to as the arginine deiminase system (ADS) (Liu

TABLE 1 Oral microbiota, their metabolites, and functions.

Metabolites	Bacteria	Function in oral health	Function in oral disorder
SCFAs	*Streptococcus* *Lactobacillus* *F. nucleatum* *T. forsythia* *P. intermedia* *P. gingivalis* *T. denticola*	Regulating the immune equilibrium between the host defense system and the maintenance of commensal immune tolerance (Jiao et al., 2020)	Increasing the pathogenicity of another oral microbiota Reconstruction of the biofilm species Developing the SCFAs producing bacteria Inducing tissue inflammation in subgingival and supragingival Reducing the expression of ICAM-1 and the transmigration of immune cells Reduction the production of mediators and phagocytosis of neutrophils Reactivation of latent viruses (Roslund et al., 2021)
VSCs	*P. gingivalis* *P. intermedia* *P. nigrescence* *T. forsythia*	Promoting the antitumor immune responses Suppressing the H2S-mediated proliferative capacity on oral cancer cells	Stimulation of the inflammatory cytokines IL-1β and IL-18 Promoting the inflammatory response Oral halitosis (Feng et al., 2023)
Bacteriocin	*Streptococcus* *Lactobacillus* *Fusobacterium* *Prevotella*	Probiotic effects Anticancer effects	Interfering with the growth of others microbial competitor Improving the colonization and establishment of bacteria in oral biofilms (Barbour et al., 2022)
Organic acid	*Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Bifidobacterium Streptococcus*, *Pediococcus*, *Leuconostoc*	Antagonistic activities against adverse microorganisms (Zhang G. et al., 2020)	Affecting the ecology of the oral microbiome and demineralization of enamel surface (Saha et al., 2023)
Nitrate	*Rothia* *Neisseria* *Porphyromonas* *Actinomyces* *Granulicatella* *Veillonella* *Prevotella* *Haemophilus* *Fusobacterium* *Brevibacillu* *Kingella*	Maintaining the pH of oral cavity in normal range Reducing lactic acid accumulation Controlling the development of caries	It increases in response to as a response of salivary glands to the inflammatory process (Sánchez et al., 2014)

SCFAs, Short chain fatty acids; VSCs, volatile sulfur compounds.

et al., 2012). The alkalinity created by this process can eliminate the acidification produced by sugar metabolism. In fact, some findings showed that lower arginine catabolic capacity, as determined by measuring ADS activity, had correlated with the formation of carious lesions in children. This event is the result of ammonia production by ADS, which leads to the elimination of glycolytic demineralizing acids, thereby promoting the development of a microbiota that protects dental health (Nascimento, 2018; Nascimento et al., 2019).

Therefore, using ADS+ strains as probiotics and adding arginine to oral health products can be a helpful strategy for preventing and treating dental caries (Nascimento et al., 2019). Oral alkaline producing species such as *S. gordonii* and *Actinomyces* spp. can be used to neutralize the acidity of the oral cavity in oral cancer and limit the development of acidogenic bacteria (Chattopadhyay et al., 2019). Also, the oral microbiota produces several classes of metabolites, such as bacteriocins, lantipeptides (or lantibiotics) and sactipeptides that are classified as Ribosomally synthesized and post-translationally modified peptides (RiPPs). They can integrate into the target cell membrane and induce membrane perforation, which is their main mode of action. Because of their cell-killing activity, these peptides have been indicated for use in the treatment of cancer (Barbour et al., 2022).

The most extensively studied examples of RiPPs are bacteriocins, which are synthesized by some bacteria. They are typically described as antimicrobial peptides or proteins with the ability to inhibit or kill specific pathogens. The most widely studied oral microbial clusters that produce bacteriocins are members of *Prevotella* spp., *Streptococcus* spp., *Fusobacterium* spp., and *Lactobacillus* spp., which secrete one or more bacteriocin-like inhibitory factors (Heilbronner et al., 2021). On the other hand, it has been shown that lantibiotics, as one of the categories of bacteriocins, interfere with the membrane potential of target cells. An example of lantibiotics synthesized by oral community microbiota is salivaricin A. This is the primary lantibiotic produced by *S. salivarius* (Wescombe et al., 2012). Sactipeptides are a type of RiPP that possess a sactionine thioether bridge that is post-translationally incorporated and required for their ability to exert antimicrobial activity. Streptosactin is an example of a sactipeptide synthesized by *Streptococcus thermophilus* and oral microbiota, such as *Streptococcus constellatus*, *S. gordonii*, *S. oralis*, and *S. parasanguini* (Bushin et al., 2020).

6 Imbalance of oral microbiota in oral disorders

An imbalance between the microbiota and the host caused by certain factors can lead to diversity in the microbial community. This can cause to oral and dental diseases in which beneficial bacteria are destroyed and pathogens proliferate. Under these conditions, the immune system is unable to fight the pathogens, exposing the host to tissue and dental damage such as dental caries, periodontal disease, and carcinogenic metabolites that can cause cancer and other systemic diseases (Verma et al., 2018; Radaic and Kapila, 2021). In addition, it has also been contributed to various types of malignancies, including gastric, colorectal, liver, lung, esophageal, and breast malignancies (Takahashi, 2015). In this section, we focus on some diseases that result from perturbations in oral microbiota.

6.1 Dental caries (cavities)

In general, investigation of the oral microbiota composition in caries revealed that the bacterial population in caries is less diverse than in the healthy state (Zhang Y. et al., 2022). Although, the genera *Streptococcus* spp. and *Lactobacillus* spp., have been studied as the most common causative agents of dental caries, current evidence using meta transcriptomics approaches has revealed a broad spectrum of microbiota contributing to dental caries (Lin et al., 2021).

Moreover, *Actinomyces* spp., *Fusobacterium* spp., *Porphyromonas* spp., *Rothia* spp., *Granulicatella* spp., *Gemella* spp., *Selenomonas* spp., *Bifidobacteria* spp., *Scardovia* spp., *Corynebacterium* spp., *Granulicatella* spp., *Propionibacterium* spp., *Bifidobacterium* spp., and *Scardovia* spp. are strongly related to caries development (Zhang et al., 2018; Duran-Pinedo, 2021; Zhang J. S. et al., 2022). These microbiota interact with each other in a dynamic polymicrobial biofilm and induce the progression of caries from early-onset (primary demineralization) to deeper damage with dentin exposure (Zhang et al., 2018).

6.2 Periodontal diseases (gingivitis and periodontitis)

Chronic inflammation caused by oral microbiota can damage the structures that support the teeth, leading to gingivitis and periodontal disease (Duran-Pinedo, 2021). Gingivitis is a reversible stage that begins at the bottom of the apical gingival border and modifies the supra and subgingival sulci (Radaic and Kapila, 2021).

Molecular analyses of bacterial communities have demonstrated that a distinct cohort of oral microbiota, including the genera *Oribacterium* spp., *Leptotrichia* spp., *Tannerella* spp., *Lachnospiraceae* spp., *Lachnoanaerobaculum* spp., *Lautropia* spp. as well as *Prevotella oulorum* and *Fusobacterium nucleatum* are more abundant during gingivitis (Kistler et al., 2013; Nowicki et al., 2018). The rapid development of gingivitis leads to the accumulation of oral microorganisms in the gingival crevices, resulting in inflammation and ultimately the destruction of the periodontium and alveolar bone (Sedghi et al., 2021). Imbalance of oral microbiota is a consequence of periodontitis, leading to a shift from commensal to pathogenic species (Radaic and Kapila, 2021).

Examination of the oral microbiota of healthy individuals and those with periodontitis revealed that *Actinomyces*, *Rothia*, and *Streptococcus* spp. are predominant in the healthy oral cavity compared with periodontitis, whereas periodontitis was associated with a greater diversity of pathogenic taxa including *Treponema* spp., *P. gingivalis*, *Porphyromonas endodontalis*, *P. intermedia*, *Filifactor alocis*, *Tannerella forsythia*, *Parvimonas micra*, and *Fretibacterium* spp. (Abusleme et al., 2021). In another study, periodontitis was associated with an elevated number of pathogens such as *Parvimonas*, *Fusobacterium*, *Prevotella* as well as *F. alocis*, *P. gingivalis*, *T. forsythia*, and *Treponema denticola* (Abusleme et al., 2013; Dabdoub et al., 2016).

6.3 Oral cancer

Chronic inflammation caused by microorganisms in the oral cavity could potentially lead to the development of oral cancer by inhibiting cellular apoptotic processes and producing carcinogenic metabolites (Zhang et al., 2018). Anaerobic bacteria such as *Prevotella* spp., *Porphyromonas* spp. and *Fusobacterium* spp. can produce inflammatory cytokines such as interleukin-6 (IL-6), IL-1β, matrix metalloproteinases MMP-8 and MMP-9 and tumor necrosis factor-α (TNF-α) in response to LPS from Gram-negative bacteria, leading to chronic inflammation that is associated with invasive and aggressive tumor phenotypes, and epithelial tumor migration (Sun et al., 2020; Tuominen and Rautava, 2021). *Porphyromonas* spp. stimulates anti-apoptotic effects on tumor cells by reducing the tumor suppressor P53, activating the anti-apoptotic Jak1/Act/Stat3 signaling pathway and secreting an anti-apoptotic enzyme (nucleoside diphosphate kinase) (Karpiński, 2019).

Long-term periodontal disease is a major contributor to oral squamous cell carcinoma (OSCC). A decrease in Firmicutes and an increase in bacteria such as *Fusobacterium* spp., *Porphyromonas* spp., *Haemophilus* spp., *Peptostreptococcus* spp., *Rothia* spp., *Prevotella* spp., *Streptococcus* spp., and *Veillonella* spp. have been observed in OSCC (Lee et al., 2017; Yang et al., 2018). Furthermore, an increase in bacteria such as *Capnocytophaga leadbetteri*, *P. intermedia*, *Aggregatibacter segnis*, *Peptostreptococcus stomatis*, and *F. nucleatum* have been proved in the sites of buccal mucosal tumors in OSCC patients compared with normal tissue (Zhang L. et al., 2020). Oral microbiota can induce reactive oxygen species (ROS) and organic acids that may be involved in the development of cancer. Some species in the oral cavity, including S. oralis, *S. sanguinis*, *S. gordonii*, *Lactobacillus fermentum*, *L. minutus* and *Bifidobacterium adolescentis*, produce hydrogen peroxide (H2O2) through increased expression of NADPH oxidase activity *oxidase activity* (Abranches et al., 2018). In addition, *Alloprevotella* spp., *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *Peptostreptococcus* spp., and *F. nucleatum* produce free radicals such as hydrogen Sulfide, dimethyl Sulfide and methyl mercaptan, which affect tumor angiogenesis and growth (Hampelska et al., 2020; Wu et al., 2022).

Certain bacteria such as *Streptococcus* spp., *Lactococcus* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Pediococcus* spp. and *Leuconostoc* spp. produce lactic acid and lower the pH, which may provide an acidic microenvironment in tumor cells and increase the risk of metastasis (Vinasco et al., 2019).

7 Oral microbiota metabolites in oral disorders

There has been limited investigation into the characterization of oral microbiota metabolic products and their contribution to maintaining the balance between different types of bacteria and the human host in the oral microbiota (Ge et al., 2015) (Table 1). SCFAs are the best studied bacterial metabolites derived from the fermentation of carbohydrates and amino acids. SCFAs, including butyric, lactic, formic, acetic, and propionic acids, are necessary for reconstituting biofilm species and inducing tissue inflammation in the subgingival and supragingival oral cavity (Takahashi et al., 2010). Changes in the levels of individual metabolites due to changes in environmental conditions can lead to periodontal disease (Gomez-Arango et al., 2017).

Streptococcus and *Lactobacillus* are the major lactic acid producing bacteria in the oral cavity. The high levels of lactic acid in animal models of active caries indicate their important role in the cariogenic process (Olson et al., 2011). Lactic acid production by *S. gordonii* increases the pathogenicity of another oral microbiota such as *A. actinomycetemcomitans*. In addition, *Eubacterium* spp., *Veillonella* spp., and *Corynebacterium* spp. can catabolize lactic acid produced by *Streptococcus* spp. as a preferred substrate in oral biofilms (Zhang L. et al., 2020). A number of oral microbiota, including *P. intermedia*, *P. gingivalis*, *T. forsythia*, *F. nucleatum*, and *T. denticola* produce significant amounts of butyric acid (Mai et al., 2017). This metabolite, through an anti-inflammatory process, prevents an appropriate immune response against the bacteria involved in periodontitis and thus promotes the development of periodontal disease in the oral cavity (Lourenço et al., 2014). Butyrate is necessary in immune processes associated with periodontal disease by reducing the expression of the adhesion protein intercellular adhesion molecule-1 (ICAM-1) and the transmigration of immune cells, as well as reducing mediator production and neutrophil phagocytosis (Zhou et al., 2018). Butyrate accumulation can also stimulate the colonization of other oral microbiota such as *Actinomyces oris* in the early stages of biofilm colonization (Liu et al., 2021). It appears that SCFAs are also associated with cancer development. The acidic environment created by the acidogenic oral microbiota establishes an ideal environment for the growth and invasion of cancer cells (Abusleme et al., 2013). In addition, a number of SCFAs, including propionic, butyric, isobutyric, and isovaleric acids, can reactivate latent viruses such as Kaposi's sarcoma-associated herpesvirus and Epstein–Barr virus to initiate tumorigenic processes in host cells (Reddy et al., 2019; Di Spirito et al., 2022). On the other hand, protein metabolism by the oral microbiota occurs in subgingival bacterial niches and deep periodontal pockets where access to dietary carbohydrates is limited (Gutt et al., 2018). The amino acid metabolizing bacteria *Fusobacterium*, produces alkaline by-products that raise the pH of the oral cavity to near neutral. This alkaline environment favors the colonization and activation of the proteolytic capacity of acid-sensitive oral bacteria such as *P. gingivalis*, which contributes to the initiation of inflammatory responses (Jin Baek et al., 2017). Some oral microbiota such as *P. intermedia* and *P. gingivalis* can metabolize acidic amino acids such as glutamic acid and aspartic acid to produce propionic acid and acetic acid. Increased metabolism of these amino acids is associated with the progression of periodontal pathology, particularly during pregnancy (Beck et al., 2020). In addition, *Bifidobacterium* spp. and *Scardovia wiggsiae* can synthesize acetic acid through the metabolic pathway and mediate pathogenicity through this by-product (Barbour et al., 2022). The production of bacterial metabolites in some cases has beneficial effects in oral diseases. Catabolism of the aromatic amino acid phenylalanine by redox reactions produces alkaline metabolites such as phenylacetate and regulates the pH of the oral biofilm (Roslund et al., 2021). The alkaline environment established by bacteria can neutralize the acidification of carbohydrate metabolism and stimulate the development of dental health by maintaining the microbiota in dental caries (Feng et al., 2023). In addition, alkali-producing species can balance acidic pH and reduce acidogenic bacteria, thereby inhibiting their tumor-promoting activities in oral cancer cells (Jiao et al., 2020). Some anaerobic oral microbiota, such as *P. intermedia*, *F. nucleatum*, and *P. gingivalis*, can metabolize the aromatic amino acid tryptophan to synthesize a variety of toxic indolic and phenolic metabolites, including indole, skatole, indole pyruvate, and indole acetate. These compounds, especially skatole, are odorous and contribute to oral halitosis (Zhang G. et al., 2020). *F. nucleatum* and *P. intermedia* may catabolize lipids and fatty acids in high-fat diets by lipases (Saha et al., 2023). Lipid metabolites may have significant pathogenic capacity. The metabolism of choline by *S. sanguinis* produces trimethylamine, which is converted to trimethylamine N-oxide in liver cell. High levels of trimethylamine N-oxide in the blood can interfere with reverse cholesterol transport and induce foam cell formation (Sánchez et al., 2014) (Table 2).

8 Microbiota based therapies

8.1 Probiotics

Probiotics are live, non-pathogenic bacteria that provide health benefits to the host when consumed in adequate amounts. These microorganisms can alter the composition of the oral microbiota. The goal of probiotic therapy is to replace harmful indigenous microorganisms with non-pathogenic ones. Probiotics are often used to treat various oral and dental conditions, such as periodontal disease, halitosis, dental caries, and oral candidiasis (Bosch et al., 2012; Allaker and Ian Douglas, 2015; Mishra et al., 2020; Zhang Y. et al., 2022). Probiotics are available in various commercial forms, including tablets, toothpaste, and mouthwash, for the treatment of periodontal disease (Parcina Amizic et al., 2017; Zhang Y. et al., 2022). Systematic reviews by Inchingolo et al. (2023) and Navidifar et al. (2023) have shown the beneficial effects of probiotics on oral health conditions, including periodontitis, gingivitis, dental caries, and orthodontics. These bacteria interact directly with plaque, disrupt biofilm formation by competing for receptors on host tissue or other bacteria, and compete for nutrients. One significant mechanism involves the production of antimicrobial substances that inhibit oral microorganisms, such as organic acids, low molecular weight antimicrobial peptides, hydrogen peroxide, adhesion inhibitors, and bacteriocins. Probiotics also indirectly influence immune function by regulating both innate and adaptive responses, interacting with oral epithelial cells, and enhancing mucosal barrier function. Lactic acid bacteria can interact with immune cells like macrophages and T cells, leading to cytokine release and impacting systemic immunity (Lin T. H. et al., 2018; Mishra et al., 2020; Abdi and Ranjbar, 2022).

TABLE 2 Probiotics and their potential role in oral health.

	Bacteria	Mechanism	Outcomes	References
Probiotic	*Bifidobacterium* *Lactobacillus* *Streptococcus*		- Increase alpha diversity in the oral microbiota - Positive influence of additional probiotics - Downregulate inflammatory processes - Activate beneficial pathways such as type I and type II interferon responses	Willis and Gabaldon (2020)
	Streptococcus A12 *Streptococcus dentisani*	Arginine metabolism	- Inhibition of acidic pH produced in cariogenic biofilms	Loozen et al. (2015), Huang et al. (2016), Lopez-Lopez et al. (2017), Willis and Gabaldon (2020)
	Bdellovibrio bacteriovorus	It invades the periplasmic space of Gram-negative bacteria by attacking the prey's external surface and hydrolyzing its components, forming the bdelloplast	- Treatment of periodontal disease	Bonfiglio et al. (2020)
	L. rhamuosus GG	Deplete glucose and repress ergosterol synthesis, inhibition of the growth of pathogens by fending off adhesion to oral epithelial cells	- Prevent early caries in children	Nagaraj et al. (2012), Qiu et al. (2020)
	Bifidobacterium DN-173010, *L. rhamnosus* CG *L. reuteri* *L. casei*	Directly or indirectly alter gut microbiota through its metabolites, adhesion mechanisms, and gene regulation Reducing pro-inflammatory bacterial colonization and attenuating inflammatory responses	- Change the colonizing of cariogenic bacteria - Prevent dental caries	Busscher et al. (1999), Näse et al. (2001), Caglar et al. (2005), Caglar et al. (2006), Allaker and Ian Douglas (2015)
	L. paracasei GMNL-33	Inhibition the growth of many pathogenic microbes such as *S. mutans*	- Reduction of *S. mutans* in saliva	Chuang et al. (2011)
	L. gasseri SBT2055	Improved expression of β-defensin	- Prevention of *P. gingivalis* oral infection - Reduction of IL-6 and TNF-α mRNA levels in gingival tissue	Kobayashi et al. (2017)
	L. lactis NCC2211	Integrating into the oral biofilm	- Prevent the growth of *Streptococcus sobrinus* OMZ176	Chuang et al. (2011), Mishra et al. (2020)
	L. reuteri RC-14 *L. rhamnosus* GR-1 *L. reuteri* ATCC PTA 5289	Prevent the connection of other pathogens by co-accumulation with yeasts	- Inhibitory effect against *C. albicans* and *L. reuteri*	

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Commercial probiotics like *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* have been shown to enhance alpha diversity in the oral microbiota (Willis and Gabaldon, 2020). Various theories suggest that probiotics such as *S. salivarius* strains can reduce inflammation and induce beneficial responses, including type I and type II interferon responses. *Streptococcus* A12 and *Streptococcus dentisani* can neutralize the acidic pH in cariogenic biofilms through arginine metabolism. For probiotics to exert their cariostatic effects, they must adhere to oral tissues and become part of the biofilm. Their effectiveness is also influenced by their duration of presence in the area. Dairy products like milk, yogurt, and cheese are effective carriers of probiotics. For instance, incorporating *Lactobacillus rhamnosus* GG into milk has been shown to help prevent early tooth decay in children (Nagaraj et al., 2012; Qiu et al., 2020). *Bifidobacterium* DN-173 010, *Lactobacillus rhamnosus* CG, *Lactobacillus reuteri*, and *Lactobacillus casei* have been demonstrated to disrupt cariogenic bacteria colonization, reducing the risk of dental caries (Busscher et al., 1999; Näse et al., 2001; Caglar et al., 2005, 2006; Allaker and Ian Douglas, 2015). A combination of live lyophilized acidophilic *lactobacilli* has been used topically to treat mild to moderate periodontitis (Lin T. H. et al., 2018). Coordination *Lactococcus lactis* NCC2211 into the verbal biofilm can avoid the development of *Streptococcus sobrinus* OMZ176. A biosurfactant inferred from *L. fermentum* that employments glucosyltransferases has been appeared to avoid *S. mutans* from creating sucrose, subsequently diminishing dental caries caused by this bacterium (Mishra et al., 2020). Considerations have appeared that *L. reuteri* RC-14, *L. rhamnosus* GR-1, and *L. reuteri* ATCC PTA 5289 can hinder the development of *C. albicans* by co-aggregating with yeasts, avoiding other pathogens from following to verbal surfaces (Chuang et al., 2011; Mishra et al., 2020).

Bacillus subtilis has a metabolic advantage over human oral pathogens, including *Streptococcus*. *B. subtilis* cells perform better in utilizing sugar alcohols like mannitol and sorbitol compared to *S. mutans*, which may explain the significant decrease in *S. mutans* biofilm production during co-culture (Duanis-Assaf et al., 2020). Reducing *S. mutans* could potentially help prevent caries from developing. Research indicates that gastric feeding of *Lactobacillus gasseri* enhances mice immunity against pathogenic bacteria by increasing bone resorption, periodontal ligament separation, and disruption, and reducing *P. gingivalis* (Kobayashi et al., 2017).

Chronic inflammation is a characteristic of cancer, and altered oral flora can lead to oral inflammation that promotes mutagenesis, angiogenesis, and uncontrolled cell proliferation, resulting in oral cancer (Kay et al., 2019; La Rosa et al., 2020). Anaerobic bacteria such as *Fusobacterium*, *Porphyromonas*, and *Prevotella* are common causes of the chronic inflammation associated with periodontal disease, and cause the release of inflammatory mediators (La Rosa et al., 2020; Vyhnalova et al., 2021; Bostanghadiri et al., 2023). Evidence suggests that probiotics help maintain oral health by selectively replacing and inhibiting dysbiosis. Postbiotics, including short-chain fatty acids (SCFA), polysaccharides, microbial cell fractions, functional proteins, EPS, and cell lysates, offer anticancer activity and can serve as an alternative to conventional chemotherapy (Żółkiewicz et al., 2020). Moreover, postbiotics of *Lactobacillus acidophilus*, *L. casei* and *Bifidobacterium longum* have shown significant anticancer effects on both implanted and chemically induced tumors. Administering the cytoplasmic fraction of lactic acid bacteria (LAB) *in vivo* enhanced specific antitumor activity by altering cellular immunity without causing direct tumor cell death (Lee et al., 2004). A study by Zhang et al. showed that probiotic *L. salivarius REN* has the ability to inhibit oral cancer development in a dose-dependent manner through protection against oxidative damage and downregulation of COX-2 and proliferating cell nuclear antigen (Zhang et al., 2013). COX-2 is often up-regulated in many types of cancer, where it blocks apoptosis, alters cell adhesion, and manipulates the normal cell signaling pathway. Therefore, inhibiting COX-2 may be an effective strategy to prevent oral cancer by blocking the production of PGE 2, which promotes cancer growth (Mohan and Epstein, 2003). Also, Zhang et al. showed that oral gavage of *L. salivarius REN* or its postbiotic can potently inhibit the progression of 4-nitroquinoline-1-oxide-induced oral carcinogenesis by decomposing 4-nitroquinoline-1-oxide to a less toxic compound, preventing DNA from oxidative damage caused by the carcinogen, downregulating COX-2/PCNA expression, and inducing apoptosis. An *in vitro* study on human myeloid cells showed that probiotic *L. reuteri ATCC 6475* supernatant (Lr-S 6475) in combination with tumor necrosis factor (TNF) can induce apoptosis via NF-kB and MAPK pathways (Zhang et al., 2013). Secreted metabolites of *Acidobacter syzygii* exhibit anticancer effects by induction of apoptosis (Aghazadeh et al., 2017).

8.2 Microbiota transplantation

Growing antibiotic resistance has triggered interest in finding new treatments. Microbiota-centered interventions include a range of therapies that attempt to restore ecological balance by harnessing microorganisms (Bosch et al., 2012). Some examples of these interventions include microbiota transplantation, probiotics, engineered symbiotic bacteria, and microbiota-derived proteins and metabolites (Sorbara and Pamer, 2022). One of the alternative treatments for oral diseases is oral microbiota transplantation (OMT), a type of treatment that involves altering the dysbiosis of the microbiota to restore the microbial ecological balance.

OMT works by transferring biofilms from healthy donors to patients with caries or periodontitis. The proposed procedure, as hypothesized by Dewhirst and Hoffmann, involves three steps: (1) collecting supragingival plaque from a caries-free donor who may be a family member of the recipient patient, (2) preserving the plaque in saline solution, and (3) transferring the plaque sample to the teeth of a patient with active caries using a nylon swab (Pozhitkov et al., 2015; Mira et al., 2017). It is important to have a donor with a strong oral microbiome that is free of cariogenic bacteria, particularly *S. mutans*, and shows a slight decrease in pH when exposed to a sugar stimulus.

It is likely that interspecies interactions and immunostimulatory effects will play a significant role in OMT therapy. OMT reduced systemic inflammation induced by neck and head irradiation by reducing the levels of IL-1, IL-6, TNF-α, and TGF-β (Xiao et al., 2021). However, transplanted oral biofilms must be able to withstand selective environmental pressures and continue to exert beneficial effects. OMT is hypothesized to work by establishing colonization of oral sites, competing with pathogenic bacteria for adhesion sites and nutrient sources, producing bacteriocin and hydrogen peroxide to eliminate pathogens, and controlling local and systemic immune responses (Nascimento, 2017). The safety issues related to the use of OMT and oral probiotics are comparable. In similar to probiotics, transplanted biofilms must be disease-free and have impressive genetic

stability. However, how OMT works and the best way to deliver it is not understood (Nath et al., 2021). As of now, it is uncertain if oral biofilms should be directly transplanted from a donor in good health into a recipient, or if they should be pre-treated to reduce the levels of pathogenic bacteria before transplantation. It is also uncertain whether biofilms produced *in vitro* or naturally occurring commensal organisms would be a superior alternative (Sánchez et al., 2014). Nevertheless, biofilms of clinical strains with useful and health-promoting properties are effective in interacting with and replacing the biofilms associated with the disease (Gutt et al., 2018).

Other factors to be considered include the need to disinfect the recipient's oral cavity before OMT and whether single or several doses of oral transplants would be necessary for successful and sustained colonization of the oral cavity for the establishment and maintenance of health (Nascimento, 2017).

8.3 Antimicrobial agents targeting specific oral pathogens

The most common form of oral pathogen eradication is the use of antibiotics. Some systemic antibiotics that target specific oral pathogens include penicillin, metronidazole, macrolides, and clindamycin. Metronidazole is a topical medication that is effective against obligate anaerobic microorganisms in the oral cavity, including those from infected necrotic pulp. In *in vitro* experiments, metronidazole inhibited over 99% of the bacteria found in carious lesions and infected root dentin (Delaviz et al., 2019). Erythromycin is a macrolide commonly used to treat various bacterial infections. According to some evidence, caries-active dams can become caries-inactive when individuals receive erythromycin. In addition, the treatment with erythromycin resulted in a 35% reduction in plaque formation within one week (Qiu et al., 2020).

Penicillin has been shown to be effective against several strains of gram-positive *Streptococci* and *Staphylococci*, as well as certain gram-negative bacteria. However, bacterial resistance to β-lactam antibiotics is a major challenge (Macy, 2015). Over the years, the use of systemic antibiotics for the treatment of oral bacteria has decreased. This is because many of these antibiotics not being originally designed for this purpose, and they are lack of specificity in treating oral diseases. Instead, alternative antimicrobial agents have been introduced that specifically target oral bacteria, including chlorhexidine, fluoride, antimicrobial peptides (AMPs), and quaternary ammonium salts. Fluoride, the major anion of fluorine, has been demonstrated to be a very powerful agent in the prevention of tooth decay of dental caries, especially when added to consumed water or to oral products such as toothpastes, mouthwashes, and oral supplements. There is an ongoing debate about whether fluoride helps prevent tooth decay (O'Mullane et al., 2016). It is generally believed that the fluoride ions interact with the tooth's mineral structure, thus leading to more remineralization and avoiding demineralization due to cariogenic bacteria's acidity (Thurnheer and Belibasakis, 2018).

Chlorhexidine, a cationic polybiguanide, was an early antiseptic that was recommended for dental decay and has been demonstrated to be the most effective. It remains the most important antiplaque agent because of its ability to prevent plaque formation and bacterial adhesion. Disruption of acidic groups of glycoproteins in saliva by chlorhexidine reduces plaque adhesion. It also prevents bacterial adhesion to tooth surfaces, either by adhering to extracellular polysaccharides or by the competition with calcium ions for accumulation in plaque (Brookes et al., 2020).

Initially, quaternary ammonium salts were added to mouthwashes to combat oral plaque. Later, they have been added to dental products to provide antimicrobial properties paliguanid (Ge et al., 2015). AMPs are widely distributed in the human body and exhibit antimicrobial properties that target microorganisms (Gordon et al., 2005; Zasloff, 2019). AMPs are considered as one of effective molecules in the eradication of oral pathogens. They are derived from larger molecules and consist of a signal sequence along with various modifications such as glycosylation, proteolysis, amino acid isomerization (Khurshid et al., 2016). AMPs are classified into different categories based on their amino acid composition, size, and structural configurations (Brogden, 2005; Harris et al., 2009). Oral epithelial cells, salivary glands, and neutrophils contribute to the production of various AMPs in saliva, which together provide a powerful defense due to their antibacterial, antioxidant, and antifungal properties (Kutta et al., 2006; Güncü et al., 2015). The most common AMPs expressed in the oral cavity are including α-, and β- defensins (shown as hNP), histatins, statherin, adrenomedullin, azurocidin, and cathelicidins (LL37). The majority of hNP-1 to 3 is concentrated in saliva, gingival epithelia, tongue, palate, buccal mucosa, and salivary glands/ducts and accounting for approximately 99% in a healthy individual (Dale and Krisanaprakornkit, 2001). Dale et al. found that individuals with dental caries have reduced levels of α-defensins (hNP-1, -2, and -3) in their saliva, suggesting that these biomarkers could be used to assess caries risk in the general population (Dale et al., 2006). It has been proposed that hBD-1 is constitutively expressed and has the function of inhibiting the transformation of normal flora into harmful microbes, while hBD-2 and -3 are induced by bacterial lipopolysaccharides (LPS), proinflammatory mediators (Interleukins [IL-1β], tumor necrosis factors [TNF-α], interferons [IFN-γ]) and are more effective in the control of nearly all types of pathogens (Khurshid et al., 2016). Histatins, a group of low positively charged histidine-enriched peptides found in saliva, are produced by cells of the parotid and submandibular salivary ducts in healthy adults (De Sousa-Pereira et al., 2013). Histatins often have antifungal activity and are composed of three main members of His-1, His-3 and His-5 (Bikker, 2020). Their antifungal mechanism is exerted in several steps, including the inhibition of mitochondrial respiration by forming reactive oxygen species, entering the cell by mobilizing ions (K+, Mg2+) that results in cell death (Khurshid et al., 2016). For example, Hst-5 is used in a mouthwash (Demegen) for oral candidiasis in patients with human immunodeficiency virus HIV because of its potent limiting effects on Candida biofilm (Pusateri et al., 2009; Gorr, 2012). In humans, the oral cavity and respiratory tract have a single representative of cathelicidins called human cationic antimicrobial peptide (hCAP18) (Murakami et al., 2002). Numerous studies have shown that LL37/hCAP18 is effective against a wide range of microorganisms, including gram-negative and gram-positive bacteria, fungi, viruses, and parasites (López-García et al., 2005; Ridyard and Overhage, 2021; Aloul et al., 2022; Bhattacharjya et al., 2024). It rapidly eliminates bacteria by creating ionic channels in their cell walls and by its ability to bind to LPS in gram-negative bacteria (Sancho-Vaello et al., 2020). Another study demonstrated the enhanced bactericidal activity of synthetic peptides derived from LL37/hCAP18 against Streptococcus sanguis (Isogai et al., 2003). In addition, Ouhara et al. demonstrated a higher

activity of LL37 against a group of oral bacteria including *S. mutans*, *S. sanguinis*, *S. salivarius* and *S. mitis* when compared to human b-defensins (Ouhara et al., 2005). Adrenomedullin is a disulfide bonded cationic amphipathic antimicrobial peptide found in high concentrations in whole saliva. Adrenomedullin levels are significantly elevated in periodontally compromised regions compared to healthy regions (Kapas et al., 2004; Gorr, 2009). Statherin is a basic histidine-rich peptide of the histatin/statherin family whose antimicrobial properties are observed in its C-terminus (Wilmarth et al., 2004). Statherin is found in saliva and gingival crevicular fluid and inhibits the growth of anaerobic bacteria of the oral cavity (Denny et al., 2008). Azurocidin, a protein found in saliva, exhibits potent antibacterial activity against Gram-negative bacteria by binding strongly to lipopolysaccharide (Dhaifalah et al., 2014). Essential oils (EOs) also are one of other options in treating infection associated with oral cavity. EOs have been the focus of research for many years as effective antimicrobial agents and are applied in various medical fields, including dentistry (Radu et al., 2023). EOs derived from aromatic plants, are known for their strong odor and complex nature, containing hundreds of elements with a predominance of two or three major components. Hydrocarbon terpenes and terpenoids form the main components, with the majority of terpenoids being monoterpenes and sesquiterpenes, and the remaining group being oxygenated derivatives of hydrocarbon terpenes (Elyemni et al., 2019). Monoterpenes have been extensively researched for their potential to treat a number of diseases. Terpenes and terpenoids, along with aromatic and aliphatic elements, are responsible for the bactericidal or bacteriostatic effects of EOs (Elyemni et al., 2019; Machado et al., 2022). In addition, the antimicrobial activity of EOs depends on their unique composition, structure, amount and potential interactions (Zomorodian et al., 2015; Jafri and Ahmad, 2020). EOs have been shown to have antibacterial, antifungal, and anti-inflammatory properties that target oral pathogens (Karadağlıoğlu et al., 2019). The mechanism of action of EOs is primarily by increasing the permeability of cell membranes, leading to the leakage of ions and cellular components, ultimately causing cell lysis (Karan, 2019). EOs can also interfere with protein synthesis or cell division through stimulating the production of reactive oxygen species, leading to cell death (Lakhdar et al., 2012). Toscano-Garibay et al. indicated that among the oral pathogens tested, *S. mutans* is the most susceptible to EOs, followed by *S. sobrinus*, *S. salivarius*, *S. sanguis*, and *L. acidophilus* (Toscano-Garibay et al., 2017). There are limited studies on the efficacy of EOs against *C. albicans*. Oregano oil was found to inhibit the attachment and development of *C. albicans* biofilm and to reduce biofilm formation on surfaces pretreated with this oil (Karan, 2019). The results of a clinical study showed that regular use of an EO-based mouthrinse significantly reduced plaque, gingivitis, and periodontitis compared to a 0.05% cetylpyridinium chloride mouthrinse (Dagli and Dagli, 2014). Essential oils have the potential to inhibit plaque, providing additional protection to soft tissues from bacterial damage. While chlorhexidine (CHX) is often preferred for plaque control and the treatment of gingivitis and periodontitis, EOs are considered the most reliable substitute (Dosoky and Setzer, 2018; Kajjari et al., 2022). Sweet orange, bitter orange, lemon, lime, grapefruit, bergamot, yuzu and kumquat have been shown to have medicinal properties when used in mouthwash (Radu et al., 2023). *E. faecalis* is frequently present in root canals identified with apical periodontitis and is a main cause of secondary endodontic infections (Reiznautt et al., 2021). According to a study by Gokalp et al., a combination of calcium hydroxide and two EOs (*M. spicata* and *O. dubium*) showed significant antimicrobial effects (Nabavizade et al., 2018).

9 Conclusion and perspectives

As our understanding of the oral microbiota advances, it is becoming increasingly clear that maintaining a balanced microbial community is critical for optimal oral health. Disruption of this balance can lead to the proliferation of pathogenic bacteria, resulting in conditions such as dental caries and periodontitis. Therefore, a comprehensive approach to oral health must include both preventive and therapeutic strategies. Preventive measures, including good oral hygiene, a balanced diet and the avoidance of risk factors, are essential. However, due to the limitations and temporary efficacy of conventional treatments, there is a growing need for innovative solutions. Probiotics and microbiota transplantation are promising ways to maintain the microbial balance. Probiotics, available in various commercial formulations, have shown considerable success, while microbiota transplantation, although relatively new, offers a compelling area for further investigation. As we continue to elucidate the complex interactions between the oral microbiota and the host, it is imperative that we continually re-evaluate and improve our preventive and therapeutic approaches to ensure long-term oral health.

Author contributions

NB: Investigation, Project administration, Writing – original draft, Writing – review & editing. MK: Writing – original draft, Visualization. ET: Writing – original draft, Visualization. ZE: Writing – original draft, Visualization. AK: Writing – original draft, Visualization. TN: Methodology, Writing – original draft. DD-S: Supervision, Validation, Writing – review & editing.

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References

Abdi, M., and Ranjbar, R. (2022). A review on antiviral efficacy of Bifidobacterium species. *Rev. Res. Med. Microbiol.* 33, 74–81. doi: 10.1097/MRM.0000000000000295

Abranches, J., Zeng, L., Kajfasz, J. K., Palmer, S., Chakraborty, B., Wen, Z., et al. (2018). Biology of oral streptococci. *Microbiol. Spectr.* 6, 10–128. doi: 10.1128/microbiolspec.GPP3-0042-2018

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., et al. (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* 7, 1016–1025. doi: 10.1038/ismej.2012.174

Abusleme, L., Hoare, A., Hong, B. Y., and Diaz, P. I. (2021). Microbial signatures of health, gingivitis, and periodontitis. *Periodontology* 86, 57–78. doi: 10.1111/prd.12362

Adams, R. I., Bateman, A. C., Bik, H. M., and Meadow, J. F. (2015). Microbiota of the indoor environment: a meta-analysis. *Microbiome* 3, 1–18. doi: 10.1186/s40168-015-0108-3

Aghazadeh, Z., Pouralibaba, F., and Yari Khosroushahi, A. (2017). The prophylactic effect of *Acetobacter syzygii* probiotic species against squamous cell carcinoma. *J. Dent. Res. Dent. Clin. Dent. Prospects* 11, 208–214. doi: 10.15171/joddd.2017.037

Al Bataineh, M. T., Dash, N. R., Elkhazendar, M., Alnusairat, D. A. M. H., Darwish, I. M. I., Al-Hajjaj, M. S., et al. (2020). Revealing oral microbiota composition and functionality associated with heavy cigarette smoking. *J. Transl. Med.* 18, 1–10. doi: 10.1186/s12967-020-02579-3

Aleksandrowicz, P., Brzezińska-Błaszczyk, E., Dudko, A., and Agier, J. (2020). Archaea occurrence in the subgingival biofilm in patients with Peri-implantitis and periodontitis. *Int. J. Periodontics Restorative Dent.* 40, 677–683. doi: 10.11607/prd.4670

Allaker, R. P., and Ian Douglas, C. W. (2015). Non-conventional therapeutics for oral infections. *Virulence* 6, 196–207. doi: 10.4161/21505594.2014.983783

Aloul, K. M., Nielsen, J. E., Defensor, E. B., Lin, J. S., Fortkort, J. A., Shamloo, M., et al. (2022). Upregulating human cathelicidin antimicrobial peptide LL-37 expression May prevent severe COVID-19 inflammatory responses and reduce microthrombosis. *Front. Immunol.* 13:880961. doi: 10.3389/fimmu.2022.880961

Arboleya, S., Sánchez, B., Milani, C., Duranti, S., Solís, G., Fernández, N., et al. (2015). Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *J. Pediatr.* 166, 538–544. doi: 10.1016/j.jpeds.2014.09.041

Bäckhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., et al. (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 12, 611–622. doi: 10.1016/j.chom.2012.10.012

Bai, Y., Ma, K., Li, J., Li, J., Bi, C., and Shan, A. (2021). Deoxynivalenol exposure induces liver damage in mice: inflammation and immune responses, oxidative stress, and protective effects of *Lactobacillus rhamnosus* GG. *Food Chem. Toxicol.* 156:112514. doi: 10.1016/j.fct.2021.112514

Baker, J. L., and Edlund, A. (2018). Exploiting the Oral microbiome to prevent tooth decay: has evolution already provided the best tools? *Front. Microbiol.* 9:3323. doi: 10.3389/fmicb.2018.03323

Barbour, A., Elebyary, O., Fine, N., Oveisi, M., and Glogauer, M. (2022). Metabolites of the oral microbiome: important mediators of multikingdom interactions. *FEMS Microbiol. Rev.* 46:fuab039. doi: 10.1093/femsre/fuab039

Beck, J. D., Philips, K., Moss, K., Divaris, K., Morelli, T., and Offenbacher, S. (2020). Advances in precision oral health. *Periodontol.* 82, 268–285. doi: 10.1111/prd.12314

Bhattacharjya, S., Zhang, Z., and Ramamoorthy, A. (2024). LL-37: structures, antimicrobial activity, and influence on amyloid-related diseases. *Biomol. Ther.* 14:320. doi: 10.3390/biom14030320

Bikker, F. J. (2020). The importance of saliva to oral health; from haddock to histatin. *Ned. Tijdschr. Tandheelkd.* 127, 551–555. doi: 10.5177/ntvt.2020.10.19094

Bonfiglio, G., Neroni, B., Radocchia, G., Marazzato, M., Pantanella, F., and Schippa, S. (2020). Insight into the possible use of the predator *Bdellovibrio bacteriovorus* as a probiotic. *Nutrients* 12:2252. doi: 10.3390/nu12082252

Bosch, M., Nart, J., Audivert, S., Bonachera, M. A., Alemany, A. S., Fuentes, M. C., et al. (2012). Isolation and characterization of probiotic strains for improving oral health. *Arch. Oral Biol.* 57, 539–549. doi: 10.1016/j.archoralbio.2011.10.006

Bostanci, N., Krog, M. C., Hugerth, L. W., Bashir, Z., Fransson, E., Boulund, F., et al. (2021). Dysbiosis of the human Oral microbiome during the menstrual cycle and vulnerability to the external exposures of smoking and dietary sugar. *Front. Cell. Infect. Microbiol.* 11:625229. doi: 10.3389/fcimb.2021.625229

Bostanghadiri, N., Razavi, S., Shariati, A., Talebi, M., Mirkalantari, S., Emami Razavi, A., et al. (2023). Exploring the interplay between *Fusobacterium nucleatum* with the expression of microRNA, and inflammatory mediators in colorectal cancer. *Front. Microbiol.* 14:1302719. doi: 10.3389/fmicb.2023.1302719

Bowen, W. H., Burne, R. A., Wu, H., and Koo, H. (2018). Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol.* 26, 229–242. doi: 10.1016/j.tim.2017.09.008

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3, 238–250. doi: 10.1038/nrmicro1098

Brookes, Z. L. S., Bescos, R., Belfield, L. A., Ali, K., and Roberts, A. (2020). Current uses of chlorhexidine for management of oral disease: a narrative review. *J. Dent.* 103:103497. doi: 10.1016/j.jdent.2020.103497

Bryden, W. L. (2012). Mycotoxin contamination of the feed supply chain: implications for animal productivity and feed security. *Anim. Feed Sci. Technol.* 173, 134–158. doi: 10.1016/j.anifeedsci.2011.12.014

Bürgers, R., Hahnel, S., Reichert, T. E., Rosentritt, M., Behr, M., Gerlach, T., et al. (2010). Adhesion of *Candida albicans* to various dental implant surfaces and the influence of salivary pellicle proteins. *Acta Biomater.* 6, 2307–2313. doi: 10.1016/j.actbio.2009.11.003

Bushin, L. B., Covington, B. C., Rued, B. E., Federle, M. J., and Seyedsayamdost, M. R. (2020). Discovery and biosynthesis of Streptosactin, a Sactipeptide with an alternative topology encoded by commensal Bacteria in the human microbiome. *J. Am. Chem. Soc.* 142, 16265–16275. doi: 10.1021/jacs.0c05546

Busscher, H. J., Mulder, A. F., and Van Der Mei, H. C. (1999). *In vitro* adhesion to enamel and *in vivo* colonization of tooth surfaces by lactobacilli from a bio-yoghurt. *Caries Res.* 33, 403–404. doi: 10.1159/000016541

Caglar, E., Cildir, S. K., Ergeneli, S., Sandalli, N., and Twetman, S. (2006). Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. *Acta Odontol. Scand.* 64, 314–318. doi: 10.1080/00016350600801709

Caglar, E., Sandalli, N., Twetman, S., Kavaloglu, S., Ergeneli, S., and Selvi, S. (2005). Effect of yogurt with Bifidobacterium DN-173 010 on salivary mutans streptococci and lactobacilli in young adults. *Acta Odontol. Scand.* 63, 317–320. doi: 10.1080/00016350510020070

Camanocha, A., and Dewhirst, F. E. (2014). Host-associated bacterial taxa from Chlorobi, Chloroflexi, GN02, Synergistetes, SR1, TM7, and WPS-2 Phyla/candidate divisions. *J. Oral Microbiol.* 6. doi: 10.3402/jom.v6.25468

Caselli, E., Fabbri, C., D'accolti, M., Soffritti, I., Bassi, C., Mazzacane, S., et al. (2020). Defining the oral microbiome by whole-genome sequencing and resistome analysis: the complexity of the healthy picture. *BMC Microbiol.* 20:120. doi: 10.1186/s12866-020-01801-y

Chattopadhyay, I., Verma, M., and Panda, M. (2019). Role of Oral microbiome signatures in diagnosis and prognosis of Oral Cancer. *Technol. Cancer Res. Treat.* 18:153303381986735. doi: 10.1177/1533033819867354

Chuang, L. C., Huang, C. S., Ou-Yang, L. W., and Lin, S. Y. (2011). Probiotic *Lactobacillus paracasei* effect on cariogenic bacterial flora. *Clin. Oral Investig.* 15, 471–476. doi: 10.1007/s00784-010-0423-9

Cornejo Ulloa, P., Van Der Veen, M. H., and Krom, B. P. (2019). Modulation of the oral microbiome by the host to promote ecological balance. *Odontology* 107, 437–448. doi: 10.1007/s10266-019-00413-x

Dabdoub, S. M., Ganesan, S. M., and Kumar, P. S. (2016). Comparative metagenomics reveals taxonomically idiosyncratic yet functionally congruent communities in periodontitis. *Sci. Rep.* 6:38993. doi: 10.1038/srep38993

Dagli, N., and Dagli, R. (2014). Possible use of essential oils in dentistry. *J. Int. Oral Health* 6, i–ii.

Dale, B. A., and Krisanaprakornkit, S. (2001). Defensin antimicrobial peptides in the oral cavity. *J. Oral Pathol. Med.* 30, 321–327. doi: 10.1034/j.1600-0714.2001.300601.x

Dale, B. A., Tao, R., Kimball, J. R., and Jurevic, R. J. (2006). Oral antimicrobial peptides and biological control of caries. *BMC Oral Health* 6:S13. doi: 10.1186/1472-6831-6-S1-S13

Dame-Teixeira, N., De Cena, J. A., Côrtes, D. A., Belmok, A., Dos Anjos Borges, L. G., Marconatto, L., et al. (2020). Presence of Archaea in dental caries biofilms. *Arch. Oral Biol.* 110:104606. doi: 10.1016/j.archoralbio.2019.104606

De Sousa-Pereira, P., Amado, F., Abrantes, J., Ferreira, R., Esteves, P. J., and Vitorino, R. (2013). An evolutionary perspective of mammal salivary peptide families: cystatins, histatins, statherin and PRPs. *Arch. Oral Biol.* 58, 451–458. doi: 10.1016/j.archoralbio.2012.12.011

Delaviz, Y., Liu, T. W., Deonarain, A. R., Finer, Y., Shokati, B., and Santerre, J. P. (2019). Physical properties and cytotoxicity of antimicrobial dental resin adhesives containing dimethacrylate oligomers of ciprofloxacin and metronidazole. *Dent. Mater.* 35, 229–243. doi: 10.1016/j.dental.2018.11.016

Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., et al. (2008). The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome Res.* 7, 1994–2006. doi: 10.1021/pr700764j

Deo, P. N., and Deshmukh, R. (2019). Oral microbiome: unveiling the fundamentals. *J. Oral Maxillofac. Pathol.* 23, 122–128. doi: 10.4103/jomfp.JOMFP_304_18

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., et al. (2010). The human oral microbiome. *J. Bacteriol.* 192, 5002–5017. doi: 10.1128/JB.00542-10

Dhaifalah, I., Andrys, C., Drahosova, M., Musilova, I., Adamik, Z., and Kacerovsky, M. (2014). Azurocidin levels in maternal serum in the first trimester can predict preterm

prelabor rupture of membranes. *J. Matern. Fetal Neonatal Med.* 27, 511–515. doi: 10.3109/14767058.2013.820698

Di Spirito, F., Pelella, S., Argentino, S., Sisalli, L., and Sbordone, L. (2022). Oral manifestations and the role of the oral healthcare workers in COVID-19. *Oral Dis.* 28, 1003–1004. doi: 10.1111/odi.13688

Dosoky, N. S., and Setzer, W. N. (2018). Chemical composition and biological activities of essential oils of Curcuma species. *Nutrients* 10:1196. doi: 10.3390/nu10091196

Duanis-Assaf, D., Steinberg, D., and Shemesh, M. (2020). Efficiency of *Bacillus subtilis* metabolism of sugar alcohols governs its probiotic effect against cariogenic *Streptococcus mutans*. *Artif. Cells Nanomed. Biotechnol.* 48, 1222–1230. doi: 10.1080/21691401.2020.1822855

Duran-Pinedo, A. E. (2021). Metatranscriptomic analyses of the oral microbiome. *Periodontology* 85, 28–45. doi: 10.1111/prd.12350

Efenberger, M., Agier, J., Pawłowska, E., and Brzezińska-Błaszczyk, E. (2015). Archaea prevalence in inflamed pulp tissues. *Cent. Eur. J. Immunol.* 40, 194–200. doi: 10.5114/ceji.2015.51358

Elyemni, M., Louaste, B., Nechad, I., Elkamli, T., Bouia, A., Taleb, M., et al. (2019). Extraction of essential oils of *Rosmarinus officinalis* L. by two different methods: Hydrodistillation and microwave assisted Hydrodistillation. *ScientificWorldJournal* 2019:3659432. doi: 10.1155/2019/3659432

Feng, J., Liu, J., Jiang, M., Chen, Q., Zhang, Y., Yang, M., et al. (2023). The role of oral nitrate-reducing bacteria in the prevention of caries: a review related to caries and nitrate metabolism. *Caries Res.* 57, 119–132. doi: 10.1159/000529162

Ferrazzano, G. F., Amato, I., Ingenito, A., De Natale, A., and Pollio, A. (2009). Anti-cariogenic effects of polyphenols from plant stimulant beverages (cocoa, coffee, tea). *Fitoterapia* 80, 255–262. doi: 10.1016/j.fitote.2009.04.006

Ferreira, C. M., Vieira, A. T., Vinolo, M. A. R., Oliveira, F. A., Curi, R., and Martins, F. D. S. (2014). The central role of the gut microbiota in chronic inflammatory diseases. *J Immunol Res* 2014, 1–12. doi: 10.1155/2014/689492

Franzosa, E. A., Huang, K., Meadow, J. F., Gevers, D., Lemon, K. P., Bohannan, B. J., et al. (2015). Identifying personal microbiomes using metagenomic codes. *Proc. Natl. Acad. Sci. USA* 112, E2930–E2938. doi: 10.1073/pnas.1423854112

Fujiwara, N., Tsuruda, K., Iwamoto, Y., Kato, F., Odaki, T., Yamane, N., et al. (2017). Significant increase of oral bacteria in the early pregnancy period in Japanese women. *J. Investig. Clin. Dent.* 8:e12189. doi: 10.1111/jicd.12189

Ge, Y., Wang, S., Zhou, X., Wang, H., Xu, H. H., and Cheng, L. (2015). The use of quaternary ammonium to combat dental caries. *Materials (Basel)* 8, 3532–3549. doi: 10.3390/ma8063532

Godlewska, U., Brzoza, P., Sroka, A., Majewski, P., Jentsch, H., Eckert, M., et al. (2017). Antimicrobial and attractant roles for chemerin in the oral cavity during inflammatory gum disease. *Front. Immunol.* 8:353. doi: 10.3389/fimmu.2017.00353

Gomez-Arango, L. F., Barrett, H. L., Mcintyre, H. D., Callaway, L. K., Morrison, M., and Dekker Nitert, M. (2017). Antibiotic treatment at delivery shapes the initial oral microbiome in neonates. *Sci. Rep.* 7:43481. doi: 10.1038/srep43481

Gordon, Y. J., Romanowski, E. G., and Mcdermott, A. M. (2005). A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr. Eye Res.* 30, 505–515. doi: 10.1080/02713680590968637

Gorr, S. U. (2009). Antimicrobial peptides of the oral cavity. *Periodontol.* 51, 152–180. doi: 10.1111/j.1600-0757.2009.00310.x

Gorr, S. U. (2012). Antimicrobial peptides in periodontal innate defense. *Front. Oral Biol.* 15, 84–98. doi: 10.1159/000329673

Grant, M., Kilsgård, O., Åkerman, S., Klinge, B., Demmer, R. T., Malmström, J., et al. (2019). The human salivary antimicrobial peptide profile according to the oral microbiota in health, periodontitis and smoking. *J. Innate Immun.* 11, 432–444. doi: 10.1159/000494146

Güncü, G. N., Yilmaz, D., Könönen, E., and Gürsoy, U. K. (2015). Salivary antimicrobial peptides in early detection of periodontitis. *Front. Cell. Infect. Microbiol.* 5:99. doi: 10.3389/fcimb.2015.00099

Gutt, B., Ren, Q., Hauser-Gerspach, I., Kardas, P., Stübinger, S., Astasov-Frauenhoffer, M., et al. (2018). Beneficial Oral biofilms as smart bioactive interfaces. *Front. Microbiol.* 9:107. doi: 10.3389/fmicb.2018.00107

Hampelska, K., Jaworska, M. M., Babalska, Z. Ł., and Karpiński, T. M. (2020). The role of oral microbiota in intra-oral halitosis. *J. Clin. Med.* 9:2484. doi: 10.3390/jcm9082484

Hannig, C., Hannig, M., Kensche, A., and Carpenter, G. (2017). The mucosal pellicle–an underestimated factor in oral physiology. *Arch. Oral Biol.* 80, 144–152. doi: 10.1016/j.archoralbio.2017.04.001

Harris, F., Dennison, S. R., and Phoenix, D. A. (2009). Anionic antimicrobial peptides from eukaryotic organisms. *Curr. Protein Pept. Sci.* 10, 585–606. doi: 10.2174/138920309789630589

Heilbronner, S., Krismer, B., Brötz-Oesterhelt, H., and Peschel, A. (2021). The microbiome-shaping roles of bacteriocins. *Nat. Rev. Microbiol.* 19, 726–739. doi: 10.1038/s41579-021-00569-w

Herremans, K. M., Riner, A. N., Cameron, M. E., Mckinley, K. L., Triplett, E. W., Hughes, S. J., et al. (2022). The oral microbiome, pancreatic cancer and human diversity in the age of precision medicine. *Microbiome* 10:93. doi: 10.1186/s40168-022-01262-7

Holmes, A. R., Gilbert, C., Wells, J. M., and Jenkinson, H. F. (1998). Binding properties of *Streptococcus gordonii* SspA and SspB (antigen I/II family) polypeptides expressed on the cell surface of *Lactococcus lactis* MG1363. *Infect. Immun.* 66, 4633–4639. doi: 10.1128/IAI.66.10.4633-4639.1998

Hu, H., Burrow, M. F., and Leung, W. K. (2023). Proteomic profile of in situ acquired pellicle on tooth and restorative material surfaces. *J. Dent.* 129:104389. doi: 10.1016/j.jdent.2022.104389

Huang, X., Palmer, S. R., Ahn, S. J., Richards, V. P., Williams, M. L., Nascimento, M. M., et al. (2016). A highly Arginolytic Streptococcus species that potently antagonizes *Streptococcus mutans*. *Appl. Environ. Microbiol.* 82, 2187–2201. doi: 10.1128/AEM.03887-15

Inchingolo, F., Inchingolo, A. M., Malcangi, G., De Leonardis, N., Sardano, R., Pezzolla, C., et al. (2023). The benefits of probiotics on Oral health: Systematic Review of the Literature. *Pharmaceuticals (Basel)* 16:1313. doi: 10.3390/ph16091313

Isogai, E., Hirata, M., Isogai, H., Matuo, K., Kimura, K., Yokota, K., et al. (2003). Antimicrobial activity of synthetic human CAP18 peptides to Streptococcus sanguis isolated from patients with Behçet's disease. *Adv. Exp. Med. Biol.* 528, 195–200. doi: 10.1007/0-306-48382-3_38

Jafri, H., and Ahmad, I. (2020). *Thymus vulgaris* essential oil and thymol inhibit biofilms and interact synergistically with antifungal drugs against drug resistant strains of Candida albicans and *Candida tropicalis*. *J. Mycol. Med.* 30:100911. doi: 10.1016/j.mycmed.2019.100911

Jang, H., Patoine, A., Wu, T. T., Castillo, D. A., and Xiao, J. (2021). Oral microflora and pregnancy: a systematic review and meta-analysis. *Sci. Rep.* 11:16870. doi: 10.1038/s41598-021-96495-1

Jaspers, I. (2014). Cigarette smoke effects on innate immune mechanisms in the nasal mucosa. Potential effects on the microbiome. *Ann. Am. Thorac. Soc.* 11, S38–S42. doi: 10.1513/AnnalsATS.201306-154MG

Jia, Y.-J., Liao, Y., He, Y.-Q., Zheng, M.-Q., Tong, X.-T., Xue, W.-Q., et al. (2021). Association between oral microbiota and cigarette smoking in the Chinese population. *Front. Cell. Infect. Microbiol.* 11:658203. doi: 10.3389/fcimb.2021.658203

Jiang, W. X., Hu, Y. J., Gao, L., He, Z. Y., Zhu, C. L., Ma, R., et al. (2015). The impact of various time intervals on the supragingival plaque dynamic core microbiome. *PLoS One* 10:e0124631. doi: 10.1371/journal.pone.0124631

Jiao, Y., Wu, L., Huntington, N. D., and Zhang, X. (2020). Crosstalk between gut microbiota and innate immunity and its implication in autoimmune diseases. *Front. Immunol.* 11:282. doi: 10.3389/fimmu.2020.00282

Jin Baek, K., Choi, Y. S., Kang, C. K., and Choi, Y. (2017). The proteolytic activity of *Porphyromonas gingivalis* is critical in a murine model of periodontitis. *J. Periodontol.* 88, 218–224. doi: 10.1902/jop.2016.160262

Kajjari, S., Joshi, R. S., Hugar, S. M., Gokhale, N., Meharwade, P., and Uppin, C. (2022). The effects of lavender essential oil and its clinical implications in dentistry: a review. *Int. J. Clin. Pediatr. Dent.* 15, 385–388. doi: 10.5005/jp-journals-10005-2378

Kapas, S., Pahal, K., Cruchley, A. T., Hagi-Pavli, E., and Hinson, J. P. (2004). Expression of adrenomedullin and its receptors in human salivary tissue. *J. Dent. Res.* 83, 333–337. doi: 10.1177/154405910408300412

Karadağlıoğlu, Ö., Ulusoy, N., Başer, K. H. C., Hanoğlu, A., and Şık, İ. (2019). Antibacterial activities of herbal toothpastes combined with essential oils against *Streptococcus mutans*. *Pathogens* 8:8. doi: 10.3390/pathogens8010020

Karan, N. B. (2019). Influence of lavender oil inhalation on vital signs and anxiety: a randomized clinical trial. *Physiol. Behav.* 211:112676. doi: 10.1016/j.physbeh.2019.112676

Karpiński, T. M. (2019). Role of oral microbiota in cancer development. *Microorganisms* 7:20. doi: 10.3390/microorganisms7010020

Kay, J., Thadhani, E., Samson, L., and Engelward, B. (2019). Inflammation-induced DNA damage, mutations and cancer. *DNA Repair (Amst)* 83:102673. doi: 10.1016/j.dnarep.2019.102673

Khurshid, Z., Naseem, M., Sheikh, Z., Najeeb, S., Shahab, S., and Zafar, M. S. (2016). Oral antimicrobial peptides: types and role in the oral cavity. *Saudi Pharm. J.* 24, 515–524. doi: 10.1016/j.jsps.2015.02.015

Kistler, J. O., Booth, V., Bradshaw, D. J., and Wade, W. G. (2013). Bacterial community development in experimental gingivitis. *PLoS One* 8:e71227. doi: 10.1371/journal.pone.0071227

Kobayashi, R., Kobayashi, T., Sakai, F., Hosoya, T., Yamamoto, M., and Kurita-Ochiai, T. (2017). Oral administration of *Lactobacillus gasseri* SBT2055 is effective in preventing *Porphyromonas gingivalis*-accelerated periodontal disease. *Sci. Rep.* 7:545. doi: 10.1038/s41598-017-00623-9

Kutta, H., May, J., Jaehne, M., Münscher, A., and Paulsen, F. P. (2006). Antimicrobial defence mechanisms of the human parotid duct. *J. Anat.* 208, 609–619. doi: 10.1111/j.1469-7580.2006.00567.x

La Rosa, G. R. M., Gattuso, G., Pedullà, E., Rapisarda, E., Nicolosi, D., and Salmeri, M. (2020). Association of oral dysbiosis with oral cancer development. *Oncol. Lett.* 19, 3045–3058. doi: 10.3892/ol.2020.11441

Lakhdar, L., Hmamouchi, M., Rida, S., and Ennibi, O. (2012). Antibacterial activity of essential oils against periodontal pathogens: a qualitative systematic review. *Odontostomatol. Trop.* 35, 38–46.

Lamont, R. J., Koo, H., and Hajishengallis, G. (2018). The oral microbiota: dynamic communities and host interactions. *Nat. Rev. Microbiol.* 16, 745–759. doi: 10.1038/s41579-018-0089-x

Lee, W.-H., Chen, H.-M., Yang, S.-F., Liang, C., Peng, C.-Y., Lin, F.-M., et al. (2017). Bacterial alterations in salivary microbiota and their association in oral cancer. *Sci. Rep.* 7:16540. doi: 10.1038/s41598-017-16418-x

Lee, J. W., Shin, J. G., Kim, E. H., Kang, H. E., Yim, I. B., Kim, J. Y., et al. (2004). Immunomodulatory and antitumor effects *in vivo* by the cytoplasmic fraction of Lactobacillus casei and *Bifidobacterium longum*. *J. Vet. Sci.* 5, 41–48. doi: 10.4142/jvs.2004.5.1.41

Li, C. L., Liu, D. L., Jiang, Y. T., Zhou, Y. B., Zhang, M. Z., Jiang, W., et al. (2009). Prevalence and molecular diversity of Archaea in subgingival pockets of periodontitis patients. *Oral Microbiol. Immunol.* 24, 343–346. doi: 10.1111/j.1399-302X.2009.00514.x

Li, D., Xi, W., Zhang, Z., Ren, L., Deng, C., Chen, J., et al. (2020). Oral microbial community analysis of the patients in the progression of liver cancer. *Microb. Pathog.* 149:104479. doi: 10.1016/j.micpath.2020.104479

Lin, Y., Chen, J., Zhou, X., and Li, Y. (2021). Inhibition of *Streptococcus mutans* biofilm formation by strategies targeting the metabolism of exopolysaccharides. *Crit. Rev. Microbiol.* 47, 667–677. doi: 10.1080/1040841X.2021.1915959

Lin, W., Jiang, W., Hu, X., Gao, L., Ai, D., Pan, H., et al. (2018). Ecological shifts of supragingival microbiota in association with pregnancy. *Front. Cell. Infect. Microbiol.* 8:24. doi: 10.3389/fcimb.2018.00024

Lin, T. H., Lin, C. H., and Pan, T. M. (2018). The implication of probiotics in the prevention of dental caries. *Appl. Microbiol. Biotechnol.* 102, 577–586. doi: 10.1007/s00253-017-8664-z

Lippi, G., and Mattiuzzi, C. (2020). The global burden of pancreatic cancer. *Arch. Med. Sci.* 16, 820–824. doi: 10.5114/aoms.2020.94845

Liu, Y.-L., Nascimento, M., and Burne, R. A. (2012). Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries. *Int. J. Oral Sci.* 4, 135–140. doi: 10.1038/ijos.2012.54

Liu, X., Tong, X., Zhu, J., Tian, L., Jie, Z., Zou, Y., et al. (2021). Metagenome-genome-wide association studies reveal human genetic impact on the oral microbiome. *Cell Discov.* 7:117. doi: 10.1038/s41421-021-00356-0

Loozen, G., Boon, N., Pauwels, M., Slomka, V., Rodrigues Herrero, E., Quirynen, M., et al. (2015). Effect of *Bdellovibrio bacteriovorus* HD100 on multispecies oral communities. *Anaerobe* 35, 45–53. doi: 10.1016/j.anaerobe.2014.09.011

López-García, B., Lee, P. H., Yamasaki, K., and Gallo, R. L. (2005). Anti-fungal activity of cathelicidins and their potential role in *Candida albicans* skin infection. *J. Invest. Dermatol.* 125, 108–115. doi: 10.1111/j.0022-202X.2005.23713.x

Lopez-Lopez, A., Camelo-Castillo, A., Ferrer, M. D., Simon-Soro, A., and Mira, A. (2017). Health-associated niche inhabitants as Oral probiotics: the case of Streptococcus dentisani. *Front. Microbiol.* 8:379. doi: 10.3389/fmicb.2017.00379

Lourenço, T. G., Heller, D., Silva-Boghossian, C. M., Cotton, S. L., Paster, B. J., and Colombo, A. P. (2014). Microbial signature profiles of periodontally healthy and diseased patients. *J. Clin. Periodontol.* 41, 1027–1036. doi: 10.1111/jcpe.12302

Machado, T. Q., Da Fonseca, A. C. C., Duarte, A. B. S., Robbs, B. K., and De Sousa, D. P. (2022). A narrative review of the antitumor activity of monoterpenes from essential oils: an update. *Biomed. Res. Int.* 2022, 1–20. doi: 10.1155/2022/6317201

Macy, E. (2015). Penicillin allergy: optimizing diagnostic protocols, public health implications, and future research needs. *Curr. Opin. Allergy Clin. Immunol.* 15, 308–313. doi: 10.1097/ACI.0000000000000173

Mai, S., Mauger, M. T., Niu, L. N., Barnes, J. B., Kao, S., Bergeron, B. E., et al. (2017). Potential applications of antimicrobial peptides and their mimics in combating caries and pulpal infections. *Acta Biomater.* 49, 16–35. doi: 10.1016/j.actbio.2016.11.026

Mark Welch, J. L., Rossetti, B. J., Rieken, C. W., Dewhirst, F. E., and Borisy, G. G. (2016). Biogeography of a human oral microbiome at the micron scale. *Proc. Natl. Acad. Sci.* 113, E791–E800. doi: 10.1073/pnas.1522149113

Meena, N., Verma, P., Pande, R., Kumar, M., Watts, A., and Gupta, O. (2020). Bioavailability and nutritional analysis of flavonoids. *Plant Phenol. Sustain. Agricult.* 1, 135–156. doi: 10.1007/978-981-15-4890-1_6

Mira, A., Simon-Soro, A., and Curtis, M. A. (2017). Role of microbial communities in the pathogenesis of periodontal diseases and caries. *J. Clin. Periodontol.* 44, S23–s38. doi: 10.1111/jcpe.12671

Mishra, S., Rath, S., and Mohanty, N. (2020). Probiotics-a complete oral healthcare package. *J. Integr. Med.* 18, 462–469. doi: 10.1016/j.joim.2020.08.005

Mohan, S., and Epstein, J. B. (2003). Carcinogenesis and cyclooxygenase: the potential role of COX-2 inhibition in upper aerodigestive tract cancer. *Oral Oncol.* 39, 537–546. doi: 10.1016/S1368-8375(03)00035-6

Murakami, M., Ohtake, T., Dorschner, R. A., and Gallo, R. L. (2002). Cathelicidin antimicrobial peptides are expressed in salivary glands and saliva. *J. Dent. Res.* 81, 845–850. doi: 10.1177/154405910208101210

Nabavizade, M., Sobhnamayan, F., Bahrami, H., Rafieian-Kopaei, M., and Abbaszadegan, A. (2018). Evaluation of the wettability of a resin-based sealer in contact with some herbal irrigants. *Dent. Res. J. (Isfahan)* 15, 130–135. doi: 10.4103/1735-3327.226527

Nagaraj, T., Ravi, B., Sankara, S. N., Madhu, K., and Kailasam, S. (2012). Probiotics and Oral health. *J. Ind. Acad. Oral Med. Radiol.* 24, 146–148. doi: 10.5005/jp-journals-10011-1281

Nascimento, M. (2017). Oral microbiota transplant: a potential new therapy for oral diseases. *J. Calif. Dent. Assoc.* 45, 565–568. doi: 10.1080/19424396.2017.12222506

Nascimento, M. M. (2018). Potential uses of arginine in dentistry. *Adv. Dent. Res.* 29, 98–103. doi: 10.1177/0022034517735294

Nascimento, M. M., Alvarez, A. J., Huang, X., Hanway, S., Perry, S., Luce, A., et al. (2019). Arginine metabolism in Supragingival Oral biofilms as a potential predictor of caries risk. *JDR Clin. Trans. Res.* 4, 262–270. doi: 10.1177/2380084419834234

Näse, L., Hatakka, K., Savilahti, E., Saxelin, M., Pönkä, A., Poussa, T., et al. (2001). Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. *Caries Res.* 35, 412–420. doi: 10.1159/000047484

Nath, S., Zilm, P., Jamieson, L., Kapellas, K., Goswami, N., Ketagoda, K., et al. (2021). Development and characterization of an oral microbiome transplant among Australians for the treatment of dental caries and periodontal disease: a study protocol. *PLoS One* 16:e0260433. doi: 10.1371/journal.pone.0260433

Navidifar, T., Mahdizade Ari, M., Alipourkermani, A., Afifirad, R., Asadollahi, P., Veisi, A., et al. (2023). Clinical efficacy of probiotics on Oral health: a systematic review of clinical trials. *Curr. Pharm. Biotechnol.* 24, 1916–1927. doi: 10.2174/1389201024666230405135457

Nowicki, E. M., Shroff, R., Singleton, J. A., Renaud, D. E., Wallace, D., Drury, J., et al. (2018). Microbiota and metatranscriptome changes accompanying the onset of gingivitis. *MBio* 9:e00575-18. doi: 10.1128/mBio.00575-18

O'Mullane, D. M., Baez, R. J., Jones, S., Lennon, M. A., Petersen, P. E., Rugg-Gunn, A. J., et al. (2016). Fluoride and oral health. *Community Dent. Health* 33, 69–99.

Oliveira, S. G., Nishiyama, R. R., Trigo, C. A. C., Mattos-Guaraldi, A. L., Dávila, A. M. R., Jardim, R., et al. (2021). Core of the saliva microbiome: an analysis of the MG-RAST data. *BMC Oral Health* 21:351. doi: 10.1186/s12903-021-01719-5

Olson, J. C., Cuff, C. F., Lukomski, S., Lukomska, E., Canizales, Y., Wu, B., et al. (2011). Use of 16S ribosomal RNA gene analyses to characterize the bacterial signature associated with poor oral health in West Virginia. *BMC Oral Health* 11:7. doi: 10.1186/1472-6831-11-7

Ouhara, K., Komatsuzawa, H., Yamada, S., Shiba, H., Fujiwara, T., Ohara, M., et al. (2005). Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, {beta}-defensins and LL37, produced by human epithelial cells. *J. Antimicrob. Chemother.* 55, 888–896. doi: 10.1093/jac/dki103

Parcina Amizic, I., Cigic, L., Gavic, L., Radic, M., Biocina Lukenda, D., Tonkic, M., et al. (2017). Antimicrobial efficacy of probiotic-containing toothpastes: an *in vitro* evaluation. *Med. Glas. (Zenica)* 14, 139–144. doi: 10.17392/870-16

Pozhitkov, A. E., Leroux, B. G., Randolph, T. W., Beikler, T., Flemmig, T. F., and Noble, P. A. (2015). Towards microbiome transplant as a therapy for periodontitis: an exploratory study of periodontitis microbial signature contrasted by oral health, caries and edentulism. *BMC Oral Health* 15:125. doi: 10.1186/s12903-015-0109-4

Pusateri, C. R., Monaco, E. A., and Edgerton, M. (2009). Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Arch. Oral Biol.* 54, 588–594. doi: 10.1016/j.archoralbio.2009.01.016

Qiu, W., Zhou, Y., Li, Z., Huang, T., Xiao, Y., Cheng, L., et al. (2020). Application of antibiotics/antimicrobial agents on dental caries. *Biomed. Res. Int.* 2020, 1–11. doi: 10.1155/2020/5658212

Radaic, A., and Kapila, Y. L. (2021). The oralome and its dysbiosis: new insights into oral microbiome-host interactions. *Comput. Struct. Biotechnol. J.* 19, 1335–1360. doi: 10.1016/j.csbj.2021.02.010

Radu, C. M., Radu, C. C., Bochiș, S. A., Arbănași, E. M., Lucan, A. I., Murvai, V. R., et al. (2023). Revisiting the therapeutic effects of essential oils on the oral microbiome. *Pharmacy (Basel)* 11:33. doi: 10.3390/pharmacy11010033

Reddy, M. S., Shetty, S. R., and Vannala, V. (2019). Embracing personalized medicine in dentistry. *J. Pharm. Bioallied Sci.* 11, S92–s96. doi: 10.4103/JPBS.JPBS_297_18

Reiznautt, C. M., Ribeiro, J. S., Kreps, E., Da Rosa, W. L. O., De Lacerda, H., Peralta, S. L., et al. (2021). Development and properties of endodontic resin sealers with natural oils. *J. Dent.* 104:103538. doi: 10.1016/j.jdent.2020.103538

Ridyard, K. E., and Overhage, J. (2021). The potential of human peptide LL-37 as an antimicrobial and anti-biofilm agent. *Antibiotics (Basel)* 10:650. doi: 10.3390/antibiotics10060650

Rosier, B., Marsh, P., and Mira, A. (2018). Resilience of the oral microbiota in health: mechanisms that prevent dysbiosis. *J. Dent. Res.* 97, 371–380. doi: 10.1177/0022034517742139

Rosier, B. T., Moya-Gonzalvez, E. M., Corell-Escuin, P., and Mira, A. (2020). Isolation and characterization of nitrate-reducing bacteria as potential probiotics for oral and systemic health. *Front. Microbiol.* 11:555465. doi: 10.3389/fmicb.2020.555465

Roslund, K., Lehto, M., Pussinen, P., Hartonen, K., Groop, P.-H., Halonen, L., et al. (2021). Identifying volatile *in vitro* biomarkers for oral bacteria with proton-transfer-reaction mass spectrometry and gas chromatography–mass spectrometry. *Sci. Rep.* 11:16897. doi: 10.1038/s41598-021-96287-7

Saadaoui, M., Singh, P., and Al Khodor, S. (2021). Oral microbiome and pregnancy: a bidirectional relationship. *J. Reprod. Immunol.* 145:103293. doi: 10.1016/j.jri.2021.103293

Saha, S., Chopra, A., Kamath, S. U., and Kashyap, N. N. (2023). Can acid produced from probiotic bacteria alter the surface roughness, microhardness, and elemental composition of enamel? An *in vitro* study. *Odontology* 111, 929–941. doi: 10.1007/s10266-023-00804-1

Sánchez, G. A., Miozza, V. A., Delgado, A., and Busch, L. (2014). Total salivary nitrates and nitrites in oral health and periodontal disease. *Nitric Oxide* 36, 31–35. doi: 10.1016/j.niox.2013.10.012

Sancho-Vaello, E., Gil-Carton, D., François, P., Bonetti, E. J., Kreir, M., Pothula, K. R., et al. (2020). The structure of the antimicrobial human cathelicidin LL-37 shows oligomerization and channel formation in the presence of membrane mimics. *Sci. Rep.* 10:17356. doi: 10.1038/s41598-020-74401-5

Sedghi, L., Dimassa, V., Harrington, A., Lynch, S. V., and Kapila, Y. L. (2021). The oral microbiome: role of key organisms and complex networks in oral health and disease. *Periodontology* 87, 107–131. doi: 10.1111/prd.12393

Sorbara, M. T., and Pamer, E. G. (2022). Microbiome-based therapeutics. *Nat. Rev. Microbiol.* 20, 365–380. doi: 10.1038/s41579-021-00667-9

Sun, J., Tang, Q., Yu, S., Xie, M., Xie, Y., Chen, G., et al. (2020). Role of the oral microbiota in cancer evolution and progression. *Cancer Med.* 9, 6306–6321. doi: 10.1002/cam4.3206

Takahashi, N. (2015). Oral microbiome metabolism: from "who are they?" to "what are they doing?". *J. Dent. Res.* 94, 1628–1637. doi: 10.1177/0022034515606045

Takahashi, D., Shukla, S. K., Prakash, O., and Zhang, G. (2010). Structural determinants of host defense peptides for antimicrobial activity and target cell selectivity. *Biochimie* 92, 1236–1241. doi: 10.1016/j.biochi.2010.02.023

Thurnheer, T., and Belibasakis, G. N. (2018). Effect of sodium fluoride on oral biofilm microbiota and enamel demineralization. *Arch. Oral Biol.* 89, 77–83. doi: 10.1016/j.archoralbio.2018.02.010

Toscano-Garibay, J. D., Arriaga-Alba, M., Sánchez-Navarrete, J., Mendoza-García, M., Flores-Estrada, J. J., Moreno-Eutimio, M. A., et al. (2017). Antimutagenic and antioxidant activity of the essential oils of Citrus sinensis and Citrus latifolia. *Sci. Rep.* 7:11479. doi: 10.1038/s41598-017-11818-5

Tuominen, H., and Rautava, J. (2021). Oral microbiota and cancer development. *Pathobiology* 88, 116–126. doi: 10.1159/000510979

Verma, D., Garg, P. K., and Dubey, A. K. (2018). Insights into the human oral microbiome. *Arch. Microbiol.* 200, 525–540. doi: 10.1007/s00203-018-1505-3

Vinasco, K., Mitchell, H. M., Kaakoush, N. O., and Castano-Rodriguez, N. (2019). Microbial carcinogenesis: lactic acid bacteria in gastric cancer. *Biochim. Biophys. Acta* 1872:188309. doi: 10.1016/j.bbcan.2019.07.004

Vyhnalova, T., Danek, Z., Gachova, D., and Linhartova, P. B. (2021). The role of the oral microbiota in the etiopathogenesis of oral squamous cell carcinoma. *Microorganisms* 9:1549. doi: 10.3390/microorganisms9081549

Wade, W. G. (2013). The oral microbiome in health and disease. *Pharmacol. Res.* 69, 137–143. doi: 10.1016/j.phrs.2012.11.006

Wade, W. G. (2021). Resilience of the oral microbiome. *Periodontology* 86, 113–122. doi: 10.1111/prd.12365

Welch, J. L. M., Ramírez-Puebla, S. T., and Borisy, G. G. (2020). Oral microbiome geography: micron-scale habitat and niche. *Cell Host Microbe* 28, 160–168. doi: 10.1016/j.chom.2020.07.009

Wescombe, P. A., Hale, J. D., Heng, N. C., and Tagg, J. R. (2012). Developing oral probiotics from *Streptococcus salivarius*. *Future Microbiol.* 7, 1355–1371. doi: 10.2217/fmb.12.113

Willis, J. R., and Gabaldon, T. (2020). The human Oral microbiome in health and disease: from sequences to ecosystems. *Microorganisms* 8:308. doi: 10.3390/microorganisms8020308

Wilmarth, P. A., Riviere, M. A., Rustvold, D. L., Lauten, J. D., Madden, T. E., and David, L. L. (2004). Two-dimensional liquid chromatography study of the human whole saliva proteome. *J. Proteome Res.* 3, 1017–1023. doi: 10.1021/pr049911o

Wu, D.-D., Ngowi, E. E., Zhai, Y.-K., Wang, Y.-Z., Khan, N. H., Kombo, A. F., et al. (2022). Role of hydrogen sulfide in Oral disease. *Oxidative Med. Cell. Longev.* 2022, 1–14. doi: 10.1155/2022/1886277

Wu, J., Peters, B. A., Dominianni, C., Zhang, Y., Pei, Z., Yang, L., et al. (2016). Cigarette smoking and the oral microbiome in a large study of American adults. *ISME J.* 10, 2435–2446. doi: 10.1038/ismej.2016.37

Xiao, H., Fan, Y., Li, Y., Dong, J., Zhang, S., Wang, B., et al. (2021). Oral microbiota transplantation fights against head and neck radiotherapy-induced oral mucositis in mice. *Comput. Struct. Biotechnol. J.* 19, 5898–5910. doi: 10.1016/j.csbj.2021.10.028

Yang, C.-Y., Yeh, Y.-M., Yu, H.-Y., Chin, C.-Y., Hsu, C.-W., Liu, H., et al. (2018). Oral microbiota community dynamics associated with oral squamous cell carcinoma staging. *Front. Microbiol.* 9:862. doi: 10.3389/fmicb.2018.00862

Ye, C., and Kapila, Y. (2021). Oral microbiome shifts during pregnancy and adverse pregnancy outcomes: hormonal and immunologic changes at play. *Periodontology* 87, 276–281. doi: 10.1111/prd.12386

Zasloff, M. (2019). Antimicrobial peptides of multicellular organisms: my perspective. *Adv. Exp. Med. Biol.* 1117, 3–6. doi: 10.1007/978-981-13-3588-4_1

Zaura, E., Keijser, B. J., Huse, S. M., and Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol.* 9:259. doi: 10.1186/1471-2180-9-259

Zhang, J. S., Chu, C.-H., and Yu, O. Y. (2022). Oral microbiome and dental caries development. *Dent. J.* 10:184. doi: 10.3390/dj10100184

Zhang, Y., Ding, Y., and Guo, Q. (2022). Probiotic species in the Management of Periodontal Diseases: an overview. *Front. Cell. Infect. Microbiol.* 12:806463. doi: 10.3389/fcimb.2022.806463

Zhang, L., Liu, Y., Zheng, H. J., and Zhang, C. P. (2020). The oral microbiota may have influence on oral cancer. *Front. Cell. Infect. Microbiol.* 9:476. doi: 10.3389/fcimb.2019.00476

Zhang, G., Lu, M., Liu, R., Tian, Y., Vu, V. H., Li, Y., et al. (2020). Inhibition of *Streptococcus mutans* biofilm formation and virulence by *Lactobacillus plantarum* K41 isolated from traditional Sichuan pickles. *Front. Microbiol.* 11:774. doi: 10.3389/fmicb.2020.00774

Zhang, M., Tang, H., Yuan, Y., Ou, Z., Chen, Z., Xu, Y., et al. (2023). The role of indoor microbiome and metabolites in shaping Children's nasal and Oral microbiota: a pilot multi-Omic analysis. *Meta* 13:1040. doi: 10.3390/metabo13101040

Zhang, M., Wang, F., Jiang, L., Liu, R., Zhang, L., Lei, X., et al. (2013). *Lactobacillus salivarius* REN inhibits rat oral cancer induced by 4-nitroquioline 1-oxide. *Cancer Prev. Res. (Phila.)* 6, 686–694. doi: 10.1158/1940-6207.CAPR-12-0427

Zhang, Y., Wang, X., Li, H., Ni, C., Du, Z., and Yan, F. (2018). Human oral microbiota and its modulation for oral health. *Biomed. Pharmacother.* 99, 883–893. doi: 10.1016/j.biopha.2018.01.146

Zhou, Z., Ling, G., Ding, N., Xun, Z., Zhu, C., Hua, H., et al. (2018). Molecular analysis of oral microflora in patients with primary Sjögren's syndrome by using high-throughput sequencing. *PeerJ* 6:e5649. doi: 10.7717/peerj.5649

Żółkiewicz, J., Marzec, A., Ruszczyński, M., and Feleszko, W. (2020). Postbiotics-a step beyond pre- and probiotics. *Nutrients* 12:2189. doi: 10.3390/nu12082189

Zomorodian, K., Ghadiri, P., Saharkhiz, M. J., Moein, M. R., Mehriar, P., Bahrani, F., et al. (2015). Antimicrobial activity of seven essential oils from Iranian aromatic plants against common causes of oral infections. *Jundishapur J. Microbiol.* 8:e17766. doi: 10.5812/jjm.17766

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Evaluation of efficacy and safety of *Lacticaseibacillus rhamnosus* LRa05 in the eradication of *Helicobacter pylori*: a randomized, double-blind, placebo-controlled trial

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Aim: This study aims to evaluate the efficacy of *Lacticaseibacillus rhamnosus* LRa05 supplementation in enhancing *Helicobacter pylori* (*H. pylori*) eradication rate and alleviating the gastrointestinal side effects associated with bismuth quadruple therapy.

Methods: *H. pylori*-positive patients were randomized to receive levofloxacin-based bismuth quadruple therapy combined either probiotic LRa05 or a placebo for two weeks, followed by LRa05 (1×10^{10} CFU) or maltodextrin for the next two weeks. *H. pylori* infection was detected by ^{13}C breath test pre- and post-treatment. Blood and stool samples were collected at week 0 and week 4 for routine and biochemical analysis, and serum inflammatory markers. Gastrointestinal symptoms were evaluated using the gastrointestinal symptom rating scale (GSRS). Intestinal microbiota was analyzed using 16S rRNA sequencing. The research was listed under the Chinese Clinical Trial Registry (ChiCTR2300072220), and written informed consent was obtained from all participants.

Results: The LRa05 group exhibited a trend toward higher *H. pylori* eradication rates (86.11%) compared to the placebo group (82.86%), though the difference was not statistically significant. Significant reductions in neutrophil count, alanine aminotransferase, aspartate aminotransferase, pepsinogen I, interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) ($p < 0.05$) suggest that LRa05 supplementation may mitigate inflammation, enhance liver function, and potential aid in early cancer prevention. GSRS symptom scores showed that LRa05 alleviated abdominal pain, acid reflux, bloating, and diarrhea, enhancing patient compliance. Furthermore, 16S rRNA sequencing showed that LRa05 countered the antibiotic-induced disruption of gut microbiota diversity, primarily by increasing beneficial bacteria.

Conclusion: Although LRa05 did not significantly improve the success rate of *H. pylori* eradication therapy, it has the potential to improve liver function and reduced levels of inflammatory markers such as IL-6 and TNF-α in the body,

regulating the inflammatory response. In addition, it played a positive role in alleviating the adverse symptoms and gut microbiota disturbances caused by eradication therapy, providing a possible way to improve the overall health of patients and demonstrating promising clinical potential.

Clinical Trial Registration: http://www.chictr.org.cn, identifier ChiCTR2300072220.

KEYWORDS

***Helicobacter pylori*, eradication, bismuth quadruple therapy, *Lacticaseibacillus rhamnosus* LRa05, gut microbiota**

Introduction

Helicobacter pylori (*H. pylori*) is widely recognized as a human pathogen (1). It has been linked to numerous gastrointestinal disorders including chronic active gastritis, peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma (2) and stomach cancer. Furthermore, research has indicated that *H. pylori* is not only associated with gastric diseases, but also with a series of extra-gastric conditions (3), such as vitamin B12 deficiency, iron deficiency anemia, non-alcoholic fatty liver disease, idiopathic thrombocytopenic purpura, coronary atherosclerosis, Alzheimer's disease, etc. A meta-analysis report indicates that the estimated global prevalence of *H. pylori* infection has decreased from 58. 2% (95% CI 50.7 - 65.8) during the period from 1980 to 1990 to 43.1% (40.3 - 45.9) during the period of 2011-2022 (4). Although the prevalence of *H. pylori* infection has decreased in the past decade, it still remains at a relatively high level. Consequently, the prevention and treatment of *H. pylori* -related diseases continue a prominent topic in the field of gastroenterology, controlling *H. pylori* poses a significant public health and global societal challenge.

It is well known that intestinal microbiota stability is crucial for human health. However, several studies have demonstrated that eradicating *H. pylori* may alter the gastric and intestinal microbial milieu in the short term (5, 6). In patients who have been unsuccessful in eliminating *H. pylori*, there will inevitably be alterations in the intestinal microbiota following one or even multiple eradication treatments (7). The inclusion of probiotics on the basis of bismuth quadruple therapy represents a novel and effective therapy (8, 9). Investigations have demonstrated that probiotic-assisted therapy can construct a beneficial profile of gut microbiota (10, 11). Nevertheless, there exists a variety of probiotics, and there is no uniform consensus on the timing for combining them with *H. pylori* eradication drugs (whether before, after, or simultaneously). Additionally, while it is generally agreed upon that probiotics can mitigate gastrointestinal adverse effects associated with eradication therapy (12, 13), further investigation is warranted to validate this claim.

The bismuth quadruple regimen is currently the recommended primary empirical treatment plan against *H. pylori* infections by consensus (14, 15). While it is effective for most infections produced by this bacterium, there are a number of challenges that lead to an unsatisfactory eradication rate (16), including the emergence of antibiotic-resistant strains (17), increased adverse reactions, and decreased host CYP2C19 gene polymorphism. Furthermore, widespread and irregular use of antibiotics may result in various effects, such as antibiotic-associated diarrhea (AAD), toxic side effects, and the short-term selection and spread of drug-resistant bacteria. In the long term, it may also result in alterations to the mucosal immune response pattern, the induction of gastric and intestinal microbiota imbalance (18), and the potential contribution to the development of certain diseases. In conclusion, these issues present significant difficulties in clinical *H. pylori* treatment and highlight the urgent need for improved therapeutic strategies.

Some probiotics such as *Bifidobacterium, Lactobacillus crispatus* FSCDJY67L3 and *Lactobacillus reuteri* DSM 17648 have been used in clinical studies related to the eradication of *H. pylori* (13, 19–23). In these relevant clinical trials, there is controversy about whether probiotics supplementation can improve the eradication rate of *H. pylori*. Some studies believe that probiotics supplementation can improve the eradication rate, while others hold the opposite attitude. However, most articles believe that probiotics supplementation can effectively alleviate the adverse gastrointestinal symptoms caused by eradication therapy, which is also the view supported by our study.

Lacticaseibacillus rhamnosus LRa05, originating from the feces of healthy infants, has been extensively utilized in preclinical studies, *in vitro* tests, and animal models (24–28). Research indicates that LRa05 can effectively regulate the gut microbiota, improving glucose metabolism (29) and alleviating the lipid accumulation of high-fat diet mice (27), and its safety has been confirmed by studies. It is considered to be a potential probiotic for the food industry (28), but few articles have used LRa05 for *H. pylori* eradication therapy. Therefore, in this study, we employed LRa05 as a supplementary treatment for *H. pylori* eradication, comprehensively evaluating its impact on eradication rates, blood biochemistry parameters, inflammatory markers, adverse gastrointestinal symptoms (such as nausea, vomiting, diarrhea, dyspepsia, and other symptoms), and changes in the gut microbiome.

Materials and methods

Study design and ethical statement

This study utilized a double-blind, placebo-controlled design conformant with the Declaration of Helsinki principles. We enrolled 72 patients infected with *H. pylori* from the outpatient department of Jinshan Hospital. Subjects were randomly assigned in a 1:1 ratio to either the LRa05 group or the placebo group. The overview of the trial design and process is illustrated in Figures 1 , 2. This trial was conducted at Jinshan Hospital affiliated to Fudan University in China from June 2023 to April 2024.

Population recruitment

Only individuals who fulfill the following criteria were eligible for selection: (1) Written informed consent has been obtained, ensuring that patients have a comprehensive understanding of the experiment's content, procedures, and potential adverse reactions; (2) Subjects (including males) must not have any family planning intentions and should voluntarily adopt effective contraceptive measures from 14 days prior to screening until 6 months after completion of the trial; (3) Patients aged between 18 and 65 years old are included; (4) Male volunteers must weigh not less than 50 kg, while female subjects should have a minimum weight of 45 kg with a BMI of ≥ 19.0 kg/m^2; (5) *H. pylori* infections were diagnosed using either ^{13}C/^{14}C-UBT or gastric mucosal tissue immunohistochemical detection of Hp (IHC for Hp). The exclusion criteria were determined as follows: (1) Contraindication or allergy to any component of the study drugs; (2) Substance abuse (drug or alcohol, consuming more than 14 units of alcohol per week: 1 unit = 285 mL of beer, 25 mL of spirits, or 100 mL of wine); (3) A history of prior *H. pylori* eradication therapy; (4) Use of antibiotics or probiotics within the preceding 3 months; (5) Presence of severe concurrent organic disease such as gastrointestinal bleeding, obstruction, perforation or tumor; (6) Patients with liver disease indicated by an aminotransferase index exceeding 1.5 times the upper limit of normal and kidney disease indicated by a creatinine index surpassing the upper limit of normal; (7) Positive fecal occult blood test results; (8) Participants with a severe mental illness impeding collaboration and communication necessary to meet study requirements; (9) Pregnant or breastfeeding women; (10) Individuals deemed unsuitable by investigators.

Intervention and monitoring protocol

Participants were administered a standard antibiotic regimen comprising Rabeprazole (20 mg bid), Levofloxacin (250 mg bid), Metronidazole (400 mg bid), and Bismuth pectin (200 mg tid). Additionally, individuals in the LRa05 group received *Lacticaseibacillus rhamnosus* LRa05 (1×10^{10} CFU), whereas those in the placebo group were given a placebo consisting of maltodextrin, 1 packet of 3 g per day (Figure 2). Both the probiotic and placebo products were supplied by Wecare Probiotics Co., Ltd, ensuring consistency and quality control throughout the study. The intervention spanned four weeks, with the first two weeks involving combined treatment of antibiotics and probiotic/placebo, followed by a two-week period where only probiotic LRa05 or placebo were administered.

Outcomes

The primary outcome is the eradication rate. The secondary outcome measures of this study encompassed the impact of combined probiotic LRa05 on blood routine examination, serum biochemical index, the total Gastrointestinal Symptom Rating Scale (GSRS) score, the incidence of adverse reactions, to evaluate

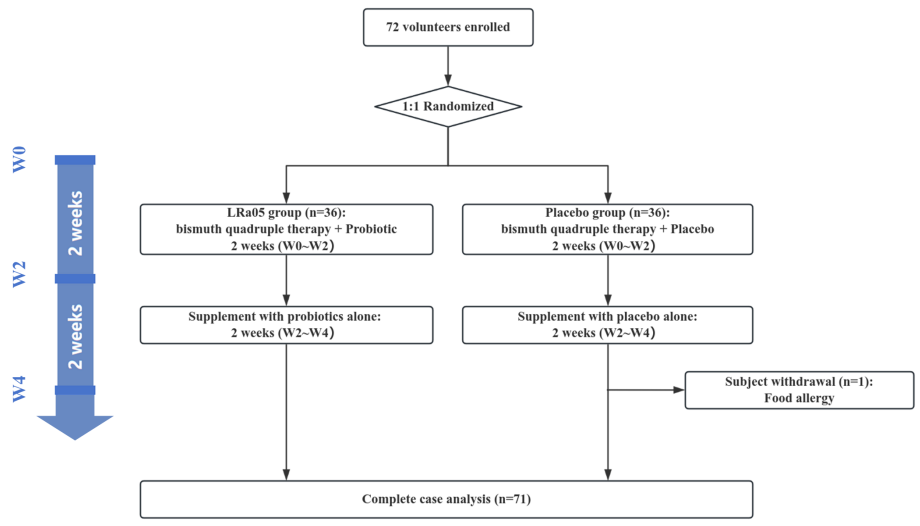


FIGURE 1
Flow diagram showing the study design. LRa05, *Lacticaseibacillus rhamnosus*.

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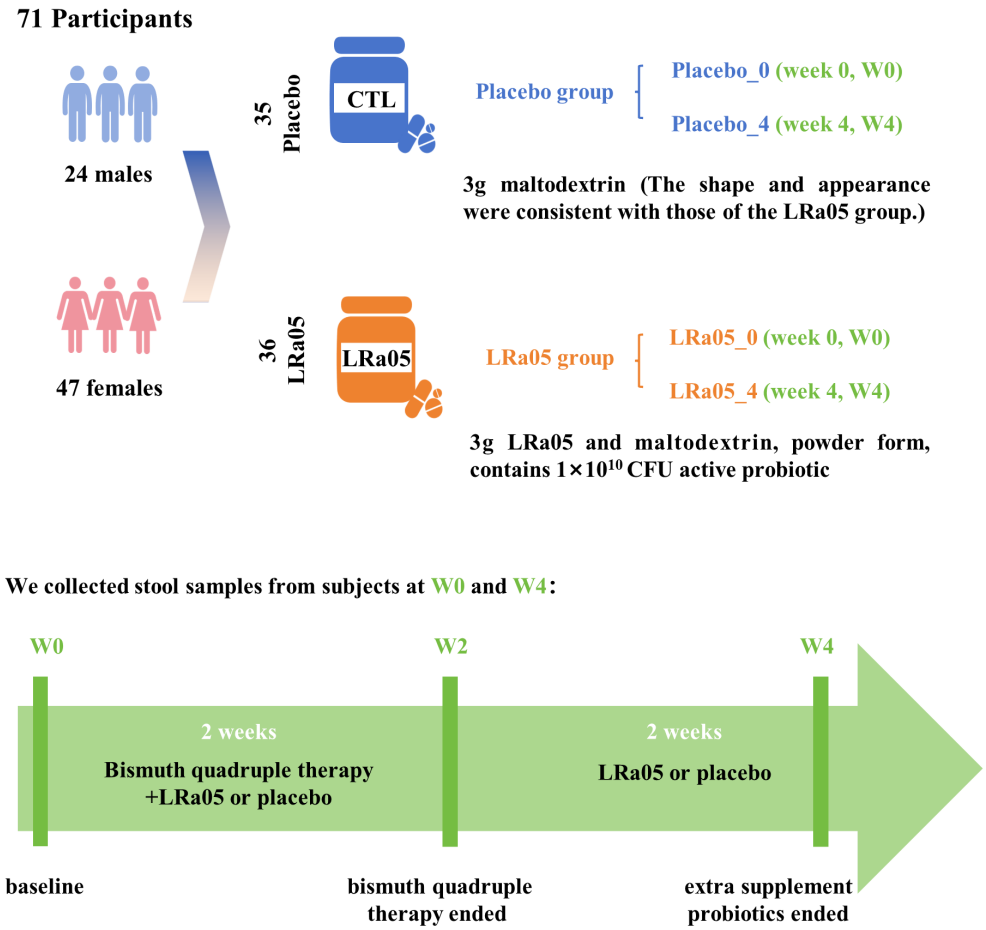


FIGURE 2
Study design details. Seventy-two subjects were recruited and 71 participants completed the trial. We collected stool and blood samples at baseline (W0) and 4 weeks after the intervention (W4), and conducted questionnaires. LRa05, *Lacticaseibacillus rhamnosus*.

compliance and security levels. Additionally, we assessed alterations in intestinal microbiota, including short-term variations in microbiota diversity (at week 0 and week 4), modifications in gut microbiota.

H. pylori and hematology detection

We assisted patients with confirmed *H. pylori* infection and inclusion in the study by either of the following methods: gastric antrum mucosal tissue immunohistochemical detection of Hp (IHC for Hp) positive, ^{13}C-UBT positive, or ^{14}C-UBT positive. IHC for Hp were performed after gastric mucosal tissue was obtained by gastroscopy (30). And the ^{13}C/^{14}C-UBT test is simpler and more convenient, requiring the patient to take orally an isotopically labeled urea solution and blow at regular intervals (31). In addition, we collected blood samples from patients, sent the samples to the laboratory for blood routine (such as RBC: red blood cell, NEUT: neutrophil, PLT: platelet, HGB: hemoglobin) and blood biochemical tests (including AST: spartate aminotransferase, ALT: alanine aminotransferase, TBIL: total bilirubin, UREA: urea, CREA: creatinine) (Mindray BS-2800M, China). We also detected the level of blood inflammatory factors (IL-6: Interleukin-6, TNF-α: Tumor Necrosis Factor-alpha, IL-17: Interleukin-17, IL-8: Interleukin-8), gastrin-17 (GT17), and pepsinogen I (PGI) by ELISA method. All measurements were performed according to the manufacturer's instructions (Jiangsu Meimian industrial Co., Ltd Yancheng).

Fecal sample collection

Fresh stool samples were collected from all patients at baseline week 0 (W0) and at week 4 (W4). Participants were instructed to promptly deliver the fecal specimen to the research assistant within the hospital on the day of collection. Subsequently, all stool samples were immediately frozen and stored at a temperature of -80°C for preservation.

The 16s RNA sequencing

The total genomic DNA of the fecal samples was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany)

according to the manufacturer's instructions. The extracted DNA was kept in a refrigerator at a temperature of -80°C until processed. Polymerase chain reaction (PCR) was used to amplify the V3V4 16s rRNA bacterial gene. Upstream (5'-CCTACGGGNGGCWGCAG-3') and downstream (5'-GACTACHVGGGTATCTAATCC-3') primer, bacterial genomic template DNA and Premix Taq constitutes the PCR reaction system. The amplifications were carried out using the program: initial denaturation at 95°C for 3min, followed by 25 cycles of denaturation at 94°C for 15s, annealing at 55°C for 30s, and extension at 72°C for 45s, with a final elongation step at 72°C for 10min. The PCR products were combined in equal proportions and subsequently purified using the Qiagen Gel Extraction Kit (Qiagen, Germany) to ensure uniformity and quality for downstream applications. The amplified DNA fragments were subsequently subjected to sequencing using an Illumina MiSeq platform (Illumina, San Diego, USA). After the sequencing was completed, the software cutadapt v2.6 was used to filter the raw date according to the window to remove the low quality, and the clean date was obtained. The filtered high-quality clean data was used for subsequent analysis. Reads were spliced into tags according to the overlap between reads, and tags were grouped into OTU, compared with the database and annotated by species. Based on the OTU and annotated results, sample species complexity analysis, inter-group species difference analysis and association analysis were carried out.

Microbiota analysis

The Bray-Curtis distance analysis from the vegan package in R (4.2) was utilized to evaluate the ecological diversity between the placebo and LRa05 groups based on the relative abundance of bacterial genera. Chao1 and ACE, Shannon and Simpson indices were used to evaluate richness and diversity of gut microbiota in participants following the intervention with LRa05. β diversity was assessed using Bray-Curtis distance, and the results were visualized through principal coordinates analysis (PCoA) plot.

Statistical analysis

Statistical analyses were conducted using SPSS software, version 27.0. For this study, quantitative data that conformed to a normal distribution are expressed as mean ± standard deviation (SD). Results were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to determine statistical significance. Quantitative data not following a normal distribution are presented as median and interquartile range. Nonparametric tests were used for analysis, with between-group comparisons performed using the Mann-Whitney U test, and within-group comparisons using the Wilcoxon signed-rank test. Categorical data were compared using the chi-square test. Correlation between two variables was assessed using Fisher's exact test. The level of significance was set at $\alpha = 0.05$, with $p < 0.05$ indicating statistically significant differences.

Results

Baseline characteristics of the subjects

Of the 72 participants enrolled, 71 completed the trial. Unfortunately, one participant in the placebo group was withdrawn early due to sea-food allergy (Figure 1). Ultimately, we collected stool and blood samples from 71 patients (Figure 2). The baseline information of enrolled patients is shown in Table 1. There were no significant differences in gender composition, age, BMI, occupation, marital status, smoking habits and medical history between the two groups ($p > 0.05$). Therefore, we believe that the biochemical indicators, microbiota composition, the GSRS score and other test indicators of the two groups of patients are comparable.

H. pylori eradication rate and adverse events

The *H. pylori* eradication rates of the LRa05 and placebo group were 86.11% and 82.86% (Figure 3), respectively. This indicates that probiotics could improve the eradication rate to a certain extent and that probiotics would not have a negative impact on the eradication therapy. However, there was no statistically significant difference ($\chi 2 = 0.144$, $p = 0.7048$).

Changes in biochemical indicators before and after intervention

NEUT showed a significant reduction in the LRa05 group before and after treatment ($p = 0.0009$), while there was no

TABLE 1 Baseline characteristics of participants in the two groups.

Characteristics	Placebo (n=35)	LRa05 (n=36)	*p* value
Gender			0.932
Male	12 (34.29%)	12 (33.33%)	
Female	23 (65.71%)	24 (66.67%)	
Age [a]	45.23 ± 11.95	48.03 ± 9.61	0.486
BMI [b]	22. 86 (21.78~26.33)	22.27 (20.96~24.24)	0.173
Job			0.307
Manual worker	1 (2.86%)	0	
Mental laborers	34 (97.14%)	36 (100%)	
Marriage			0.307
Unmarried	1 (2.86%)	0	
Married	34 (97.14%)	36 (100%)	
Smoking	0 (100%)	0 (100%)	≈1
Medical History	0 (100%)	0 (100%)	≈1

[a]Data are presented as mean ± standard deviation (SD).
[b]Data are presented as median (P_{25}, P_{75}).
LRa05, *Lacticaseibacillus rhamnosus*.

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A

Group	Eradicated	Uneradicated
LRa05	31	5
Placebo	29	6

B

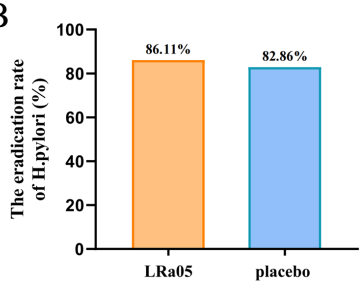


FIGURE 3
Eradication rate of *H. pylori* in patients treated with probiotics or placebo. The number of people in both groups with or without *H. pylori* eradication after treatment **(A)**. The LRa05 group had a higher eradication rate than the placebo group **(B)**, but there was no significant difference in the eradication rate between the two groups (Chi square = 0.144, p = 0.7048). LRa05, *Lacticaseibacillus rhamnosus*.

significant difference in the placebo group. Similarly, ALT and AST in the placebo group changed significantly before and after treatment (p = 0.0054, p = 0.0005, respectively), while the difference was not significant in the LRa05 group (Figure 4). Compared with the placebo group, the LRa05 group is more effective in regulating the level of PGI (p = 0.033, Figure 5).

Regulation of serum inflammatory factors

The comparison of inflammatory markers showed that the levels of IL-6 (p = 0.0012) and TNF-α (p = 0.0277) in the placebo group significantly increased after treatment, and the level of IL-8 also increased significantly, though not to a statistically significant level (p = 0.081) (Figure 6). However, there were no significant changes in the levels of these inflammatory factors in LRa05 group ($p > 0.05$).

Adverse gastrointestinal symptoms

The gastrointestinal adverse symptoms in the patients were assessed using the GSRS. Measurements were taken at baseline (W0) and four weeks post-intervention (W4). The analysis demonstrated a significant reduction in GSRS scores from baseline to follow-up in both the placebo and LRa05 group (Table 2). A more granular analysis indicated that, relative to the placebo, the LRa05 group was significantly more effective in alleviating gastrointestinal symptoms, including abdominal pain, acid reflux, abdominal distension, and loose stools ($p < 0.05$, Table 3).

Effects of the intervention on gut microbiota diversity

The species accumulation curve (Figure 7A) shows a continuous increase in the number of species in each group, indicating that species accumulation has reached saturation. In addition, the Venn diagram in Figure 7A shows 140 common species between the placebo group and the LRa05 group, accounting for 67% of the 209 species found. Further analysis (Figure 7B) showed that both placebo and LRa05 intervention reduced the species richness of intestinal microbiota (Chao1 and ACE), and did not cause significant alterations in community diversity (Shannon and Simpson). Furthermore, for the Shannon index, the LRa05 intervention significantly decreased, while there was a decrease in the placebo group, but the change was small and not statistically significant. These findings are consistent with our species accumulation analysis and the results of the Venn Diagram.

Although α diversity analysis did not reveal significant changes in gut microbiota resulting from placebo and LRa05 interventions, β diversity analysis showed (Figure 7C) that placebo and LRa05 interventions had significant effects on gut microbiota composition (p = 0. 003). The adonis assay was used to assess the effects of LRa05 and placebo interventions on gut microbiota composition. However, at the end of the study, the difference between the LRa05 and placebo groups was not significant (p = 0. 491) (Figure 7D).

Changes at the phylum and genus levels in intestinal microbiota

At the phylum level, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteriota, and Actinobacteria are the five most dominant bacterial phyla (accounting 99.9%) in the intestinal microbiota of the subjects (Figure 8A). Our analysis of the gut microbiota data at time points W0 and W4 revealed that there were no significant differences in the intestinal microbiota at the phylum level between the Placebo and LRa05 groups at any given time point (Figure 8B), except for Bacteroidetes. At the W4 point, the level of Bacteroidetes significantly decreased in the placebo group. Regarding Proteobacteria, both groups showed an upward trend at the end of the intervention period after antibiotic treatment.

In terms of genus classification, although most beneficial bacteria such as *Faecalibacterium*, *Roseburia* and *Bifidobacterium* have generally decreased in all patients after treatment, the reduction of these bacteria groups is less pronounced in patients receiving LRa05 intervention. For example, although the content of *Faecalibacterium* in the LRa05 group has decreased, the rate of

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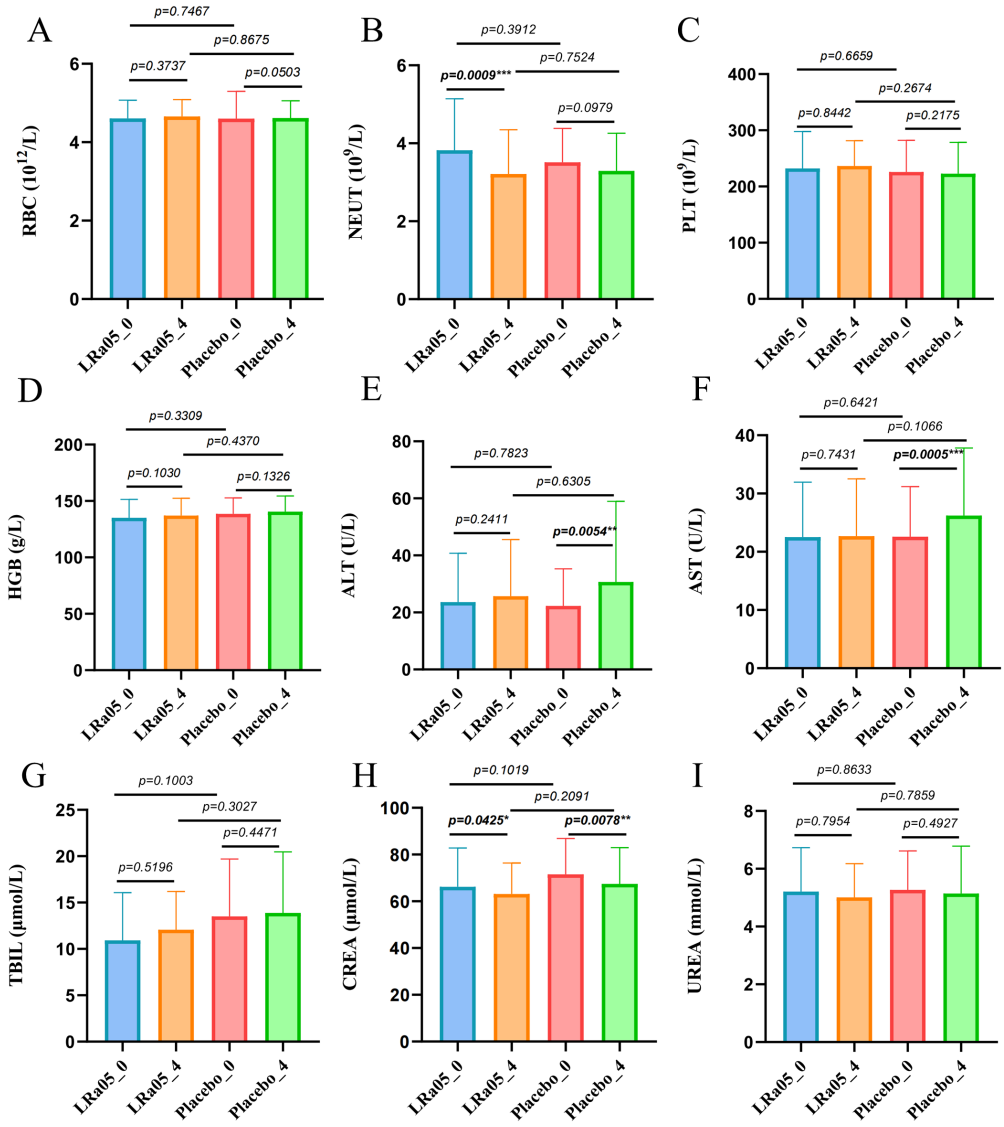


FIGURE 4
Results of statistical analysis of blood biochemical indexes of patients. **(A)** RBC, red blood cell, **(B)** NEUT, neutrophil, **(C)** PLT, platelet, **(D)** HGB, hemoglobin, **(E)** ALT, alanine aminotransferase, **(F)** AST, spartate aminotransferase, **(G)** TBIL, total bilirubin, **(H)** CREA, creatinine, **(I)** UREA, urea. LRa05, *Lacticaseibacillus rhamnosus*. _0: week 0, _4: week 4. **p<0.01, ***p<0.001.

decline in *Faecalibacterium* is significantly smaller than in the placebo group (p = 0.008). *Bacteroides* and *Blautia* genera in the LRa05 group also showed a decline, but to a similarly small extent compared to the placebo group, suggesting a protective effect of LRa05 intervention. The *Lachnospira* and *Roseburia* genera decreased significantly in the placebo group, whereas the decrease was more moderate in the LRa05 group. After antibiotic treatment, the abundance of both beneficial and harmful bacteria decreased, but the following bacteria groups increased in the LRa05 group compared to the placebo group: *Faecalibacterium, Lachnospira, Parabacteroides, Subdoligranulum, Phascolarctobacterium, Agathobacter, Fusicatenibacter, Alistipes, Coprococcus, Oscillibacter, Parasutterella, Megamonas* (Supplementary Table 1).

Correlation analysis between gut microbiota and inflammatory markers after LRa05 intervention

The *Megamonas* genus exhibits a positive correlation with PGI, GT17, and C13, while showing a negative correlation with inflammatory factors such as IL-6, IL-8, and TNF-α. *Phascolarctobacterium* has a positive correlation with indicators like CREA, UREA, TBIL, and C13, while displaying a negative correlation with inflammatory factors like IL-6 and IL-8. *Faecalibacterium* and *Fusicatenibacter* demonstrate a negative correlation with IL-8 and a positive correlation with C13. Therefore, LRa05 combination therapy adjusts the abundance of *Megamonas, Phascolarctobacterium,*

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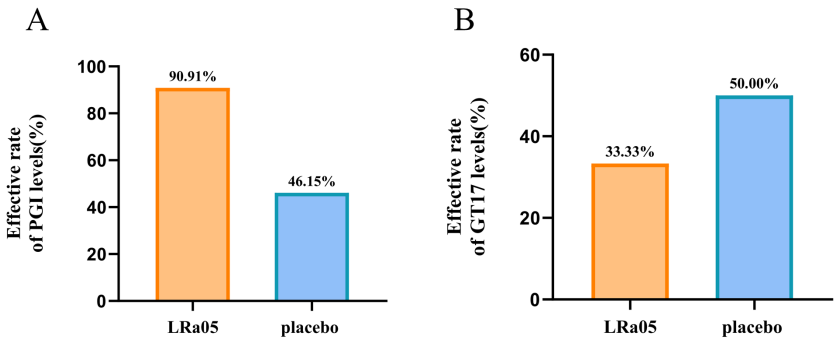


FIGURE 5
Changes of PGI levels in patients. The formula for calculating the effect rate of PGI level: (week0 number of abnormal PGI indicators - week4 number of abnormal PGI indicators)/week0 number of abnormal PGI indicators. GT17, gastrin-17; PGI, pepsinogen I; LRa05, *Lacticaseibacillus rhamnosus*.

Faecalibacterium, and *Fusicatenibacter* to reduce intestinal inflammation and promote intestinal health (Figure 9).

Discussion

The results demonstrate that although LRa05 does not significantly enhance the *H. pylori* eradication rate, it effectively improves liver function, modulates inflammatory responses, and significantly optimizes gastrointestinal symptoms and dysbiosis caused by eradication treatment. This suggests that LRa05 can serve as a potent adjunct to conventional *H. pylori* eradication therapies, mitigating the discomfort caused by pharmacological treatments.

Several similar clinical studies have shown that probiotic supplementation has no significant effect on *H. pylori* eradication

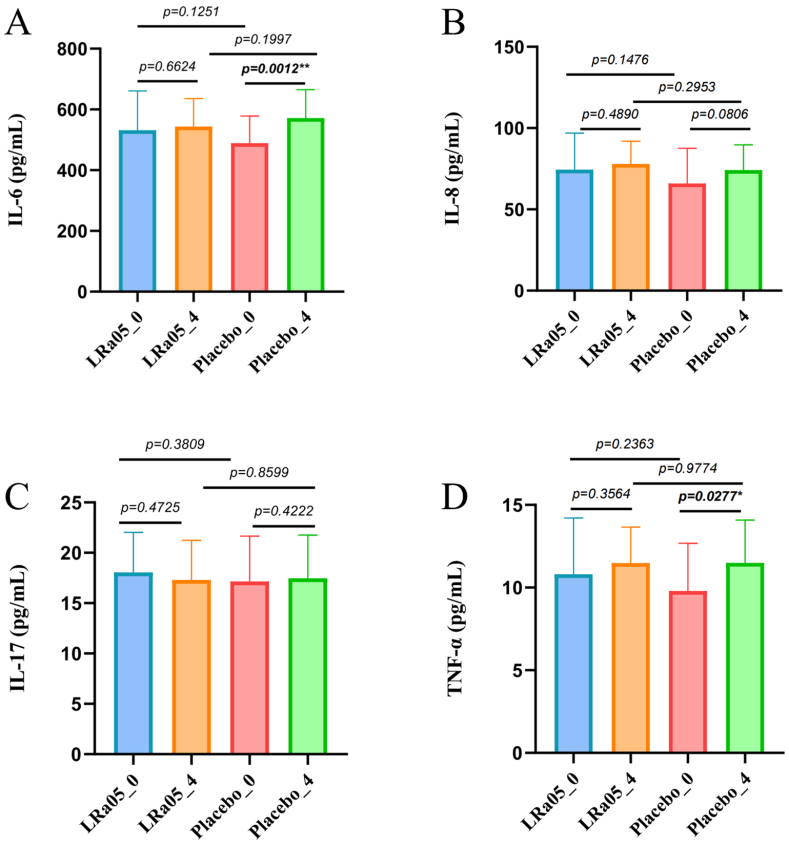


FIGURE 6
Serum inflammatory marker dynamics in patients. **(A)** Interleukin-6 (IL-6), **(B)** Tumor Necrosis Factor-α (TNF-α), **(C)** Interleukin-17 (IL-17), **(D)** Interleukin-8 (IL-8). LRa05, *Lacticaseibacillus rhamnosus*. Changes measured at Week 0 (_0) and Week 4 (_4) for both LRa05 and placebo groups. *p<0.05, **p<0.01.

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TABLE 2 Efficacy of patients with improved GSRS scores.

GSRS Score	LRa05 group (n = 36)	Placebo group (n = 35)	*p* value
week 0	6.61 ± 5.42	7.00 ± 3.99	0.4257
week 4	3.69 ± 3.61	4.89 ± 5.33	0.3728
p value	0.0006***	0.0058**	–

**p < 0.01, ***p < 0.001. LRa05: *Lacticaseibacillus rhamnosus*.

TABLE 3 Subcategorize gastrointestinal symptoms.

gastrointestinal symptoms	LRa05 group	Placebo group	*p* value
abdominal pain	91.67%(11/12)	36.36%(4/11)	0.009**
acid reflux	73.33%(11/15)	33.33%(5/15)	0.028*
abdominal distension	60.00%(12/20)	28.57%(6/21)	0.043*
loose stools	50.00%(9/18)	13.33%(2/15)	0.026*

*p < 0.05, **p < 0.01. LRa05, *Lacticaseibacillus rhamnosus*. The proportion in the table refers to the proportion of people with gastrointestinal symptoms in remission.

rates (22, 32). However, some scholars argue that probiotics offer a potential benefit in enhancing the eradication rate of *H. pylori* infection (33, 34). In this study, the eradication rate of the two groups changed numerically, and the eradication rate of LRa05 group was higher than that of the placebo group, but the difference was not significant. Therefore, we do not recommend probiotics as a separate drug to eradicate *H. pylori*, but adding probiotics as a supplement to the original eradication therapy may be a beneficial approach.

The results showed that LRa05 can regulate the blood neutrophil count, as well as ALT and AST levels, which indicates that as a probiotic supplement, it has a certain effect on regulating inflammation, reducing neutrophil count and improving liver function. Other studies on *Lactobacillus rhamnose*-treated liver disease also support our conclusion (35, 36). Although there were significant differences in CREA levels between the two groups before and after treatment, which may be related to antibiotic treatment, CREA values were within the normal reference range for creatinine in adults (44-133 μmol/L). There were no significant changes in other blood routine and blood biochemical indexes, indicating that the safety of LRa05 as a probiotic supplement is guaranteed. To our surprise, there was a significant difference in the frequency of Pepsinogen I (PGI) abnormalities between the two groups at week 4. Pepsinogen I is strongly associated with atrophic gastritis and can be used as an indicator to assess stomach cancer risk (37, 38). Those results indicate that probiotics may have the potential to improve gastric mucosa atrophy and preventing gastric cancer.

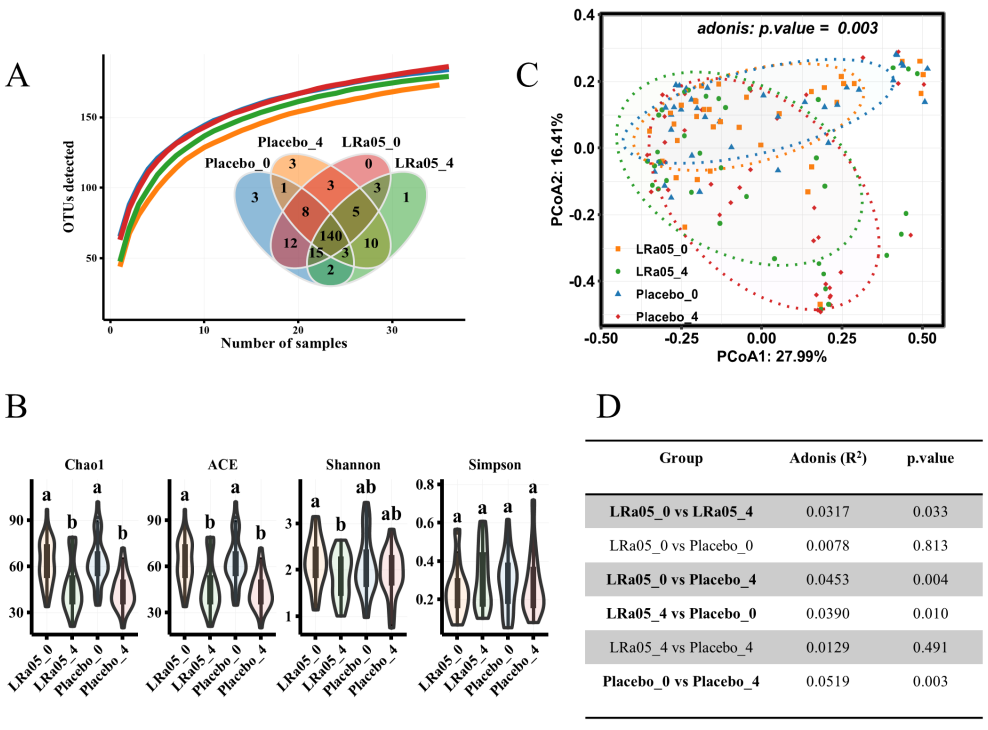


FIGURE 7
Changes in gut microbiota diversity pre- and post-intervention. **(A)** Species accumulation curve and Venn diagram depicting species distribution across placebo and LRa05 groups. **(B)** α diversity alterations measured pre- and post-intervention. **(C)** β diversity changes evaluated using principal component analysis based on the Bray–Curtis distance, with each point representing an individual subject's gut microbiota composition. **(D)** Statistical comparison of β diversity differences between groups. LRa05, *Lacticaseibacillus rhamnosus*. _0: week 0, _4: week 4. All the averages are listed in order from largest to smallest, and then the largest average is marked with the letter a; This average is compared with the following averages, where the difference is not significant, marked with the letter a, until a certain average with a significant difference, marked with the letter b. Where there is an identical marking letter, the difference is not significant, and where there is a different marking letter, the difference is significant.

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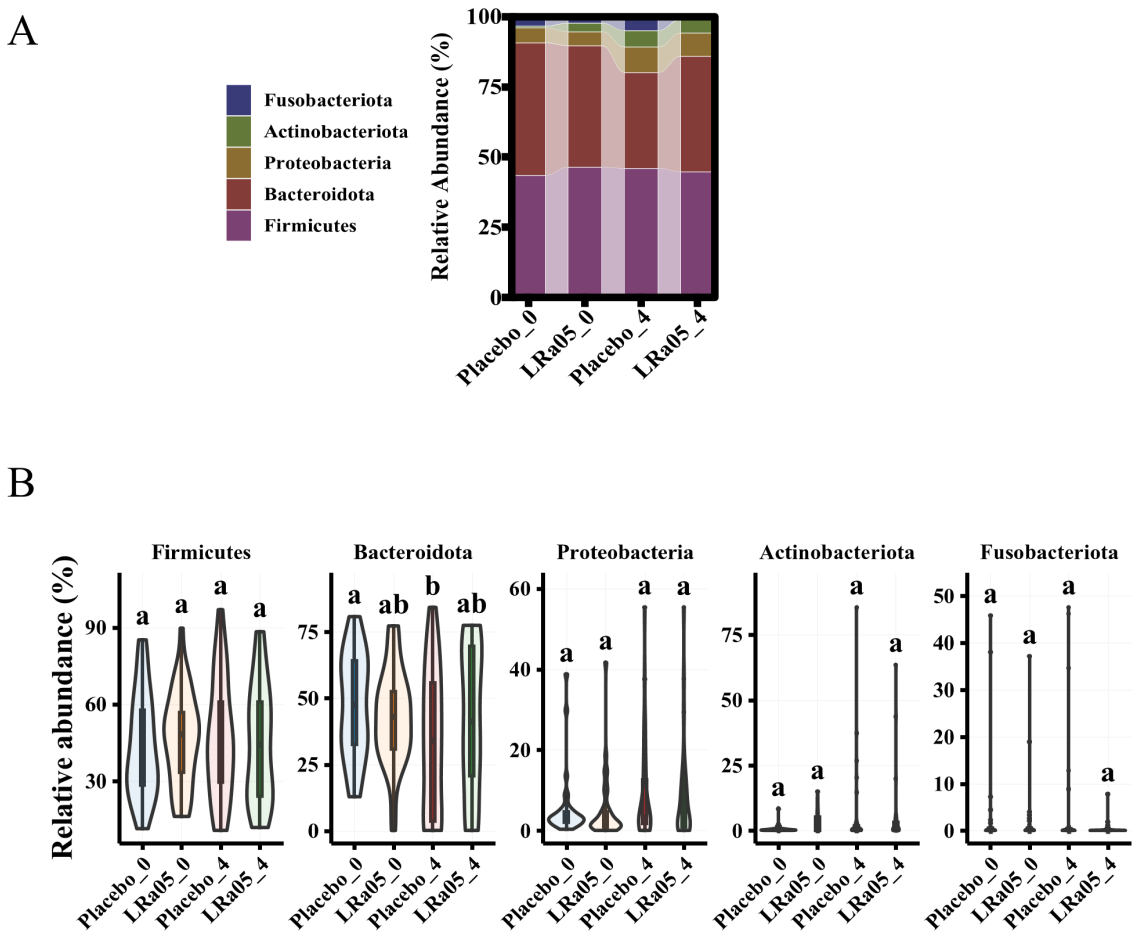


FIGURE 8
Changes in gut microbiota composition in placebo and LRa05 groups pre- and post-intervention. **(A)** Distribution of microbiota abundance at the phylum level across placebo and LRa05 groups. **(B)** Changes in phylum-level abundance from baseline (W0) to post-intervention (W4). LRa05, *Lacticaseibacillus rhamnosus*, _0: week 0, _4: week 4. All the averages are listed in order from largest to smallest, and then the largest average is marked with the letter a; This average is compared with the following averages, where the difference is not significant, marked with the letter a, until a certain average with a significant difference, marked with the letter b. Where there is an identical marking letter, the difference is not significant, and where there is a different marking letter, the difference is significant.

Many previous studies have shown that probiotics play an important role in human immune regulation and regulate the expression levels of inflammatory factors such as IL-6, IL-8, TNF-α, etc (39, 40). This coincides with the conclusion of this study. The regulation of the human immune microenvironment and enhancement of immune system function are among the mechanisms that probiotics can contribute to eradicating *H. pylori* infection (10). This may explain why probiotic supplementation in this study led to improvements in gastrointestinal adverse symptoms.

Previous research studies have shown that probiotic supplementation can improve the adverse gastrointestinal symptoms caused by *H. pylori* eradication (6, 23), it can effectively relieve diarrhea, abdominal pain, nausea, constipation and other symptoms. Contrary to some other studies (13), the total GSRS score did not differ significantly between the two groups in this study after treatment. However, through further statistics, we found that after treatment, patients in the LRa05 group had significant improvements in abdominal pain, abdominal distension, acid reflux and loose stool compared with the placebo group. The positive effect of probiotics on gastrointestinal symptoms can effectively enhance patient compliance and improve the safety of eradication therapy, which is of great significance for the eradication of *H. pylori*.

Overall, after antibiotic treatment, the abundance of both beneficial and harmful bacteria decreased while in the probiotic (LRa05) group, it increased compared to the placebo group. This is consistent with some previous clinical trials (9, 25, 32). The relatively increased beneficial bacteria in the probiotic group are *Faecalibacterium*, *Lachnospira*, *Parabacteroides*, *Subdoligranulum*, *Phascolarctobacterium*, *Agathobacter*, *Fusicatenibacter*, *Alistipes*, *Coprococcus*, *Oscillibacter*, *Parasutterella*, *Megamonas*, etc. Among them, *Faecalibacterium*, *Parabacteroides*, *Phascolarctobacterium*, *Oscillibacter* and *Parasutterella* are all related to immune regulation and can inhibit inflammatory response (41–45). In this study, the changes of IL-6 and TNF-α levels may be related to these microbial changes. The abundance of most beneficial bacteria genera generally decreased after treatment, but the decrease of these microorganisms

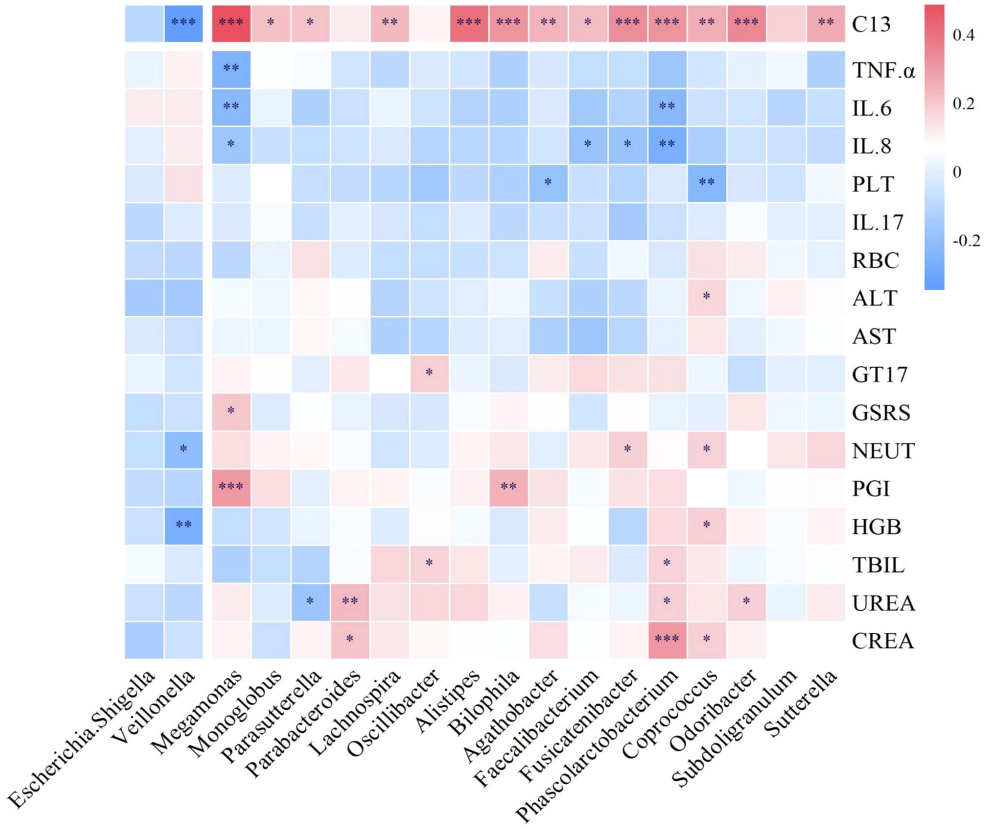


FIGURE 9
Correlation between intestinal microbiota and serum biochemistry, inflammatory factors and ^{13}C. ^{13}C, ^{13}C-urea breath test; TNF-α, tumor necrosis factor α; IL-6, interleukin-6; IL-8, interleukin-8; PLT, platelet; IL-17, interleukin-17; RBC, red blood cell; ALT, alanine aminotransferase; AST, spartate aminotransferase; GT17, gastrin-17; GSRS, gastrointestinal symptom rating scale; NEUT, neutrophil; PGI, pepsinogen I; HGB, hemoglobin; TBIL, total bilirubin; UREA, urea; CREA, creatinine. *p<0.05, **p<0.01, ***p<0.001.

was smaller in patients receiving probiotics, indicating that supplementation of probiotics has a protective effect on intestinal microbiota homeostasis. Antibiotic treatment resulted in a decrease in the abundance of most bacteria, but a few species showed an increasing trend at the end of treatment. In this study, it was observed that in both the probiotic-assisted antibiotic treatment and the control (placebo) group, *Proteobacteria* showed an increasing trend at the end of treatment. *Proteobacteria* is a genus of bacteria, including some important pathogenic bacteria such as *Enterobacteriales* and *H. pylori* (46). This upward trend may indicate that antibiotic treatment has affected the microbiota balance in the gut, reducing some of the competing beneficial bacteria and thus creating conditions for the growth of potentially pathogenic bacteria such as *Proteobacteria*. This is consistent with the conclusions of some previous studies (47–49). Therefore, it is reasonable to think that antibiotic treatment can affect the diversity of gut microbiota, including beneficial and harmful bacteria. Probiotic intervention can reverse the trend of antibiotic microbiota changes, mainly by increasing the abundance of beneficial bacteria. From this perspective, it is particularly important to supplement probiotics while eradicating *H. pylori* to increase the abundance of beneficial bacteria in the gut and reduce the increase of harmful bacteria.

In summary, while LRa05 intervention demonstrated some efficacy in adjunctive *H. pylori* eradication therapy, our study faces several limitations. Firstly, our sample size was insufficient. Secondly, this study lacked uniform requirements for diagnosing *H. pylori* infection during the enrollment and screening preventing scientific statistical analysis of *H. pylori* bacterial load before and after treatment. Further analysis of ^{13}C breath test values may offer deeper insights into probiotics' eradication effects on *H. pylori*. Additionally, although we supplemented probiotics for an additional 2 weeks post-therapy, this duration might have been insufficient, resulting in insignificant changes in some indicators. Hence, extending probiotic supplementation duration could potentially yield better therapeutic outcomes.

Conclusion

This study confirmed the effectiveness of LRa05 as a supplement for *H. pylori* eradication therapy. Although it does not improve the eradication rate, it can alleviate the adverse symptoms and intestinal microbiota disturbances caused by eradication treatment, and can also regulate the inflammatory response in the body and improve liver function.

Data availability statement

The datasets generated during this study are included in this article. The 16S rRNA gene sequence from human fecal samples is accessible in the NCBI genome database under accession PRJNA1111965.

Ethics statement

The studies involving humans were approved by the Chinese Clinical Trial Registry. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YN: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. JL: Investigation, Writing – original draft, Writing – review & editing. HQ: Writing – original draft, Writing – review & editing. CL: Writing – original draft, Writing – review & editing. XS: Writing – original draft, Writing – review & editing. SB: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1450414/full#supplementary-material

References

1. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet*. (1984) 1:1311–5. doi: 10.1016/s0140-6736(84)91816-6

2. C.Wotherspoon A, Ortiz-Hidalgo C, Falzon M, Xxxg.Isaacson P. Helicobacter pylori-associated gastritis and primary B-cell gastric lymphoma. *Lancet*. (1991) 338:1175–6. doi: 10.1016/0140-6736(91)92035-z

3. Suerbaum S, Michetti P. Helicobacter pylori infection. *N Engl J Med*. (2002) 347:1175–86. doi: 10.1056/NEJMra020542

4. Li Y, Choi H, Leung K, Jiang F, Graham DY, Leung WK. Global prevalence of Helicobacter pylori infection between 1980 and 2022: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol*. (2023) 8:553–64. doi: 10.1016/s2468-1253(23)00070-5

5. Yap TW, Gan HM, Lee YP, Leow AH, Azmi AN, Francois F, et al. Helicobacter pylori eradication causes perturbation of the human gut microbiome in young adults. *PloS One*. (2016) 11:e0151893. doi: 10.1371/journal.pone.0151893

6. Xu W, Xu L, Xu C. Relationship between Helicobacter pylori infection and gastrointestinal microecology. *Front Cell Infect Microbiol*. (2022) 12:2022.938608. doi: 10.3389/fcimb.2022.938608

7. Liu A, Wang Y, Song Y, Du Y. Treatment with compound Lactobacillus acidophilus followed by a tetracycline- and furazolidone-containing quadruple regimen as a rescue therapy for Helicobacter pylori infection. *Saudi J Gastroenterol*. (2020) 26:78–83. doi: 10.4103/sjg.SJG_589_19

8. Fallone CA, Moss SF, Malfertheiner P. Reconciliation of recent helicobacter pylori treatment guidelines in a time of increasing resistance to antibiotics. *Gastroenterology*. (2019) 157:44–53. doi: 10.1053/j.gastro.2019.04.011

9. Ji J, Yang H. Using probiotics as supplementation for helicobacter pylori antibiotic therapy. *Int J Mol Sci 21*. (2020) 21:1136. doi: 10.3390/ijms21031136

10. Nabavi-Rad A, Sadeghi A, Asadzadeh Aghdaei H, Yadegar A, Smith SM, Zali MR. The double-edged sword of probiotic supplementation on gut microbiota structure in Helicobacter pylori management. *Gut Microbes*. (2022) 14:2108655. doi: 10.1080/19490976.2022.2108655

11. FitzGerald J, Patel S, Eckenberger J, Guillemard E, Veiga P, Schäfer F, et al. Improved gut microbiome recovery following drug therapy is linked to abundance and replication of probiotic strains. *Gut Microbes*. (2022) 14:2094664. doi: 10.1080/19490976.2022.2094664

12. Buckley M, Lacey S, Doolan A, Goodbody E, Seamans K. The effect of Lactobacillus reuteri supplementation in Helicobacter pylori infection: a placebo-controlled, single-blind study. *BMC Nutr*. (2018) 4:48. doi: 10.1186/s40795-018-0257-4

13. Hong Q, Wang J, Zhang H, Liu X, Liu Z. Study of the effect of Lactobacillus crispatus FSCDJY67L3 on Helicobacter Pylori eradication: a double-blind randomized controlled clinical trial. *Front Immunol*. (2023) 14:2023.1265995. doi: 10.3389/fimmu.2023.1265995

14. Malfertheiner P, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, et al. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. *Gut*. (2017) 66:6–30. doi: 10.1136/gutjnl-2016-312288

15. Ding SZ, Du YQ, Lu H, Wang WH, Cheng H, Chen SY, et al. Chinese consensus report on family-based helicobacter pylori infection control and management (2021 edition). *Gut*. (2022) 71:238–53. doi: 10.1136/gutjnl-2021-325630

16. Hu Y, Zhu Y, Lu NH. Novel and effective therapeutic regimens for helicobacter pylori in an era of increasing antibiotic resistance. *Front Cell Infect Microbiol*. (2017) 7:2017.00168. doi: 10.3389/fcimb.2017.00168

17. Liu DS, Wang YH, Zeng ZR, Zhang ZY, Lu H, Xu JM, et al. Primary antibiotic resistance of Helicobacter pylori in Chinese patients: a multiregion prospective 7-year study. *Clin Microbiol Infect*. (2018) 24:780. doi: 10.1016/j.cmi.2017.11.010

18. Sung JJY, Coker OO, Chu E, Szeto CH, Luk STY, Lau HCH, et al. Gastric microbes associated with gastric inflammation, atrophy and intestinal metaplasia 1 year after Helicobacter pylori eradication. *Gut*. (2020) 69:1572–80. doi: 10.1136/gutjnl-2019-319826

19. Elghannam MT, Hassanien MH, Ameen YA, Turky EA, GM EL, AA EL, et al. Helicobacter pylori and oral-gut microbiome: clinical implications. *Infection*. (2023) 52:289–300. doi: 10.1007/s15010-023-02115-7

20. Peng R, Zhang Z, Qu Y, Chen W. The impact of Helicobacter pylori eradication with vonoprazan-amoxicillin dual therapy combined with probiotics on oral microbiota: a randomized double-blind placebo-controlled trial. *Front Microbiol*. (2023) 14:2023.1273709. doi: 10.3389/fmicb.2023.1273709

21. Ismail NI, Nawawi KNM, Hsin DCC, Hao KW, Mahmood N, Chearn GLC, et al. Probiotic containing Lactobacillus reuteri DSM 17648 as an adjunct treatment for Helicobacter pylori infection: A randomized, double-blind, placebo-controlled trial. *Helicobacter*. (2023) 28:e13017. doi: 10.1111/hel.13017

22. Marinelli P, Scalese G, Covelli A, Ruffa A, Bedetti G, Bruno G, et al. Lactobacillus rhamnosus GG supplementation on eradication rate and dyspepsia in Helicobacter pylori infection treated with three-in-one bismuth quadruple therapy. *Front Microbiol*. (2022) 13:2022.932331. doi: 10.3389/fmicb.2022.932331

23. Plomer M, Iii Perez M, Greifenberg DM. Effect of Bacillus clausii Capsules in Reducing Adverse Effects Associated with Helicobacter pylori Eradication Therapy: A Randomized, Double-Blind, Controlled Trial. *Infect Dis Ther*. (2020) 9:867–78. doi: 10.1007/s40121-020-00333-2

24. Dong Y, Han M, Fei T, Liu H, Gai Z. Utilization of diverse oligosaccharides for growth by Bifidobacterium and Lactobacillus species and their in *vitro* co-cultivation characteristics. *Int Microbiol*. (2024) 27:941–52. doi: 10.1007/s10123-023-00446-x

25. Gai Z, Dong Y, Xu F, Zhang J, Yang Y, Wang Y. Changes in the gut microbiota composition of healthy young volunteers after administration of Lacticaseibacillus rhamnosus LRa05: A placebo-controlled study. *Front Nutr*. (2023) 10:2023.1105694. doi: 10.3389/fnut.2023.1105694

26. Gai Z, Liao W, Huang Y, Dong Y, Feng H, Han M. Effects of Bifidobacterium BL21 and Lacticaseibacillus LRa05 on gut microbiota in type 2 diabetes mellitus mice. *AMB Express*. (2023) 13:97. doi: 10.1186/s13568-023-01603-1

27. Sun M, Wu T, Zhang G, Liu R, Sui W, Zhang M, et al. Lactobacillus rhamnosus LRa05 improves lipid accumulation in mice fed with a high fat diet via regulating the intestinal microbiota, reducing glucose content and promoting liver carbohydrate metabolism. *Food Funct*. (2020) 11:9514–25. doi: 10.1039/d0fo01720e

28. Chen T, Shao Y, Zhang Y, Zhao Y, Han M, Gai Z. *In vitro* and in *vivo* genome-based safety evaluation of Lacticaseibacillus rhamnosus LRa05. *Food Chem Toxicol*. (2024) 186:114600. doi: 10.1016/j.fct.2024.114600

29. Wu T, Zhang Y, Li W, Zhao Y, Long H, Muhindo EM, et al. Lactobacillus rhamnosus LRa05 Ameliorate Hyperglycemia through a Regulating Glucagon-Mediated Signaling Pathway and Gut Microbiota in Type 2 Diabetic Mice. *J Agric Food Chem*. (2021) 69:8797–806. doi: 10.1021/acs.jafc.1c02925

30. Lash RH, Genta RM. Routine anti-helicobacter immunohistochemical staining is significantly superior to reflex staining protocols for the detection of helicobacter in gastric biopsy specimens. *Helicobacter*. (2016) 21:581–5. doi: 10.1111/hel.12315

31. Logan RP. Urea breath tests in the management of Helicobacter pylori infection. *Gut*. (1998) 43 Suppl 1:S47–50. doi: 10.1136/gut.43.2008.s47

32. He C, Xie Y, Zhu Y, Zhuang K, Huo L, Yu Y, et al. Probiotics modulate gastrointestinal microbiota after Helicobacter pylori eradication: A multicenter randomized double-blind placebo-controlled trial. *Front Immunol*. (2022) 13:2022.1033063. doi: 10.3389/fimmu.2022.1033063

33. Liang B, Yuan Y, Peng XJ, Liu XL, Hu XK, Xing DM. Current and future perspectives for Helicobacter pylori treatment and management: From antibiotics to probiotics. *Front Cell Infect Microbiol*. (2022) 12:2022.1042070. doi: 10.3389/fcimb.2022.1042070

34. Tang B, Tang L, Huang C, Tian C, Chen L, He Z, et al. The effect of probiotics supplementation on gut microbiota after helicobacter pylori eradication: A multicenter randomized controlled trial. *Infect Dis Ther*. (2021) 10:317–33. doi: 10.1007/s40121-020-00372-9

35. Vajro P, Mandato C, Licenziati MR, Franzese A, Vitale DF, Lenta S, et al. Effects of Lactobacillus rhamnosus strain GG in pediatric obesity-related liver disease. *J Pediatr Gastroenterol Nutr*. (2011) 52:740–3. doi: 10.1097/MPG.0b013e31821f9b85

36. Vatsalya V, Feng W, Kong M, Hu H, Szabo G, McCullough A, et al. The beneficial effects of lactobacillus GG therapy on liver and drinking assessments in patients with moderate alcohol-associated hepatitis. *Am J Gastroenterol*. (2023) 118:1457–60. doi: 10.14309/ajg.0000000000002283

37. Malfertheiner P. Editorial: the non-invasive diagnosis of atrophic gastritis. *Aliment Pharmacol Ther*. (2017) 46:1112–3. doi: 10.1111/apt.14340

38. Kitahara F, Kobayashi K, Sato T, Kojima Y, Araki T, Fujino MA. Accuracy of screening for gastric cancer using serum pepsinogen concentrations. *Gut*. (1999) 44:693–7. doi: 10.1136/gut.44.5.693

39. Suez J, Zmora N, Segal E, Elinav E. The pros, cons, and many unknowns of probiotics. *Nat Med*. (2019) 25:716–29. doi: 10.1038/s41591-019-0439-x

40. Thomas CM, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. *Gut Microbes*. (2010) 1:148–63. doi: 10.4161/gmic.1.3.11712

41. Mart416 R, Rios-Covian D, Huillet E, Auger S, Khazaal S, Bermaal,ian,c.1. LG, et al. Faecalibacterium: a bacterial genus with promising human health applications. *FEMS Microbiol Rev 47*. (2023) 47:fuad039. doi: 10.1093/femsre/fuad039

42. Lei Y, Tang L, Liu S, Hu S, Wu L, Liu Y, et al. Parabacteroides produces acetate to alleviate heparanase-exacerbated acute pancreatitis through reducing neutrophil infiltration. *Microbiome*. (2021) 9:115. doi: 10.1186/s40168-021-01065-2

43. Yang Z, Su H, Lv Y, Tao H, Jiang Y, Ni Z, et al. Inulin intervention attenuates hepatic steatosis in rats via modulating gut microbiota and maintaining intestinal barrier function. *Food Res Int*. (2023) 163:112309. doi: 10.1016/j.foodres.2022.112309

44. Yang J, Li Y, Wen Z, Liu W, Meng L, Huang H. Oscillospira - a candidate for the next-generation probiotics. *Gut Microbes*. (2021) 13:1987783. doi: 10.1080/19490976.2021.1987783

45. Chen YJ, Wu H, Wu SD, Lu N, Wang YT, Liu HN, et al. Parasutterella, in association with irritable bowel syndrome and intestinal chronic inflammation. *J Gastroenterol Hepatol*. (2018) 33:1844–52. doi: 10.1111/jgh.14281

46. Taylor JA, Sichel SR, Salama NR. Bent bacteria: A comparison of cell shape mechanisms in proteobacteria. *Annu Rev Microbiol*. (2019) 73:457–80. doi: 10.1146/annurev-micro-020518-115919

47. Becattini S, Taur Y, Pamer EG. Antibiotic-induced changes in the intestinal microbiota and disease. *Trends Mol Med*. (2016) 22:458–78. doi: 10.1016/j.molmed.2016.04.003

48. Ianiro G, Tilg H, Gasbarrini A. Antibiotics as deep modulators of gut microbiota: between good and evil. *Gut*. (2016) 65:1906–15. doi: 10.1136/gutjnl-2016-312297

49. Korpela K, Salonen A, Vepsäläinen O, Suomalainen M, Kolmeder C, Varjosalo M, et al. Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. *Microbiome*. (2018) 6:182. doi: 10.1186/s40168-018-0567-4

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Distinct lower respiratory tract microbiota profiles linked to airway mucus hypersecretion in children with *Mycoplasma pneumoniae* pneumonia

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Background: Airway mucus hypersecretion (AMH) can occur in children with acute respiratory diseases, but its underlying mechanisms and relationship with the lower respiratory tract microbiota (LRTM) are not yet fully understood. This study investigates the characteristics of LRTM in children with *Mycoplasma pneumoniae* pneumonia (MPP) and its impact on AMH.

Methods: We collected bronchoalveolar lavage fluid and related clinical indicators from 202 children with MPP. 16S rRNA gene amplicon sequencing was used for detection and identification. Microbial diversity and characteristic genera were compared, and their abundance was analyzed for correlations with clinical factors.

Results: As the disease course (days from onset to bronchoscopy, grouped into T1, T2, T3) extended, α-diversity of the LRTM gradually increased, particularly in the T3 hypersecretion group. Moreover, significant differences were observed in the incidence of AMH, co-infection rates, peripheral white blood cell (WBC) count, and C-reactive protein levels. In AMH, *Mycoplasmoides* and *Veillonella* abundance and peripheral neutrophils were risk factors for increased secretions. In addition, in the T3 co-infection group, *Streptococcus* and *Prevotella* increased, replacing *Stenotrophomonas* as the dominant genus, possibly due to β-lactam antibiotic use. *Prevotella* abundance was strongly correlated with WBC.

Conclusion: The composition and structure of LRTM in children with MPP played a crucial role in AMH and disease progression.

KEYWORDS

***Mycoplasma pneumoniae* pneumonia, lower respiratory tract microbiota, airway mucus hypersecretion, co-infection, children**

1 Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is a common pathogen responsible for community-acquired pneumonia in children (Wang et al., 2022). In recent years, there was an increase in the incidence of *Mycoplasma pneumoniae* pneumonia (MPP), with more than 50% of children admitted to respiratory wards having been diagnosed with MPP (Yan et al., 2024). The known pathogenic mechanisms of *M. pneumoniae* infection include adhesion, oxidative stress, community-acquired respiratory distress syndrome (CARDS) toxin, and immune-mediated inflammatory damage, all of which have the potential to lead to airway mucus hypersecretion (AMH) (Yao and Han, 2021). Excessive airway mucus secretion not only produces highly viscous and elastic mucus, which is difficult to remove, leading to mucus retention, even mucus plug formation. This subsequently exacerbates infections, causes airway obstruction, and airflow limitation, ultimately resulting in progressive decline in lung function (Bao and Zhang, 2018).

In children with MPP, AMH may be closely associated with the imbalance of lower respiratory tract microbiota (LRTM). Studies showed that bronchoscopy was one of the methods for evaluating airway mucus secretion, and the bronchoscopy mucus score system (BS score) for children quantified the amount and distribution of airway mucus (Chang et al., 2006; Xia and Luo, 2022). Moreover, studies on pediatric non-cystic fibrosis bronchiectasis demonstrated that the microbial growth rate in bronchoalveolar lavage fluid (BALF) increased with higher BS scores (Nursoy et al., 2021).

The LRTM not only plays a crucial role in maintaining respiratory homeostasis but may also influence the onset and progression of respiratory diseases through interactions with the host (Wang et al., 2023). Under certain conditions, bacteria that are normally symbiotic can become involved in pathogenic processes, triggering inflammatory responses (Kim et al., 2016). Conversely, pulmonary microbiota may suppress other pathogenic microorganisms (Wu and Segal, 2018) by competing for nutrients (Spiga et al., 2023) or producing metabolites (Hardy and Merrell, 2021). Changes in the structure and composition of these microbial communities may affect mucus secretion in the airways, thereby influencing the course and prognosis of MPP.

However, studies on the correlation between LRTM and AMH remain limited. Therefore, this study employed 16S rRNA amplicon sequencing to investigate the structure and compositional trends of LRTM under varying mucus secretion states in children with MPP. We analyzed the impact of characteristic bacterial genera on AMH to provide new insights and evidence for clinical interventions.

2 Materials and methods

2.1 Study population and sample collection

In this cross-sectional study, we included pediatric patients who had been hospitalized for MPP at the Foshan Maternal and Child Health Hospital between October 2023 and January 2024. Electronic bronchoscopy (EOB) was performed on these patients, and BALF was collected for subsequent 16S rRNA sequencing. Additionally, demographic characteristics, clinical features, laboratory results, and imaging results were documented. The following criteria were used to include participants in the study: (1) A diagnosis of pneumonia in accordance with the "Guidelines for Diagnosis and Treatment of *Mycoplasma pneumoniae* Pneumonia in Children (2023 Edition)"(National Health Commission of the People's Republic of China, 2023); (2) The age of the children was ≤13 years; (3) The children underwent EOB during their period of hospitalization. The following criteria were used to exclude participants from the study: (1) Children with chronic respiratory diseases, and those whose duration from disease onset to EOB exceeded one month; (2) Those with severe diseases such as heart, liver, kidney diseases, or tumors; (3) Those with congenital diseases such as congenital heart disease; (4) Those with epilepsy or other mental disorders; (5) Cases without BS scoring under bronchoscopy; (6) Cases with insufficient sample volume to extract respiratory microbiota DNA or failure to construct a sequencing library.

In accordance with the BS scoring system and as detailed in the studies (Chang et al., 2006; Cai et al., 2023) on airway mucus hypersecretion, BS scores of 1–3 entered into the non-hypersecretion group, and scores of 4–6 entered the hypersecretion group. The pre-EOB period refers to the time from illness onset to the day before EOB. The co-infection refers to the simultaneous infection of children with *M. pneumoniae* and other pathogens.

With the consent of the patient's guardian, one chest CT or X-ray was performed before admission or bronchoscopy. Additionally, a follow-up chest X-ray was conducted 3 to 5 days after bronchoscopy to assess the resolution of lung inflammation. Oxygen therapy was administered to children with pulse oximetry levels below 95%. In the cases we collected, almost all children received nebulized corticosteroid treatment, such as budesonide suspension, prior to bronchoscopy. Therefore, this study focused more on the duration of oral and intravenous corticosteroid use, such as methylprednisolone sodium succinate. According to the guidelines (National Health Commission of the People's Republic of China, 2023), for children with a confirmed diagnosis of mild MPP, we administered oral or intravenous azithromycin at a dose of 10 mg/kg/day for 3–5 days per course, followed by a second course after an interval of 3–4 days. For severe MPP, intravenous azithromycin was administered at 10 mg/kg/day for 7 consecutive days, along with routine administration of methylprednisolone at 2 mg/kg/day. For children with a drug reaction to azithromycin, erythromycin was used at a dose of 30–45 mg/kg/day for 10–14 days per course. The cases included patients recently referred from external hospitals after unsuccessful treatment. They met the inclusion and exclusion criteria and had detailed diagnostic and treatment records.

BALF should be collected from pediatric patients meeting the specified criteria, in quantities of 2–4 mL. The sample should then be placed in a sterile centrifuge tube and centrifuged at 15,600 × g at 4°C for 30 min. The supernatant should be discarded, and the pellet should be stored immediately at −80°C for future use in 16S rRNA gene amplicon sequencing.

2.2 DNA extraction and 16S rRNA gene sequencing

The total bacterial DNA in BALF was extracted using the cetyltrimethylammonium bromide (CTAB) method. The concentration and quality of DNA were evaluated through the use of 2% agarose gel electrophoresis. Following the completion of quality

checks on the DNA samples, amplification of the V4 hypervariable region of the bacterial 16S rRNA gene was conducted using universal primers: forward primer 515F and reverse primer 806R. Paired-end sequencing was conducted on the Illumina NovaSeq-PE250 platform. Raw sequencing data were processed with QIIME2 (version 2023.9.1). Demultiplexing and quality inspection of paired-end reads were performed using the "demux" plugin, while trimming of Illumina adapter sequence was performed using the "cutadapt trim-paired" plugin. Denoising of paired-end reads was performed using the "dada2 denoised-paired" plugin, which allowed to adjust the number of 5′- and 3′-end trimmed bases to achieve a read length of 250 bases, followed by filtration and deionization to remove primer sequences or low-quality sequences (minimum quality score of 30). This led to an approximately 70% good-merged reads output. Amplicon sequence variants (ASVs) were assembled using the "feature-table summarize" plugin, while the taxonomy annotation was classified by the pretrained naive Bayes classifier based on the Greengenes database (version 2022.10) with an identity cutoff of 99%.

2.3 Data processing and statistical analysis

Alpha diversity was estimated using the Shannon index; while beta diversity was calculated using weighted-UniFrac distances and sample clustering was visualized with Principal Component Analysis (PCA) plots. The online CMAP Microbial Analysis Platform[1] was utilized. The organization and statistical analysis of the data were conducted using Zstats 1.0[2] and R version 4.4.0.

Continuous variables were described using median and interquartile range (IQR), and comparisons between groups were made using Mann–Whitney *U* test or Kruskal–Wallis rank-sum tests, as appropriate. Categorical variables were presented as frequencies and percentages and analyzed using the chi-square test or Fisher's exact test, as appropriate. A multivariate binary logistic regression model was employed to examine the relationship between the predictor variables and the outcomes variables. All statistical tests were conducted with a two-sided hypothesis and a significance level of $p < 0.05$.

3 Results

3.1 Increased LRTM diversity with the extended pre-EOB periods in AMH

The study included 230 children with MPP following the exclusion of 28 cases due to the presence of other medical histories or interfering factors, the remaining 202 cases were included in the analysis. Amongst these cases, there were 90 males and 112 females, with 97 cases assigned to the hypersecretion group and 105 cases assigned to the non-hypersecretion group. The median of the pre-EOB period was 9 days, with a minimum of 4 days and a maximum of 24 days. Based on tertiles, patients were grouped as follows: T1 (≤7 days), T2 (8–10 days), and T3 (≥11 days).

16S rRNA amplicon sequencing was conducted on BALF samples, yielding a total of 23,678,356 high-quality tags. Following normalization to the lowest sequences count (42,017), 10,420,216 sequences (51.62%) and 4,394 ASVs were obtained. Subsequently, a total of 59 phyla and 1,264 genera of microbiota were identified. A comparison of microbial diversity between the hypersecretion and *non-hyper*secretion groups at different pre-EOB periods showed distinct changes (Figure 1). The alpha diversity of both groups exhibited a gradual increase with the pre-EOB period progression, with a notably greater increase observed in the hypersecretion group (Figure 1a). The Shannon index demonstrated a notable increase from 0.459 (0.131, 0.898) at the T1 period to 1.735 (1.076, 2.099) at the T3 period, representing a nearly fourfold expansion. By the T3 period, the alpha diversity in the hypersecretion group was significantly higher than that of the non-hypersecretion group (Figure 1b), with the latter having a Shannon index of only 1.022 (0.674, 2.030). However, in the non-hypersecretion group, there was an increase in alpha diversity from 0.549 (0.080, 0.986) at the T1 period to 0.936 (0.411, 1.527) at the T2 period. This remained relatively stable at the T3 period, with no significant increase observed (Figure 1b).

The results of the PCA analysis indicate significant differences in the microbial community structure across the three pre-EOB periods in both the hypersecretion group (Adonis $R^2 = 0.133$, $p = 0.001$) and the non-hypersecretion group (Adonis $R^2 = 0.066$, $p = 0.013$). At the T1 period, the microbial composition was relatively concentrated. As the disease progressed, inter-individual variation increased, with microbial structure distribution being most dispersed during the T3 period (Figures 1c,d). In the PCA analysis of the hypersecretion group, PC1 and PC2 explained 83.69% of the total variance, indicating that these components effectively represent the data's variation. At T1, microbial communities were mainly distributed in the positive direction of PC1, reflecting higher diversity and dominance of certain species. In the non-hypersecretion group, T1 communities were primarily in the negative direction of PC1, suggesting a simpler structure with more pathogens lacking competitive advantage. Adonis analysis in this group showed lower explanatory power for microbial differences, but significant changes were still observed.

A comparison of the top 20 genera by relative abundance revealed significantly higher levels of *Veillonella* (1.137 vs. 0.500, $p = 0.037$) and *Alloprevotella* (0.277 vs. 0.159, $p = 0.022$) in the hypersecretion group (Figure 1e). Comparison of the top 10 genera across the three periods showed significant abundance changes, with *Veillonella* notably different (Supplementary Figure S1).

3.2 Increased incidence of AMH with the extended pre-EOB periods

Analysis of clinical characteristics across the three pre-EOB groups (Table 1) revealed a notable increase in the incidence of AMH as the disease progresses ($p = 0.048$). This phenomenon reached its peak at the T3 period, where approximately 60% of children experience AMH (Figure 2a). Furthermore, the prevalence of the co-infection was highest during the T3 period ($p = 0.020$), reaching 30% (Figure 2b). This suggests that the probability of co-infection with other pathogens increases with disease progresses.

The three pre-EOB periods exhibited significant differences in peak fever ($p = 0.017$), duration of fever ($p < 0.001$), and days of macrolide antibiotics use ($p < 0.001$) (Table 1). It was noteworthy that demonstrate

1 http://www.cmap-zjyy.com:10548/app/cmap

2 www.zstats.net

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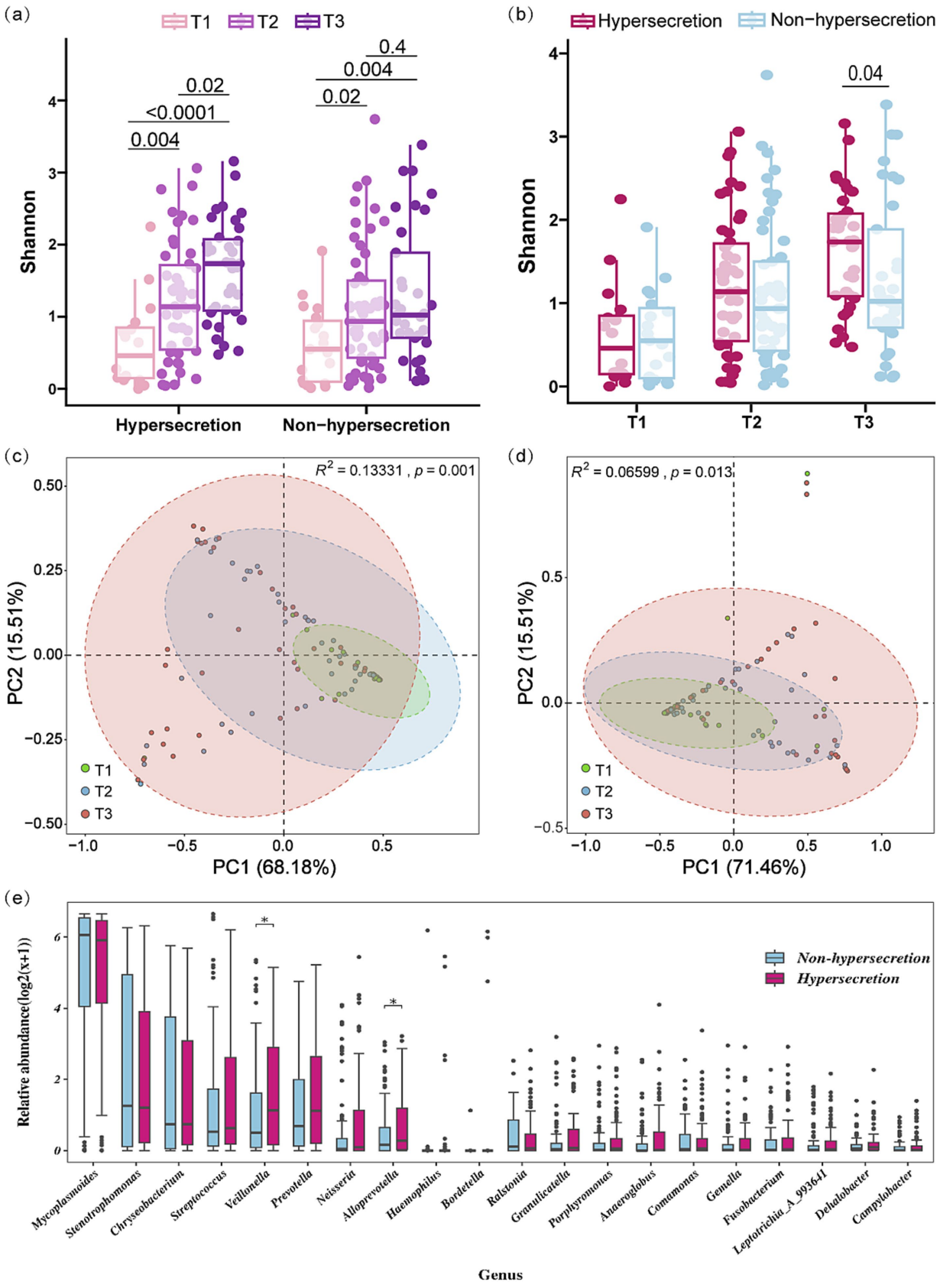


FIGURE 1
Microbial diversity characteristics across different pre-EOB periods in the hypersecretion and non-hypersecretion groups. (a,b) Multi-group comparison of Shannon index between the hypersecretion and non-hypersecretion groups at different pre-EOB periods. Differences were assessed by

(Continued)

FIGURE 1 (Continued)
Mann–Whitney *U* test with significant *p*-values shown. (c) PCA based on weighted-UniFrac distance among the three pre-EOB periods in the hypersecretion group (T1 = 12, T2 = 45, T3 = 40). Significant differences assessed by Adonis. (d) PCA based on weighted-UniFrac distance among the three pre-EOB periods in the non-hypersecretion group (T1 = 23, T2 = 54, T3 = 28). Differences assessed by Adonis. (e) Differences in the top 20 genera abundance between the hypersecretion (*n* = 97) and non-hypersecretion groups (*n* = 105) were assessed by Mann–Whitney *U* test. *$p < 0.05$.

TABLE 1 Clinical characteristics and differences of MPP across the three pre-EOB periods.

Variables	T1 (*n* = 35)	T2 (*n* = 99)	T3 (*n* = 68)	Statistic	*p*
Age (years), *n* (%)				$\chi^2 = 2.07$	0.355
<7	13 (37.14)	49 (49.49)	35 (51.47)		
≥7	22 (62.86)	50 (50.51)	33 (48.53)		
Peak body temperature (°C)	40.00 (39.40, 40.20)	39.70 (39.30, 40.00)	39.50 (39.00, 40.00)	$\chi^2 = 8.19^{\#}$	**0.017**
Duration of fever (days)	6.00 (5.00, 7.00)	7.00 (6.00, 7.50)	8.00 (5.00, 10.00)	$\chi^2 = 15.77^{\#}$	**<0.001**
Macrolides (days)	3.00 (2.00, 4.50)	4.00 (3.00, 6.00)	6.00 (4.75, 7.00)	$\chi^2 = 32.51^{\#}$	**<0.001**
β-lactams (days)	1.00 (0.00, 3.00)	2.00 (0.00, 3.00)	2.00 (0.00, 5.00)	$\chi^2 = 2.36^{\#}$	0.308
GCs (days)	0.00 (0.00, 1.00)	0.00 (0.00, 1.00)	0.00 (0.00, 1.00)	$\chi^2 = 2.88^{\#}$	0.237
NE%	58.4 (49.10, 66.90)	62.40 (56.40, 69.15)	60.75 (51.63, 72.00)	$\chi^2 = 1.08^{\#}$	0.584
LDH (U/L)	290.00 (250.50, 356.50)	298.00 (260.00, 344.50)	319.50 (290.25, 371.50)	$\chi^2 = 5.06^{\#}$	0.080
Mucus plug, *n* (%)				$\chi^2 = 0.71$	0.701
No	31 (88.57)	87 (87.88)	57 (83.82)		
Yes	4 (11.43)	12 (12.12)	11 (16.18)		
Tree-in-bud sign, *n* (%)				$\chi^2 = 1.02$	0.600
No	15 (66.67)	41 (70.21)	21 (78.95)		
Yes	15 (33.33)	44 (29.79)	31 (21.05)		
Pleural effusion, *n* (%)				$\chi^2 = 0.54$	0.765
No	29 (87.88)	79 (83.16)	51 (82.26)		
Yes	4 (12.12)	16 (16.84)	11 (17.74)		

Quantitative skewness data are presented as the median (percentile: P25, P75), and categorical data are expressed as the frequency. Macrolides, preoperative macrolide antibiotic days; β-lactams, preoperative β-lactams antibiotic days; GCs, preoperative glucocorticoid days; NE%, percentage of peripheral neutrophils; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; Co-MP, cooperative infection of *M. pneumoniae* with other pathogens.
[#]Kruskal-Wailis test.
The bold values highlight $P < 0.05$, indicating statistically significant differences.

a gradual increase in WBC with disease progresses (Figure 2c), reaching a peak at the T3 period. The WBC was significantly higher in children with co-infection than in those with single *M. pneumoniae* infection ($p = 0.02$), particularly in the hypersecretion group ($p = 0.01$) (Figure 2d). A significant decrease in CRP levels was observed between the T2 and T3 periods ($p = 0.030$) (Figure 2e).

3.3 Risk factors for increased mucus secretion: *Mycoplasmoides, Veillonella,* and neutrophil percentage

From the previous analysis, it was evident that microbial diversity is relatively higher in the hypersecretion group. We further analyzed the differences in genera and clinical indicators across different BS scores within the hypersecretion group to illustrate the impact of microbiota on mucus production. Due to having only one case with BS 6, it was excluded for better comparison. We performed a multivariate logistic regression analysis for BS scores 4 and 5, focusing on lung characteristics, inflammation indicators, and differential genera, with *p*-values adjusted for age, gender, and days of macrolide, β-lactam, and glucocorticoid use. As shown in Table 2, NE% (OR 1.07, 95% CI 1.01–1.13, $p = 0.032$), *Mycoplasmoides* (OR 1.03, 95% CI 1.01–1.06, $p = 0.042$), and *Veillonella* abundance (OR 1.11, 95% CI 1.01–1.23, $p = 0.037$) were identified as risk factors for increased mucus secretion. Interestingly, pleural effusion was negatively correlated with BS score 5 (OR 0.09, 95% CI 0.01–0.82, $p = 0.033$).

3.4 Shift of dominant LRT genera to *Stenotrophomonas* and *Chryseobacterium* in mucus hypersecretion with the extended pre-EOB periods

In the hypersecretion group, the five most prevalent microbial genera was *Mycoplasmoides* (53.3%), *Stenotrophomonas* (12.0%), *Chryseobacterium* (6.5%), *Streptococcus* (5.1%), and *Veillonella* (4.6%), as illustrated in Figure 3a. As the pre-EOB period progresses, there was a notable decline in the relative abundance of *Mycoplasmoides*, from 92.0% in T1 period to 35.3% in T3 period ($p < 0.001$) (Figure 3b).

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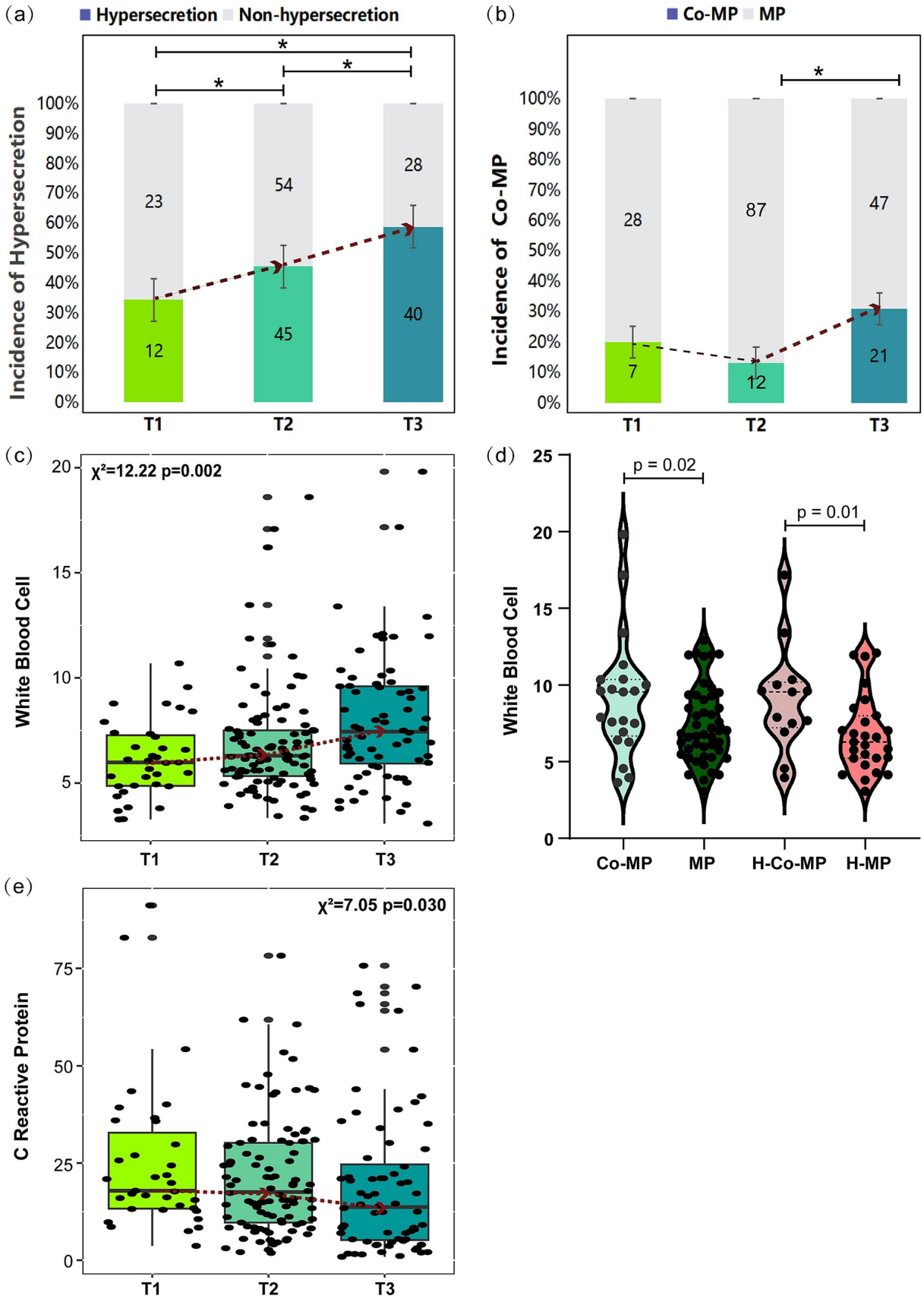


FIGURE 2
Comparison of selected clinical indicators across pre-EOB periods. (a,b) Incidence of mucus hypersecretion and co-infection across pre-EOB periods. Differences were assessed by chi-square test (χ^2). The dashed line represents the median trend and *$p < 0.05$ shown T1 ($n = 35$), T2 ($n = 99$), T3 ($n = 68$).

(Continued)

FIGURE 2 (Continued)
(c,e) Changes in WBC and CRP across different pre-EOB periods were assessed by Kruskal–Wallis test (d) Changes in WBC between children with co-infection and only *M. pneumoniae* infection were assessed by Mann–Whitney *U* test. Co-MP ($n = 40$), cooperative infection of *M. pneumoniae* with other pathogens; MP ($n = 162$), *M. pneumoniae* only; H-Co-MP ($n = 22$), cooperative infection of *M. pneumoniae* with other pathogens in hypersecretion group; H-MP ($n = 75$), *M. pneumoniae* in hypersecretion group. WBC, peripheral white blood cells count; CRP, C reactive protein.

TABLE 2 Multivariate logistic regression analysis of BS score.

Variables	*β*	*p*	OR (95% CI)
Pre EOB period			
T1 ($n = 12$)			1.00 (Reference)
T2 ($n = 44$)	1.36	0.151	3.91 (0.61–25.15)
T3 ($n = 40$)	2.10	0.069	8.20 (0.85–78.99)
Mucus plug			
No			1.00 (Reference)
Yes	0.54	0.581	1.72 (0.25–11.74)
Pleural effusion			
No			1.00 (Reference)
Yes	−2.46	**0.033**	0.09 (0.01–0.82)
Tree-in-bud sign			
No			1.00 (Reference)
Yes	0.25	0.700	1.29 (0.36–4.62)
NE%	0.06	**0.032**	1.07 (1.01–1.13)
Mycoplasmoides	0.03	**0.042**	1.03 (1.01–1.06)
Veillonella	0.11	**0.037**	1.11 (1.01–1.23)
Shannon index	−0.86	0.192	0.42 (0.12–1.54)

OR, odds ratio; CI, confidence interval; Adjust: age (month), gender, macrolides, β-lactams, glucocorticoid.
The bold values highlight $P < 0.05$, indicating statistically significant differences.

Conversely, the relative abundances of *Stenotrophomonas* (0.2% vs. 3.4%, $p = 0.009$) (Figure 3c) and *Chryseobacterium* (0.1% vs. 2.7%, $p = 0.007$) (Figure 3d) increased. The correlation analysis of the top 10 genera (Figure 3e) indicated a negative correlation between *Mycoplasmoides* and both *Stenotrophomonas* ($r = -0.56$, $p < 0.05$) and *Chryseobacterium* ($r = -0.57$, $p < 0.05$) (Figures 3f,g). In contrast, *Stenotrophomonas* and *Chryseobacterium* was found to be highly positively correlated ($r = 0.95$, $p < 0.05$) (Figure 3h). These findings indicate that as disease course progresses or preoperative antibiotic were administered, the abundance of *Mycoplasmoides* decreased, was replaced by *Stenotrophomonas* and *Chryseobacterium*. These two genera increased synergistically and gradually become the dominant genera in children with MPP. Furthermore, a highly positive correlation was observed between *Prevotella* and other genera, including *Streptococcus* ($r = 0.77$, $p < 0.05$), *Veillonella* ($r = 0.89$, $p < 0.05$), and *Alloprevotella* ($r = 0.86$, $p < 0.05$).

3.5 *Prevotella* enrichment and increased WBC in T3 co-infection group with β-lactam use

During the T3 period, children with co-infections had significantly higher abundance of *Streptococcus* (11.75% vs. 0.82%, $p < 0.001$) and *Prevotella* (4.37% vs. 0.88%, $p < 0.001$) compared to those with single *M. pneumoniae* infection, especially in the hypersecretion group (Figures 4a,b). At this point, *Mycoplasmoides* abundance was significantly lower in the co-MP group ($n = 21$), than in the MP group ($n = 47$) (4.18% vs. 49.33%, $p < 0.001$).

Spearman correlation analysis examined the relationship between microbial abundance and clinical indicators in the T3-co-MP group. The analysis revealed a significant positive correlation between *Prevotella* abundance and WBC ($r = 0.50$, $p = 0.022$) (Figures 4c,e), as well as between *Alloprevotella* abundance and LDH ($r = 0.46$, $p = 0.035$) (Figures 4c,f). Additionally, *Prevotella* ($r = -0.58$, $p = 0.006$) and *Veillonella* ($r = -0.55$, $p = 0.010$) were found to be negatively correlated with the duration of fever, while *Stenotrophomonas* ($r = 0.51$, $p = 0.035$) was positively correlated with the duration of fever.

The correlation analysis indicated a significant link between microbial composition and the duration of β-lactam antibiotic use in the T3-co-infection group (Figure 4c). As longer β-lactam antibiotic use, *Stenotrophomonas* ($r = -0.449$, $p = 0.041$) gradually declined (Figures 4d,g) and was replaced by *Streptococcus* ($r = 0.513$, $p = 0.017$) and *Prevotella* ($r = 0.526$, $p = 0.014$) (Figures 4h,i).

4 Discussion

Airway mucus hypersecretion has received extensive attention in studies of chronic respiratory diseases such as asthma (Jaramillo et al., 2024), COPD (Tian and Wen, 2015), cystic fibrosis (Mall, 2016), and bronchiectasis (Chen and Zheng, 2021; Abrami et al., 2024). While AMH was also common in acute respiratory infections in children, particularly with infections like *M. pneumoniae*, *Adenovirus* (Cai et al., 2023), *Influenza virus*, respiratory *Syncytial virus* (Yan and Deng, 2024), and *Bordetella pertussis* (Yao and Han, 2021), research on LRTM in children with MPP and mucus hypersecretion is very limited. AMH refered to the pathophysiological process in which various pathogenic factors cause hyperplasia and hypertrophy of airway mucosal goblet cells and submucosal glands, leading to excessive mucus production (Chen and Zheng, 2021). Its severity was related to the quantity and viscosity of airway secretions and the presence of small airway mucus plugs (Chen and Zheng, 2021). This study examined the composition and structural characteristics of the LRTM from two perspectives: disease progression and mucus hypersecretion. We observed that as the pre-EOB period extended, α-diversity gradually increased, particularly in the hypersecretion group. β-diversity indicated significant spatial distribution changes in the microbial community. Importantly, the incidence of AMH increased with disease progression, suggesting a potential relationship between LRTM and AMH, beyond just *M. pneumoniae* infection. Interestingly, the T1 period in the hypersecretion group had the lowest Shannon index, which seems to present a potential contradiction with the sample distribution in the PCA plot at T1. The Shannon index measures species richness and

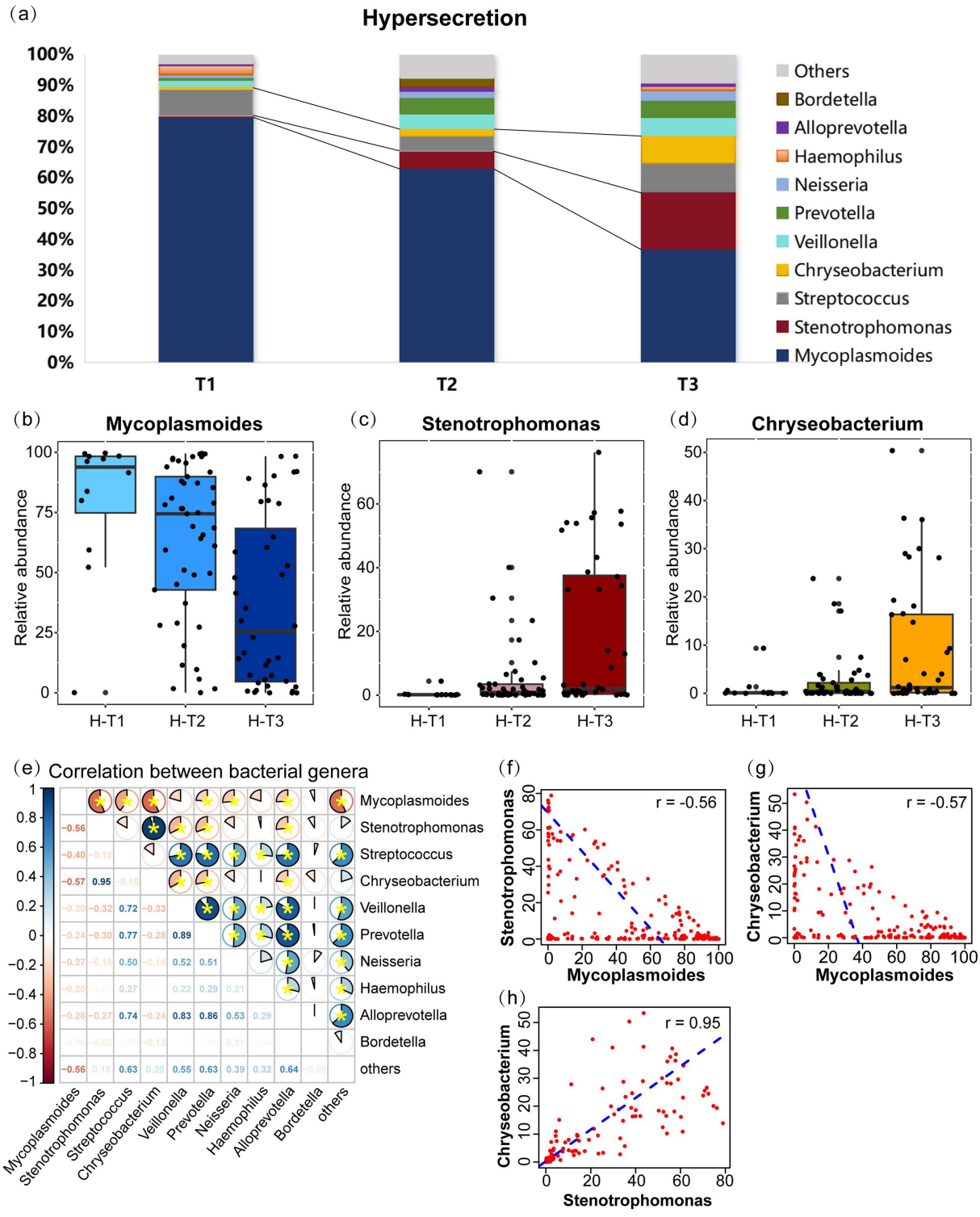


FIGURE 3
Microbial communities changes and inter-genus correlations in the hypersecretion group as the pre-EOB period progresses. (a) The relative abundances of the top 10 bacterial genera are presented in a stacked bar chart across the three pre-EOB periods T1 ($n = 12$), T2 ($n = 45$), T3 ($n = 40$). (b–d) Changes in the relative abundance of *Mycoplasmoides*, *Stenotrophomonas*, *Chryseobacterium* with the pre-EOB periods. (e) Inter-genus correlations among the top 10 genera by abundance. The pie chart slices represent the correlation and yellow * indicates $p < 0.05$. (f–h) The linear fit graph showing the Spearman correlations between *Mycoplasmoides*, *Stenotrophomonas*, *Chryseobacterium*. H-T1/T2/T3, the three pre-EOB periods in the hypersecretion group.

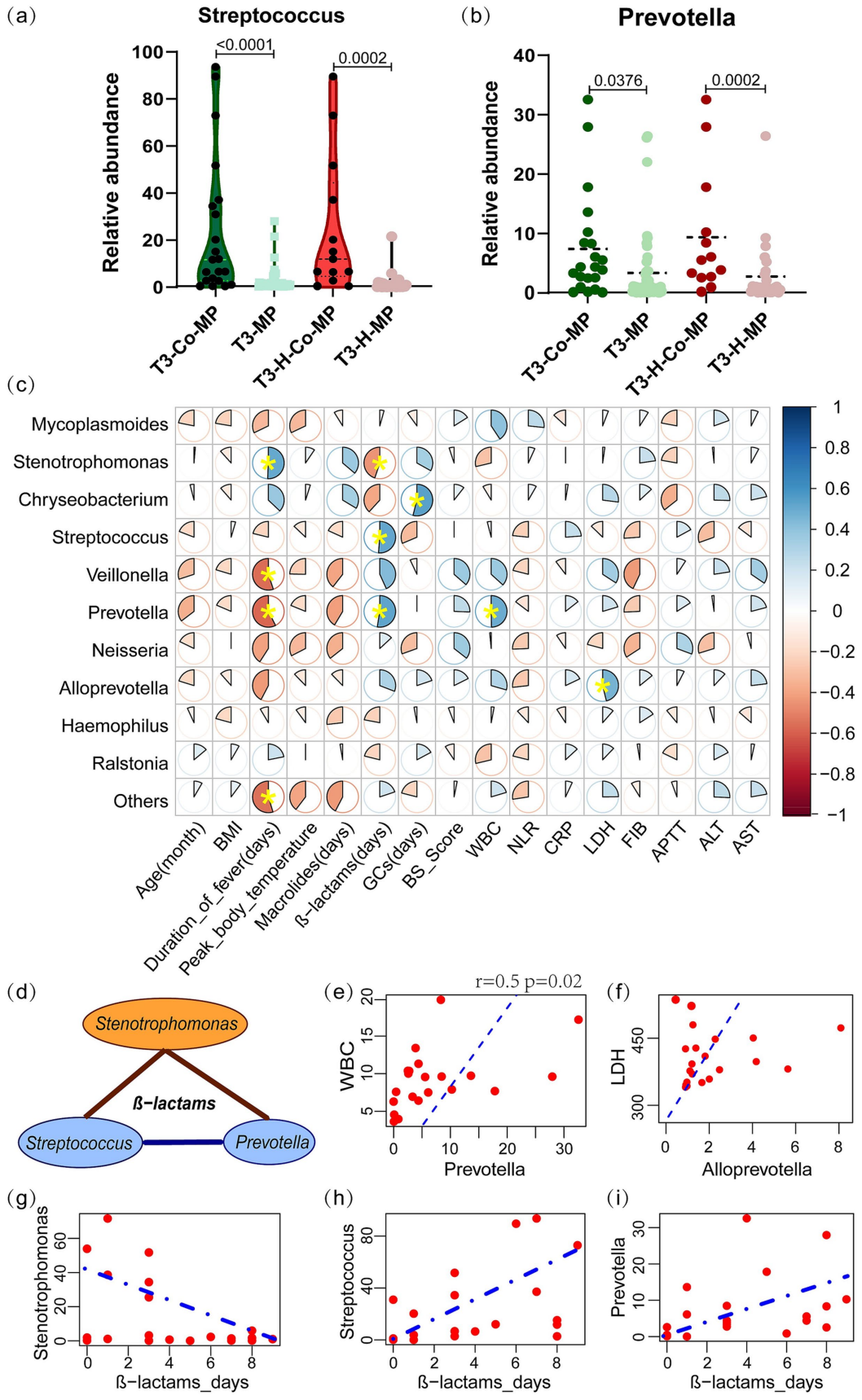


FIGURE 4
The characteristic microbial communities of co-infection group in the T3 period and their correlation with clinical indicators. (a,b) The *Streptococcus* and *Prevotella* abundances between the co-MP and MP-only groups, as well as within the hypersecretion state, were compared using the Mann–

(Continued)

FIGURE 4 (Continued)
Whitney *U* test. (c) Spearman correlation analysis between the microbial abundance and clinical indicators in T3-co-MP group. The pie chart slices represent the correlation and yellow * indicates $p < 0.05$. (d) Spearman correlation analysis of *Stenotrophomonas* with *Streptococcus* and *Prevotella* under β-lactam antibiotics. The brown line represents a negative correlation, while the blue line represents a positive correlation. (e,f) Linear correlation plots of WBC with *Prevotella*, and LDH with *Alloprevotella*; the dashed line represents the linear fit for the Spearman correlation. (g–i) Linear regression plots showing the correlation between β-lactam antibiotic days and the abundance of *Chryseobacterium*, *Streptococcus*, and *Prevotella*. T3-Co-MP ($n = 21$), co-infection in T3 period; T3-MP ($n = 47$), single *M. pneumoniae* infection in T3 period; T3-H-Co-MP ($n = 13$), co-infection in T3 hypersecretion group; T3-H-MP ($n = 8$), single *M. pneumoniae* infection in T3 hypersecretion group. GCs, preoperative glucocorticoid days; WBC, white blood cells count; NLR, the ratio of neutrophils to lymphocytes; CRP, C reactive protein; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; FIB, fibrinogen; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase.

evenness within a sample, reflecting the species diversity of a particular community. PCA analysis measures differences between samples, with a focus on community structure and heterogeneity between samples. The two indexes are complementary to each other.

We focused on analyzing the characteristic genera in AMH. The LRTM of children in T1 hypersecretion group, dominated by *Mycoplasmoides*. *M. pneumoniae* inhibits commensal bacteria by competing for nutrients (Chen et al., 2021) and inducing production of host cytokine, nitric oxide, antimicrobial peptides (Yang et al., 2004). It also invades host cells, depletes nutrients, and releases CARDS toxin, superoxide radicals, and hydrogen peroxide, causing direct epithelial cell damage (Hu et al., 2023). The exotoxin CARDS TX released by *M. pneumoniae* induces "asthma-like" histopathological changes similar to allergic diseases, characterized by elevated eosinophil levels, enhanced Th2 cell responses, increased chemokines expression, increased mucus production, and increased airway resistance (Xu et al., 2024) *M. pneumoniae* can promoting mucin upregulation via STAT6-STAT3 and epidermal growth factor receptor (EGFR) signaling pathways (Hao et al., 2014), and reduces ciliary clearance rate by directly damaging airway epithelial cells (Lu and Zhou, 2018), leading to mucus blockage. *M. pneumoniae* infection increases neutrophil counts, and the neutrophil elastase (NE) released activates the MAPK/ERK pathway and EGFR, stimulating airway epithelial cells to secrete more mucins, particularly MUC5AC and MUC5B (Chen and Tong, 2016). In T1 period, *M. pneumoniae* breaks down glucose through the glycolysis pathway to produce lactate. In T3 period, increased abundances of *Streptococcus* and *Prevotella* provide lactate as the preferred carbon source for the growth of *Veillonella*. *Veillonella* metabolizes lactate into short-chain fatty acids (SCFAs), such as acetate and propionate. It is well known that SCFAs have anti-inflammatory effects. However, studies have also shown that, *in vitro*, propionate in combination with TNF-α can synergistically increase the production of IL-6 and CXCL8 by primary human lung fibroblasts and airway smooth muscle cells (Whiteside et al., 2021). The enrichment of *Streptococcus* and *Veillonella* in airway washings from cancer patients is associated with increased inflammatory cell infiltration and upregulation of the PI3K/ERK signaling pathway in bronchial epithelium (Tsay et al., 2018). *Veillonella* in the oral cavity and respiratory tract forms a multispecies biofilm through symbiosis with late-colonizing pathogens, such as *Streptococcus* (Zhou et al., 2021). *Veillonella* promotes the increase of inflammatory factors through its metabolic products and pro-inflammatory signaling pathways, leading to excessive mucus secretion. The abundance of *Mycoplasmoides* decreased in the T3 period, while *Stenotrophomonas* and *Chryseobacterium* significantly increased. This outcome may result from a combination of microbial interactions, antibiotic use, immune responses, and prolonged disease duration. After all, increased mucin secretion directly suppress pathogen virulence, aiding in microbial coexistence (Wang B. X. et al., 2021). However, the causal relationships require further investigation.

Respiratory microbiota are influenced by migration, elimination, and microbial proliferation rates (Pérez-Cobas et al., 2023). Using a stepwise forward method, we selected clinical indicators and characteristic genera to perform a multivariate logistic regression analysis on BS grades 4 and 5 in the hypersecretion group. The results identified the peripheral neutrophil percentage, *Mycoplasmoides*, and *Veillonella* as risk factors for increased mucus secretion. Studies have shown increased neutrophil infiltration in both peripheral blood and BALF in children with MPP or refractory MPP (Wang B. X. et al., 2021; Cai et al., 2023; Abrami et al., 2024), and that MPP severity is associated with a neutrophil-mediated immune response. In MPP children with mucus plugs, higher serum IFN-γ levels were found in those with pleural effusion, which correlated positively with *Veillonella* abundance (Xu et al., 2020). Overabundance of *Veillonella parvula* induce macrophage-associated inflammation through the LPS-TLR4 pathway, impairing gut motility and further exacerbating gut dysbiosis, which promotes the development of Hirschsprung's disease-associated enterocolitis (Zhan et al., 2022). These suggest that *Veillonella* may also play a role in disease progression. On the other hand, certain *Prevotella* species, such as *Prevotella timonensis*, have a multitude of predicted α/β-galactosidases, α/β-N-acetylgalactosaminidases, and α/β-N-acetylglucosaminidases, which hydrolyze glycosidic bonds in sialic acid and fucoses. Sialidases and fucosidases are key enzymes that initiate the degradation of O-glycan structures in secreted and epithelial-bound mucins, enabling further mucin degradation after the removal of these outer monosaccharides (Segui-Perez et al., 2024). This plays a regulatory role in mucus production.

Co-infection of *M. pneumoniae* with other pathogens is common in children with respiratory syndromes (Chiu et al., 2015). In this study, 40 co-infection cases were identified through BALF bacterial culture and pathogen DNA testing during the T3 period, with 61.9% of cases involving *Streptococcus pneumoniae*, 19% *Haemophilus influenzae*, 38.1% virus. Co-infection rates were highest in the T3 period, and elevated WBC levels compared to case with only *M. pneumoniae*. Additionally, the abundance of *Streptococcus* and *Prevotella* increased significantly with β-lactam antibiotics use, and *Prevotella* was linearly correlated with WBC. The rise of pathogenic *Streptococcus* and *Prevotella* replaced the previously dominant *Stenotrophomonas*. In certain environments, such as CF lung microbial communities, *Prevotella* has been reported to produce β-lactamase, protecting *P. aeruginosa* from ceftazidime. This leads to antibiotic-resistant strains dominating the microbial community (Vandeplassche et al., 2019). As the disease progresses, the likelihood of co-infection increased. This may be due to airway epithelial damage from *M. pneumoniae* (Zhou et al., 2022; Hu et al., 2023) and the rise of opportunistic pathogens

caused by prolonged antibiotic use, leading to microbiota imbalance and increased infection risk (Grice and Segre, 2012; Wang et al., 2024). A study of bronchial mucus plugs revealed that the most prevalent genera in BALF of children with MPP were *Mycoplasmoides*, *Prevotella*, and *Streptococcus* (Xu et al., 2020). Several species of *Prevotella* are known to cause opportunistic infections (Larsen, 2017). Micro-aspiration of *Prevotella* during subclinical airway inflammation may occur (Huffnagle et al., 2017), though the specific mechanism linking *Prevotella* and inflammatory cells remains unclear and requires further study. The dominant genera that increased during the T3 period, *Stenotrophomonas* and *Chryseobacterium*, were also considered common opportunistic pathogens resistant to most β-lactam and macrolide antibiotics (Klimkaitė et al., 2023), showed a strong correlation, suggesting potential synergism. *Stenotrophomonas* was found to utilize various carbon sources, inhibit other microorganisms, and stimulate T lymphocytes to secrete IL-2, IFN-γ, and TNF-α, promoting lymphocyte apoptosis and immune suppression (Wang M. et al., 2021). A 9-year retrospective study found that most *Chryseobacterium indologenes* infections were hospital-acquired. The bacterium was found to colonize and spread via contaminated, moist medical equipment, and its prevalence has increased with the use of broad-spectrum antibiotics and invasive procedures (Zhang et al., 2021). *Veillonella* depends on lactate-producing bacteria such as *Streptococcus* and *Prevotella* for survival, which represents a typical metabolic symbiotic relationship. *Streptococcus pneumoniae i*s one of the common co-infecting species in MPP patients, capable of exacerbating lung inflammation and increasing secretions and mucus production. *Veillonella* secretes catalase to break down the H_2O_2 produced by the metabolism of Streptococcus, providing a stable anaerobic environment that facilitates the growth of oxygen-sensitive genera (Zhou et al., 2017) such as *Prevotella* and *Alloprevotella*. This also explains the positive correlation between *Veillonella* and *Prevotella* and *Alloprevotella*. *Stenotrophomonas* and *Chryseobacterium* are opportunistic pathogens that are resistant to multiple broad-spectrum antibiotics. They coexist under antibiotic use, potentially forming synergistic resistance to antibiotics, which contributes to disease progression and creates competition with other anaerobic genera such as *Veillonella* and *Prevotella* The guidelines (National Health Commission of the People's Republic of China, 2023) for the diagnosis and treatment of MPP suggest that if macrolide treatment fails to resolve fever within 72 h and clinical signs and chest imaging show no improvement or worsening, it is diagnosed as macrolide unresponsive MPP (MUMPP). In such cases, timely replacement with drugs such as doxycycline is recommended. The aim is timely intervention to reduce the risk of severe disease and complications. However, diagnosing MUMPP is not easy. From our study, it can be observed that in children with MPP, as the disease progresses and antibiotics are used, including macrolides and empirical β-lactams, airway microbial diversity begins to increase after 7 days of infection. After 10 days of infection, under conditions of mucus hypersecretion, both the likelihood of infection with drug-resistant opportunistic pathogens and common pathogens begins to rise, affecting mucus production and disease progression.

In certain environments, such as cystic fibrosis lung microbial communities, *Prevotella* has been reported to produce β-lactamase, protecting *P. aeruginosa* from ceftazidime. This leads to antibiotic-resistant strains dominating the microbial community (Vandeplassche et al., 2019). Among children with T3-period infections ($n = 21$), 13 (13/21) received β-lactam antibiotics for more than 7 days before EOB, with seemingly limited efficacy against Streptococcus. Additionally, 5 (5/21) received glucocorticoid treatment before EOB, with 3 using glucocorticoids for ≤3 days, all of whom experienced recurrent fever. This may be linked to dosage and duration, but also suggests heightened inflammatory responses. *Stenotrophomonas maltophilia*, a pathogen causing hospital-acquired infections, produces cytokines like IL-2, IFN-γ, and TNF-α, triggering systemic inflammation (Wang M. et al., 2021). TNF-α, a key fever mediator, induces fever via the hypothalamic thermoregulatory center, explaining the positive correlation between *Stenotrophomonas* and febrile days. Increased *Stenotrophomonas* abundance in sputum has been linked to poor antibiotic outcomes in acute exacerbations of COPD (Liu, 2020). In our study, *Stenotrophomonas* showed a negative correlation with β-lactam antibiotics, suggesting sensitivity to these drugs. As *Stenotrophomonas* levels declined, *Prevotella* and *Veillonella* abundance increased, with *Veillonella*-produced SCFAs playing an anti-inflammatory role, resulting in fewer febrile days. In later disease stages, microbial diversity increased, complicating the relationship due to antibiotic use, warranting further data analysis.

The complex use of antibiotics before admission can affect the BALF microbiota structure, and there are currently no clinical indicators and methods to correct for these factors. In the future, conducting controlled longitudinal studies with adequate sample sizes and integrating multiomics approaches could provide robust data for comprehensive statistical analyses of antibiotics and the LRTM (Pérez-Cobas et al., 2023). Due to the limitations of 16S rRNA sequencing, higher-resolution metagenomic sequencing is planned for the next phase to explore the mechanisms by which bacterial species (Bars-Cortina et al., 2024) in the LRT influence mucus secretion and host immunity.

In conclusion, we analyzed the diversity of LRTM in children with MPP from two perspectives: disease progression and mucus hypersecretion. Longer disease courses showed richer diversity, especially in the AMH conditions. We identified *Mycoplasmoides*, and *Veillonella*, along with peripheral neutrophils, as risk factors for increased mucus production. In co-infected MPP, *Streptococcus* and *Prevotella* were dominant in the LRTM, particularly under AMH conditions, and were significantly positively correlated with β-lactam antibiotic use. *Prevotella* was also positively correlated with WBC during this period. These suggested that characteristic genera in the lower respiratory tract of MPP children played a crucial role in influencing AMH and disease progression.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: https://ngdc.cncb.ac.cn/gsa/, PRJCA029662.

Ethics statement

The studies involving humans were approved by Ethics Committee of Foshan Women and Children Health Hospital (No: FSFY-MEC-2024-091). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

XW: Project administration, Writing – original draft, Writing – review & editing. WW: Software, Visualization, Writing – original draft, Writing – review & editing. HC: Data curation, Writing – review & editing. YH: Investigation, Writing – review & editing. QZ: Methodology, Writing – review & editing. XY: Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1491506/full#supplementary-material

References

Abrami, M., Biasin, A., Tescione, F., Tierno, D., Dapas, B., Carbone, A., et al. (2024). Mucus structure, viscoelastic properties, and composition in chronic respiratory diseases. *Int. J. Mol. Sci.* 25:1933. doi: 10.3390/ijms25031933

Bao, Y., and Zhang, P. (2018). Recognition and reasonable therapy of airway mucus hypersecretion in children. *Chin. J. Pract. Pediatr.* 33, 171–174. doi: 10.19538/j.ek2018030602

Bars-Cortina, D., Ramon, E., Rius-Sansalvador, B., Guinó, E., Garcia-Serrano, A., Mach, N., et al. (2024). Comparison between 16S rRNA and shotgun sequencing in colorectal cancer, advanced colorectal lesions, and healthy human gut microbiota. *BMC Genomics* 25:730. doi: 10.1186/s12864-024-10621-7

Cai, J. Y., Yan, C. Y., Wang, X. Q., Luo, Z. X., Luo, J., Li, Q. B., et al. (2023). Clinical characteristics and risk factors for bronchoscopic airway mucus hypersecretion in childhood pneumonia infected by different pathogens. *Zhonghua Er Ke Za Zhi* 61, 719–725. doi: 10.3760/cma.j.cn112140-20230315-00184

Chang, A. B., Faoagali, J., Cox, N. C., Marchant, J. M., Dean, B., Petsky, H. L., et al. (2006). A bronchoscopic scoring system for airway secretions—airway cellularity and microbiological validation. *Pediatr. Pulmonol.* 41, 887–892. doi: 10.1002/ppul.20478

Chen, Y., and Tong, J. (2016). Research progress of mucus high-secretion mechanisms in airway. *Basic Clin. Med.* 36, 1573–1577. doi: 10.16352/j.issn.1001-6325.2016.11.020

Chen, J., Xi, Z., Shi, Y., Liu, L., Wang, L., Qian, L., et al. (2021). Highly homogeneous microbial communities dominated by *Mycoplasma pneumoniae* instead of increased resistance to macrolide antibiotics is the characteristic of lower respiratory tract microbiome of children with refractory *Mycoplasma pneumoniae* pneumonia. *Transl. Pediatr.* 10, 604–615. doi: 10.21037/tp-20-404

Chen, R. C., and Zheng, Z. G. (2021). Chinese experts consensus on pulmonary rehabilitation therapy for airway mucus hypersecretion. *Int. J. Respir.* 41, 1686–1696. doi: 10.3760/cma.j.cn131368-20210921-00705

Chiu, C.-Y., Chen, C.-J., Wong, K.-S., Tsai, M.-H., Chiu, C.-H., and Huang, Y.-C. (2015). Impact of bacterial and viral coinfection on mycoplasmal pneumonia in childhood community-acquired pneumonia. *J. Microbiol. Immunol. Infect.* 48, 51–56. doi: 10.1016/j.jmii.2013.06.006

Grice, E. A., and Segre, J. A. (2012). The human microbiome: our second genome. *Annu. Rev. Genomics Hum. Genet.* 13, 151–170. doi: 10.1146/annurev-genom-090711-163814

Hao, Y., Kuang, Z., Jing, J., Miao, J., Mei, L. Y., Lee, R. J., et al. (2014). *Mycoplasma pneumoniae* modulates STAT3-STAT6/EGFR-FOXA2 signaling to induce overexpression of airway mucins. *Infect. Immun.* 82, 5246–5255. doi: 10.1128/IAI.01989-14

Hardy, B. L., and Merrell, D. S. (2021). Friend or foe: interbacterial competition in the nasal cavity. *J. Bacteriol.* 203:e00480. doi: 10.1128/JB.00480-20

Hu, J., Ye, Y., Chen, X., Xiong, L., Xie, W., and Liu, P. (2023). Insight into the pathogenic mechanism of *Mycoplasma pneumoniae*. *Curr. Microbiol.* 80:14. doi: 10.1007/s00284-022-03103-0

Huffnagle, G., Dickson, R., and Lukacs, N. (2017). The respiratory tract microbiome and lung inflammation: a two-way street. *Mucosal Immunol.* 10, 299–306. doi: 10.1038/mi.2016.108

Jaramillo, A. M., Vladar, E. K., Holguin, F., Dickey, B. F., and Evans, C. M. (2024). Emerging cell and molecular targets for treating mucus hypersecretion in asthma. *Allergol. Int.* 73, 375–381. doi: 10.1016/j.alit.2024.04.002

Kim, Y. J., Jeon, H., Na, S. H., Kwon, H. I., Selasi, G. N., Nicholas, A., et al. (2016). *Stenotrophomonas maltophilia* outer membrane vesicles elicit a potent inflammatory response *in vitro* and *in vivo*. *Pathog. Dis.* 74:ftw104. doi: 10.1093/femspd/ftw104

Klimkaitė, L., Ragaišis, I., Krasauskas, R., Ružauskas, M., Sužiedėlienė, E., and Armalytė, J. (2023). Novel antibiotic resistance genes identified by functional gene library screening in *Stenotrophomonas maltophilia* and *Chryseobacterium* spp. Bacteria of soil origin. *Int. J. Mol. Sci.* 24:6037. doi: 10.3390/ijms24076037

Larsen, J. M. (2017). The immune response to *Prevotella* bacteria in chronic inflammatory disease. *Immunology* 151, 363–374. doi: 10.1111/imm.12760

Liu, H. (2020). Association of sputum microbiome with clinical outcome of initial antibiotic treatment in hospitalized patients with acute exacerbations of COPD. *Pharmacol. Res.* 160:105095. doi: 10.1016/j.phrs.2020.105095

Lu, Y. M., and Zhou, W. J. (2018). Mechanism of airway mucus hypersecretion and airway clearance in children. *Chin. J. Pract. Pediatr.* 33, 175–179. doi: 10.19538/j.ek2018030603

Mall, M. A. (2016). Unplugging mucus in cystic fibrosis and chronic obstructive pulmonary disease. *Ann. Am. Thorac. Soc.* 13, S177–S185. doi: 10.1513/AnnalsATS.201509-641KV

National Health Commission of the People's Republic of China (2023). Guidelines for the diagnosis and treatment of *Mycoplasma pneumoniae* pneumonia in children (2023 edition). *Intern. J. Epid. Infec. Dis.* 50, 79–85. doi: 10.3760/cma.j.cn331340-20230217-00023

Nursoy, M. A., Kilinc, A. A., Abdillahi, F. K., Ustabas Kahraman, F., Al Shadfan, L. M., Sumbul, B., et al. (2021). Relationships between bronchoscopy, microbiology, and radiology in noncystic fibrosis bronchiectasis. *Pediatr. Allergy Immunol. Pulmonol.* 34, 46–52. doi: 10.1089/ped.2020.1319

Pérez-Cobas, A. E., Rodríguez-Beltrán, J., Baquero, F., and Coque, T. M. (2023). Ecology of the respiratory tract microbiome. *Trends Microbiol.* 31, 972–984. doi: 10.1016/j.tim.2023.04.006

Segui-Perez, C., de Jongh, R., Jonkergouw, R. L. W., Pelayo, P., Balskus, E. P., Zomer, A., et al. (2024). *Prevotella timonensis* degrades the vaginal epithelial glycocalyx through high fucosidase and sialidase activities. *mBio* 15:e0069124. doi: 10.1128/mbio.00691-24

Spiga, L., Fansler, R. T., Perera, Y. R., Shealy, N. G., Munneke, M. J., David, H. E., et al. (2023). Iron acquisition by a commensal bacterium modifies host nutritional immunity during Salmonella infection. *Cell Host Microbe* 31, 1639–1654.e10. doi: 10.1016/j.chom.2023.08.018

Tian, P.-W., and Wen, F.-Q. (2015). Clinical significance of airway mucus hypersecretion in chronic obstructive pulmonary disease. *J. Transl. Intern. Med.* 3, 89–92. doi: 10.1515/jtim-2015-0013

Tsay, J.-C. J., Wu, B. G., Badri, M. H., Clemente, J. C., Shen, N., Meyn, P., et al. (2018). Airway microbiota is associated with upregulation of the PI3K pathway in lung cancer. *Am. J. Respir. Crit. Care Med.* 198, 1188–1198. doi: 10.1164/rccm.201710-2118OC

Vandeplassche, E., Tavernier, S., Coenye, T., and Crabbé, A. (2019). Influence of the lung microbiome on antibiotic susceptibility of cystic fibrosis pathogens. *Eur. Respir. Rev.* 28:190041. doi: 10.1183/16000617.0041-2019

Wang, X., Li, M., Luo, M., Luo, Q., Kang, L., Xie, H., et al. (2022). *Mycoplasma pneumoniae* triggers pneumonia epidemic in autumn and winter in Beijing: a multicentre, population-based epidemiological study between 2015 and 2020. *Emerg. Microbes Infect.* 11, 1508–1517. doi: 10.1080/22221751.2022.2078228

Wang, W., Qiu, Z., Li, H., Wu, X., Cui, Y., Xie, L., et al. (2024). Patient-derived pathogenic microbe deposition enhances exposure risk in pediatric clinics. *Sci. Total Environ.* 924:171703. doi: 10.1016/j.scitotenv.2024.171703

Wang, B. X., Wu, C. M., and Ribbeck, K. (2021). Home, sweet home: how mucus accommodates our microbiota. *FEBS J.* 288, 1789–1799. doi: 10.1111/febs.15504

Wang, M., Yin, S., Qin, Q., Peng, Y., Hu, Z., Zhu, X., et al. (2021). *Stenotrophomonas-maltophilia* inhibits host cellular immunity by activating PD-1/PD-L1 signaling pathway to induce T-cell exhaustion. *Mol. Immunol.* 130, 37–48. doi: 10.1016/j.molimm.2020.12.019

Wang, Y., Yu, X., Liu, F., Tian, X., Quan, S., Jiao, A., et al. (2023). Respiratory microbiota imbalance in children with *Mycoplasma pneumoniae* pneumonia. *Emerg. Microbes Infect.* 12:2202272. doi: 10.1080/22221751.2023.2202272

Whiteside, S. A., McGinniss, J. E., and Collman, R. G. (2021). The lung microbiome: progress and promise. *J. Clin. Invest.* 131:e150473. doi: 10.1172/JCI150473

Wu, B. G., and Segal, L. N. (2018). The lung microbiome and its role in pneumonia. *Clin. Chest Med.* 39, 677–689. doi: 10.1016/j.ccm.2018.07.003

Xia, Y., and Luo, J. (2022). Research progress of airway mucus hypersecretion in children. *J. Clin. Pediatr.* 40, 950–954. doi: 10.12372/jcp.2022.21e1630

Xu, N., Fan, L., Li, L., and Guo, Y. (2024). Exploring the pathogenicity of *Mycoplasma pneumoniae*: focus on community-acquired respiratory distress syndrome toxins. *Microb. Pathog.* 195:106865. doi: 10.1016/j.micpath.2024.106865

Xu, X., Wu, L., Sheng, Y., Liu, J., Xu, Z., Kong, W., et al. (2020). Airway microbiota in children with bronchial mucus plugs caused by *Mycoplasma pneumoniae* pneumonia. *Respir. Med.* 170:105902. doi: 10.1016/j.rmed.2020.105902

Yan, C., and Deng, Y. (2024). Progress on risk factors involved in airway mucus hypersecretion in children with community acquired pneumonia. *Int. J. Pediatr.* 51, 38–42. doi: 10.3760/cma.j.issn.1673-4408.2024.01.008

Yan, C., Xue, G.-H., Zhao, H.-Q., Feng, Y.-L., Cui, J.-H., and Yuan, J. (2024). Current status of *Mycoplasma pneumoniae* infection in China. *World J. Pediatr.* 20, 1–4. doi: 10.1007/s12519-023-00783-x

Yang, J., Hooper, W. C., Phillips, D. J., and Talkington, D. F. (2004). Cytokines in *Mycoplasma pneumoniae* infections. *Cytokine Growth Factor Rev.* 15, 157–168. doi: 10.1016/j.cytogfr.2004.01.001

Yao, H. S., and Han, X. H. (2021). Management of airway mucus hypersecretion in children. *Chin. J. Pract. Pediatr.* 36, 178–184. doi: 10.19538/j.ek2021030604

Zhan, Z., Liu, W., Pan, L., Bao, Y., Yan, Z., and Hong, L. (2022). Overabundance of *Veillonella parvula* promotes intestinal inflammation by activating macrophages via LPS-TLR4 pathway. *Cell Death Discov.* 8, 251–212. doi: 10.1038/s41420-022-01015-3

Zhang, Y., Li, D., Yang, Y., Su, J., Xu, X., Wang, M., et al. (2021). Clinical and molecular characteristics of *Chryseobacterium indologenes* isolates at a teaching hospital in Shanghai, China. *Ann. Transl. Med.* 9:668. doi: 10.21037/atm-21-933

Zhou, W., Chen, J., Xi, Z., Shi, Y., Wang, L., and Lu, A. (2022). Characteristics of lung microbiota in children's refractory *Mycoplasma pneumoniae* pneumonia coinfected with *Human Adenovirus* B. *Can. J. Infect. Dis. Med. Microbiol.* 2022, 1–8. doi: 10.1155/2022/7065890

Zhou, P., Li, X., Huang, I.-H., and Qi, F. (2017). *Veillonella* catalase protects the growth of *Fusobacterium nucleatum* in microaerophilic and *Streptococcus gordonii*-resident environments. *Appl. Environ. Microbiol.* 83, e01079–e01017. doi: 10.1128/AEM.01079-17

Zhou, P., Manoil, D., Belibasakis, G. N., and Kotsakis, G. A. (2021). Veillonellae: beyond bridging species in oral biofilm ecology. *Front. Oral Health* 2:774115. doi: 10.3389/froh.2021.774115

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Relationship between the risk of intestinal mucosal Epstein–Barr virus and/or cytomegalovirus infection and peripheral blood NK cells numbers in patients with ulcerative colitis: a cross-sectional study in Chinese population

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Objective: This study aimed to analyze the relationship between the risk of common opportunistic pathogens Epstein–Barr virus (EBV) and cytomegalovirus (CMV) infection in intestinal mucosal tissues of Ulcerative Colitis (UC) patients and the number of peripheral blood NK cells.

Methods: UC patients admitted to a third-grade class-A hospital from January 2018 to December 2023 were selected as research population. Clinical data of the patients were collected from the electronic medical record system. Additionally, samples of intestinal mucosal tissues were obtained for real-time fluorescence quantitative PCR to detect and analyze the viral load of CMV and EBV. Blood samples were collected for lymphocyte subsets analysis. Multivariable logistic regression models analyses was used to determine the odds ratio (OR) and 95% confidence interval (95% CI) for the independent association between NK cells and EBV/CMV infections in UC. We further applied the restricted cubic spline analysis and smooth curve fitting to examine the non-linear relationship between them.

Results: 378 UC patients were enrolled. Of these patients, there were 194 patients (51.32%) with EBV /CMV infection. In multivariable logistic regression analyses NK cells was independently associated with EBV and/or CMV infection after adjusted potential confounders (OR 8.24, 95% CI 3.75–18.13, $p < 0.001$). A nonlinear relationship was found between NK cells and EBV/CMV infections, which had a threshold around 10.169. The effect sizes and CIs below and above the threshold were 0.535 (0.413–0.692), $p < 0.001$ and 1.034 (0.904–1.183), $p > 0.05$, respectively.

Conclusion: There was a non-linear relationship between NK cells and EBV/CMV infections. The risk for EBV/CMV infections was not increased with increasing

NK cells in individuals with NK cells ≥ 10.169%, whereas the risk for EBV and/or CMV infection was increased with an decreasing NK cells in those with NK cells < 10.169%. The risk of EBV/CMV infections increases when NK cells were below a certain level.

KEYWORDS

ulcerative colitis, cytomegalovirus, Epstein–Barr virus, opportunistic infections, NK cells

1 Introduction

Since the end of the 20th century, the incidence and prevalence of inflammatory bowel disease (IBD) have increased in Asian countries, and China is one of the regions with the highest incidence of IBD in Asia (Ran et al., 2021). UC is a refractory, chronic, non-specific inflammatory response disease of the bowel, and is characterized by alternating exacerbations and remissions (Zhang et al., 2022). The main therapeutic drugs such as glucocorticoids, immunosuppressive agents, and biological agents have immunosuppressive effects (Ungaro et al., 2017). The immune function of most patients is suppressed for a long time due to the disease itself and the action of therapeutic drugs, resulting in an increased risk of opportunistic infections (Ljungman et al., 2002). The most common pathogens of opportunistic infections in UC populations include EBV and CMV (Groves et al., 2021; Kerr, 2019; Albatati et al., 2020). EBV, a member of the herpes virus family, has been reported to infect more than 90% of people in childhood (Zhang et al., 2022). Because they co-evolved with humans, both viruses have developed a unique ability to establish lifelong infections (McGeoch et al., 2000).

In fact, after initial infection, they lie dormant in specific cell populations, namely memory B cells of EBV (Chiu and Sugden, 2016) and monocytes/bone marrow/endothelial cells precursors of CMV (Jarvis and Nelson, 2002). That is, latent infection, it is defined as a state in which the viral genome is preserved, but the viral gene expression is highly restricted, and the viral replication process does not occur (Ciccocioppo et al., 2021). The reversibility of latent infection largely depends on t cell-mediated immune control and changes in the differentiation/ activation state of latent virus-infected cells (Murata and Tsurumi, 2014; Jarvis and Nelson, 2002). Reactivation of latent EBV/CMV infection in UC patients can complicate the condition and may manifest hormonal nonresponse or hormonal resistance. A large amount of evidence shows that viral reactivation is the main culprit leading to the development of the disease, which is easy to develop into acute and severe UC, and significantly increases the incidence of complications such as colectomy (Papadakis et al., 2001; Kishore et al., 2004; Domènech et al., 2008; Maher and Nassar, 2009; Núñez Ortiz et al., 2022) and mortality (Kishore et al., 2004; Gauss et al., 2015). Therefore, antiviral therapy is required after CMV and/or EBV reactivation in UC patients, and treatment can reduce hormone resistance and increase the efficacy of immunosuppressant use.

However, some studies (Huang et al., 2024; Pillet et al., 2016; Hsieh et al., 2024) still show that antiviral therapy is controversial in reducing the risk of serious complications such as colectomy, which may be related to the immune status of patients. A variety of immune factors in the body can reflect immune function to a certain extent. NK cells are activated after viral infection and play a role in the early stages before inducing the T cell response and in the latency period after the T cell response is established. Depletion of NK cells and T cells has been shown to lead to EBV/CMV reactivation, suggesting that these two lymphocyte subpopulations are critical for suppressing infection (Vieira Braga et al., 2015). Moreover, early studies have shown that natural killer cells play a major role in controlling CMV infection in the early and latent stages of infection (Kuijpers et al., 2008). At present, whether the number of NK cells in a certain immune state is an important factor affecting the increased risk of opportunistic infection in UC patients, and the quantification relationship remains to be verified. Therefore, early inference of the risk of viral reactivation through quantified NK cells analysis in UC patients, which is crucial to explore the pathogenesis of common opportunistic infections associated with UC and delay the development of adverse prognosis.

2 Materials and methods

2.1 Study population

In this study, a cross-section of UC patients admitted to a third-grade class-A hospital from January 2018 to December 2023 were selected as research population. The inclusion criteria were as following: All patients met the diagnostic criteria for UC in the Guidelines for the Diagnosis and Treatment of Adult Ulcerative Colitis issued by PANCCO in July 2022 (Juliao-Baños et al., 2022); age > 18 years old; be able to communicate well and be willing to accept follow up. Establish a follow-up file for each patient. Exclusion criteria: pregnant or lactating women; Unstable vital signs and inability to complete the interview; Patients with immunodeficiency disease, malignant tumor; Patients with acute and chronic infectious enteritis, ischemic colitis, toxic megacolon, intestinal perforation and other serious diseases; Patients with a history of total (secondary) intestinal resection; The patient's clinical data were incomplete; Patients with opportunistic infections other than CMV and EBV were excluded because there were fewer than 5 cases in the population. The inclusion and exclusion of patients in this study were shown in Figure 1. This study was approved by the Ethics Committee of the hospital (approval number: 2022SYKLSZD442), and all patients signed informed consent.

Abbreviations: UC, Ulcerative Colitis; OIs, Opportunistic Infections; NK cells, Natural Killer cells; CMV, Cytomegalovirus; EBV, Epstein-Bar virus; PANCCO, Pan American Crohn's and Colitis Organization; IQR, Interquartile; SD, Standard Deviation; OR, Odds Ratio; 95% CI, 95% Confidence Interval; ALB, Albumin; HGB, Hemoglobin; IECs, Intestinal Epithelial Cells.

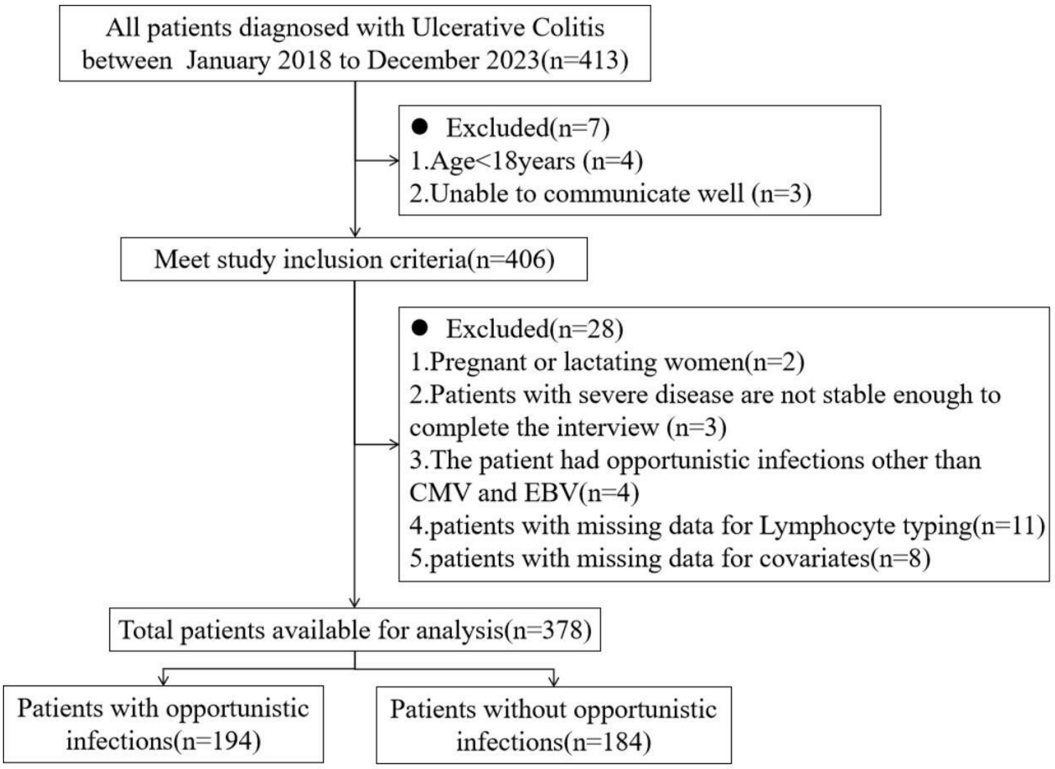


FIGURE 1
Flow diagram of inclusion and exclusion.

2.2 Methods

2.2.1 Clinical data collection and activity score

Follow-up files of patients were established, and clinical data of the patients were collected from the electronic medical record system, including sex, age, course of disease, disease activity, lesion scope, routine physical examination data, laboratory examination, imaging examination, endoscopy, histological and pathological examination results, drug treatment history, nutritional status, etc. The improved Mayo score system (Xing et al., 2021) was used to evaluate the UC activity of hospitalized patients, and UC patients were divided into remission stage (Mayo score ≤ 2 and no single score > 1) and active stage (Mayo score > 2) according to the comprehensive results of patients' symptoms and signs, relevant laboratory results, endoscopic findings and overall physician evaluation. The Mayo scores of 3 to 5 were classified as mildly active, 6 to 10 as moderately active, and 11 to 12 as severely active. According to the results of UC activity assessment, UC patients in remission were excluded from the study. Therefore, the number of such patients was very small, and the analysis results were not enough to represent the situation of this group. Montreal classification was used to evaluate the extent of the lesions, with E1 being rectal type, E2 left half colon type, and E3 extensive colon type (Satsangi et al., 2006).

2.2.2 The patients' intestinal mucosal tissue samples were taken for quantitative real-time PCR detection of CMV and EBV viral load analysis

Quantitative Real-time PCR detection of colon mucosal tissue is the gold standard in the diagnosis of UC combined with CMV/EBV infection, which is helpful for correct diagnosis and treatment (Ciccocioppo et al., 2015; Jena et al., 2022). It has been reported that the sensitivity and specificity of CMV DNA detected by qPCR in colon mucosal tissue are 92.0–96.7% and 93.0–98.7% (Groves et al., 2021). During the endoscopic examination, the patient was under anesthesia, and the doctor inserted an endoscope through the anus to observe the intestinal cavity through a monitor. Then located the appropriate site to obtain tissue specimens for colonic mucosal lesions. Gently scraped 2–3 small pieces of mucosal tissue from the surface of the colon using a biopsy forceps and placed them in a clean EP tube. When sampling, attention should be paid to sampling in the granulation tissue around ulcers or deep ulcer. The samples were immediately sent for examination after being frozen in liquid nitrogen to ensure the timeliness of the test results. Inspectors used EBV PCR fluorescence assay quantitative diagnostic kit (Sanure Biotech Inc. Hunan, China) and CMV PCR fluorescence quantitative diagnostic kit (Sanure Biotech Inc. Hunan, China) and Bio-Rad CFX-96 real-time fluorescence quantitative PCR. The reaction volume was 50 μL, including 37 μL reaction liquid, 2 μL enzyme mixture, 1 μL internal standard and 10 μL DNA extract. PCR reaction conditions were: 50°C for 2 min, 94°C for 5 min, then 45 cycles at 94°C for 15 s and 57°C for 30 s. The tissue results were expressed as the copy number of the viral genome per mg of tissue (Wang et al., 2022). Through the study of reference values, it was determined that the Ct positive judgment value of the target gene detected by this kit was 39, and the samples whose Ct value was less than 39 were reported as positive DNA of Epstein–Barr virus or CMV virus.

2.2.3 Blood samples of patients were collected for lymphocyte subsets analysis

In the early morning, 5 mL of peripheral venous blood was extracted from all enrolled patients on an empty stomach, and red blood cell lysis was performed to prepare mononuclear cell suspension. Select specific antibodies and fluorescent dyes for labeling (matching reagents from BD Company of the United States, Lymphocyte subsets detection kit- BD MultiTEST IMK Kit). It comprises two 4-color panels, each panel in one tube, there are two reagents (CD3 FITC /CD8 PE / CD45 Per CP / CD4 APC and CD3 FITC / CD16 PE + CD56 PE / CD45 PerCP /CD19 APC). Briefly, to stain a sample, 50 μL whole blood was dispensed into a specimen ID labeled Trucount tube, 20 μL respective reagent was added, and the tube was vortexed and incubated for 15 min in the dark. At the end of the incubation time, 450 μL of a BD proprietary buffered lysing solution containing <15% formaldehyde and < 50% diethylene glycol (Lysis Buffer, 10X, BD Bio-sciences, San Jose, CA) was added to each tube, and tubes were incubated again in the dark for 15 min, the labeled cells were fixed and washed; The types and numbers of lymphocyte subsets, including total T lymphocytes (CD3+), total B lymphocytes (CD3-CD19+) and NK cells (CD3-CD16 + CD56+), were measured by BD FACSAria2 flow cytometry, all expressed as percentages.

2.2.4 Statistical analysis

Patient characteristics were analyzed using quartile stratification of NK cell numbers. The quartile stratification of the number of NK cells was conducted by grouping continuous variables. Calculated the values of P25, P50, and P75. According to these values, the data were divided into four groups. Categorical variables are expressed as numbers and percentages. Continuous variables are represented by mean and standard deviation (SD) for normal distributions and median and interquartile (IQR) for skewered distributions. We used Chi-square tests, one-way ANOVA and Kruskal-Wallis tests, respectively, to compare categorical, normally distributed and non-normally distributed continuous variables. Further *post-hoc* analysis, for categorical variables, Chi-square test was used, and Bonferroni x^2 segmentation method with adjusted test level was used on the basis of Chi-squared test to conduct multiple comparisons among multiple sample rates. The test level α'was 0.0083 (0.05/6groups). Severity of disease is an ordered categorical variable. We used multiple comparison of rates by Kruskal-Walli rank sum test. The measurement data were analyzed using multiple comparison LSD-t test among multiple sample means based on ANOVA.

To investigate the relationship between the number of NK cells and the incidence of EBV and/or CMV infection in UC patients, Logistic univariate linear regression analysis and multivariate regression analysis were performed to determine the odds ratio (OR) and 95% confidence interval (95% CI). Among them, univariate Logistic regression analysis was used to evaluate the magnitude and direction of the influence of a single factor on the outcome variable. And then variables that had statistically significant on the occurrence of EBV and/or CMV infection were retained. Then we selected the variables with the NK cells' adjusted effect size (β or OR) change greater than or equal to 10%, combined with clinical experience and previous studies (Li Wai Suen et al., 2024; Li Wai Suen et al., 2020) to determine that the adjusted variables included: age, clinical classification, extent of disease, severity of disease, nutritional status - indicator albumin level, medication history - use of corticosteroids or immunosuppressants. The selection process of the adjusted variables and the related regression formulas were detailed in Supplementary Table S1. These confounding factors were controlled for further multivariate regression analysis.

According to the recommendation of the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement (Vandenbroucke et al., 2007), analyses were first performed without adjustment. Further analysis was carried out by cumulative adjustment variables. So the stability of the relationship was determined by using unadjusted models and multi-factor adjusted models according to continuous variables (calculated as OR/HR per 5% NK cells' proportion reduction) and categorical variables (quartiles), respectively. Analysis was performed in a crude model (Model 1: NK as a continuous variable adjusting variable age), and further cumulative adjustments were made for clinical classification (model 2), lesion extent and disease severity (model 3), nutritional status-indicator albumin level (model 4), and medication history (use of glucocorticoids or immunosuppressants) of the analysis variables.

To examine the non-linear relationship between the number of NK cells and the incidence of EBV and/or CMV infection, we used restricted cubic spline analysis in the Logistic regression model (Kong et al., 2018). Restricted cubic spline (RCS) regression was performed with 4 predefined knots at the 5th, 35th, 65th, and 95th percentile of NK cells to assess nonlinearity and examine the dose–response curve between the number of NK cells and the incidence of EBV and/or CMV infection after adjusting variables in Model 5. Spline regression fits smooth polynomial functions between predefined knots on a graph and joins them in a piecewise manner. Splines could be used to produce a non-linear model between a continuous prognostic variable and an outcome. If a nonlinear correlation was observed, a two-piecewise linear regression model was conducted to calculate the threshold effect of the number of NK cells on the incidence of EBV and/or CMV infection in terms of the smoothing plot/. When the number of NK cells and the incidence of EBV and/or CMV infection were evident in the smoothed curve, the recursive method automatically calculated the threshold, The likelihood-ratio test and the bootstrap resampling method were used to determine inflection points (Yu et al., 2018; Park et al., 2017; Liu et al., 2022).

We performed subgroup analyses for key characteristics that might modify the association between the number of NK cells and EBV and/or CMV infection assessed the potential confounding effects of sex, age, course of disease, clinical type, lesion extent, and disease severity on the number of NK cells. In the process of selecting subgroup variables, the variables with uneven distribution of subgroup sample size are excluded to reduce the deviation of results (Variables with too small sample size after grouping: Complication:Electrolyte imbalance, Complication:Pulmonary disease, Complication:Thyroid disease, Nutrition support:Human Albumin Solution, Medication history:Glucocorticoids OR immuno-suppressant). This study takes into account the sex variable as a grouping variable, which have a balanced sample size after stratification. $p < 0.05$ was considered significant. Multivariate logistic regression was used to assess the heterogeneity among subgroups, and likelihood ratio tests were used to examine the interaction between the number of NK cells and the incidence of EBV and/or CMV infection in each subgroup. If P for interaction<0.05, the relationship between the number of NK cells and the occurrence of EBV

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TABLE 1 Common opportunistic infections in UC patients.

Demographic characteristics	Total (n = 378)	Types of Infection				p	F/x^2
		CMV(−) EBV(−) (n = 184)	CMV(−) EBV(+) (n = 114)	CMV(+) EBV(−) (n = 12)	CMV(+) EBV(+) (n = 68)		
Sex, n (%)						0.443	2.686
Female	174 (46.0)	91 (49.5)	51 (44.7)	6 (50)	26 (38.2)		
Male	204 (54.0)	93 (50.5)	63 (55.3)	6 (50)	42 (61.8)		
Age, n (%)						0.088	Fisher
≤40 years	100 (26.5)	41 (22.3)	30 (26.3)	3 (25)	26 (38.2)		
>40 years	278 (73.5)	143 (77.7)	84 (73.7)	9 (75)	42 (61.8)		
Course of disease, n (%)						0.003	Fisher
<1 year	128 (33.9)	77 (41.8)	32 (28.1)	5 (41.7)	14 (20.6)		
1–3 years	129 (34.1)	57 (31)	35 (30.7)	2 (16.7)	35 (51.5)		
>3 years	121 (32.0)	50 (27.2)	47 (41.2)	5 (41.7)	19 (27.9)		
Clinical type, n (%)						<0.001	35.303
First episode type1	171 (45.2)	107 (58.2)	49 (43)	2 (16.7)	13 (19.1)		
Chronic relapsing type2	207 (54.8)	77 (41.8)	65 (57)	10 (83.3)	55 (80.9)		
Lesion range: Montreal typing, n (%)						<0.001	Fisher
E1	130 (34.4)	91 (49.5)	30 (26.3)	1 (8.3)	8 (11.8)		
E2	79 (20.9)	38 (20.7)	22 (19.3)	2 (16.7)	17 (25)		
E3	169 (44.7)	55 (29.9)	62 (54.4)	9 (75)	43 (63.2)		
Severity of disease, n (%)						<0.001	Fisher
Remission	167 (44.2)	122 (66.3)	35 (30.7)	2 (16.7)	8 (11.8)		
Medium	94 (24.9)	35 (19)	33 (28.9)	4 (33.3)	22 (32.4)		
Severe	117 (31.0)	27 (14.7)	46 (40.4)	6 (50)	38 (55.9)		
NK cells, Median (IQR)	9.8 (7.8, 13.8)	10.8 (9.1, 14.9)	9.7 (7.8, 13.7)	9.2 (7.1, 15.2)	6.4 (3.8, 8.8)	< 0.001	58.328

and/or CMV infection in the subgroup was statistically significant. If P for interaction>0.05, there was no statistical difference in the relationship between the two in the subgroup. The OR value and 95%CI measure the extent to which factors influence the outcome.

Statistical analysis was performed using R software (version4.2.1: The R Foundation for Statistical Computing, Vienna, Austria)[1] and the Free Statistics software (version 1.9.2: Beijing Free Clinical Medical Technology Co. Ltd., Beijing, China). p values less than 0.05 (two-sided) were considered statistically significant. The formulas that were used for all the regression analyses were detailed in the Supplementary material.

3 Results

3.1 EBV and/or CMV infection in UC patients

The basic situation of ulcerative colitis patients with EBV and/or CMV infection is shown in Table 1. Of these patients, 184 patients did not have common EBV and/or CMV infection. There were 194 patients (51.32%) with common opportunistic bacterial infection CMV and/or EBV infection, of which, the number of people with one infection is 126 (64.95%). The number of people with two infections is 68 (35.05%) 0.204 were male and 174 were female, 278 patients over 40 years old (73.5%). The number of NK cells in patients with two common opportunistic bacterial infections was significantly lower than in other patients ($p < 0.001$).

3.2 Baseline characteristics of study patients

Table 2 illustrates the baseline characteristics of all patients according to their NK cells stratified quartiles. The average age of the study participants was (49.5 ± 14.5) years. The post-hoc analysis (Table 3) showed that people with fewer NK cells tend to be the clinical type was chronic relapsing type; the lesion were classified as E1 or E3 in Montreal; the severity of the disease was severe, low ALB; the history of glucocorticoids or immunosuppressants; human albumin or nutritional support therapy was required.

1 http://www.Rproject.org

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TABLE 2 Patient characteristics and outcome parameters.

Patient characteristics	Total (n = 378)	NK cells stratified quartile				P	F/x^2
		Q1 (n = 95) (0.8–7.8)	Q2 (n = 93) (7.9–9.7)	Q3 (n = 93) (9.8–13.7)	Q4 (n = 97) (13.8–32.8)		
Sex, n (%)						0.941	0.395
Female	174 (46.0)	43 (45.3)	43 (46.2)	41 (44.1)	47 (48.5)		
Male	204 (54.0)	52 (54.7)	50 (53.8)	52 (55.9)	50 (51.5)		
Age, Mean ± SD	49.5 ± 14.5	47.3 ± 15.2	51.6 ± 13.9	49.1 ± 14.3	50.0 ± 14.3	0.235	1.425
Course of disease, n (%)						0.506	5.302
<1 year	128 (33.9)	31 (32.6)	36 (38.7)	31 (33.3)	30 (30.9)		
1–3 years	129 (34.1)	37 (38.9)	23 (24.7)	33 (35.5)	36 (37.1)		
>3 years	121 (32.0)	27 (28.4)	34 (36.6)	29 (31.2)	31 (32)		
Complication: high blood pressure, n (%)						0.534	2.188
No	332 (87.8)	86 (90.5)	78 (83.9)	83 (89.2)	85 (87.6)		
Yes	46 (12.2)	9 (9.5)	15 (16.1)	10 (10.8)	12 (12.4)		
Complication: electrolyte imbalance, n (%)						0.248	4.13
No	342 (90.5)	85 (89.5)	83 (89.2)	89 (95.7)	85 (87.6)		
Yes	36 (9.5)	10 (10.5)	10 (10.8)	4 (4.3)	12 (12.4)		
Complication: diabetes, n (%)						0.652	Fisher
No	365 (96.6)	92 (96.8)	88 (94.6)	90 (96.8)	95 (97.9)		
Yes	13 (3.4)	3 (3.2)	5 (5.4)	3 (3.2)	2 (2.1)		
Complication: pulmonary disease, n (%)						0.523	2.247
No	294 (77.8)	71 (74.7)	73 (78.5)	77 (82.8)	73 (75.3)		
Yes	84 (22.2)	24 (25.3)	20 (21.5)	16 (17.2)	24 (24.7)		
Complication: Cerebrovascular disease, n (%)						0.976	Fisher
No	368 (97.4)	93 (97.9)	90 (96.8)	91 (97.8)	94 (96.9)		
Yes	10 (2.6)	2 (2.1)	3 (3.2)	2 (2.2)	3 (3.1)		
Complication: heart disease, n (%)						0.064	Fisher
No	362 (95.8)	89 (93.7)	86 (92.5)	91 (97.8)	96 (99)		
Yes	16 (4.2)	6 (6.3)	7 (7.5)	2 (2.2)	1 (1)		
Complication: thyroid disease, n (%)						0.695	1.447
No	323 (85.4)	78 (82.1)	79 (84.9)	81 (87.1)	85 (87.6)		
Yes	55 (14.6)	17 (17.9)	14 (15.1)	12 (12.9)	12 (12.4)		
Clinical type, n (%)						0.045	8.037
First episode type1	171 (45.2)	32 (33.7)	46 (49.5)	49 (52.7)	44 (45.4)		
Chronic relapsing type2	207 (54.8)	63 (66.3)	47 (50.5)	44 (47.3)	53 (54.6)		
Lesion range: Montreal typing, n (%)						< 0.001	21.564
E1	130 (34.4)	21 (22.1)	28 (30.1)	48 (51.6)	33 (34)		
E2	79 (20.9)	25 (26.3)	26 (28)	10 (10.8)	18 (18.6)		
E3	169 (44.7)	49 (51.6)	39 (41.9)	35 (37.6)	46 (47.4)		
Severity of disease, n (%)						< 0.001	27.075
Remission	167 (44.2)	28 (29.5)	40 (43)	60 (64.5)	39 (40.2)		
Medium	94 (24.9)	26 (27.4)	24 (25.8)	14 (15.1)	30 (30.9)		
Severe	117 (31.0)	41 (43.2)	29 (31.2)	19 (20.4)	28 (28.9)		

(Continued)

TABLE 2 (Continued)

Patient characteristics	Total (n = 378)	NK cells stratified quartile				P	F/x^2
		Q1 (n = 95) (0.8–7.8)	Q2 (n = 93) (7.9–9.7)	Q3 (n = 93) (9.8–13.7)	Q4 (n = 97) (13.8–32.8)		
Extra intestinal manifestations: Joint damage, n (%)						0.576	Fisher
No	369 (97.6)	91 (95.8)	91 (97.8)	91 (97.8)	96 (99)		
Yes	9 (2.4)	4 (4.2)	2 (2.2)	2 (2.2)	1 (1)		
Extra intestinal manifestations: skin and mucosa reactions, n (%)						0.876	0.689
No	343 (90.7)	85 (89.5)	85 (91.4)	86 (92.5)	87 (89.7)		
Yes	35 (9.3)	10 (10.5)	8 (8.6)	7 (7.5)	10 (10.3)		
Extra intestinal manifestations: Ocular lesion, n (%)						0.12	Fisher
No	371 (98.1)	93 (97.9)	93 (100)	89 (95.7)	96 (99)		
Yes	7 (1.9)	2 (2.1)	0 (0)	4 (4.3)	1 (1)		
Extra intestinal manifestations: Hepatobiliary disease, n (%)						0.152	5.286
No	348 (92.1)	90 (94.7)	82 (88.2)	89 (95.7)	87 (89.7)		
Yes	30 (7.9)	5 (5.3)	11 (11.8)	4 (4.3)	10 (10.3)		
ALB, Mean ± SD	35.9 ± 6.8	34.3 ± 7.1	35.9 ± 6.3	36.7 ± 7.1	36.7 ± 6.5	0.049	2.645
HGB, Mean ± SD	120.5 ± 25.1	115.7 ± 25.0	123.6 ± 22.2	122.5 ± 28.5	120.2 ± 23.8	0.138	1.847
Medication.history: Glucocorticoids OR Immunosuppressants, n (%)						0.01	11.373
No	316 (83.6)	69 (72.6)	80 (86)	81 (87.1)	86 (88.7)		
Yes	62 (16.4)	26 (27.4)	13 (14)	12 (12.9)	11 (11.3)		
Medication history: Glucocorticoids and Immunosuppressants, n (%)						0.873	Fisher
No	364 (96.3)	91 (95.8)	89 (95.7)	91 (97.8)	93 (95.9)		
Yes	14 (3.7)	4 (4.2)	4 (4.3)	2 (2.2)	4 (4.1)		
Medication history: Biological agents, n (%)						0.655	Fisher
No	370 (97.9)	92 (96.8)	92 (98.9)	92 (98.9)	94 (96.9)		
Yes	8 (2.1)	3 (3.2)	1 (1.1)	1 (1.1)	3 (3.1)		
Nutrition support: Enteral nutrition, n (%)						0.048	7.918
No	310 (82.0)	70 (73.7)	76 (81.7)	83 (89.2)	81 (83.5)		
Yes	68 (18.0)	25 (26.3)	17 (18.3)	10 (10.8)	16 (16.5)		
Nutrition support: Human Albumin Solution, n (%)						0.038	8.445
No	321 (84.9)	72 (75.8)	83 (89.2)	81 (87.1)	85 (87.6)		
Yes	57 (15.1)	23 (24.2)	10 (10.8)	12 (12.9)	12 (12.4)		

ALB, albumin; HGB, hemoglobin.

3.3 Univariate analysis of risk factor associated with EBV and/or CMV infection in UC patients

Univariate analysis of risk factor associated with EBV and/or CMV infection in UC patients, reported as ORs and 95% CIs for the risk of that among patients with UC was summarized in Table 4. Course of disease, complication:electrolyte imbalance, pulmonary disease, thyroid disease, Clinical type, Lesion range, severity of disease, ALB, HGB, Medication history:glucocorticoids or immunosuppressants, nutrition support:human albumin solution, NK cells were significantly associated with EBV and/or CMV infection ($p < 0.01$). Other factors were not significantly associated with that.

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TABLE 3 Multiple comparisons among NK cells stratified quartile groups.

Patient characteristics	NK cells stratified quartile groups											
	Q1 vs. Q2		Q1 vs. Q3		Q1 vs. Q4		Q2 vs. Q3		Q2 vs. Q4		Q3 vs. Q4	
	Value	*p*	Value	*p*	Value	*p*	Value	*p*	Value	*p*	Value	*p*
Clinical type, n (%)												
First episode type1	4.593	0.040	7.270	0.007	2.736	0.098	0.356	0.551	0.251	0.617	1.187	0.276
Chronic relapsing type2												
Lesion range: Montreal typing, n (%)												
E1	2.513	0.292	18.346	<0.001	3.881	0.144	10.784	0.005	2.011	0.366	5.971	0.051
E2												
E3												
Severity of disease, n (%)[a]												
Remission	1.950	0.307	4.852	<0.001	1.978	0.287	2.942	0.020	0.024	1.000	−2.926	0.021
Medium												
Severe												
ALB, Mean ± SD[b]	−1.541	0.116	−2.502	0.012	−2.370	0.016	−0.962	0.991	−0.829	0.394	0.133	0.894
Medication.history: Glucocorticoids OR Immunosuppressants, n (%)												
No	5.618	0.020	5.578	0.018	7.926	0.005	0.002	0.967	0.215	0.643	0.172	0.678
Yes												
Nutrition support: Enteral nutrition, n (%)												
No	1.567	0.229	8.202	0.004	2.756	0.097	2.866	0.090	0.169	0.681	1.700	0.192
Yes												
Nutrition support: Human Albumin Solution, n (%)												
No	6.357	0.013	3.565	0.059	4.513	0.034	0.379	0.538	0.182	0.669	0.039	0.844
Yes												

[a]is the multiple comparison Kruskal-Walli rank sum test of rates.
[b]is the multiple comparison LSD-t test of the mean.

3.4 Multivariate logistic regression analysis of NK cells on the EBV and/or CMV infection in UC

In multivariable logistic regression analyses with NK cells (%)-stratified quartile, there was a 5.80 times increased risk of EBV and/or CMV infection in the lowest NK cells (%) quartile compared to highest quartile (OR 8.24, 95% CI 3.75–18.13, $p < 0.001$), independent of potential confounders (Table 5, model 5). In multivariable logistic regression analyses, with adjustment for age, NK cells (%) expressed as a continuous variable was inversely associated with EBV and/or CMV infection [for per 5% decrease of NK cells (%): OR 1.60, 95% CI 1.28–2.01, $p < 0.001$; Table 2]. This association remained significant (OR 1.49 95% CI 1.16–1.93, $p < 0.001$), independent of potential confounders (Table 5, model 5). The statistical results were robust among all models.

3.5 Nonlinear relationship analysis between NK cells and EBV and/or CMV infection in UC

Through restricted cubic spline model for smooth curve fitting, we found that the relationship between NK cells and EBV and/or CMV infection in UC was nonlinear (Figure 2), the *p* value for the likelihood ratio test was less than 0.001 (Table 6); therefore, we used a two-part model to fit the association between NK cells and EBV and/or CMV infection in UC. We found an inflection point at approximately 10.169%. On the left side of the inflection point, the effect size was 0.535 [OR: 0.535, 95% CI (0.413–0.692), $p < 0.001$], and on the right side of the inflection point, the effect size was 1.034 [OR: 1.034, 95% CI (0.904–1.183), $p > 0.05$]. This means that the risk of EBV and/or CMV infection was reduced by 46.5% with every 1% increase in NK cells. There was no association between NK cells and EBV and/or CMV infection when NK cells was ≥10.169% (Table 6). This means that the risk of EBV and/or CMV infection no longer decreases with increasing NK cells.

3.6 Subgroup analyses of factors influencing the association between NK cells and the EBV and/or CMV infection in UC

To detect whether the association between NK cells number and incidence of EBV and/or CMV infection was stable in different subgroups, analyses and interactive analyses were stratified according to confounders. No significant interaction was observed in any subgroups (*p*-value for interaction>0.05). Nevertheless, in the female subgroup, with age ≤ 40 years, course of disease>3 years, clinical type was chronic

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TABLE 4 Univariate analysis of risk factor associated with EBV and/or CMV infection in UC patients.

Variables	OR (95%CI)	*p*-value
Sex		
Male	Ref.	
Female	1.31 (0.87–1.96)	0.194
Age	0.99 (0.98–1.01)	0.270
Course of disease		
<1 year	Ref.	
1–3 years	1.91 (1.16–3.13)	0.011
>3 years	2.14 (1.29–3.56)	0.003
Complication: High blood pressure		
No	Ref.	
Yes	1.27 (0.68–2.36)	0.452
Complication: Electrolyte imbalance		
No	Ref.	
Yes	2.69 (1.26–5.76)	0.011
Complication: Diabetes		
No	Ref.	
Yes	1.11 (0.37–3.37)	0.853
Complication: Pulmonary disease		
No	Ref.	
Yes	2.59 (1.55–4.34)	<0.001
Complication: Cerebrovascular disease		
No	Ref.	
Yes	1.44 (0.4–5.17)	0.58
Complication: Heart disease		
No	Ref.	
Yes	2.97 (0.94–9.37)	0.064
Complication: Thyroid disease		
No	Ref.	
Yes	2.39 (1.3–4.41)	0.005
Clinical type		
First episode type1	Ref.	
Chronic relapsing type2	2.82 (1.86–4.29)	<0.001
Lesion range		
E1	Ref.	
E2	2.52 (1.41–4.49)	0.002
E3	4.84 (2.95–7.93)	<0.001
Severity of disease		
Remission	Ref.	
Medium	4.57 (2.66–7.84)	<0.001
Severe	9.04 (5.22–15.65)	<0.001
Extra intestinal manifestations: Joint damage		
No	Ref.	
Yes	1.19 (0.31–4.5)	0.797

(Continued)

TABLE 4 (Continued)

Variables	OR (95%CI)	*p*-value
Extra intestinal manifestations: Skin and mucosa reactions		
No	Ref.	
Yes	1.68 (0.82–3.45)	0.155
Extra intestinal manifestations: Ocular lesion		
No	Ref.	
Yes	1.27 (0.28–5.75)	0.756
Extra intestinal manifestations: Hepatobiliary disease		
No	Ref.	
Yes	1.26 (0.6–2.68)	0.542
ALB	0.92 (0.89–0.95)	<0.001
HGB	0.99 (0.98–1.00)	0.003
Medication history: Glucocorticoids OR immunosuppressants		
No	Ref.	
Yes	8.49 (3.91–18.42)	<0.001
Medication history: Glucocorticoids AND immunosuppressants		
No	Ref.	
Yes	3.63 (1.00–13.21)	0.051
Medication history: Biological agents		
No	Ref.	
Yes	2.9 (0.58–14.58)	0.195
Nutrition support: Enteral nutrition		
No	Ref.	
Yes	1.25 (0.74–2.12)	0.407
Nutrition support: Human Albumin Solution		
No	Ref.	
Yes	2.81 (1.52–5.22)	0.001
TOTEL B cells	1.02 (0.98–1.06)	0.441
TOTEL T cells	1.02 (0.99–1.05)	0.146
NK cells.	0.91 (0.87–0.95)	<0.001

ALB, albumin; HGB, hemoglobin; NK cells, natural killer cells.

TABLE 5 Multivariate logistic regression analysis of NK cells on the EBV and/or CMV infection in UC.

Model	NK cells (%)[a]		NK cells (%)-stratified quartile			
	(*n* = 378)		Q1 (*n* = 95)	Q2 (*n* = 93)	Q3 (*n* = 93)	Q4 (*n* = 97)
	OR (95%CI)	*P*	OR (95%CI)	OR (95%CI)	OR (95%CI)	Reference
Model1[b]	1.60 (1.28–2.01)	<0.001	7.86 (3.96–15.60)*	1.08 (0.61–1.93)	0.94 (0.52–1.68)	1.00
Model2[c]	1.58 (1.25–1.99)	<0.001	7.69 (3.82–15.49)*	1.13 (0.62–2.06)	1.01 (0.55–1.82)	1.00
Model3[d]	1.59 (1.25–2.03)	<0.001	9.57 (4.44–20.65)*	1.17 (0.61–2.23)	1.52 (0.77–2.97)	1.00
Model4[e]	1.55 (1.21–1.98)	<0.001	8.90 (4.12–19.24)*	1.15 (0.60–2.21)	1.49 (0.75–2.94)	1.00
Model5[f]	1.49 (1.16–1.93)	0.002	8.24 (3.75–18.13)*	1.10 (0.56–2.15)	1.42 (0.71–2.84)	1.00

Multivariable logistic regression to assess the association of NK cells' proportion with incidence of EBV and/or CMV infection. **p* for logistic regression analyses is < 0.001.
[a]NK cells'proportion was entered as a continuous variable per 5% decrease.
[b]Model 1: adjusted for age in continuous analyses, no adjustment for NK cells' proportion-stratified quartile.
[c]Model 2: adjusted as for model 1, additionally adjusted for Clinical type.
[d]Model 3: adjusted as for model 2, additionally adjusted for Lesion range and Severity of disease.
[e]Model 4: adjusted as for model 3, additionally adjusted for ALB (Nutritional status index).
[f]Model 5: adjusted as for model 4, additionally adjusted for Medication history (glucocorticoids or immunosuppressants).

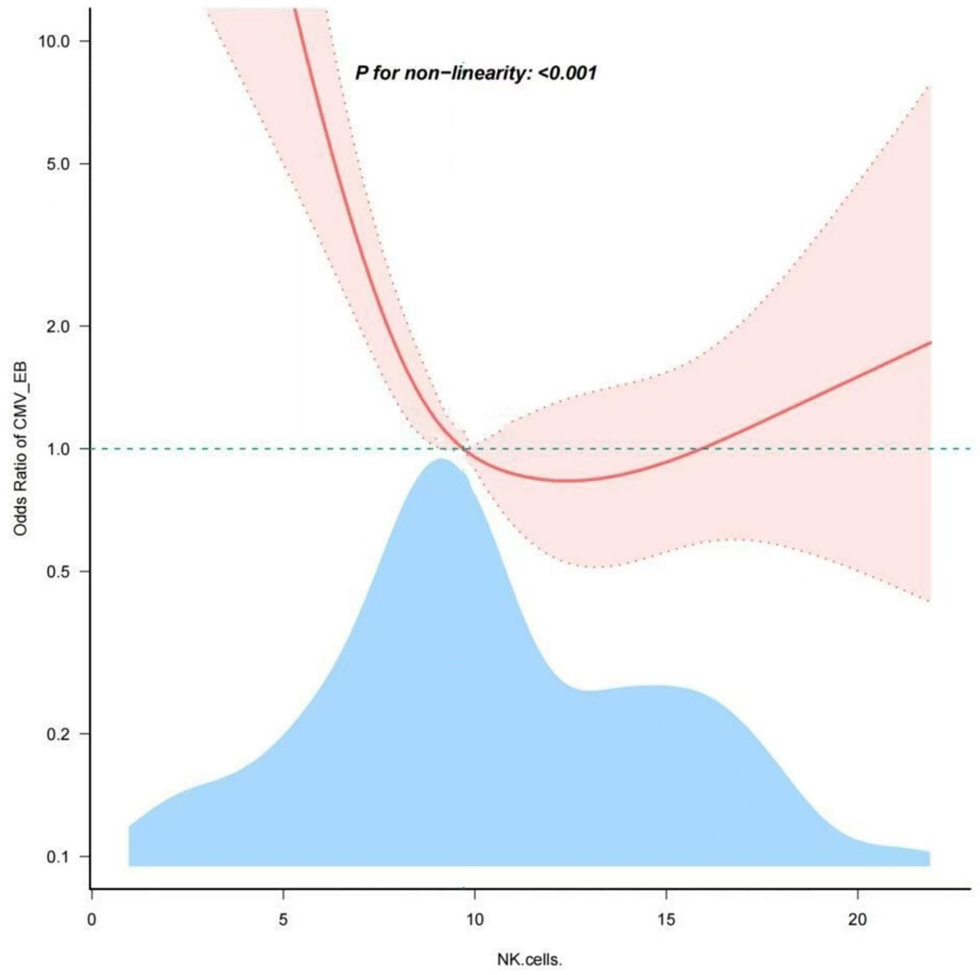


FIGURE 2
Nonlinear dose–response relationship of NK cells and the opportunistic infections. Adjustment factors included age, clinical type, lesion range, severity of disease, ALB and medication history. The red line and pink area represent the estimated values and their corresponding 95% confidence intervals, respectively.

TABLE 6 Threshold effect analysis of the relationship of NK cells and the EBV and/or CMV infection.

Item	OR	95%CI	*p*-value
Inflection point (%)	10.169	(9.959, 10.379)	
NK cells (%) < 10.169%	0.535	(0.413–0.692)	<0.001
NK cells (%) ≥ 10.169%	1.034	(0.904–1.183)	0.6242
Likelihood Ratio test			<0.001
Non-linear Test*1			<0.001

Adjusted for age, Clinical type, Lesion range, Severity of disease, ALB and Medication history.

relapsing, lesion range was E1 or E3 were associated with a greater risk for EBV and/or CMV infection compared with the corresponding subgroup. Subgroup analyses were adjusted for age, clinical type, lesion range, severity of disease, ALB and medication history (Table 7; Figure 3).

4 Discussion

To maintain intestinal homeostasis, a balanced interplay between the intestinal microbiota, intestinal epithelial cells (IECs), and immune cells is required. In UC, homeostasis is disrupted and destruction of the epithelium, atrophy of crypts and immune cell infiltration is observed (Baumdick et al., 2023), opportunistic infections are very likely to occur. Despite opportunistic infection have attracted more and more attention in UC, several key issues remain unresolved, such as their role in UC, the possible risk factors and the effects on immune function. The research so far has only revealed that dysregulated responses of innate immune cells are observed and implicated in the pathogenesis of UC (Leppkes and Neurath, 2020). This study measured EBV and/or CMV viral loads in the intestinal mucosa to accurately diagnose patients with

TABLE 7 The association between NK cells and the EBV and/or CMV infection in subgroups.

Subgroup	Variable[a]	n	n.event%	crude. OR95CI	crude.P	adj. OR95CI	adj.P	P for interaction
Sex[b]								
Female	NK cells	174	83 (47.7)	1.61 (1.15–2.26)	0.006	1.69 (1.16–2.48)	0.007	0.425
Male	NK cells	204	111 (54.4)	1.61 (1.19–2.19)	0.002	1.34 (0.92–1.96)	0.129	
Age[b]								
≤40 years	NK cells	100	59 (59)	1.95 (1.2–3.15)	0.007	2.32 (1.26–4.26)	0.007	0.194
>40 years	NK cells	278	135 (48.6)	1.5 (1.16–1.94)	0.002	1.45 (1.07–1.96)	0.017	
Course of disease[b]								
<1 year	NK cells	128	51 (39.8)	1.54 (1–2.37)	0.051	1.31 (0.76–2.24)	0.334	0.581
1-3 years	NK cells	129	72 (55.8)	1.47 (1.05–2.05)	0.023	1.47 (0.98–2.21)	0.062	
>3 years	NK cells	121	71 (58.7)	2.13 (1.33–3.42)	0.002	2.17 (1.26–3.75)	0.005	
Clinical type[c]								
First episode	NK cells	171	64 (37.4)	1.52 (1.03–2.25)	0.037	1.41 (0.88–2.24)	0.152	0.774
Chronic relapsing	NK cells	207	130 (62.8)	1.63 (1.23–2.18)	0.001	1.64 (1.17–2.28)	0.004	
Lesion range[d]								
E1	NK cells	130	39 (30)	2.18 (1.25–3.78)	0.006	1.95 (1.07–3.53)	0.028	0.437
E2	NK cells	79	41 (51.9)	1.67 (0.98–2.85)	0.059	1.75 (0.88–3.48)	0.111	
E3	NK cells	169	114 (67.5)	1.45 (1.09–1.94)	0.011	1.42 (1.01–2.01)	0.045	
Severity of disease[e]								
Remission	NK cells	167	45 (26.9)	2.38 (1.42–4.01)	0.001	2.25 (1.27–3.99)	0.006	0.107
Medium	NK cells	94	59 (62.8)	1.55 (1.02–2.34)	0.04	1.5 (0.96–2.34)	0.073	
Severe	NK cells	117	90 (76.9)	1.26 (0.88–1.82)	0.212	1.24 (0.79–1.94)	0.348	

[a]NK cells' proportion was entered as a continuous variable per 5% decrease.
[b]Adjusted for age, Clinical type, Lesion range, Severity of disease, ALB and Medication history.
[c]Adjusted for age, Lesion range, Severity of disease, ALB and Medication history.
[d]Adjusted for age, Clinical type, Severity of disease, ALB and Medication history.
[e]Adjusted for age, Clinical type, Lesion range, ALB and Medication history.

positive infections. The research shows that there were 51.32% of UC patients with common opportunistic infection CMV and/or EBV virus. The prevalence was slightly lower than previously reported study (Wang et al., 2022). However, the prevalence is higher for the population as a whole. From the perspective of immune cells, our study reveals the intrinsic relationship between the number of immune cells and opportunistic infection with viruses. Natural killer (NK) cells are innate lymphocytes that play an integral role in the host's immune defense against pathogens. Some studies have pointed out that depletion of NK cells is linked with dramatic increases in colonic damage, leukocyte infiltration and pro-inflammatory profiles (Hall et al., 2013; Baumdick et al., 2023). It also suggests that there may be a certain relationship between the internal changes of NK cells and the occurrence and development of the disease in UC patients with EBV and/or CMV infection. In this cross-sectional study, we tried to examine the NK cells associated with EBV and/or CMV infection in UC. An non-linear L-shaped association between them was found in the study, with an inflection point of almost 10.169%. The correlations between NK cells and EBV and/or CMV infection of UC patients were totally different below and above the inflection point which was around 10.169%. The association remained robust in sensitivity and subgroup analyses.

In recent publication using mass cytometry on blood samples of UC patients reported that innate NK cells (CD3-CD16 + CD56+) were significant decreased in overall UC patients (Pappa et al., 2023). Another study found that the reduction of these potential regulatory immune subsets (innate NK-like T cells among others) was observed in UC, and did not associate with disease severity (Van Unen et al., 2022). Current studies have not focused on the trend of NK cells in UC patients with EBV and/or CMV infection. Compared with previous studies, the present study analyzed data from the last 5 years after adjusting for potential confounders using multivariable regression analysis to make the results generalizable to the UC adult population. Dose response analysis revealed a non-linear relationship between NK cells and EBV and/or CMV infection. In particular, the risk for EBV and/or CMV infection was not increased with increasing NK cells in individuals with NK cells≥10.169%, whereas the risk for EBV and/or CMV infection was increased with an decreasing NK cells in those with NK cells <10.169%. In other words, the risk of EBV and/or CMV infection increases only when NK cells are below a certain level. Additionally, the association between NK cells and EBV and/or CMV infection remained stable in multivariate adjustment of logistics regression analysis.

Previous studies have suggested potential biological mechanisms underlying the association between the NK cells and EBV and/or CMV infection in UC. NK cells have immunomodulatory properties *in vivo* during chronic inflammation and autoimmunity (Zitti and Bryceson, 2018; Poggi et al., 2019;Lees, 2015). NK cells do not express

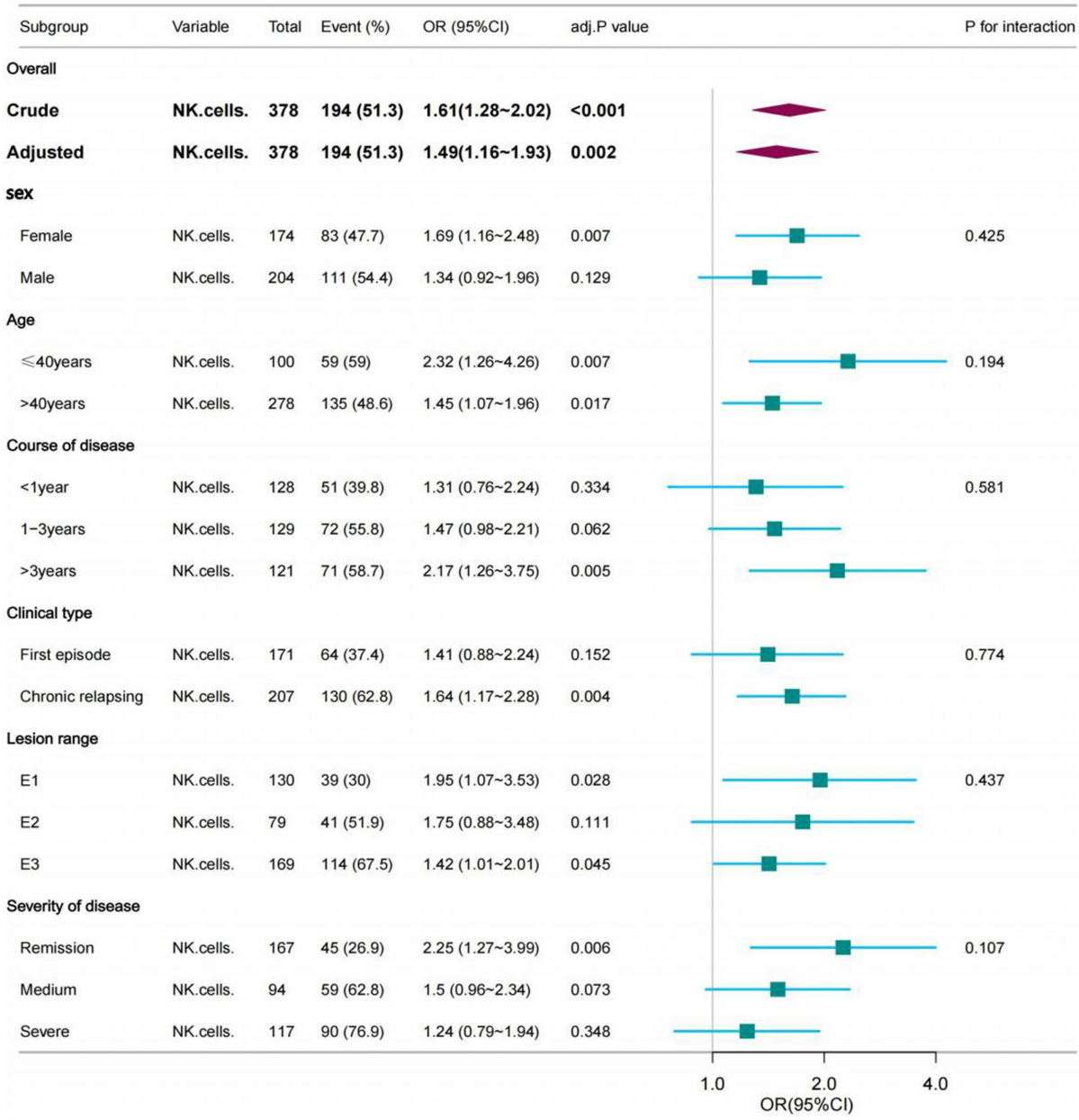


FIGURE 3
The association between NK cells and the opportunistic infections in subgroups. The *p*-value for interaction represents the likelihood of interaction between the variable and NK cells. Age was entered as a categorical variable. Adjusted for age, Clinical type, Lesion range, Severity of disease, ALB and Medication history.

specific antigen recognition receptors, but recognize "self" and "non-self" through surface activated receptors and inhibitory receptors, and directly kill the target cells infected by the virus. When virus infection occurs, the expression of MHC class I molecules on the surface of virus-infected cells is deleted or down-regulated, while some non-MHC Class I molecular ligands that can be recognized by NK cells on the surface are abnormal or up-regulated, and NK cells are further activated to kill the target cells of virus infection by releasing perforin, granase, TNF-α and expressing FasL (Cao, 2017). The above are the recognition, activation and cytotoxic effects of NK cells on the target cells of virus infection under physiological state. Cytokine stimulation of NK cells of UC patients results in reduced activation of mTORC1 and impaired IFN-γ production (Pålsson-McDermott and O'Neill, 2020). However, additional well-designed longitudinal investigations are required to further evaluate the association between the NK cells and inflammatory potential of EBV and/or CMV infection. Understanding how NK cells behave in the peripheral blood of UC patients is important not only in terms of understanding cell defects in the course of disease, but may also inform new therapeutic approaches for these patients (O'Brien and Finlay, 2019).

NK cells are key immune cells in the fight against infection and cancer. They can directly kill pathogens as well as modulate the innate and adaptive immune response. But circulating NK cells of UC patients have an unbalanced metabolic profile, with faulty mitochondria and

reduced capacity to kill. These aberrations in NK cell metabolism may contribute to defective killing and thus the secondary infections and increased risk of cancer observed in UC patients (Zaiatz Bittencourt et al., 2021). In this study, the correlation between the number of NK cells in peripheral blood of UC patients and the risk of EBV and/or CMV infection was analyzed. It can be seen that in the multivariate logistic regression analysis of NK cells (%) quartile stratified, compared with the highest quartile, after correcting for all potential confounding factors, Patients with the lowest quartile of NK cells (%) had a 7.24-fold increase in the risk of opportunistic infection (OR 8.24, 95% CI 3.75–18.13, $p < 0.001$), which fully revealed the widespread reduction in the number of peripheral NK cells in UC patients with EBV and/or CMV infection. Our study highlighted the urgent need to further explore immune defects of different cell types in UC patients and target to regulate immune cell function to clarify if this may be a future therapeutic way to treat UC with EBV and/or CMV infection patients.

Previous studies did not investigate the association and interaction between NK cells and EBV and/or CMV infection in different subgroups. Our subgroup analysis showed that female, age ≤ 40 years, course of disease>3 years, clinical type was chronic relapsing, lesion range was E1 or E3 had a higher risk of EBV and/or CMV. Previous studies (Altunal et al., 2023) have shown that long disease duration were significant risk factors for CMV colitis among patients with UC activation. This is consistent with our research. For the differences in disease susceptibility caused by sex, we can try to reveal them in subsequent studies on susceptibility genes. Owing to the limited sample size of participants with UC patients in our study, this result should be interpreted with caution, and more well-designed prospective studies in this field are warranted.

Further investigation on the functional role of NK cells in UC is necessary. NK cells from UC patients with increased viral loads should also be investigated based on the importance of NK cells in the elimination of pathogens. Our present study had certain limitations. Due to the relatively small number of UC patients with opportunistic infection, we collected data for 5 years, which may have statistical biases, and the results need to be further confirmed by expanding the sample size and additional research is required to confirm whether our results can be generalized to other diseases associated with EBV and/or CMV infection populations. Second, residual confounding effects could not be excluded. We constructed multivariable logistic regression models and performed subgroup analyses to control for the effects of potential confounders on the relationship between NK cells and the EBV and/or CMV infection. Third, because this was a cross-sectional study, the causality of the relationship between the inflammatory potential of NK cells and the EBV and/or CMV infection could not be determined. Therefore, longitudinal studies are required to determine whether the observed relationship between NK cells and the EBV and/or CMV infection is causal.

5 Conclusion

There was a non-linear relationship between NK cells and EBV and/or CMV infection. In particular, the risk for EBV and/or CMV infection was not increased with increasing NK cells in individuals with NK cells≥10.169%, whereas the risk for EBV and/or CMV infection was increased with an decreasing NK cells in those with NK cells <10.169%. The risk of EBV and/or CMV infection increases only when NK cells are below a certain level.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving humans were approved by Ethical Committee of Shanxi Provincial People's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YT: Project administration, Writing – original draft. JD: Formal analysis, Writing – review & editing. YY: Investigation, Writing – review & editing. XG: Data curation, Writing – review & editing. WW: Investigation, Writing – review & editing. FL: Conceptualization, Funding acquisition, Writing – original draft. JW: Resources, Visualization, Writing – review & editing. RL: Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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References

Albatati, S., Sharma, A., Haubrich, K., Wright, A., Gantt, S., and Blydt-Hansen, T. D. (2020). Valganciclovir prophylaxis delays onset of EBV viremia in high-risk pediatric solid organ transplant recipients. *Pediatr. Res.* 87, 892–896. doi: 10.1038/s41390-019-0523-4

Altunal, L. N., Ozel, A. S., and AK, C. (2023). Cytomegalovirus reactivation in ulcerative colitis patients: early indicators. *Niger. J. Clin. Pract.* 26, 765–770. doi: 10.4103/njcp.njcp_616_22

Baumdick, M. E., Niehrs, A., Degenhardt, F., Schwerk, M., Hinrichs, O., Jordan-Paiz, A., et al. (2023). HLA-DP on epithelial cells enables tissue damage by NKp44+ natural killer cells in ulcerative colitis. *Gastroenterology* 165, 946–962.e13. doi: 10.1053/j.gastro.2023.06.034

Cao, X. T. (2017). Medical immunology. Beijing: People's Medical Publishing House.

Chiu, Y. F., and Sugden, B. (2016). Epstein-Barr virus: the path from latent to productive infection. *Annu Rev Virol.* 3, 359–372. doi: 10.1146/annurev-virology-110615-042358

Ciccocioppo, R., Mengoli, C., Betti, E., Comolli, G., Cassaniti, I., Piralla, A., et al. (2021). Human cytomegalovirus and Epstein-Barr virus specific immunity in patients with ulcerative colitis. *Clin. Exp. Med.* 21, 379–388. doi: 10.1007/s10238-021-00702-2

Ciccocioppo, R., Racca, F., Paolucci, S., Campanini, G., Pozzi, L., Betti, E., et al. (2015). Human cytomegalovirus and Epstein-Barr virus infection in inflammatory bowel disease: need for mucosal viral load measurement. *World J. Gastroenterol.* 21, 1915–1926. doi: 10.3748/wjg.v21.i6.1915

Domènech, E., Vega, R., Ojanguren, I., Hernández, A., Garcia-Planella, E., Bernal, I., et al. (2008). Cytomegalovirus infection in ulcerative colitis: a prospective, comparative study on prevalence and diagnostic strategy. *Inflamm. Bowel Dis.* 14, 1373–1379. doi: 10.1002/ibd.20498

Gauss, A., Rosenstiel, S., Schnitzler, P., Hinz, U., Rehlen, T., Kadmon, M., et al. (2015). Intestinal cytomegalovirus infection in patients hospitalized for exacerbation of inflammatory bowel disease: a 10-year tertiary referral center experience. *Eur. J. Gastroenterol. Hepatol.* 27, 712–720. doi: 10.1097/MEG.0000000000000361

Groves, I. J., Jackson, S. E., Poole, E. L., Nachshon, A., Rozman, B., Schwartz, M., et al. (2021). Bromodomain proteins regulate human cytomegalovirus latency and reactivation allowing epigenetic therapeutic intervention. *Proc Natl Acad SciUSA.* 118:e2023025118. doi: 10.1073/pnas.2023025118

Hall, L. J., Murphy, C. T., Quinlan, A., Hurley, G., Shanahan, F., Nally, K., et al. (2013). Natural killer cells protect mice from DSS-induced colitis by regulating neutrophil function via the NKG2A receptor. *Mucosal Immunol.* 6, 1016–1026. doi: 10.1038/mi.2012.140

Hsieh, C. R., Wu, R. C., Kuo, C. J., Yeh, P. J., Yeh, Y. M., Chen, C. L., et al. (2024). Adequate antiviral treatment lowers overall complications of cytomegalovirus colitis among inpatients with inflammatory bowel diseases. *BMC Infect. Dis.* 24:443. doi: 10.1186/s12879-024-09317-w

Huang, D., Rennie, M., Krasovec, A., Nagubandi, S., Liu, S., Ge, E., et al. (2024). Impact of cytomegalovirus on outcomes in acute severe ulcerative colitis: a retrospective observational study. *Ther Adv Chronic Dis.* 15:20406223241233203. doi: 10.1177/20406223241233203

Jarvis, M. A., and Nelson, J. A. (2002). Human cytomegalovirus persistence and latency in endothelial cells and macrophages. *Curr. Opin. Microbiol.* 5, 403–407. doi: 10.1016/S1369-5274(02)00334-X

Jena, A., Mishra, S., Singh, A. K., Sekar, A., and Sharma, V. (2022). Cytomegalovirus in ulcerative colitis: an evidence-based approach to diagnosis and treatment. *Expert Rev. Gastroenterol. Hepatol.* 16, 109–120. doi: 10.1080/17474124.2022.2032662

Juliao-Baños, F., Grillo-Ardila, C. F., Alfaro, I., Andara-Ramírez, M. T., Avelar-Escobar, O., Barahona-Garrido, J., et al. (2022). Update of the PANCCO clinical practice guidelines for the treatment of ulcerative colitis in the adult population. *Rev Gastroenterol Mex (Engl Ed).* 87, 342–361. doi: 10.1016/j.rgmxen.2022.04.006

Kerr, J. R. (2019). Epstein-Barr virus(EBV)reactivation and therapeutic inhibitors. *J. Clin. Pathol.* 72, 651–658. doi: 10.1136/jclinpath-2019-205822

Kishore, J., Ghoshal, U., Ghoshal, U. C., Krishnani, N., Kumar, S., Singh, M., et al. (2004). Infection with cytomegalovirus in patients with inflammatory bowel disease: prevalence, clinical significance and outcome. *J. Med. Microbiol.* 53, 1155–1160. doi: 10.1099/jmm.0.45629-0

Kong, X., Huang, X., Zhao, M., Xu, B., Xu, R., Song, Y., et al. (2018). Platelet count affects efficacy of folic acid in preventing first stroke. *J. Am. Coll. Cardiol.* 71, 2136–2146. doi: 10.1016/j.jacc.2018.02.072

Kuijpers, T. W., Baars, P. A., Dantin, C., van den Burg, M., van Lier, R. A., and Roosnek, E. (2008). Human NK cells can control CMV infection in the absence of T cells. *Blood* 112, 914–915. doi: 10.1182/blood-2008-05-157354

Lees, J. R. (2015). Interferon gamma in autoimmunity: a complicated player on a complex stage. *Cytokine* 74, 18–26. doi: 10.1016/j.cyto.2014.10.014

Leppkes, M., and Neurath, M. F. (2020). Cytokines in inflammatory bowel diseases - update 2020. *Pharmacol. Res.* 158:104835. doi: 10.1016/j.phrs.2020.104835

Li Wai Suen, C. F. D., Choy, M. C., and De Cruz, P. (2020). Letter:how can we reduce mortality in elderly patients with acute severe ulcerative colitis? *Aliment. Pharmacol. Ther.* 51, 1444–1445. doi: 10.1111/apt.15702

Li Wai Suen, C. F. D., Seah, D., Choy, M. C., and De Cruz, P. (2024). Factors associated with response to rescue therapy in acute severe ulcerative colitis. *Inflamm. Bowel Dis.* 30, 1389–1405. doi: 10.1093/ibd/izad183

Liu, H., Wang, L., Chen, C., Dong, Z., and Yu, S. (2022). Association between dietary niacin intake and migraine among American adults: National Health and nutrition examination survey. *Nutrients* 14:3052. doi: 10.3390/nu14153052

Ljungman, P., Griffiths, P., and Paya, C. (2002). Definitions of cytomegalovirus infection and disease in transplant recipients. *Clin. Infect. Dis.* 34, 1094–1097. doi: 10.1086/339329

Maher, M. M., and Nassar, M. I. (2009). Acute cytomegalovirus infection is a risk factor in refractory and complicated inflammatory bowel disease. *Dig. Dis. Sci.* 54, 2456–2462. doi: 10.1007/s10620-008-0639-6

McGeoch, D. J., Dolan, A., and Ralph, A. C. (2000). Toward a comprehensive phylogeny for mammalian and avian herpesviruses. *J. Virol.* 74, 10401–10406. doi: 10.1128/jvi.74.22.10401-10406.2000

Murata, T., and Tsurumi, T. (2014). Switching of EBV cycles between latent and lytic states. *Rev. Med. Virol.* 24, 142–153. doi: 10.1002/rmv.1780

Núñez Ortiz, A., Rojas Feria, M., de la Cruz Ramírez, M. D., Gómez Izquierdo, L., Trigo Salado, C., Herrera Justiniano, J. M., et al. (2022). Impact of Epstein-Barr virus infection on inflammatory bowel disease (IBD) clinical outcomes. *Rev. Esp. Enferm. Dig.* 114, 259–265. doi: 10.17235/reed.2021.7915/2021

O'Brien, K. L., and Finlay, D. K. (2019). Immunometabolism and natural killer cell responses. *Nat. Rev. Immunol.* 19, 282–290. doi: 10.1038/s41577-019-0139-2

Pålsson-McDermott, E. M., and O'Neill, L. A. J. (2020). Targeting immunometabolism as an anti-inflammatory strategy. *Cell Res.* 30, 300–314. doi: 10.1038/s41422-020-0291-z

Papadakis, K. A., Tung, J. K., Binder, S. W., Kam, L. Y., Abreu, M. T., Targan, S. R., et al. (2001). Outcome of cytomegalovirus infections in patients with inflammatory bowel disease. *Am. J. Gastroenterol.* 96, 2137–2142. doi: 10.1111/j.1572-0241.2001.03949.x

Pappa, A., Mühler, J., Gast, P., Hebbar Subramanyam, S., Ohl, K., Muschaweck, M., et al. (2023). Pediatric IBD patients show medication and disease activity dependent changes in NK cell and CD4 memory T cell populations. *Front. Pediatr.* 11:1123873. doi: 10.3389/fped.2023.1123873

Park, S. Y., Freedman, N. D., Haiman, C. A., Le Marchand, L., Wilkens, L. R., and Setiawan, V. W. (2017). Association of Coffee Consumption with Total and Cause-Specific Mortality among Nonwhite Populations. *Ann. Intern. Med.* 167, 228–235. doi: 10.7326/M16-2472

Pillet, S., Pozzetto, B., and Roblin, X. (2016). Cytomegalovirus and ulcerative colitis: place of antiviral therapy. *World J. Gastroenterol.* 22, 2030–2045. doi: 10.3748/wjg.v22.i6.2030

Poggi, A., Benelli, R., Venè, R., Costa, D., Ferrari, N., and Tosetti, F. (2019). Human gut-associated natural killer cells in health and disease. *Front. Immunol.* 10:961. doi: 10.3389/fimmu.2019.00961

Ran, Z., Wu, K., Matsuoka, K., Jeen, Y. T., Wei, S. C., Ahuja, V., et al. (2021). Asian Organization for Crohn's and colitis and Asia Pacific Association of Gastroenterology practice recommendations for medical management and monitoring of inflammatory bowel disease in Asia. *J. Gastroenterol. Hepatol.* 36, 637–645. doi: 10.1111/jgh.15185

Satsangi, J., Silverberg, M. S., Vermeire, S., and Colombel, J. F. (2006). The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 55, 749–753. doi: 10.1136/gut.2005.082909

Ungaro, R., Mehandru, S., Allen, P. B., Peyrin-Biroulet, L., and Colombel, J. F. (2017). Ulcerative colitis. *Lancet* 389, 1756–1770. doi: 10.1016/S0140-6736(16)32126-2

van Unen, V., Ouboter, L. F., Li, N., Schreurs, M., Abdelaal, T., Kooy-Winkelaar, Y., et al. (2022). Identification of a disease-associated network of intestinal immune cells in treatment-naive inflammatory bowel disease. *Front. Immunol.* 23:893803. doi: 10.3389/fimmu.2022.893803IF:5.7Q1

Vandenbroucke, J. P., von Elm, E., Altman, D. G., Gøtzsche, P. C., Mulrow, C. D., Pocock, S. J., et al. (2007). Strengthening the reporting of observational studies in epidemiology (STROBE): explanation and elaboration. *Ann. Intern. Med.* 147, W163–W194. doi: 10.7326/0003-4819-147-8-200710160-00010-w1

Vieira Braga, F. A., Hertoghs, K. M., van Lier, R. A., and van Gisbergen, K. P. (2015). Molecular characterization of HCMV-specific immune responses: parallels between CD8(+) T cells, CD4(+) T cells, and NK cells. *Eur. J. Immunol.* 45, 2433–2445. doi: 10.1002/eji.201545495

Wang, W., Chen, X., Pan, J., Zhang, X., and Zhang, L. (2022). Epstein-Barr virus and human cytomegalovirus infection in intestinal mucosa of Chinese patients with

inflammatory bowel disease. *Front. Microbiol.* 13:915453. doi: 10.3389/fmicb.2022.915453

Xing, R. X., Wang, H. Q., and Zhang, W. H. (2021). Clinical significance of the ratio of serum copper to zinc in patients with inflammatory bowel disease. *China J Dig* 41, 665–670. doi: 10.3760/cma.j.cn311367-20210107-00017

Yu, X., Chen, J., Li, Y., Liu, H., Hou, C., Zeng, Q., et al. (2018). Threshold effects of moderately excessive fluoride exposure on children's health: a potential association between dental fluorosis and loss of excellent intelligence. *Environ. Int.* 118, 116–124. doi: 10.1016/j.envint.2018.05.042

Zaiatz Bittencourt, V., Jones, F., Tosetto, M., Doherty, G. A., and Ryan, E. J. (2021). Dysregulation of metabolic pathways in circulating natural killer cells isolated from inflammatory bowel disease patients. *J. Crohns Colitis* 15, 1316–1325. doi: 10.1093/ecco-jcc/jjab014

Zhang, H., Zhao, S., and Cao, Z. (2022). Impact of Epstein-Barr virus infection in patients with inflammatory bowel disease. *Front. Immunol.* 13:1001055. doi: 10.3389/fimmu.2022.1001055

Zitti, B., and Bryceson, Y. T. (2018). Natural killer cells in inflammation and autoimmunity. *Cytokine Growth Factor Rev.* 42, 37–46. doi: 10.1016/j.cytogfr.2018.08.001

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The nasal mycobiome of individuals with allergic rhinitis and asthma differs from that of healthy controls in composition, structure and function

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Allergic rhinitis (AR) and asthma (AS) are two of the most common chronic respiratory diseases and a major public health concern. Multiple studies have demonstrated the role of the nasal bacteriome in AR and AS, but little is known about the airway mycobiome and its potential association to airway inflammatory diseases. Here we used the internal transcriber spacers (ITS) 1 and 2 and high-throughput sequencing to characterize the nasal mycobiome of 339 individuals with AR, AR with asthma (ARAS), AS and healthy controls (CT). Seven to ten of the 14 most abundant fungal genera (*Malassezia, Alternaria, Cladosporium, Penicillium, Wallemia, Rhodotorula, Sporobolomyces, Naganishia, Vishniacozyma*, and *Filobasidium*) in the nasal cavity differed significantly ($p \leq 0.049$) between AS, AR or ARAS, and CT. However, none of the same genera varied significantly between the three respiratory disease groups. The nasal mycobiomes of AR and ARAS patients showed the highest intra-group diversity, while CT showed the lowest. Alpha-diversity indices of microbial richness and evenness only varied significantly ($p \leq 0.024$) between AR or ARAS and CT, while all disease groups showed significant differences ($p \leq 0.0004$) in microbial structure (i.e., beta-diversity indices) when compared to CT samples. Thirty metabolic pathways (PICRUSt2) were differentially abundant (Wald's test) between AR or ARAS and CT patients, but only three of them associated with 5-aminoimidazole ribonucleotide (AIR) biosynthesis were over abundant (log2 Fold Change >0.75) in the ARAS group. AIR has been associated to fungal pathogenesis in plants. Spiec-Easi fungal networks varied among groups, but AR and ARAS showed more similar interactions among their members than with those in the CT mycobiome; this suggests chronic respiratory allergic diseases may disrupt fungal connectivity in the nasal cavity. This study contributes valuable fungal data and results to understand the relationships between the nasal mycobiome and allergy-related conditions. It demonstrates for the first time that the nasal

mycobiota varies during health and allergic rhinitis (with and without comorbid asthma) and reveals specific taxa, metabolic pathways and fungal interactions that may relate to chronic airway disease.

KEYWORDS

allergy, asthma, ITS, mycobiome, nasal cavity, Portugal, rhinitis

1 Introduction

Allergic rhinitis and asthma are two of the most common chronic airway diseases in Western countries inflicting a relevant health and economic burden to society (Todo-Bom et al., 2007; Sa-Sousa et al., 2012; Fonseca et al., 2021). In Portugal, allergic rhinitis has a prevalence of 9–10% in children and adolescents and 26.1% in adults (Todo-Bom et al., 2007; Falcão et al., 2008; Muc et al., 2014); while asthma has a prevalence of 8.4% in children and adolescents and 6.8% in adults (Sa-Sousa et al., 2012; Muc et al., 2014; Ferreira-Magalhaes et al., 2016).

Allergic rhinitis is considered an inflammation of the nasal mucosa, characterized by sneezing, congestion, itching, and rhinorrhea (Steelant et al., 2016; Steelant et al., 2018; Acevedo-Prado et al., 2022; Savoure et al., 2022). Similarly, asthma is a multifactorial condition of the airways characterized by obstruction, inflammation, and mucous production (Mims, 2015; Licari et al., 2018; Dharmage et al., 2019). Allergic rhinitis and asthma frequently coexist (Compalati et al., 2010; Pite et al., 2014; Ferreira-Magalhaes et al., 2015; Small et al., 2018; Bousquet et al., 2019)—more than 46% of the Portuguese patients with asthma also show allergic rhinitis (Valero et al., 2009; Acevedo-Prado et al., 2022). This suggests that they may represent a combined airway inflammatory disease with several pathophysiological, epidemiological, and clinical connections within the concept of a united airway disease (Bergeron and Hamid, 2005; Pawankar, 2006; Kim et al., 2008; Compalati et al., 2010; Bousquet et al., 2023).

Multiple metataxonomic and metagenomic studies have already demonstrated that the upper airway bacteriome is a gatekeeper of respiratory health and plays a significant role in the onset, development, and severity of both allergic rhinitis (Lal et al., 2017; Bender et al., 2020; Gan et al., 2021; Chen et al., 2022; Kim et al., 2022; Azevedo et al., 2023; Pérez-Losada et al., 2023a,b) and asthma (Bogaert et al., 2011; Brar et al., 2012; Huang and Boushey, 2014; Castro-Nallar et al., 2015; Dickson and Huffnagle, 2015; Huang and Boushey, 2015; Pérez-Losada et al., 2015; Teo et al., 2015; Pérez-Losada et al., 2016a,b; Pérez-Losada et al., 2017; Dinwiddie et al., 2018; Frati et al., 2018; Pérez-Losada et al., 2018; Hufnagl et al., 2020; Losol et al., 2021; Raita et al., 2021). These same studies have also shown that the nasal cavity is a major reservoir for opportunistic bacterial pathogens, which can spread to other sections of the respiratory tract and potentially induce respiratory illnesses (Garcia-Rodriguez and Fresnadillo Martinez, 2002; Hilty et al., 2010; Bogaert et al., 2011; Dickson et al., 2013; Biesbroek et al., 2014; Huang and Boushey, 2015; Pérez-Losada et al., 2015; Teo et al., 2015; Pérez-Losada et al., 2016b; Prevaes et al., 2016; Huang, 2017; Lal et al., 2017; Pérez-Losada et al., 2017; Esposito and Principi, 2018; Pérez-Losada et al., 2018; Gan et al., 2021; Chen et al., 2022; Kim et al., 2022).

Less is known, however, about the human mycobiome and its role in chronic airway diseases (Goldman et al., 2018; Rick et al., 2020; van Tilburg Bernardes et al., 2020; Oliveira et al., 2023). The recent inclusion of fungi in human microbiome research has revealed that they are also implicated in asthma onset and development in susceptible individuals (Carpagnano et al., 2016; Goldman et al., 2018; Rick et al., 2020; van Tilburg Bernardes et al., 2020; Yuan et al., 2023), although very few studies have surveyed the upper airways (Jung et al., 2015; Yuan et al., 2023). Similarly, to the best of our knowledge, only one study so far has characterized the airway mycobiome of patients with allergic rhinitis (Jung et al., 2015); hence, the taxonomic composition and interactions, and functional diversity of the fungal communities inhabiting the nose remain unknown, or poorly understood at best, in both asthmatic and rhinitic patients.

In this study, we have used the internal transcriber spacer (ITS) 1 and 2 and next-generation sequencing to characterize the nasal mycobiomes of children and adults with allergic rhinitis (with and without asthma comorbidity), asthma and healthy controls. We describe unique fungal taxonomic and functional profiles across those four clinical groups and compare their composition, structure, metabolism, and network interactions.

2 Materials and methods

2.1 Participants

All participants enrolled in this study were part of the ASMAPORT Project (PTDC/SAU-INF/27953/2017). This study was approved by the "Comissão de Ética para a Saúde" of the Centro Hospitalar Universitário São João/Faculdade de Medicina (Porto, Portugal) in March 2017, Parecer_58-17. Written consent was obtained from all independent participants or their legal guardians using the informed consent documents approved by the Comissão de Ética.

ASMAPORT was a cross-sectional study of Portuguese children and adults designed to investigate host-microbe during asthma and rhinitis. Participants were recruited from northern Portugal while attending the outpatient clinic of the Serviço de Imunoalergologia in the Centro Hospitalar Universitário São João from July 2018 to January 2020. Healthy volunteers from the Porto area with no history of respiratory illness were also enrolled but did not complete the questionnaire or provide clinical information. The diagnosis of allergic rhinitis was confirmed by an allergy specialist based on clinical criteria and a positive skin prick or specific IgE test to at least one clinically relevant inhalant allergen in the region (Pereira et al., 2006; Bousquet et al., 2009). Diagnosis of asthma was confirmed by the attending physician based in the presence of typical symptoms in the previous 12 months or a positive bronchodilator responsiveness testing with salbutamol (Silva et al., 2019). Further details are provided in Pérez-Losada et al. (2023a,b).

2.2 Sampling

A total of 339 individuals participated in this study (Supplementary Table S1). They were categorized into four clinical groups: allergic rhinitis (AR = 47), allergic rhinitis with asthma (ARAS = 155), asthma (AS = 12), and healthy controls (CT = 125 individuals). Samples were collected by swabbing the right and left nostrils. Further detail is provided in Pérez-Losada et al. (2023a). Because of the sample size of the AS group, we have only used AS in some of the pairwise comparisons and applied statistical tests that are moderately robust to small sample sizes (see below). Similar considerations were also implemented in other microbiome studies of asthma and rhinitis including small groups or cohorts (Hilty et al., 2010; Castro-Nallar et al., 2015; Pérez-Losada et al., 2015; Lal et al., 2017; Fazlollahi et al., 2018; Pérez-Losada et al., 2023a,b).

2.3 High-throughput sequencing

Total DNA was extracted from swabs using the ZymoBIOMICS™ DNA Miniprep Kit D4300. DNA extractions were prepared for sequencing using the Schloss' MiSeq_WetLab_SOP protocol in Kozich et al. (2013). DNA samples were amplified and sequenced for the ITS1-ITS2 region (~230 bp) following the protocols used in the Earth Microbiome Project (Thompson et al., 2017) and primer ITS1F Fwd: CTTGGTCATTTAGAGGAAGTAA and primer ITS2 Rev: GCTGCGTTCTTCATCGATGC—https://earthmicrobiome.org. All samples were sequenced in a single run of the Illumina MiSeq sequencing platform at the University of Michigan Medical School. Negative controls processed as above showed no PCR band on an agarose gel. We used eight water and reagent negative controls and five mock communities (i.e., reference samples with a known composition) to detect contaminating microbial DNA within reagents and measure the sequencing error rate. We did not find evidence of contamination and our sequencing error rate was as low as 0.0051%.

2.4 Mycobiome analyses

Internal transcriber spacer amplicon sequence variants (ASV) in each sample were inferred using dada2 version 1.18 (Callahan et al., 2016) and following author's recommendations for the ITS region.[1] Reads were filtered using standard parameters, with no uncalled bases, maximum of two expected errors and truncating reads at a quality score of 2 or less. Forward and reverse reads were merged and chimeras were identified. Taxonomic assignment was performed against the UNITE v9.0 2023-07-18 database (Nilsson et al., 2019) using the implementation of the RDP naive Bayesian classifier available in the dada2 R package (Wang et al., 2007; Quast et al., 2013). ASV sequences were aligned in MAFFT (Katoh and Standley, 2013) and used to build a tree with FastTree (Price et al., 2010). The resulting ASV tables and phylogenetic tree were imported into phyloseq (McMurdie and Holmes, 2013) for further analysis. Sequence files and associated metadata and BioSample attributes for all samples used in this study have been deposited in the NCBI (PRJNA1107919). Metadata and ASV abundances with corresponding taxonomic classifications are presented in Supplementary Tables S1, S2, respectively.

We normalized our samples using the negative binomial distribution (McMurdie and Holmes, 2014) implemented in the Bioconductor package DESeq2 (Love et al., 2014). This approach simultaneously accounts for library size differences and biological variability and has increased sensitivity if groups include less than 20 samples (Weiss et al., 2017). Taxonomic and phylogenetic alpha-diversity (within-sample) were estimated using Chao1 richness and Shannon, Abundance-based Coverage Estimator (ACE), and Phylogenetic Diversity (PD) indices. Beta-diversity (between-sample) was estimated using phylogenetic Unifrac (unweighted and weighted), Bray–Curtis and Jaccard distances, and dissimilarity between samples was explored using principal coordinates analysis (PCoA).

Differences in taxonomic composition (phyla and genera) and alpha-diversity indices between disease groups (AR, ARAS, and AS) and healthy individuals (CT) were assessed using linear models (mixed and standard) analysis to account for the non-independence of subjects (random effect)—lmer4 R package (Bates et al., 2015). We also included age, season and sex as covariables in all our initial model comparisons. Lineal models with randomized subjects were not better than those without random effects, as suggested by their similar or lower scores for the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Additionally, none of the covariables were significant for any of the taxonomic and diversity indices compared. Beta-diversity indices were compared using permutational multivariate ANOVA (adonis)—vegan R package (Dixon, 2003). We applied the Benjamini–Hochberg method at alpha = 0.05 to correct for multiple hypotheses testing (Cook, 1977; Benjamini and Hochberg, 1995). All the analyses were performed in R (R Development Core Team, 2008) and RStudio (RStudio Team, 2015).

2.5 Functional analyses

Metabolic pathways were predicted by imputation of gene families and genomes as implemented in PICRUSt2 (Douglas et al., 2020). Briefly, we used the fungi ITS reference database provided by the developers to align our ITS sequences (minimum alignment 0.6) and then place them onto an ITS phylogenetic tree. Using ASV abundances obtained in dada2, we predicted gene family profiles and ultimately sample pathway abundances. Pathways were annotated using the MetaCyc database (Caspi et al., 2020) and differential pathway abundance among groups was determined in DESeq2 (Wald test; adjusted p value <0.01). Statistical analyses and visualization were conducted using functions in the ggpicrust R package (Yang et al., 2023).

2.6 Network analyses

Changes in fungal community structure were explored using covariation network analysis as implemented in Spiec-Easi (Kurtz et al., 2015). We estimated networks for AR, ARAS, and CT at the genus level (abundance filter threshold = 0.0005; mb method;

1 https://benjjneb.github.io/dada2/ITS_workflow.html

greedy clustering). Network estimation, statistics, and visualization was carried out in the microeco R package (Liu et al., 2021).

3 Results

We collected nasal swabs from a cohort of 339 participants (214 individuals with respiratory disease and 125 healthy controls) from northern Portugal comprised mainly of children and young adults (Supplementary Table S1). The median age of the participants was 12.5 ± 5.0 years and 53.7% were female. Subjects with respiratory disease were subdivided into three groups: AR (47), ARAS (155), and AS (12 subjects). We sequenced the ITS1-ITS2 gene to characterize the nasal mycobiome of each participant. Twenty-two samples (i.e., technical replicates) from the following groups were sequenced twice due to seemingly faint PCR bands in agarose gels: AR (seven samples), ARAS (13 samples), and CT (two samples). ASV singletons and samples with <1,014 reads were eliminated, rendering a final data set of 306 samples with the following distribution: AR (42 samples from 36 individuals), ARAS (142 samples from 130 individuals), AS (12 samples from 12 individuals), and CT (110 samples from 108 individuals).

3.1 Mycobiome taxonomic diversity and structure

Our nasal mycobiome (306 samples after quality control) dataset comprised 6,145,342 clean reads, ranging from 1,014 to 223,989 sequences per sample (mean = 20,082.8) and a total of 5,635 ASVs (Supplementary Table S2). AR samples had 570 unique ASVs, ARAS samples had 2,202, AS samples had 138 and CT samples had 1,615 (Supplementary Figure 1). The four groups shared 122 ASVs, the disease groups shared 78 ASVs, while other pairs and trios shared a variable number, ranging from 1 to 323 ASVs (Supplementary Figure 1).

The nasal mycobiome sequences across all 306 filtered samples were classified into two dominant (>1% abundance) Phyla: Ascomycota (54.0%) and Basidiomycota (44.9%) (Table 1). Those Phyla comprised 14 dominant (>1%) genera (Table 1; Figure 1), being the most abundant *Cladosporium* (23.0%), *Wallemia* (8.9%), *Malassezia* (8.3%), and *Rhodotorula* (8.2%). All the other detected phyla and genera accounted for <1% of the total ITS sequences each.

ASV2 of the genus *Cladosporium* comprised the nasal core microbiome (prevalence ≥90%) of the respiratory disease patients and accounted for 12.8% of their total reads. No core mycobiome was detected for the control samples. ASV2 may represent the more stable and consistent member of the nasal mycobiomes (Backhed et al., 2012; Shade and Handelsman, 2012) in the disease patients.

We also compared the mean relative abundance of specific taxa in subjects with respiratory disease and healthy controls. None of the two dominant fungal phyla (Ascomycota and Basidiomycota) comprising the nasal microbiome showed significant differences in their mean relative proportions between the groups compared (Table 1). Of the 14 dominant fungal genera comprising the nasal microbiome (Figure 1; Table 1), 7–10 genera showed significant differences in their mean relative proportions between all respiratory disease group (AS, AR or ARAS) and CT after FDR correction. However, none of the same genera varied significantly between the three respiratory disease groups (Table 1).

Alpha-diversity indices (Shannon, Chao1, ACE, and PD) of microbial community richness and evenness varied among clinical groups (Figure 2; Supplementary Table S3). AR and ARAS showed the highest diversity for all indices, while CT showed the lowest. ARAS–CT and AR–CT comparisons were significantly distinct for Shannon, Chao1, and ACE after FDR correction (Wilcoxon test; $p \leq 0.024$). All the other pairwise comparisons, including those of PD estimates, were not significant.

To characterize the structure of the nasal mycobiomes (beta diversity), we applied principal coordinates analysis (PCoAs) to Unifrac (unweighted and weighted), Bray–Curtis and Jaccard distance matrices. All the PCoAs showed partial segregation of the mycobiotas from each clinical group (Figure 3). Subsequently, adonis analyses detected significant differences ($p \leq 0.0004$) in beta-diversity between each of the respiratory disease groups (AS, AR and ARAS) and the healthy controls for all the distances. None of the pairwise comparisons between respiratory disease groups resulted significant. This suggests that the nasal mycobiomes of AS, AR and ARAS participants may differ from those of healthy individuals in a similar compositional manner.

3.2 Mycobiome functional diversity

To understand whether different disease groups exhibited differences in the nasal mycobiome functional capabilities, we inferred the functional potential of AR, ARAS, and CT groups. We found significant differences (adjusted *p*-value <0.01) in abundance in 30 pathways (MetaCyc annotated) between AR and CT or ARAS (Figure 4). Most changes in pathway abundance represented pathways enriched in CT compared to AR or ARAS with negative or nearly zero log2 Fold Change (FC). Only three pathways associated with 5-aminoimidazole ribonucleotide biosynthesis were over abundant (log2 FC > 0.75) in ARAS patients (Figure 4B). These pathways are associated with the *de novo* biosynthesis of purine nucleotides and of thiamin (PWY-6121; PWY-6122; PWY-6277). Interestingly, the comparison AR versus ARAS yielded no significant results (*p*-value >0.1), suggesting both conditions share a similar nasal mycobiome functional signature.

3.3 Mycobiome interactions

We further wanted to investigate potential direct or indirect interactions among fungal groups. We inferred inverse covariance networks using the Spiec-Easi model to compare the structure and connectivity of the nasal mycobiome. In the CT network, we identified seven modules of interacting fungi (Figure 5) with a degree of connectivity between 1 and 2, indicating very low connectivity. Likewise, betweenness centrality, a measure of importance of a node in a network, was also low (range 0–2). In turn, in the ARAS network (Figure 5), degree of connectivity ranged between 1 and 7, indicating higher connectivity. Some fungal genera were connected up to other 7 genera, and of those, *Cystobasidium*, *Pseudopithomyces*, *Peniophora*, and *Debaryomyces*, presented high betweenness centrality (e.g., *Peniophora* > 90), highlighting their role as hubs in the ARAS

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TABLE 1 Mean relative proportions (%) of fungal phyla and genera in the nasal mycobiome of participants with allergic rhinitis (AR), AR with comorbid asthma (ARAS), asthma (AS) and healthy controls (CT).

		Mean relative proportions (%)						Linear model test significance			
	All	AR	ARAS	AS	CT	AR-CT	ARAS-CT	AS-CT	AR-ARAS	AR-AS	ARAS-AS
Phylum											
Ascomycota	54	49.2	56.2	79.2	48.6	ns	ns	ns	ns	ns	ns
Basidiomycota	44.9	49.6	43.3	20.4	49.4	ns	ns	ns	ns	ns	ns
Genus											
Malassezia	8.3	0.3	1.3	0.2	23.7	<0.0001	<0.0001	<0.0001	ns	ns	ns
Alternaria	3.2	3.6	3.6	1.6	2.7	0.024	0.0003	ns	ns	ns	ns
Cladosporium	23	23.3	28.6	63.6	7.5	<0.0001	<0.0001	<0.0001	ns	ns	ns
Penicillium	2.4	1.8	3.3	1.4	1.6	0.049	<0.0001	0.031	ns	ns	ns
Aspergillus	4	3.6	3.4	1.9	5.4	ns	ns	ns	ns	ns	ns
Candida	4.7	4.2	4.7	4.6	5.2	ns	ns	ns	ns	ns	ns
Aleurina	2.4	0.1	0.2	0.3	7	ns	ns	ns	ns	ns	ns
Wallemia	8.9	15.8	11.8	2.7	2.5	<0.0001	<0.0001	0.0063	ns	ns	ns
Rhodotorula	8.2	12.2	12.1	3.5	1.6	<0.0001	<0.0001	0.0008	ns	ns	ns
Sporobolomyces	1.2	1.1	2	0.9	0.1	<0.0001	<0.0001	ns	ns	ns	ns
Naganishia	1.4	2.9	1.2	0.4	1	0.016	0.0026	ns	ns	ns	ns
Vishniacozyma	1.4	2.9	1.5	0.9	0.6	<0.0001	<0.0001	0.0023	ns	ns	ns
Sistotrema	1	0.5	0.6	1.5	1.7	ns	ns	ns	ns	ns	ns
Filobasidium	1.4	1.5	1.4	2.8	1.1	0.0009	<0.0001	0.019	ns	ns	ns

p values for significant pairwise comparisons (linear model test) between groups are also displayed. ns, not significant.

mycobiome. The AR network showed a degree of connectivity and betweenness centrality of 1–4 and 0–30, respectively (Figure 5). It shared similarities with the ARAS network, where *Phlebia* and *Debaryomyces* were also highly connected (4 and 5 in AR; 2 and 5 in ARAS). Node overlap between the three networks varied; ARAS and AR shared 23.6% of the nodes, ARAS and CT shared 14.6% and AR and CT shared 9.1%. Edge overlap was limited between networks (<5%), suggesting the overall structure of the networks is different.

4 Discussion

The role of the fungal communities residing in the upper airways in allergic rhinitis and asthma is practically unknown (Jung et al., 2015; Yuan et al., 2023). Here we present the results of a cross-sectional study comparing the nasal mycobiome of 339 individuals with allergic rhinitis (with and without comorbid asthma), asthma and healthy controls.

The nasal mycobiomes were composed of basically two phyla (Ascomycota and Basidiomycota) and 14 genera (Figure 1; Table 1). These two phyla and all dominant genera have been previously described in the airways of both healthy, asthmatic and rhinitic individuals, although with different abundances (Jung et al., 2015; Carpagnano et al., 2016; Goldman et al., 2018; Rick et al., 2020; van Tilburg Bernardes et al., 2020; Yuan et al., 2023). We detected common opportunistic pathogenic fungi like *Malassezia, Aspergillus, Candida*, and *Penicillium* (Badiee and Hashemizadeh, 2014). Moreover, exposure to *Alternaria* spores has been associated with AR symptoms (Andersson et al., 2003; Oliveira et al., 2023). This confirms at fungal level what is already known for bacteria, that the nasal cavity is a major reservoir for opportunistic pathogens that can cause allergic rhinitis and asthma (Garcia-Rodriguez and Fresnadillo Martinez, 2002; Hilty et al., 2010; Bogaert et al., 2011; Dickson et al., 2013; Biesbroek et al., 2014; Huang and Boushey, 2015; Pérez-Losada et al., 2015; Teo et al., 2015; Pérez-Losada et al., 2016b; Prevaes et al., 2016; Huang, 2017; Lal et al., 2017; Pérez-Losada et al., 2017; Esposito and Principi, 2018; Pérez-Losada et al., 2018; Gan et al., 2021; Chen et al., 2022; Kim et al., 2022; Pérez-Losada et al., 2023a).

Healthy participants differed greatly in fungal composition from those with chronic respiratory illnesses. The nasal mycobiome of healthy controls contained 28.7% unique ASVs, while the AR, ARAS and AS mycobiomes contained 10.1, 39.1, and 2.4% unique ASVs, respectively (Supplementary Table S2; Supplementary Figure 1). These ASVs are potential biomarkers of disease for each group. Further metataxonomic and metagenomic studies are needed to confirm these results and their potential as therapeutic targets for rhinitis and asthma (Castro-Nallar et al., 2015; Pérez-Losada et al., 2015; Pérez-Losada et al., 2023a,b).

Fungal phyla (Ascomycota and Basidiomycota) did not vary significantly in their mean relative proportions between groups, but up to 71% of the dominant genera varied significantly between healthy samples and respiratory disease groups (Table 1). The most striking differences were observed between AR or ARAS and CT, where 10 of 14 genera varied in their mean relative abundances, respectively. *Alternaria, Cladosporium, Penicillium, Wallemia, Rhodotorula, Sporobolomyces, Naganishia, Vishniacozyma* and *Filobasidium* were

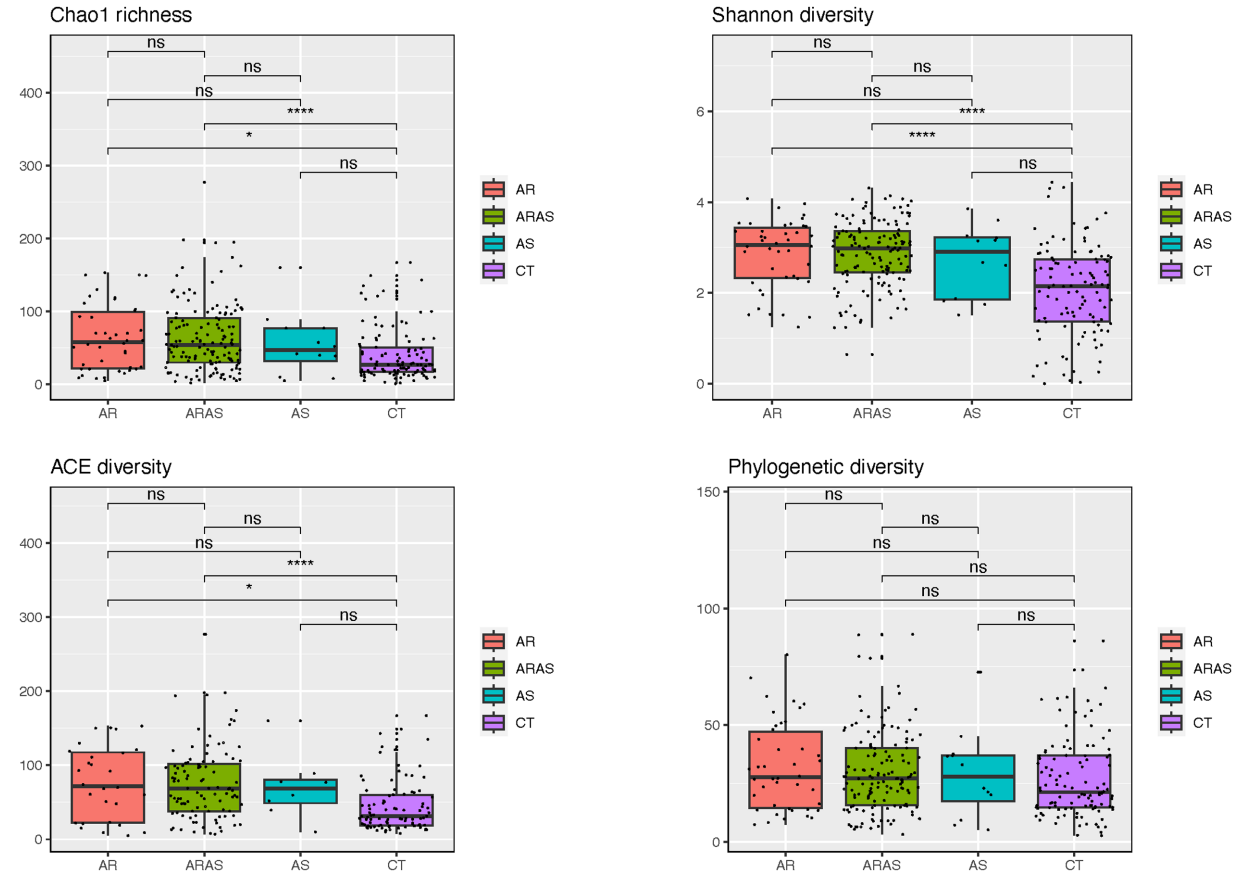


FIGURE 1
Alpha-diversity estimates (Chao1, Shannon, ACE, and phylogenetic diversity) and statistical significance (linear model test) in nasal fungal communities from participants with allergic rhinitis (AR), AR with comorbid asthma (ARAS), asthma (AS), and healthy controls (CT). ns, not significant; $*p \leq 0.05$; $****p \leq 0.0001$.

significantly more abundant in AR and ARAS, while *Malassezia* was significantly more abundant in healthy controls. A previous study of the nasal vestibule (Jung et al., 2015) in four patients with allergic rhinitis and four controls showed that Basidiomycota and *Malassezia* were highly abundant in all samples (>92%); nonetheless, no significant differences were reported among groups. This disagrees with our findings here and could result from the low sample size, fungal gene sequenced (i.e., large ribosomal subunit) or geographic region (i.e., Seoul metropolitan area) in Jung et al.'s study. Seven fungal genera varied significantly between AS and CT, despite the small sample size of this group. As before, *Malassezia* was also much more abundant in CT, while the other genera varied less in their mean relative abundances. Previous studies have also revealed significant differences in the mycobiota of asthmatic patients for *Cladosporium, Rhodotorula, Malassezia* or *Penicillium* (van Woerden et al., 2013; Goldman et al., 2018; Sharma et al., 2019; Yuan et al., 2023). Compositional changes in these fungal groups may provide insights into the pathobiology of allergic rhinitis and asthma. Further studies are required to confirm our findings and untangle the relationship between fungal colonization, dysbiosis and chronic inflammatory disease (Nguyen et al., 2015; Goldman et al., 2018; Rick et al., 2020; van Tilburg Bernardes et al., 2020; Yuan et al., 2023).

Fungal alpha-diversity (species richness and evenness) was significantly higher in ARAS and AR compared to CT for all indices but PD (Figure 2). The only study that explored the diversity of the nasal mycobiota in individuals with rhinitis (Jung et al., 2015) also reported higher estimates of Shannon diversity for the AR group. If confirmed, this may suggest that allergic rhinitis (with or without asthma comorbidity) may increase microbial diversity in the upper airways, as seen in previous studies of the bacteriome (Choi et al., 2014; Gan et al., 2021; Kim et al., 2022; Pérez-Losada et al., 2023a,b).

AR, ARAS, and AS samples displayed significant differences in community structure (i.e., beta-diversity) compared to those of healthy controls (Figure 3). This pattern held for all the distance metrics used, whether accounting for phylogenetic diversity or not. No differences were observed between AR and ARAS groups. A previous study of the nasal mycobiota (Jung et al., 2015) has also revealed that AR and CT communities were considerably differentiated. Another study (2020) has also shown specific community structuring associated with distinct bacterial composition of the lung in AS vs. CT. Hence, as indicated before (Pérez-Losada et al., 2023a,b), these results suggest that fungal compositional shifts may be a reliable predictor of allergic rhinitis or asthma in the upper airways, given their lower stochasticity associated to dysbiosis (Ma et al., 2019; Ma, 2020).

The functional component of the allergic rhinitis mycobiome is largely underexplored. Here, we used an imputation method to

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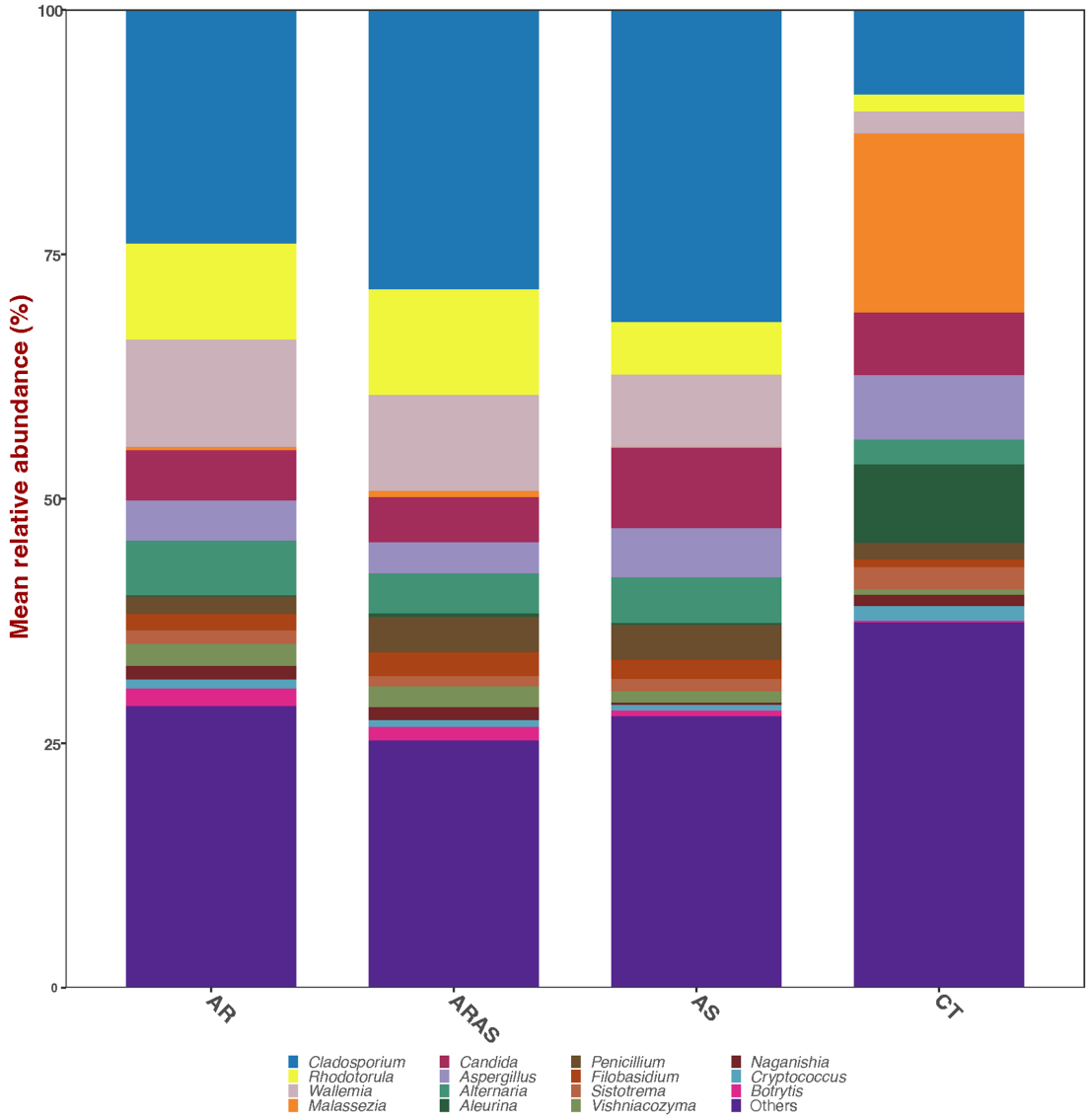


FIGURE 2
Bar plots of mean relative proportions of the top fungal genera in the nasal cavity of participants with allergic rhinitis (AR), AR with comorbid asthma (ARAS), asthma (AS), and healthy controls (CT).

indirectly explore the functional potential of the nasal mycobiome (Figure 4). We found modest yet significant differences in metabolic pathway abundance when comparing the AR to CT groups. Pathway relative overexpression was high for three pathways related to 5-aminoimidazole ribonucleotide (AIR) biosynthesis in the ARAS group (log2 FC > 0.75). AIR is a key intermediate for purine nucleotide biosynthesis and a precursor to 4-amino-2-methyl-5-hydroxymethylpyrimidine, the first product of pyrimidine biosynthesis. No studies so far have investigated AIR biosynthesis in the airway microbiome, but, interestingly, studies of the gut bacteriome have related AIR biosynthesis to several clinical conditions and diseases (hyperuricemia, inflammatory bowel disease and colorectal cancer) (Ma et al., 2021; Sheng et al., 2021). The impact (if any) of fungal AIR biosynthesis in human health has not been investigated. Purine metabolism is necessary to synthesize DNA and RNA, and in plant pathogenic fungi is associated with fungal growth and pathogenesis (Sun et al., 2024). Some authors (Chitty and Fraser, 2017) have reviewed the literature regarding purine acquisition and synthesis in human pathogenic fungi, finding that purines are essential in diverse processes such as signal transduction, energy metabolism and DNA synthesis, turning AIR biosynthesis into a potential therapeutic target. More studies are needed to test whether AIR biosynthesis in the human airway mycobiome is associated with respiratory diseases such as allergic rhinitis or asthma.

We have also explored mycobiome interactions to better understand the role of fungi in the nasal cavity (Figure 5). Direct or indirect interactions are usually inferred based on co-occurrence or co-variation of microbes' abundance. For instance, positive interactions might be indicative of syntrophy (a relationship in which one or both organisms benefit nutritionally from the presence of the other), while negative interactions may indicate competition. The CT and AR groups showed fewer significant interactions, all of which

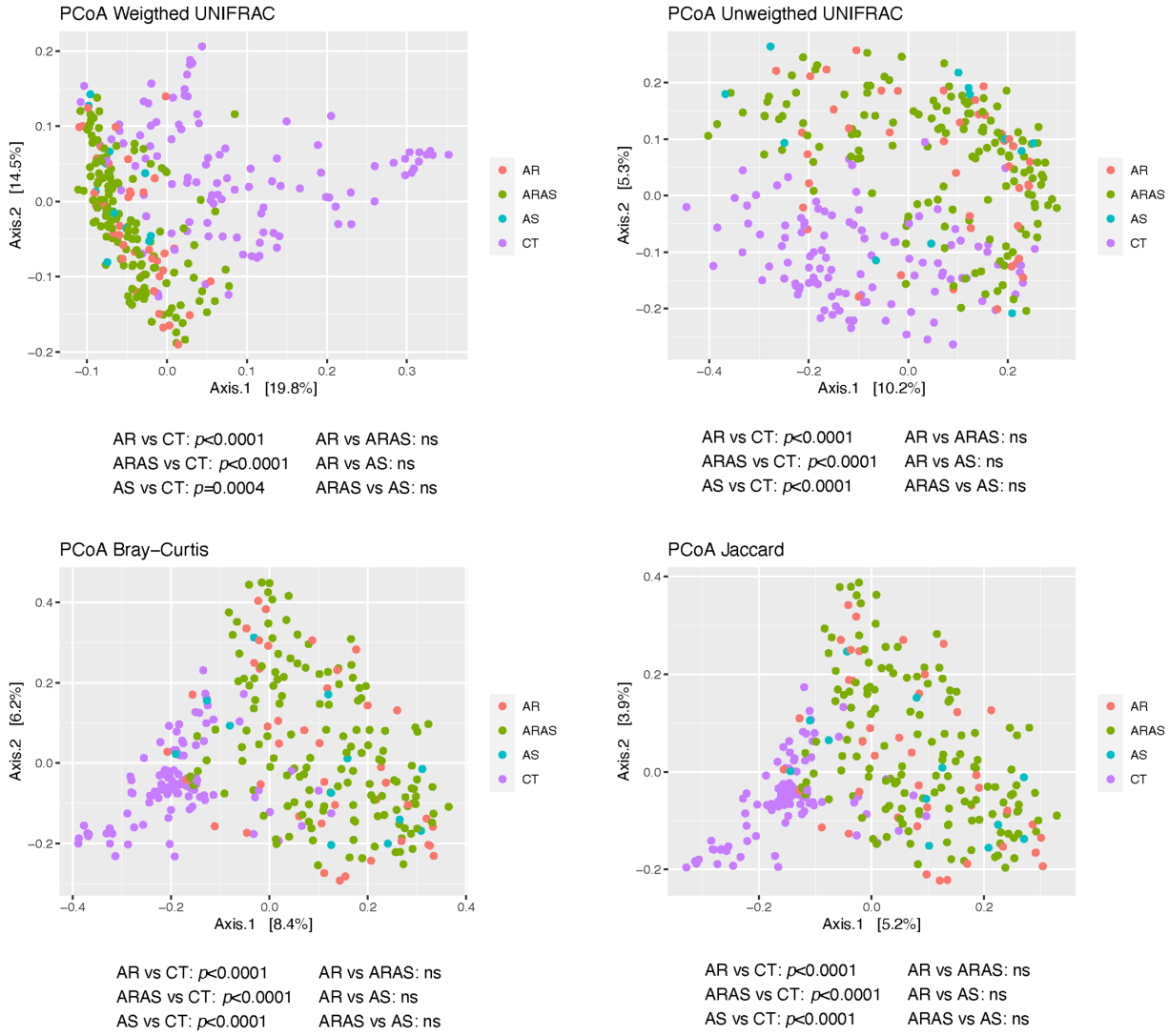


FIGURE 3
Principal coordinates analysis (PCoA) plots of beta-diversity estimates (Unifrac, Bray-Curtis and Jaccard indices) and statistical significance (Adonis test) in nasal fungal communities from participants with allergic rhinitis (AR), AR with comorbid asthma (ARAS), asthma (AS), and healthy controls (CT). ns, not significant.

were positive, suggesting either similar roles of fungi in the community or syntrophy. In turn, the ARAS group exhibited more diverse relationships with multiple modules with positive and negative interactions among fungal taxa. Previous research has shown that these patterns of co-abundance and exclusion seem to be stable across body sites in the healthy human microbiome and that its alteration can be indicative of underlying disease processes (Faust et al., 2012). In previous studies of the bacteriome in patients with allergic rhinitis (Pérez-Losada et al., 2018; Pérez-Losada et al., 2023a,b) or of the mycobiome in asthmatics (Huang et al., 2020; Liu et al., 2020), co-occurrence networks in diseased participants exhibited different interactions than in healthy controls. Our novel analyses of the airway mycobiome in rhinitic patients seem to confirm those results, although with the allergic rhinitis and comorbid asthma group (ARAS) exhibiting a higher and more diverse mycobiome network. Interestingly, in spite of the multiple connections of rhinitis and asthma and the proposed concept of a united airway disease (Compalati et al., 2010), recent omic data (Dizier et al., 2007; Lemonnier et al., 2020) suggest that rhinitis alone and rhinitis with comorbid asthma may represent two distinct diseases with different allergen sensitization and disease onset (Siroux et al., 2018), rhinitis severity (Savoure et al., 2023) and treatment response (Sousa-Pinto et al., 2022). Moreover, the hypothesis that these two distinct diseases are possibly modulated by the microbiome has been recently proposed (Bousquet et al., 2023). Further research is needed to explore the role of fungi in chronic inflammation, particularly in allergic individuals.

Our study highlights significant differences in the nasal mycobiome composition, structure, and function between individuals with allergic rhinitis and asthma and healthy controls. These findings have profound implications for understanding innate and adaptive host immune responses to fungi in the airways (Bartemes and Kita, 2018; Silva-Gomes et al., 2024). The nasal mycobiome can modulate the local immune environment. Fungal components, such as cell wall polysaccharides (e.g., β-glucans), are known to interact with pattern

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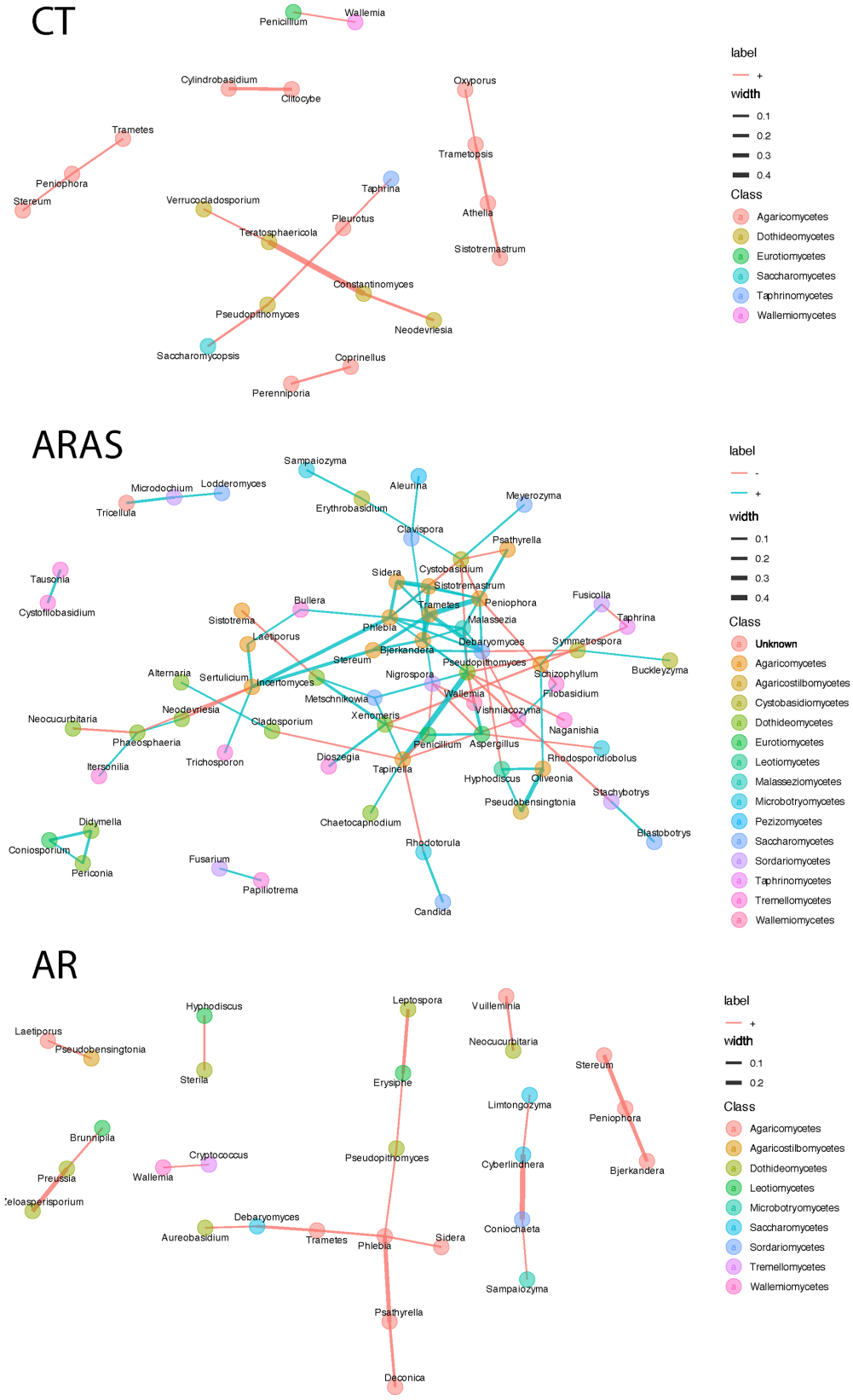


FIGURE 4
Spiec-Easi networks of fungal taxa in the nasal mycobiomes of participants with allergic rhinitis (AR), AR with comorbid asthma (ARAS) and healthy controls (CT). Nodes represent taxa connected by edges whose width (0.1–0.4) is proportional to the strength of their association. Cyan and pink edges indicate positive and negative correlations, respectively.

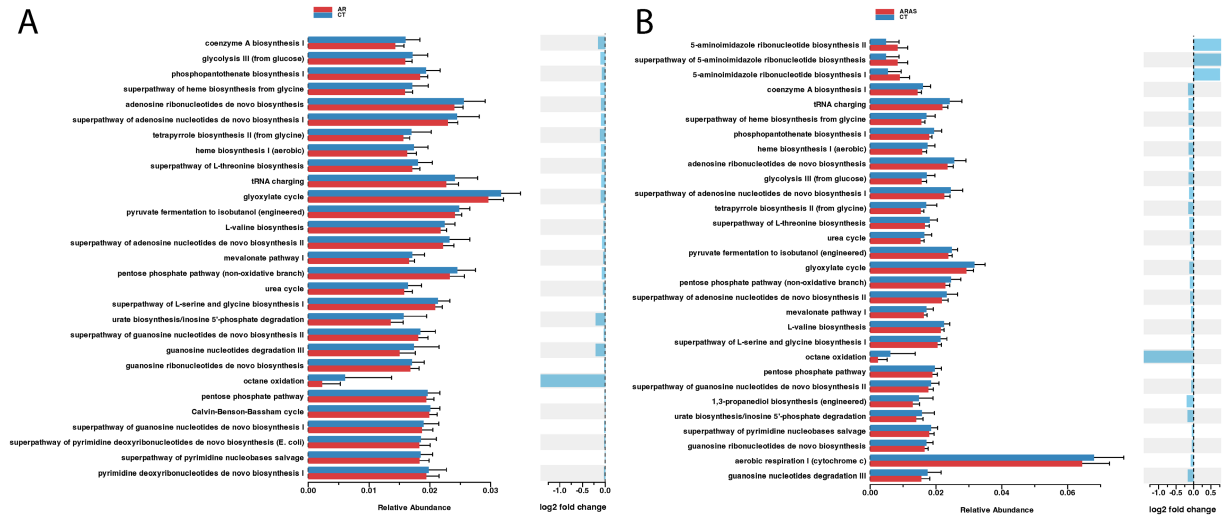


FIGURE 5
Differential abundance analysis (Wald's test; adjusted *p* value <0.01) of functional profiles in the nasal mycobiomes of participants with allergic rhinitis (AR) and healthy controls (CT) **(A)**, and AR participants with comorbid asthma (ARAS) and CT **(B)**.

recognition receptors on immune cells, leading to the activation of various immune pathways (Brown et al., 2012; Bartemes and Kita, 2018). This interaction can exacerbate or alleviate inflammation in the respiratory tract, influencing the severity of allergic reactions. Altered nasal mycobiome profiles, such as those revealed here, may contribute to allergic sensitization. Fungi can also produce potent allergens that trigger Th2-mediated immune responses, characterized by increased production of IgE and activation of mast cells and eosinophils (Noverr and Huffnagle, 2004; Noverr et al., 2004; Bartemes and Kita, 2018). This immune activation plays a critical role in the pathogenesis of allergic rhinitis and asthma. The nasal epithelial barrier's integrity and the effectiveness of innate immune defenses are closely linked to mycobiome composition. Dysmycobiosis, i.e., imbalance in the fungal community, can compromise these defenses, making individuals more susceptible to infections and exacerbations of allergic conditions (Iliev and Leonardi, 2017). Our findings are supported by previous research demonstrating that distinct nasal mycobiome profiles can activate different immunological responses. For instance, van Woerden et al. (2013) showed that fungal dysbiosis in asthmatic patients correlates with altered immune responses, including increased airway inflammation. A recent review (Jafarlou, 2024) has highlighted that fungal diseases are emerging as a significant global health threat, with the potential to cause a pandemic with widespread outbreaks and significant morbidity and mortality. There is already growing evidence that the lung mycobiome has a significant impact on clinical outcome of chronic respiratory diseases such as asthma (Nguyen et al., 2015). Little is known, however, about allergic rhinitis or the role of the nasal cavity mycobiota (Jung et al., 2015). We showed that nasal dysmycobiosis may contribute to allergic rhinitis with or without asthma comorbidity and warrants further research to elucidate the relationship between the nasal mycobiota and airway pathology.

This study has several limitations. Metataxonomic approaches suffer from the inherent limitations of collecting sequence data from a single gene target (ITS here) (Hilton et al., 2016; Pérez-Losada et al., 2022). PCR amplification biases can also impact microbial compositional assessments. ITS1-2 has limited resolution at the species and sometimes genus level for taxonomic assignment. Although the composition of the described nasal mycobiomes is similar to those reported by others in the nasal cavity of healthy and diseased individuals (Jung et al., 2015; Carpagnano et al., 2016; Goldman et al., 2018; Rick et al., 2020; van Tilburg Bernardes et al., 2020; Yuan et al., 2023). The sample size of the asthmatic (AS) group is relatively small, although we have tried to account for it using statistical approaches moderately robust to small sample sizes. The metabolic potential of the mycobiomes was predicted by imputation of gene families and genomes in PICRUSt2 instead of inferred using shotgun metagenomics; hence functional profiles should be interpreted with caution. This study focuses on a cohort of Portuguese individuals for whom we have collected limited demographic and clinical data (i.e., heath status, season, age, and sex) for all the participants. It is uncertain to what extent our results can be generalized to other countries and cohorts, but since clinical practices in Portugal for treating rhinitis and asthma follow international guidelines and recommendations and nasal mycobiomes characterized here resembled those described in other studies of cohorts from United States, Europe, and Asia, we feel like our insights are broadly applicable. Nonetheless, future research should address the impact of other demographic, clinical and environmental factors on the diversity of airway mycobiomes (Cavaleiro Rufo et al., 2017; Zhang et al., 2023; Paciencia et al., 2024). The relevance of detecting fungi associated with specific phenotypes of disease is unknown, dual-transcriptomic studies coupled with longitudinal sampling (as opposed to the cross-sectional sampling design used here) can help to clarify whether specific microbes are drivers or bystanders in rhinitic and asthmatic patients. Future microbiome research should address this issue.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession

number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA1107919.

Ethics statement

The studies involving humans were approved by Comissão de Ética para a Saúde of the Centro Hospitalar Universitário São João/Faculdade de Medicina (Porto, Portugal) in March 2017 (Parecer_58-17). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MP-L: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EC-N: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. JG-H: Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. JB: Data curation, Methodology, Writing – original draft, Writing – review & editing. LD: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. TR: Data curation, Methodology, Writing – original draft, Writing – review & editing. MO: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1464257/full#supplementary-material

SUPPLEMENTARY FIGURE 1
UpSet plots of amplicon sequence variants (ASVs) in the nasal mycobiome of participants with allergic rhinitis (AR), AR with comorbid asthma (ARAS), asthma (AS) and healthy controls (CT).

References

Acevedo-Prado, A., Seoane-Pillado, T., Lopez-Silvarrey-Varela, A., Salgado, F. J., Cruz, M. J., Faraldo-Garcia, A., et al. (2022). Association of rhinitis with asthma prevalence and severity. *Sci. Rep.* 12:6389. doi: 10.1038/s41598-022-10448-w

Andersson, M., Downs, S., Mitakakis, T., Leuppi, J., and Marks, G. (2003). Natural exposure to Alternaria spores induces allergic rhinitis symptoms in sensitized children. *Pediatr. Allergy Immunol.* 14, 100–105. doi: 10.1034/j.1399-3038.2003.00031.x

Azevedo, A. C., Hilario, S., and Goncalves, M. F. M. (2023). Microbiome in nasal mucosa of children and adolescents with allergic rhinitis: a systematic review. *Children* 10:226. doi: 10.3390/children10020226

Backhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., et al. (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 12, 611–622. doi: 10.1016/j.chom.2012.10.012

Badiee, P., and Hashemizadeh, Z. (2014). Opportunistic invasive fungal infections: diagnosis & clinical management. *Indian J. Med. Res.* 139, 195–204

Bartemes, K. R., and Kita, H. (2018). Innate and adaptive immune responses to fungi in the airway. *J. Allergy Clin. Immunol.* 142, 353–363. doi: 10.1016/j.jaci.2018.06.015

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Bender, M. E., Read, T. D., Edwards, T. S., Hargita, M., Cutler, A. J., Wissel, E. F., et al. (2020). A comparison of the bacterial nasal microbiome in allergic rhinitis patients before and after immunotherapy. *Laryngoscope* 130, E882–E888. doi: 10.1002/lary.28599

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Bergeron, C., and Hamid, Q. (2005). Relationship between asthma and rhinitis: epidemiologic, pathophysiologic, and therapeutic aspects. *Allergy, Asthma Clin. Immunol.* 1, 81–87. doi: 10.1186/1710-1492-1-2-81

Biesbroek, G., Tsivtsivadze, E., Sanders, E. A., Montijn, R., Veenhoven, R. H., Keijser, B. J., et al. (2014). Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am. J. Respir. Crit. Care Med.* 190, 1283–1292. doi: 10.1164/rccm.201407-1240OC

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., van Gils, E., et al. (2011). Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One* 6:e17035. doi: 10.1371/journal.pone.0017035

Bousquet, P. J., Burbach, G., Heinzerling, L. M., Edenharter, G., Bachert, C., Bindslev-Jensen, C., et al. (2009). GA2LEN skin test study III: minimum battery of test inhalent allergens needed in epidemiological studies in patients. *Allergy* 64, 1656–1662. doi: 10.1111/j.1398-9995.2009.02169.x

Bousquet, J., Hellings, P. W., Agache, I., Amat, F., Annesi-Maesano, I., Ansotegui, I. J., et al. (2019). Allergic rhinitis and its impact on asthma (ARIA) phase 4 (2018): change management in allergic rhinitis and asthma multimorbidity using mobile technology. *J. Allergy Clin. Immunol.* 143, 864–879. doi: 10.1016/j.jaci.2018.08.049

Bousquet, J., Melen, E., Haahtela, T., Koppelman, G. H., Togias, A., Valenta, R., et al. (2023). Rhinitis associated with asthma is distinct from rhinitis alone: the ARIA-MeDALL hypothesis. *Allergy* 78, 1169–1203. doi: 10.1111/all.15679

Brar, T., Nagaraj, S., and Mohapatra, S. (2012). Microbes and asthma: the missing cellular and molecular links. *Curr. Opin. Pulm. Med.* 18, 14–22. doi: 10.1097/MCP.0b013e32834dccc0

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G., and White, T. C. (2012). Hidden killers: human fungal infections. *Sci. Transl. Med.* 4:165rv113. doi: 10.1126/scitranslmed.3004404

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Carpagnano, G. E., Malerba, M., Lacedonia, D., Susca, A., Logrieco, A., Carone, M., et al. (2016). Analysis of the fungal microbiome in exhaled breath condensate of patients with asthma. *Allergy Asthma Proc.* 37, 41–46. doi: 10.2500/aap.2016.37.3943

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., et al. (2020). The MetaCyc database of metabolic pathways and enzymes—a 2019 update. *Nucleic Acids Res.* 48, D445–D453. doi: 10.1093/nar/gkz862

Castro-Nallar, E., Shen, Y., Freishtat, R. J., Pérez-Losada, M., Manimaran, S., Liu, G., et al. (2015). Integrating metagenomics and host gene expression to characterize asthma-associated microbial communities. *BMC Med. Genet.* 8:50. doi: 10.1186/s12920-015-0121-1

Cavaleiro Rufo, J., Madureira, J., Paciencia, I., Aguiar, L., Pereira, C., Silva, D., et al. (2017). Indoor fungal diversity in primary schools may differently influence allergic sensitization and asthma in children. *Pediatr. Allergy Immunol.* 28, 332–339. doi: 10.1111/pai.12704

Chen, M., He, S., Miles, P., Li, C., Ge, Y., Yu, X., et al. (2022). Nasal bacterial microbiome differs between healthy controls and those with asthma and allergic rhinitis. *Front. Cell. Infect. Microbiol.* 12:841995. doi: 10.3389/fcimb.2022.841995

Chitty, J. L., and Fraser, J. A. (2017). Purine acquisition and synthesis by human fungal pathogens. *Microorganisms* 5:33. doi: 10.3390/microorganisms5020033

Choi, C. H., Poroyko, V., Watanabe, S., Jiang, D., Lane, J., deTineo, M., et al. (2014). Seasonal allergic rhinitis affects sinonasal microbiota. *Am. J. Rhinol. Allergy* 28, 281–286. doi: 10.2500/ajra.2014.28.4050

Compalati, E., Ridolo, E., Passalacqua, G., Braido, F., Villa, E., and Canonica, G. W. (2010). The link between allergic rhinitis and asthma: the united airways disease. *Expert Rev. Clin. Immunol.* 6, 413–423. doi: 10.1586/eci.10.15

Cook, R. D. (1977). Detection of influential observation in linear regression. *Technometrics* 19, 15–18. doi: 10.1080/00401706.1977.10489493

Dharmage, S. C., Perret, J. L., and Custovic, A. (2019). Epidemiology of asthma in children and adults. *Front. Pediatr.* 7:246. doi: 10.3389/fped.2019.00246

Dickson, R. P., Erb-Downward, J. R., and Huffnagle, G. B. (2013). The role of the bacterial microbiome in lung disease. *Expert Rev. Respir. Med.* 7, 245–257. doi: 10.1586/ers.13.24

Dickson, R. P., and Huffnagle, G. B. (2015). The lung microbiome: new principles for respiratory bacteriology in health and disease. *PLoS Pathog.* 11:e1004923. doi: 10.1371/journal.ppat.1004923

Dinwiddie, D. L., Denson, J. L., and Kennedy, J. L. (2018). Role of the airway microbiome in respiratory infections and asthma in children. *Pediatr. Allergy Immunol. Pulmonol.* 31, 236–240. doi: 10.1089/ped.2018.0958

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. doi: 10.1111/j.1654-1103.2003.tb02228.x

Dizier, M. H., Bouzigon, E., Guilloud-Bataille, M., Genin, E., Oryszczyn, M. P., Annesi-Maesano, I., et al. (2007). Evidence for a locus in 1p31 region specifically linked to the co-morbidity of asthma and allergic rhinitis in the EGEA study. *Hum. Hered.* 63, 162–167. doi: 10.1159/000099828

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Esposito, S., and Principi, N. (2018). Impact of nasopharyngeal microbiota on the development of respiratory tract diseases. *Eur. J. Clin. Microbiol. Infect. Dis.* 37, 1–7. doi: 10.1007/s10096-017-3076-7

Falcão, H., Ramos, E., Marques, A., and Barros, H. (2008). Prevalence of asthma and rhinitis in 13 year old adolescents in Porto, Portugal. *Rev. Port. Pneumol.* 14, 747–768. doi: 10.1016/S0873-2159(15)30285-3

Faust, K., Sathirapongsasuti, J. F., Izard, J., Segata, N., Gevers, D., Raes, J., et al. (2012). Microbial co-occurrence relationships in the human microbiome. *PLoS Comput. Biol.* 8:e1002606. doi: 10.1371/journal.pcbi.1002606

Fazlollahi, M., Lee, T. D., Andrade, J., Oguntuyo, K., Chun, Y., Grishina, G., et al. (2018). The nasal microbiome in asthma. *J. Allergy Clin. Immunol.* 142, 834–843.e2. doi: 10.1016/j.jaci.2018.02.020

Ferreira-Magalhaes, M., Pereira, A. M., Sa-Sousa, A., Morais-Almeida, M., Azevedo, I., Azevedo, L. F., et al. (2015). Asthma control in children is associated with nasal symptoms, obesity, and health insurance: a nationwide survey. *Pediatr. Allergy Immunol.* 26, 466–473. doi: 10.1111/pai.12395

Ferreira-Magalhaes, M., Sa-Sousa, A., Morais-Almeida, M., Pite, H., Azevedo, L. F., Azevedo, M. I., et al. (2016). Asthma-like symptoms, diagnostic tests, and asthma medication use in children and adolescents: a population-based nationwide survey. *J. Asthma* 53, 269–276. doi: 10.3109/02770903.2015.1095926

Fonseca, J., Taveira-Gomes, T., Pereira, A. M., Branco-Ferreira, M., Carreiro-Martins, P., Alves-Correia, M., et al. (2021). ARIA 2019: an integrated care pathway for allergic rhinitis in Portugal. *Acta Medica Port.* 34, 144–157. doi: 10.20344/amp.13777

Frati, F., Salvatori, C., Incorvaia, C., Bellucci, A., Di Cara, G., Marcucci, F., et al. (2018). The role of the microbiome in asthma: the gut(−)lung Axis. *Int. J. Mol. Sci.* 20:123. doi: 10.3390/ijms20010123

Gan, W., Yang, F., Meng, J., Liu, F., Liu, S., and Xian, J. (2021). Comparing the nasal bacterial microbiome diversity of allergic rhinitis, chronic rhinosinusitis and control subjects. *Eur. Arch. Otorrinolaringol.* 278, 711–718. doi: 10.1007/s00405-020-06311-1

Garcia-Rodriguez, J. A., and Fresnadillo Martinez, M. J. (2002). Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J. Antimicrob. Chemother.* 50, 59–73. doi: 10.1093/jac/dkf506

Goldman, D. L., Chen, Z., Shankar, V., Tyberg, M., Vicencio, A., and Burk, R. (2018). Lower airway microbiota and mycobiota in children with severe asthma. *J. Allergy Clin. Immunol.* 141, 808–811.e7. doi: 10.1016/j.jaci.2017.09.018

Hilton, S. K., Castro-Nallar, E., Pérez-Losada, M., Toma, I., McCaffrey, T. A., Hoffman, E. P., et al. (2016). Metataxonomic and metagenomic approaches vs. culture-based techniques for clinical pathology. *Front. Microbiol.* 7:484. doi: 10.3389/fmicb.2016.00484

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., et al. (2010). Disordered microbial communities in asthmatic airways. *PLoS One* 5:e8578. doi: 10.1371/journal.pone.0008578

Huang, Y. J. (2017). Nasopharyngeal microbiota: gatekeepers or fortune tellers of susceptibility to respiratory tract infections? *Am. J. Respir. Crit. Care Med.* 196, 1504–1505. doi: 10.1164/rccm.201707-1470ED

Huang, Y. J., and Boushey, H. A. (2014). The microbiome and asthma. *Ann. Am. Thorac. Soc.* 11, S48–S51. doi: 10.1513/AnnalsATS.201306-187MG

Huang, Y. J., and Boushey, H. A. (2015). The microbiome in asthma. *J. Allergy Clin. Immunol.* 135, 25–30. doi: 10.1016/j.jaci.2014.11.011

Huang, C., Yu, Y., Du, W., Liu, Y., Dai, R., Tang, W., et al. (2020). Fungal and bacterial microbiome dysbiosis and imbalance of trans-kingdom network in asthma. *Clin. Transl. Allergy* 10:42. doi: 10.1186/s13601-020-00345-8

Hufnagl, K., Pali-Scholl, I., Roth-Walter, F., and Jensen-Jarolim, E. (2020). Dysbiosis of the gut and lung microbiome has a role in asthma. *Semin. Immunopathol.* 42, 75–93. doi: 10.1007/s00281-019-00775-y

Iliev, I. D., and Leonardi, I. (2017). Fungal dysbiosis: immunity and interactions at mucosal barriers. *Nat. Rev. Immunol.* 17, 635–646. doi: 10.1038/nri.2017.55

Jafarlou, M. (2024). Unveiling the menace: a thorough review of potential pandemic fungal disease. *Front. Fungal Biol.* 5:1338726. doi: 10.3389/ffunb.2024.1338726

Jung, W. H., Croll, D., Cho, J. H., Kim, Y. R., and Lee, Y. W. (2015). Analysis of the nasal vestibule mycobiome in patients with allergic rhinitis. *Mycoses* 58, 167–172. doi: 10.1111/myc.12296

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kim, H., Bouchard, J., and Renzi, P. M. (2008). The link between allergic rhinitis and asthma: a role for antileukotrienes? *Can. Respir. J.* 15, 91–98. doi: 10.1155/2008/416095

Kim, H. J., Kim, J. H., Han, S., and Kim, W. (2022). Compositional alteration of the nasal microbiome and *Staphylococcus aureus*-characterized dysbiosis in the nasal mucosa of patients with allergic rhinitis. *Clin. Exp. Otorhinolaryngol.* 15, 335–345. doi: 10.21053/ceo.2021.01928

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi: 10.1128/AEM.01043-13

Kurtz, Z. D., Muller, C. L., Miraldi, E. R., Littman, D. R., Blaser, M. J., and Bonneau, R. A. (2015). Sparse and compositionally robust inference of microbial ecological networks. *PLoS Comput. Biol.* 11:e1004226. doi: 10.1371/journal.pcbi.1004226

Lal, D., Keim, P., Delisle, J., Barker, B., Rank, M. A., Chia, N., et al. (2017). Mapping and comparing bacterial microbiota in the sinonasal cavity of healthy, allergic rhinitis, and chronic rhinosinusitis subjects. *Int. Forum Allergy Rhinol.* 7, 561–569. doi: 10.1002/alr.21934

Lemonnier, N., Melen, E., Jiang, Y., Joly, S., Menard, C., Aguilar, D., et al. (2020). A novel whole blood gene expression signature for asthma, dermatitis, and rhinitis multimorbidity in children and adolescents. *Allergy* 75, 3248–3260. doi: 10.1111/all.14314

Licari, A., Brambilla, I., Marseglia, A., De Filippo, M., Paganelli, V., and Marseglia, G. L. (2018). Difficult vs. severe asthma: definition and limits of asthma control in the pediatric population. *Front. Pediatr.* 6:170. doi: 10.3389/fped.2018.00170

Liu, C., Cui, Y., Li, X., and Yao, M. (2021). Microeco: an R package for data mining in microbial community ecology. *FEMS Microbiol. Ecol.* 97:fiaa255. doi: 10.1093/femsec/fiaa255

Liu, H. Y., Li, C. X., Liang, Z. Y., Zhang, S. Y., Yang, W. Y., Ye, Y. M., et al. (2020). The interactions of airway bacterial and fungal communities in clinically stable asthma. *Front. Microbiol.* 11:1647. doi: 10.3389/fmicb.2020.01647

Losol, P., Choi, J. P., Kim, S. H., and Chang, Y. S. (2021). The role of upper airway microbiome in the development of adult asthma. *Immune Netw.* 21:e19. doi: 10.4110/in.2021.21.e19

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Ma, Z. S. (2020). Testing the Anna Karenina principle in human microbiome-associated diseases. *iScience* 23:101007. doi: 10.1016/j.isci.2020.101007

Ma, Z. S., Li, L., and Gotelli, N. J. (2019). Diversity-disease relationships and shared species analyses for human microbiome-associated diseases. *ISME J.* 13, 1911–1919. doi: 10.1038/s41396-019-0395-y

Ma, Y., Zhang, Y., Xiang, J., Xiang, S., Zhao, Y., Xiao, M., et al. (2021). Metagenome analysis of intestinal bacteria in healthy people, patients with inflammatory bowel disease and colorectal cancer. *Front. Cell. Infect. Microbiol.* 11:599734. doi: 10.3389/fcimb.2021.599734

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

McMurdie, P. J., and Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput. Biol.* 10:e1003531. doi: 10.1371/journal.pcbi.1003531

Mims, J. W. (2015). Asthma: definitions and pathophysiology. *Int. Forum Allergy Rhinol.* 5, S2–S6. doi: 10.1002/alr.21609

Muc, M., Mota-Pinto, A., and Padez, C. (2014). Prevalence of asthma and rhinitis symptoms among children living in Coimbra, Portugal. *Rev. Port. Pneumol.* 20, 208–210. doi: 10.1016/j.rppneu.2013.08.002

Nguyen, L. D., Viscogliosi, E., and Delhaes, L. (2015). The lung mycobiome: an emerging field of the human respiratory microbiome. *Front. Microbiol.* 6:89. doi: 10.3389/fmicb.2015.00089

Nilsson, R. H., Larsson, K. H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., et al. (2019). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 47, D259–D264. doi: 10.1093/nar/gky1022

Noverr, M. C., and Huffnagle, G. B. (2004). Does the microbiota regulate immune responses outside the gut? *Trends Microbiol.* 12, 562–568. doi: 10.1016/j.tim.2004.10.008

Noverr, M. C., Noggle, R. M., Toews, G. B., and Huffnagle, G. B. (2004). Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. *Infect. Immun.* 72, 4996–5003. doi: 10.1128/IAI.72.9.4996-5003.2004

Oliveira, M., Oliveira, D., Lisboa, C., Boechat, J. L., and Delgado, L. (2023). Clinical manifestations of human exposure to Fungi. *J. Fungi* 9:381. doi: 10.3390/jof9030381

Paciencia, I., Sharma, N., Hugg, T. T., Rantala, A. K., Heibati, B., Al-Delaimy, W. K., et al. (2024). The role of biodiversity in the development of asthma and allergic sensitization: a state-of-the-science review. *Environ. Health Perspect.* 132:66001. doi: 10.1289/EHP13948

Pawankar, R. (2006). Allergic rhinitis and asthma: are they manifestations of one syndrome? *Clin. Exp. Allergy* 36, 1–4. doi: 10.1111/j.1365-2222.2006.02420.x

Pereira, C., Valero, A., Loureiro, C., Davila, I., Martinez-Cocera, C., Murio, C., et al. (2006). Iberian study of aeroallergens sensitisation in allergic rhinitis. *Eur Ann Allergy Clin Immunol* 38, 186–194

Pérez-Losada, M., Alamri, L., Crandall, K. A., and Freishtat, R. J. (2017). Nasopharyngeal microbiome diversity changes over time in children with asthma. *PLoS One* 12:e0170543. doi: 10.1371/journal.pone.0170543

Pérez-Losada, M., Authelet, K. J., Hoptay, C. E., Kwak, C., Crandall, K. A., and Freishtat, R. J. (2018). Pediatric asthma comprises different phenotypic clusters with unique nasal microbiotas. *Microbiome* 6:179. doi: 10.1186/s40168-018-0564-7

Pérez-Losada, M., Castro-Nallar, E., Bendall, M. L., Freishtat, R. J., and Crandall, K. A. (2015). Dual transcriptomic profiling of host and microbiota during health and disease in pediatric asthma. *PLoS One* 10:e0131819. doi: 10.1371/journal.pone.0131819

Pérez-Losada, M., Castro-Nallar, E., Laerte Boechat, J., Delgado, L., Azenha Rama, T., Berrios-Farias, V., et al. (2023a). Nasal Bacteriomes of patients with asthma and allergic rhinitis show unique composition, structure, function and interactions. *Microorganisms* 11:683. doi: 10.3390/microorganisms11030683

Pérez-Losada, M., Castro-Nallar, E., Laerte Boechat, J., Delgado, L., Azenha Rama, T., Berrios-Farias, V., et al. (2023b). The oral bacteriomes of patients with allergic rhinitis and asthma differ from that of healthy controls. *Front. Microbiol.* 14:1197135. doi: 10.3389/fmicb.2023.1197135

Pérez-Losada, M., Crandall, K. A., and Freishtat, R. J. (2016a). Comparison of two commercial DNA extraction kits for the analysis of nasopharyngeal bacterial communities. *AIMS Microbiol.* 2, 108–119. doi: 10.3934/microbiol.2016.2.108

Pérez-Losada, M., Crandall, K. A., and Freishtat, R. J. (2016b). Two sampling methods yield distinct microbial signatures in the nasopharynges of asthmatic children. *Microbiome* 4:25. doi: 10.1186/s40168-016-0170-5

Pérez-Losada, M., Narayanan, D. B., Kolbe, A. R., Ramos-Tapia, I., Castro-Nallar, E., Crandall, K. A., et al. (2022). Comparative analysis of metagenomics and metataxonomics for the characterization of vermicompost microbiomes. *Front. Microbiol.* 13:854423. doi: 10.3389/fmicb.2022.854423

Pite, H., Pereira, A. M., Morais-Almeida, M., Nunes, C., Bousquet, J., and Fonseca, J. A. (2014). Prevalence of asthma and its association with rhinitis in the elderly. *Respir. Med.* 108, 1117–1126. doi: 10.1016/j.rmed.2014.05.002

Prevaes, S. M., de Winter-de Groot, K. M., Janssens, H. M., de Steenhuijsen Piters, W. A., Tramper-Stranders, G. A., Wyllie, A. L., et al. (2016). Development of the nasopharyngeal microbiota in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 193, 504–515. doi: 10.1164/rccm.201509-1759OC

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2--approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raita, Y., Pérez-Losada, M., Freishtat, R. J., Harmon, B., Mansbach, J. M., Piedra, P. A., et al. (2021). Integrated omics endotyping of infants with respiratory syncytial virus bronchiolitis and risk of childhood asthma. *Nat. Commun.* 12:3601. doi: 10.1038/s41467-021-23859-6

Rick, E. M., Woolnough, K. F., Seear, P. J., Fairs, A., Satchwell, J., Richardson, M., et al. (2020). The airway fungal microbiome in asthma. *Clin. Exp. Allergy* 50, 1325–1341. doi: 10.1111/cea.13722

RStudio Team (2015). Integrated development for R. RStudio, IncBoston, MA.

Sa-Sousa, A., Morais-Almeida, M., Azevedo, L. F., Carvalho, R., Jacinto, T., Todo-Bom, A., et al. (2012). Prevalence of asthma in Portugal—the Portuguese National Asthma Survey. *Clin. Transl. Allergy* 2:15. doi: 10.1186/2045-7022-2-15

Savoure, M., Bousquet, J., Jaakkola, J. J. K., Jaakkola, M. S., Jacquemin, B., and Nadif, R. (2022). Worldwide prevalence of rhinitis in adults: a review of definitions and temporal evolution. *Clin. Transl. Allergy* 12:e12130. doi: 10.1002/clt2.12130

Savoure, M., Bousquet, J., Leynaert, B., Renuy, A., Siroux, V., Goldberg, M., et al. (2023). Rhinitis phenotypes and multimorbidities in the general population: the CONSTANCES cohort. *Eur. Respir. J.* 61:2200943. doi: 10.1183/13993003.00943-2022

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi: 10.1111/j.1462-2920.2011.02585.x

Sharma, A., Laxman, B., Naureckas, E. T., Hogarth, D. K., Sperling, A. I., Solway, J., et al. (2019). Associations between fungal and bacterial microbiota of airways and asthma endotypes. *J. Allergy Clin. Immunol.* 144, 1214–1227.e7. doi: 10.1016/j.jaci.2019.06.025

Sheng, S., Chen, J., Zhang, Y., Qin, Q., Li, W., Yan, S., et al. (2021). Structural and functional alterations of gut microbiota in males with hyperuricemia and high levels of liver enzymes. *Front Med* 8:779994. doi: 10.3389/fmed.2021.779994

Silva, D., Severo, M., Paciencia, I., Rufo, J., Martins, C., Moreira, P., et al. (2019). Setting definitions of childhood asthma in epidemiologic studies. *Pediatr. Allergy Immunol.* 30, 708–715. doi: 10.1111/pai.13111

Silva-Gomes, R., Caldeira, I., Fernandes, R., Cunha, C., and Carvalho, A. (2024). Metabolic regulation of the host-fungus interaction: from biological principles to therapeutic opportunities. *J. Leukoc. Biol.* 116, 469–486. doi: 10.1093/jleuko/qiae045

Siroux, V., Ballardini, N., Soler, M., Lupinek, C., Boudier, A., Pin, I., et al. (2018). The asthma-rhinitis multimorbidity is associated with IgE polysensitization in adolescents and adults. *Allergy* 73, 1447–1458. doi: 10.1111/all.13410

Small, P., Keith, P. K., and Kim, H. (2018). Allergic rhinitis. *Allergy, Asthma Clin. Immunol.* 14:51. doi: 10.1186/s13223-018-0280-7

Sousa-Pinto, B., Schunemann, H. J., Sa-Sousa, A., Vieira, R. J., Amaral, R., Anto, J. M., et al. (2022). Comparison of rhinitis treatments using MASK-air(R) data and considering the minimal important difference. *Allergy* 77, 3002–3014. doi: 10.1111/all.15371

Steelant, B., Farre, R., Wawrzyniak, P., Belmans, J., Dekimpe, E., Vanheel, H., et al. (2016). Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression. *J. Allergy Clin. Immunol.* 137, 1043–1053.e5. doi: 10.1016/j.jaci.2015.10.050

Steelant, B., Seys, S. F., Van Gerven, L., Van Woensel, M., Farre, R., Wawrzyniak, P., et al. (2018). Histamine and T helper cytokine-driven epithelial barrier dysfunction in allergic rhinitis. *J. Allergy Clin. Immunol.* 141, 951–963.e8. doi: 10.1016/j.jaci.2017.08.039

Sun, M., Dai, P., Cao, Z., and Dong, J. (2024). Purine metabolism in plant pathogenic fungi. *Front. Microbiol.* 15:1352354. doi: 10.3389/fmicb.2024.1352354

Teo, S. M., Mok, D., Pham, K., Kusel, M., Serralha, M., Troy, N., et al. (2015). The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 17, 704–715. doi: 10.1016/j.chom.2015.03.008

Thompson, L. R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Locey, K. J., et al. (2017). A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* 551, 457–463. doi: 10.1038/nature24621

Todo-Bom, A., Loureiro, C., Almeida, M. M., Nunes, C., Delgado, L., Castel-Branco, G., et al. (2007). Epidemiology of rhinitis in Portugal: evaluation of the intermittent and the persistent types. *Allergy* 62, 1038–1043. doi: 10.1111/j.1398-9995.2007.01448.x

Valero, A., Pereira, C., Loureiro, C., Martinez-Cocera, C., Murio, C., Rico, P., et al. (2009). Interrelationship between skin sensitization, rhinitis, and asthma in patients with allergic rhinitis: a study of Spain and Portugal. *J Investig Allergol Clin Immunol* 19, 167–172

van Tilburg Bernardes, E., Gutierrez, M. W., and Arrieta, M. C. (2020). The fungal microbiome and asthma. *Front. Cell. Infect. Microbiol.* 10:583418. doi: 10.3389/fcimb.2020.583418

van Woerden, H. C., Gregory, C., Brown, R., Marchesi, J. R., Hoogendoorn, B., and Matthews, I. P. (2013). Differences in fungi present in induced sputum samples from asthma patients and non-atopic controls: a community based case control study. *BMC Infect. Dis.* 13:69. doi: 10.1186/1471-2334-13-69

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., et al. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5:27. doi: 10.1186/s40168-017-0237-y

Yang, C., Mai, J., Cao, X., Burberry, A., Cominelli, F., and Zhang, L. (2023). ggpicrust2: an R package for PICRUSt2 predicted functional profile analysis and visualization. *Bioinformatics* 39:btad470. doi: 10.1093/bioinformatics/btad470

Yuan, H., Liu, Z., Dong, J., Bacharier, L. B., Jackson, D., Mauger, D., et al. (2023). The fungal microbiome of the upper airway is associated with future loss of asthma control and exacerbation among children with asthma. *Chest* 164, 302–313. doi: 10.1016/j.chest.2023.03.034

Zhang, M., Tang, H., Yuan, Y., Ou, Z., Chen, Z., Xu, Y., et al. (2023). The role of indoor microbiome and metabolites in shaping children's nasal and oral microbiota: a pilot multi-omic analysis. *Meta* 13:1040. doi: 10.3390/metabo13101040

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