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Dietary modulation of gut microbiota-X axis

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Dietary modulation of gut microbiota-X axis

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Editorial: Dietary modulation of gut microbiota-X axis

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Editorial on the Research Topic
Dietary modulation of gut microbiota-X axis

1 The gut microbiota-X axis: an integrative physiological framework

The concept of the "Gut Microbiota-X axis" signifies a fundamental shift in our understanding of how diet systemically influences host physiology via microbial mediation (1). This axis embodies a dynamic, multidirectional communication network in which gut commensals metabolize nutritional substrates into bioactive compounds—such as vitamins, neurotransmitters, and immunomodulators—that directly engage with extra-intestinal organs (2). Accumulating evidence establishes gut microbiota as key metabolic interpreters, translating dietary intake into molecular signals that modulate the neuro-endocrine-immune network (3).

Central to this paradigm is the role of the gut microbiota—a diverse ecosystem of bacteria, archaea, viruses, and fungi—which acts as a metabolic interface, converting dietary and host-derived compounds into bioactive molecules. Notable among these are microbiota metabolites like short-chain fatty acids (SCFAs; e.g., butyrate, propionate, and acetate), produced from dietary fiber fermentation, which function as epigenetic regulators through histone deacetylase (HDAC) inhibition (4). Similarly, microbial transformation of primary bile acids into secondary bile acids activates nuclear receptors such as FXR and TGR5, thereby modulating metabolic and inflammatory pathways systemically (5).

The immune system serves as a major conduit for this cross-organ communication. Microbial components and metabolites continuously interact with gut-associated lymphoid tissue (GALT), shaping immune cell differentiation (e.g., of regulatory T cells) and cytokine production, which in turn exert distal effects on organ inflammation and functionality (6). Additionally, the vagus nerve provides a direct neural pathway through which gut signals influence central nervous system activity, while endocrine mechanisms involving gut hormones such as peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) further integrate metabolic and cognitive functions (7). Dysbiosis—a disruption of microbial homeostasis—compromises this intricate exchange, often increasing intestinal permeability and permitting leakage of microbial products like lipopolysaccharide (LPS) into circulation (8). This can initiate a state of chronic low-grade inflammation, contributing to the pathophysiology of multiple diseases across organ systems, including Alzheimer's disease (via the gut-brain axis) (9), non-alcoholic fatty liver disease (via the gut-liver axis) (10), and psoriasis (via the

gut-skin axis) (11). Thus, the gut microbiota-X axis functions as a critical physiological integrator, wherein diet-microbiota interactions fine-tune systemic homeostasis and organismal resilience.

2 Thematic advancements: mechanistic elucidation and clinical translation

This Research Topic compiles cutting-edge research exploring the intricate bidirectional relationship between dietary components and the gut microbiota-X axis, emphasizing its profound implications for host health and disease pathogenesis. The collected studies employ diverse methodologies—ranging from preclinical animal models and randomized controlled trials (RCTs) to large-scale epidemiological analyses and systematic reviews—to elucidate specific mechanisms and therapeutic potentials. A prominent theme is the targeted use of probiotics to modulate gut microbial composition and function for health benefits. Key findings demonstrate the efficacy of specific strains, such as *Bifidobacterium adolescentis* CCFM1447 in mitigating osteoporosis by enriching intestinal bacteria capable of vitamin D conversion (Yu, Tian et al.), and *Bifidobacterium longum* subsp. *infantis* CCFM1426 enhances the anti-colitic effects of vitamin A through retinoic acid restoration and microbiota modulation in murine models of ulcerative colitis (Yu, Huang et al.). The translational potential of probiotics is further supported by an RCT showing specific formulations (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*; 3×10^9 CFU) effectively mitigate stress and inflammation in malnourished adults via gut microbiota modulation (Ahmadi-Khorram et al.). Beyond live bacteria, the issue explores broader dietary strategies, including granola with multiple prebiotics increasing *Bifidobacterium* abundance and improving stress/sleepiness in Japanese participants (Sasaki et al.), and the therapeutic application of fecal microbiota transplantation (FMT) in severe food intolerance (Huang, Huang et al.).

A second major focus involves the development and application of novel indices to quantify the relationship between diet, gut microbiota, and disease risk in human populations. Several studies leverage the US National Health and Nutrition Examination Survey (NHANES) data to establish significant associations between a proposed "dietary index for gut microbiota" and specific pathologies. Zhang X. et al. link this index to female infertility, while Wang et al. demonstrate its correlation with metabolic dysfunction-associated steatotic liver disease (MASLD). Most notably, Huang, Liu et al. establish an association between this dietary index and diabetes, proposing phenotypic age and body mass index (BMI) as significant mediators in this relationship, highlighting the complex interplay of diet, microbes, metabolic health, and aging. The critical role of microbial metabolites beyond vitamins is underscored in a systematic review and meta-analysis by Xie L. et al., which synthesizes preclinical evidence supporting the therapeutic potential of microbially-derived short-chain fatty acids (SCFAs) for acute lung injury. Furthermore, a review by Zhang R. et al. examines the intricate links between dietary habits, the gut-brain axis, and cognitive function, emphasizing the microbiota's role in neurobehavioral outcomes. Supporting this, Ding et al. present evidence suggesting early childhood probiotic intake may modify the association between prenatal micronutrient supplementation and neurobehavioral development in preschoolers. Finally, Xie Q. et al. review the emerging therapeutic potential of probiotics and postbiotics in targeting apoptosis for the treatment and prevention of digestive diseases. Collectively, this Research Topic significantly advances our understanding of how dietary components—from specific foods and supplements to broader dietary patterns—can be strategically manipulated to shape gut microbial communities and their functional outputs (vitamin conversion, SCFA production, immune modulation, neuroactive metabolite generation), thereby offering novel avenues for preventing and treating a wide spectrum of conditions including metabolic diseases, gastrointestinal disorders, osteoporosis, neurological impairments, and inflammatory states. The research highlights the move toward personalized nutrition and microbiota-targeted therapeutics while also identifying key areas for future investigation, such as refining dietary indices, elucidating strain-specific effects, and translating preclinical findings into robust clinical applications.

3 Future insights

Future research on the dietary modulation of the gut microbiota-X axis will likely focus on precision nutrition strategies that account for individual variability in microbiome composition, host genetics, and environmental factors. A key direction involves the development of targeted interventions using specific dietary components—such as prebiotics, polyphenols, and fermented foods—to selectively modulate microbial taxa and metabolic pathways that influence the X axis (e.g., brain, liver, or bone). Advanced multi-omics integration, including metagenomics, metabolomics, and transcriptomics, will be essential to elucidate mechanistic links between diet, microbial metabolites (e.g., SCFAs, bile acids, and tryptophan derivatives), and host physiological outcomes. Furthermore, long-term randomized controlled trials and longitudinal studies are needed to establish causal relationships and evaluate the sustainability of dietary interventions in improving health outcomes related to the microbiota-X axis.

Another promising area is the application of artificial intelligence and machine learning to analyze complex datasets and predict individual responses to dietary interventions. This approach could enable the design of personalized dietary recommendations that dynamically adapt to shifts in an individual's gut microbiome and health status. Additionally, there is growing interest in exploring the role of chrono nutrition and meal timing in modulating microbial rhythms and their interactions with host circadian biology. Future studies should also investigate the synergistic effects of diet and other lifestyle factors, such as exercise and stress management, on the microbiota-X axis. Ultimately, translating these insights into clinical practice and public health nutrition will require interdisciplinary collaboration and the development of functional foods or nutraceuticals that are evidence-based, effective, and accessible.

Author contributions

BL: Funding acquisition, Investigation, Project administration, Visualization, Writing – original draft. FB: Project administration, Validation, Visualization, Writing – review & editing. YY: Project administration, Resources, Validation, Visualization, Writing – review & editing.

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Inflammatory diet, gut microbiota and sensorineural hearing loss: a cross-sectional and Mendelian randomization study

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Aims: Inflammatory diets can trigger chronic inflammation and affect gut microbiota. However, the relationship between dietary preferences and sensorineural hearing loss (SNHL) remains unclear. This study aims to elucidate the relationship between different dietary preferences and sensorineural deafness.

Methods: The Dietary Inflammation Index (DII) and SNHL were defined by data from the National Health and Nutrition Examination Survey (NHANES), and exploring their relationship. Using Mendelian randomization (MR) to analyze the relationship between 34 dietary preferences, 211 gut microbiota, and SNHL.

Results: Smooth curve fitting indicated that the risk of SNHL increased with increasing DII score when the DII score was greater than 5.15. MR results suggest that a diet including both oily and non-oily fish can substantially reduce the risk of SNHL. Additionally, six specific gut microbiota were found to have significant causal relationship with SNHL.

Conclusion: An inflammatory diet may increase the risk of developing SNHL. The observed relationship between fish consumption, gut microbiota, and SNHL suggests the existence of a gut-inner ear axis.

KEYWORDS

inflammatory diet, gut microbiota, sensorineural hearing loss, NHANES, Mendelian randomization

1 Introduction

Hearing plays a critical role in the animal kingdom, aiding predators in locating their prey and helping animals avoid natural predators. In human society, hearing significantly impacts language and cognition. Research indicates that hearing loss in children can lead to impaired oral expression, delayed language development, and reduced literacy skills (1). In older adults, age-related hearing loss is common and can contribute to functional decline and loss of independence. Studies suggest that long-term hearing loss may lead to atrophy in certain brain areas, particularly the temporal lobe, which is responsible for auditory perception, language, and memory functions. Additionally, hearing loss can result in social isolation, increasing the risk of dementia (2). Unfortunately, hearing loss is a highly prevalent sensory disorder globally. Over 5% of the world's population, including 34 million children—require rehabilitation for

disabling hearing loss. It is estimated that by 2050, nearly 2.5 billion people will have some degree of hearing loss. Of these, more than 700 million will have disabling hearing loss (3). These issues pose significant economic and health challenges to society and public health. The etiology of sensorineural hearing loss (SNHL) is complex, often associated with aging, genetic mutations, noise exposure, ototoxic drugs, and degenerative processes linked to chronic diseases. Therefore, developing effective interventions to reduce or delay the onset of hearing loss remains a crucial component of the solution.

The relationship between inflammation and age-related diseases has received much attention in recent years. Unlike the acute inflammatory response, which is typically associated with infection and tissue injury and involves the recruitment of leukocytes and plasma proteins to the affected tissues, tissue stress or dysfunction triggers an adaptive response known as para-inflammation (4). This response is largely dependent on tissue-resident macrophages. Para-inflammation may be the culprit associated with modern human diseases. Chronic inflammation, a hallmark of immune senescence, is a mild inflammatory condition that exacerbates with age (5). Evidence of chronic inflammation has been observed in various models of aging-related diseases, including type II diabetes, cardiovascular disease, and Alzheimer's disease (6–8). Chronic inflammation is closely linked to macrophages. Recent studies have identified resident macrophages in the cochlea, particularly within the spiral ligament, spiral ganglion, and stria vascularis. These tissue-resident macrophages play a crucial role in the detection, phagocytosis, and clearance of cellular debris and pathogens, in addition to triggering inflammation and affecting tissue repair through the production of inflammatory cytokines and chemokines. Cochlear injury can activate these macrophages, initiating an immune response (9). Previous research has established a strong association between diet and systemic inflammation. The Mediterranean diet, in particular, is renowned for its anti-inflammatory effects (10). In contrast, the gut microbiota, regulated by dietary practices, plays a key role in the host's energy homeostasis, immune activity, and interactions with other body organs (11–14). Studies have confirmed that changes in gut flora are associated with the progression of SNHL (15). Pathological stress-induced inflammatory intestinal microenvironment may lead to the disruption of the intestinal barrier, which in turn allows metabolites and pro-inflammatory factors of the intestinal microbiota to be transferred to other organs, including the inner ear, via the body circulation (16). These findings may suggest the presence of a gut-inner ear axis. Given this background, our study aims to explore the association between dietary inflammation and sensorineural deafness.

Diet influences the risk of developing chronic diseases through multiple mechanisms, such as oxidative stress modulation, energy balance regulation, and alterations in gut microbiota (17, 18). These effects are due to dietary patterns and the pro- or anti-inflammatory properties of individual dietary components. Adopting a healthy dietary pattern and consuming nutrient-rich food groups have been shown to reduce inflammatory markers (19). The Dietary Inflammation Index (DII) offers a novel tool for exploring the inflammatory contributions of various dietary components. Although previous studies have established the role of the DII in the pathophysiology of neurodegenerative diseases, the relationship between inflammation-related sensorineural hearing loss and the DII remains unclear. To address this gap, we explored the relationship between DII and SNHL using National Health and Nutrition Examination Survey (NHANES) data. Additionally, we conducted two multi-omics Mendelian randomization (MR) studies to investigate the effects of different dietary preferences and gut microbiota on SNHL.

2 Methods

Our study comprises two main parts. First, we utilized dietary and hearing data from the NHANES database to define the DII and assess the presence of SNHL in subjects. Using multivariate logistic regression, smoothed curve analysis, and subgroup analyses, we investigated the relationship between DII and SNHL. Second, the instrumental variables (IVs) were extracted from genome-wide association studies (GWAS) related to dietary preferences. We used a two-sample MR method to assess the effect of various dietary preferences on susceptibility to SNHL. Similarly, we extracted IVs for 211 species of gut microbiota to explore the causal relationship between human gut microbiota and SNHL, thereby corroborating the existence of the gut-inner ear axis.

2.1 Cross-sectional study

2.1.1 Description of data sources

NHANES is a national program designed to assess the health and nutritional status of Americans. Conducted biennially, NHANES boasts a sample size of approximately 5,000 individuals and adheres strictly to research ethics principles. To explore the relationship between DII and SNHL, data from five NHANES cycles, 2007 through 2012 and 2015 through 2018, were used. These cycles were chosen due to their comprehensive coverage of the variables necessary for both dietary and hearing data, with all data meticulously processed using standardized protocols. Our analyses adhered rigorously to predetermined exclusion criteria, which encompassed individuals under the age of 19, those exhibiting abnormal findings in the tympanic chamber or otoscopic examinations, as well as those lacking complete dietary, hearing, or covariate information. Initially, our participant pool comprised 49,667 individuals. However, after applying these stringent exclusion criteria, our study ultimately encompassed 3,673 participants (Figure 1).

2.1.2 Diet inflammatory index

The DII is a validated tool derived from literature, comprising 45 food parameters recognized for their anti-inflammatory or pro-inflammatory properties. It serves to standardize the classification of individual dietary components. This tool has been employed to forecast levels of inflammatory markers across various datasets and populations. Consistent with prior research, we extracted 26 relevant food parameters from the NHANES database and computed the DII for each dietary component using a standardized method. This calculation involved determining the standard deviation of (daily intake - global mean daily intake) divided by the global mean daily intake, multiplied by the overall inflammatory effect score of the respective dietary component (20). The summation of these individual DIIs yields the subject's overall DII. Detailed information on the dietary components utilized in calculating the DII is provided in Supplementary Table S1.

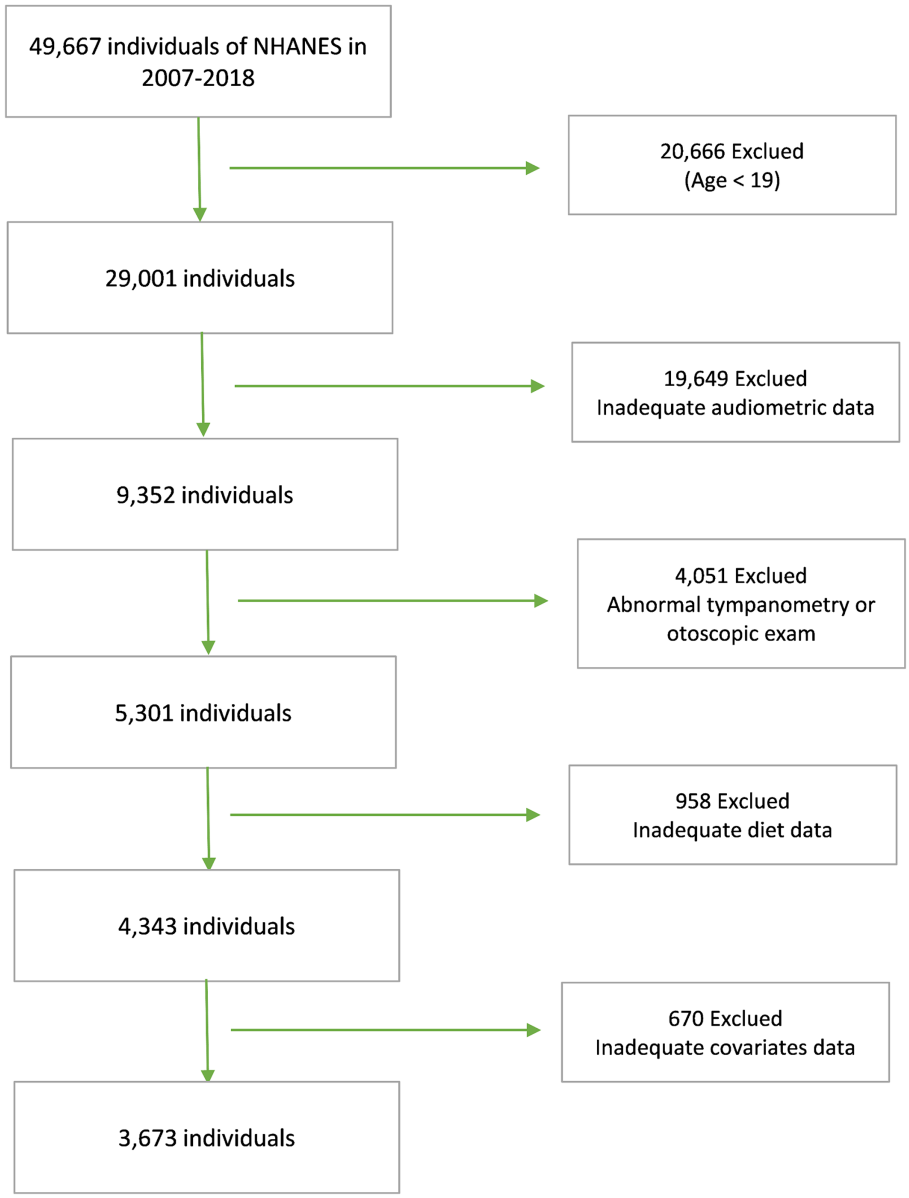

FIGURE 1
Flow chart of study participants.

2.1.3 SNHL defined

SNHL can be defined when the mean pure tone hearing threshold exceeds 20 dB and potential mixed or conductive hearing loss is excluded (21). In accordance with established criteria and prior literature, the following parameters were employed to diagnose SNHL in this study: mean pure tone hearing thresholds exceeding 20 dB, normal otoscopic findings, peak conductance of at least 0.3 mL, tympanograms indicative of type A, and denial of a history of colds within 24 h (22). The NHANES audiometric assessment program encompasses audiometric questionnaires, otoscopy, pure-tone air-conduction audiometry, and tympanometry. Trained examiners administer all components of the audiometric examination to participants within a dedicated soundproof room at the Mobile Examination Center (MEC). During audiometry, careful evaluation of air-conduction thresholds is conducted for each ear across seven frequencies, with intensity levels ranging from −10 to 120 dB. Additionally, thresholds for each ear are repeated at 1,000 Hz, spanning an intensity range of −10 to 120 dB. If the disparity between the results of the two tests exceeds 10 dB, the results are deemed unacceptable; conversely, if the variance falls within 10 dB, the outcomes of the initial 1,000 Hz test are utilized.

2.1.4 Covariates

The covariates used in this study were derived from demographic and health-related data in NHANES and included age, race, gender, household income, educational attainment, poverty rate, diabetes status, and body mass index (BMI). In addition, serum cotinine has a longer half-life in the blood and is viewed as a marker of active smoking (23, 24). Detailed data for all variables in the study are available at www.cdc.gov/nchs/nhanes/.

2.2 Mendelian randomization

2.2.1 Data availability

The MR analysis utilized publicly available data. GWAS data pertaining to dietary patterns were sourced from the GWAS Catalog, specifically entries GCST90096892-GCST90096929 (25). These datasets comprised information from 445,779 participants enrolled in the UK Biobank, identifying 283 genetic markers associated with dietary intake. To isolate direct genetic effects on food exposure, a total of 38 GWAS for dietary preferences were identified after adjusting for effects mediated through other traits. Detailed information is provided in Supplementary Table S1. GWAS datasets concerning the composition of the human gut microbiota were from the international consortium MiBioGen (26). This extensive GWAS study involved 24 ethnic cohorts and included genotyping data from 18,340 participants, exploring associations between human genetic variation and the gut microbiota. The GWAS analyses included a total of 211 taxa covering 131 genera, 35 families, 20 orders, 16 classes, and 9 phyla.

Furthermore, GWAS data related to SNHL were accessible from the FinnGen R9 consortium, comprising 32,487 cases and 331,736 controls. Within this dataset, sensory deafness was defined as hearing loss originating from the inner ear or sensory organs or the vestibular nerve.

2.2.2 Selection of IVs

We screened for independent single nucleotide polymorphisms (SNPs) that constitute instrumental variables (IVs) associated with 38 dietary preferences and 211 human gut microbiota. To select independent genetic variants, genome-wide significant SNPs were grouped by linkage disequilibrium (LD) ($r^2 < 0.001$ for SNPs within a 1 Mb genomic region). Since the gut microbiota GWAS was unable to screen a sufficient amount of IVs after the stringent screening criteria described above, we followed the same criteria as previous studies and relaxed the correlation screening criteria to $p < 1 \times 10^{-5}$ (27, 28). Finally, a two-sample MR was performed using IVs separately and SNHL GWAS data.

2.2.3 Statistical analysis

MR uses genetic variation as a tool to test the causal relationship between an exposure (dietary preference and gut microbiota) and an outcome (SNHL) that requires three core assumptions to be met. MR estimates for each risk factor were determined using inverse variance weighted (MR-IVW) analysis as the primary means of MR analysis, which uses random effects meta-analysis to combine Wald ratio estimates of causal effects obtained from each SNPs tested. We conducted a series of sensitivity analyses, including MR-Egger, weighted median, and heterogeneity tests to test the underlying assumptions of MR.

3 Results

3.1 Cross-sectional study

3.1.1 Baseline characteristics

In our study, data were collected from a total of 3,673 participants, with a mean age of 45.83 ± 16.17 years. Among these participants, 47.07% were males and 52.93% were females. As depicted in Table 1, we categorized the survey-weighted participant characteristics into two groups based on disease status: SNHL and normal. Of the total participants, 1,742 (47.43%) exhibited SNHL. A comparative analysis revealed that individuals with SNHL were more likely to belong to older age groups, male gender, non-Hispanic white ethnicity, have lower levels of educational attainment, be at risk for diabetes, have a history of tobacco use, and possess a higher body mass index, in comparison to those without SNHL.

3.1.2 The association between DII and SNHL

Our findings revealed that there was no significant correlation between DII and SNHL in either the unadjusted logistic regression model or the multivariate logistic regression model after accounting for various covariates (Table 2). However, when attempting to model the nonlinear relationship between SNHL and DII by fitting a smoothed curve, we observed a similar U-shaped correlation between DII score and SNHL, even after adjusting for different covariates. In particular, through a threshold effect analysis, we noted that the risk of hearing loss was associated with higher DII scores when the DII score exceeded 5.15 (Table 3).

TABLE 1 Clinical characteristics of all 3,673 subjects among subjects with SNHL and without SNHL.

Characteristics	Control	SNHL	*p*-value
	n = 1931	*n* = 1742	
Age, years	35.97 ± 11.04	55.15 ± 13.44	<0.0001
Gender (%)			<0.0001
Male	42.18	52.99	
Female	57.82	47.01	
Race (%)			<0.0001
Mexican American	9.51	6.35	
Other Hispanic	7.03	5.43	
Non-Hispanic White	64.64	76.06	
Non-Hispanic Black	11.27	6.94	
Other race	7.56	5.23	
Education level (%)			<0.0001
<9th grade	2.55	4.51	
9–11th grade	7.03	9.63	
High school grade/GED or equivalent	15.97	23.70	
Some college or AA degree	32.87	30.52	
College graduate or above	41.58	31.64	
Diabetes mellitus (%)	3.37	14.08	<0.0001
PIR, mean	3.03 ± 1.66	3.19 ± 1.61	0.0038
Cotinine, ng/mL	41.75 ± 103.32	56.00 ± 132.64	0.0003
BMI, kg/m^2	28.42 ± 6.94	29.84 ± 6.60	<0.0001

Mean ± SD for continuous variables: the *p*-value was calculated by weighted linear regression model. PIR, the ratio of family income to poverty; BMI, body mass index; SNHL, sensorineural hearing loss.

TABLE 2 The associations between DII and SNHL.

Exposure	Model I OR (95% CI) *P*	Model II OR (95% CI) *P*	Model III OR (95% CI) *P*
DII	0.99 (0.97, 1.00) 0.0164	1.01 (0.99, 1.02) 0.2270	0.99 (0.98, 1.01) 0.3526

Model I: None covariates were adjusted. Model II: Gender, age and race were adjusted. Model III: Gender, age, race, education level, PIR, Cotinine, BMI and Diabetes were adjusted. PIR, the ratio of family income to poverty; BMI, body mass index; DII, Dietary Inflammation Index; SNHL, sensorineural hearing loss.

TABLE 3 Threshold effect analysis of DII and SNHL.

	Adjusted HR (95% CI), *p*-value
Fitting by the standard linear model	0.99 (0.98, 1.01) 0.3526
Fitting by the two-piecewise linear model	
Inflection point	5.15
DII < 5.15	0.98 (0.96, 1.00) 0.0305
DII ≥ 5.15	1.14 (1.04, 1.26) 0.0068
P for Log-likelihood ratio	0.004

Gender, age, race, education level, BMI, PIR, Cotinine and Diabetes were adjusted. PIR, the ratio of family income to poverty; BMI, body mass index; DII, Dietary Inflammation Index; SNHL, sensorineural hearing loss.

3.1.3 Subgroup analyses

No interaction between the unfavorable correlation between DII and SNHL with age, gender and diabetes was found in this study (Table 4).

3.2 Mendelian randomization

We used three methods to assess the causal relationship between dietary preferences and SNHL, with IVW as the primary means of MR analysis. Thirty four dietary preferences and 211 gut microbiota were screened for SNPs used for genetic prediction. The F-statistics of these genetic tools were all greater than the threshold of 10, indicating stronger tools.

After a comprehensive MR analysis, we found that a fish diet significantly reduced the risk of sensorineural deafness. Among them, consumption of non-oily fish (OR = 0.068, 95%CI: 0.018–0.259, $p = 8.541 \times 10^{-5}$) as well as oily fish (OR = 0.558, 95%CI: 0.386–0.807, $p = 1.921 \times 10^{-3}$) showed a protective effect on hearing. The *p*-value of MR-Egger intercept for both positive MR analyses was greater than 0.05, indicating that no pleiotropy was detected. The p-value of the Cochran Q test for the non-oily fish diet was 0.323, but the p-value of the Cochran Q test for the oily fish diet was less than 0.05, which may indicate heterogeneity. However, the heterogeneity was acceptable because the IVW method of random effects model was used as an assessment tool in this study (29).

In addition to this, after performing 211 MR analyses, we screened out six gut microbiota that were significantly associated with SNHL, with varying effect values. The genus *RikenellaceaeRC9gutgroup* (OR = 1.056, $p = 0.025$), genus *Bifidobacterium* (OR = 1.131, $p = 0.004$), and family *Porphyromonadaceae* (OR = 1.159, $p = 0.018$) showed positive correlation for SNHL; The phylum *Verrucomicrobia* (OR = 0.912, $p = 0.015$), genus *Flavonifractor* (OR = 0.878, $p = 0.021$), and family *Streptococcaceae* (OR = 0.919, $p = 0.047$) showed negative correlation for SNHL. Detailed information on all positive endpoints is displayed in Table 5. We performed sensitivity analyses on all results, and scatter plots, funnel plots, and "leave one-out analysis" plots can be viewed in Supplementary Figure S1.

TABLE 4 Subgroup analysis for the association between DII and SNHL.

Subgroup	OR (95%CI)	*P* for interaction
Gender		0.8064
Male	0.99 (0.98, 1.01) 0.3830	
Female	1.00 (0.98, 1.02) 0.7329	
Age		0.6260
<65	0.97 (0.97, 0.99) <0.0001	
≥65	0.99 (0.92, 1.07) 0.8666	
Diabetes status		0.9231
Yes	0.98 (0.94, 1.03) 0.5155	
No	0.98 (0.97, 0.99) 0.0043	

DII, Dietary Inflammation Index; SNHL, sensorineural hearing loss.

4 Discussion

In this study, we analyzed data from large observational studies and GWAS to evaluate the influence of inflammatory dietary preferences on SNHL. We then conducted a multi-omics MR analysis to investigate the causal impact of gut microbiota on SNHL. Our analysis revealed that an inflammatory diet indeed raises the risk of SNHL. Subsequently, our MR analysis found that a diet rich in fish significantly reduced the risk of SNHL. In addition, we identified 6 specific gut microbiota that were significantly associated with SNHL. Overall, our study confirms the strong relationship between diet and SNHL and provides compelling evidence supporting the existence of a gut-inner ear axis.

Inflammation underlies many pathophysiological processes, typically triggered by infection and injury. Recently, the concept of quasi-inflammation has gained attention. This adaptive response occurs when tissues react to various stimuli—such as pathogens, cellular debris, nutrients, and intestinal microbiota—in a manner that lies between homeostasis and classical inflammation (4). Quasi-inflammation may contribute to numerous chronic diseases in modern humans, partly due to shifts in the body's homeostatic set-points (e.g., insulin sensitivity) (4). Unlike acute inflammation, which requires significant tissue damage or infection, quasi-inflammation is prompted by tissue dysfunction and aims to restore function and homeostasis. Aging and gut microbiota dysbiosis are linked to para-inflammation, with age-related immune deficiencies leading to unresolved inflammatory processes. Both aging and high-fat diets increase intestinal permeability, allowing leakage that can provoke systemic inflammation (16, 30–32). It has been proposed that inflammation from gut dysbiosis accelerates age-related cochlear degeneration (33). Diet plays a crucial role in regulating individual inflammation levels. Several randomized controlled trials have shown that the Mediterranean diet is associated with lower concentrations of the inflammatory marker C-reactive protein (CRP) (34), whereas

TABLE 5 Significant estimates for MR analysis.

Exposure	Outcome	IVW-derived *p*-value	OR (95% CI)	Cochran's Q-derived *p*-value	MR-Egger intercept derived *p*-value
Non-oily fish consumption	SNHL	8.541×10^{-5}	0.068 (0.018–0.259)	0.323	0.796
Oily fish consumption	SNHL	1.921×10^{-3}	0.558 (0.386–0.807)	5.358×10^{-4}	0.374
Verrucomicrobia	SNHL	0.015	0.912 (0.848–0.982)	0.879	0.624
RikenellaceaeRC9gutgroup	SNHL	0.025	1.056 (1.007–1.108)	0.541	0.399
Flavonifractor	SNHL	0.021	0.878 (0.786–0.980)	0.323	0.515
Bifidobacterium	SNHL	0.004	1.131 (1.039–1.230)	0.056	0.586
Streptococcaceae	SNHL	0.047	0.919 (0.845–0.999)	0.196	0.510
Porphyromonadaceae	SNHL	0.018	1.159 (1.026–1.310)	0.397	0.614

SNHL, sensorineural hearing loss; IVW, inverse variance weighted.

unhealthy foods that are high in energy, fat and sugar, and low in dietary fiber may contribute to local and systemic inflammation (35). And it has long been shown that chronic inflammation is associated with most human pathologies, such as cancer and autoimmune and neurodegenerative diseases (36). We also chose the DII because the index is universal, involving the 6 most commonly studied markers of inflammation, and can be used as a summary measure of diet-related inflammation in any population (37). According to our results, the risk of hearing impairment increases when the DII exceeds a certain threshold, which is consistent with the previous belief that an inflammatory diet leads to chronic disease. Based on previous studies and our current analysis of the NHANES data, we believe that reducing inflammatory diets and optimizing dietary strategies are essential for the prevention and control of chronic diseases, including age-related hearing loss. Moreover, the protective effect of a fish-rich diet against sensorineural deafness has been consistently supported by observational studies (38, 39). Good blood flow within the cochlea is crucial for ensuring the delivery of adequate oxygen and glucose while efficiently removing metabolic byproducts. An impaired blood supply to the cochlea can disrupt the maintenance of intracochlear potential, endolymphatic fluid balance, and the integrity of the blood-labyrinth barrier (BLB). These disruptions can lead to hypoxic–ischemic damage to hair cells and subsequent hearing loss (40). Additionally, cochlear hypoperfusion-induced mitochondrial DNA damage and chronic inflammation have been associated with age-related hearing loss (41, 42). The intake of fish and long-chain omega-3 polyunsaturated fatty acids may help mitigate these impairments and slow the progression of age-related hearing loss. This protective effect occurs through various mechanisms, including improving vascular reactivity and endothelial function, preventing thrombosis and inflammation, modulating membrane ion channels and electrophysiological responses to ischemic stress, influencing gene expression, and reducing pro-inflammatory or pre-thrombotic eicosanoids derived from arachidonic acid (43). Our study aligns with the findings of a previous prospective study that followed 1,038,093 subjects over several years. This study demonstrated that consuming two or more servings of fish per week reduced the risk of hearing loss, and that higher intake of long-chain omega-3 polyunsaturated fatty acids was also negatively associated with the risk of hearing loss (39). However, it is noteworthy that the prior study's conclusions were based on US females, and our MR analyses did not perform gender subgroup analyses. Additionally, the previous study confirmed that consumption of any type of fish tended to reduce the risk of hearing loss, suggesting that the benefits may be largely attributable to the omega-3 fatty acids found in fish. Interestingly, our study found that the consumption of non-fatty fish provided more hearing protection than fatty fish, indicating that other potential protective pathways might exist. These pathways may involve complex interactions between various nutrients in fish that act synergistically with omega-3 fatty acids to protect against hearing loss. This finding complements previous observational studies and suggests that a diet rich in fish can be an effective strategy for preserving hearing health.

The balance of the gastrointestinal tract is closely linked to the health of many distal organs. For example, the gut-brain axis and gut-lung axis are well-established, and gut microbiota play a crucial role in these interactions (44–47). Bacteria within the gut can exhibit both pro-inflammatory and anti-inflammatory properties, and the gut microbiota responds dynamically to different diets. When the balance between microorganisms is disturbed, it can lead to ecological imbalances and various inflammatory responses, increasing the host's susceptibility to diseases (48). The gut microbiota communicates with the host through multiple pathways, including microbial metabolites, tryptophan metabolism and the immune system. These interactions can trigger the secretion of chemokines, neurotransmitters, cytokines, neuropeptides, endocrine messengers and microbial byproducts. These molecules can enter the vascular and lymphatic systems, influencing neural signals transmitted by vagal and spinal afferents. Chronic systemic inflammation can result from dysbiosis of the intestinal flora, leading to disrupted gut ecology, increased intestinal barrier permeability, and the infiltration of pathogens and microbial solutes into the bloodstream (30). This chronic inflammation can compromise the integrity of the blood–brain barrier (BBB), allowing pathogens and pro-inflammatory cytokines to infiltrate the brain, which can lead to neuroinflammation and neurodegeneration (49–51).

The BLB in the stria vascularis of the inner ear consists of pericytes (PCs), vascular endothelial cells (ECs), and Perivascular-resident macrophage-like melanocytes, is analogous to the BBB, making it susceptible to the influence of metabolites from the gut microbiota and pro-inflammatory molecules. These substances can penetrate the BLB, potentially compromising cochlear integrity and leading to inflammation and injury (16, 52). Dysfunction of the BLB due to inflammation can reduce its ability to restrict the entry of inflammatory or infectious agents and immune cells into the cochlea,

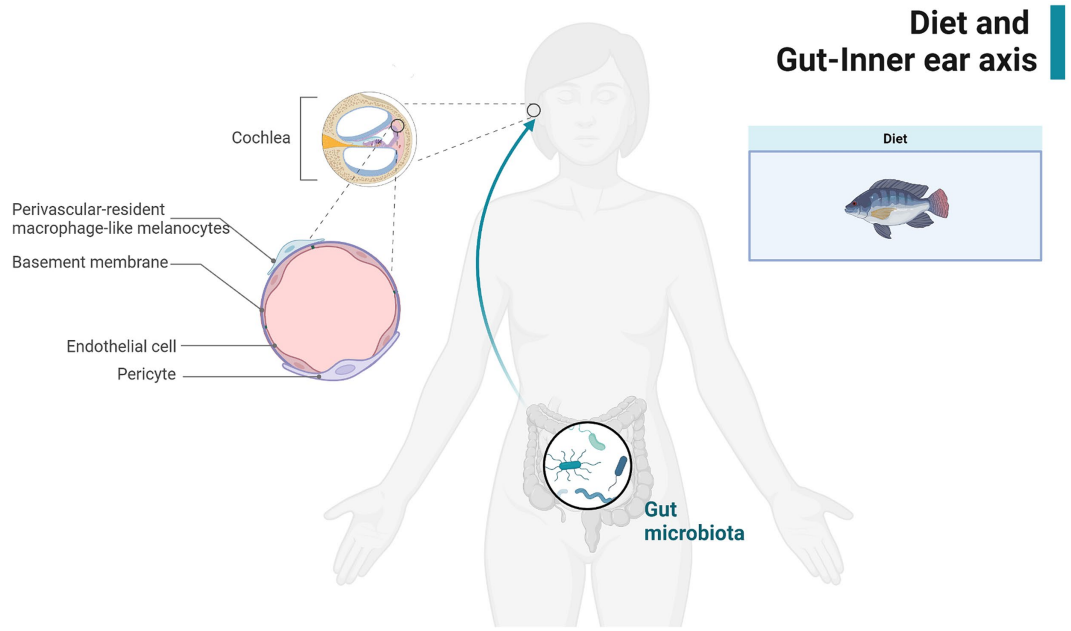

FIGURE 2
Demonstration of the gut-inner ear axis. Created with BioRender.com.

thereby exacerbating cochlear damage (53, 54). Nearly all major causes of acquired hearing loss share similar pathogenic mechanisms, suggesting a potential link between dietary preferences, gut microbes, and inner ear health (Figure 2). Three of the six gut microbiota found in our study to be significantly associated with SNHL increase susceptibility to SNHL. The association between the *RikenellaceaeRC9gutgroup* and inflammation and obesity has long been established (55, 56). The *Porphyromonas* family is also thought to be associated with increased intestinal permeability and a shift in features associated with inflammatory diseases (57). However, it is puzzling that *Bifidobacteria* associated with anti-inflammation proved in our study to also increase susceptibility to SNHL. Inflammatory diets have been shown to decrease *Flavonifractor* abundance thereby affecting glucose homeostasis and increasing the systemic inflammatory state (58). This is the same conclusion as our current study. This relationship may be explained by chronic inflammation and the emerging concept of the gut-inner ear axis. For instance, an observational study found that the prevalence of high-frequency hearing loss was significantly higher in obese adolescents compared to their normal-weight peers (59). Additionally, serum triglycerides and blood glucose levels have been associated with sensorineural hearing loss, potentially related to cochlear blood supply (60). Our study adds to the growing body of evidence supporting the gut-inner ear axis by elucidating possible mechanisms linking gut microbiota and dietary factors to cochlear health. This connection underscores the importance of maintaining gut health to prevent or mitigate hearing loss.

Our study has significant strengths. First, using a large sample from NHANES, we defined the DII index using dietary data and inferred a potential relationship between the DII index and sensorineural deafness. In addition, we further inferred the effect of dietary preference and gut microbiota on susceptibility to SNHL at the genetic level using MR analysis, excluding confounders, and based on this we confirmed and delved into the existence of the gut-inner ear axis. This is a more complete study of the correlation between diet and SNHL at present. Our study also has limitations. First, data obtained from dietary questionnaires in observational studies may be biased. Dietary questionnaires may have errors in reporting and recall, subjects with lower DII scores may have healthier dietary and lifestyle habits, and despite our adjustment for covariates, there are possible confounders that could not be controlled for. Second, the *p*-values of both multi-omics MRs for dietary preference and gut flora were not corrected for, which may present the possibility of false positives. However, previous histologic MR studies have concluded that even if the corrected p-value is greater than 0.05, it should still be considered suggestive of a potential association (61, 62). The GWAS data in this study were all from European populations, so the conclusions from the MR analyses apply only to Europeans, and similarly, the NHANES data were from US populations, and the conclusions about this section apply only to Americans. The generalizability of the conclusions would need to be verified in the future using other populations (e.g., Asian or African). In the end, the present study only explored the relationship between inflammatory diet and gut flora and SNHL from observational studies and genetic perspectives, and more basic studies are needed in the future to confirm and elucidate the specific mechanisms and pathways of these causative relationships, so as to establish a detailed mechanism of the gut-inner ear axis.

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

YW: Writing – review & editing, Writing – original draft. JN: Writing – original draft, Software, Methodology. KY: Writing – original draft, Validation, Software, Data curation. JW: Writing – original draft, Data curation. XW: Writing – review & editing. YZ: 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/fnut.2024.1458484/full#supplementary-material

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Association of dietary live microbe intake with kidney stone disease in US adults: a real-world cross-sectional study

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Background: Kidney stone disease (KSD) is a common urological condition linked with hypertension, chronic kidney disease, and other health issues. Although the gut microbiome has a notable association with KSD formation, the relationship between dietary live microbes and KSD risk remains underexplored.

Methods: This study utilized data from the NHANES surveys conducted between 2007 and 2016 to analyze the association between dietary live microbe intake and KSD. Dietary intake data were obtained through 24-h dietary recall interviews conducted by trained professionals. Participants were categorized into three groups based on Sanders' classification system of dietary live microbe intake: low, medium, and high. The intake levels were determined by estimating the live microbe content in foods. Weighted logistic regression analysis was employed to account for the complex survey design and to assess the impact of different levels of live microbe intake on KSD risk.

Results: A total of 20,380 participants were included in the study. Participants with low, medium, and high dietary microbe intake represented 33, 39, and 28% of the cohort, respectively. The adjusted odds ratios (ORs) for KSD were 0.78 (95% CI, 0.65–0.93) in the high dietary live microbe group compared to the low group ($p < 0.05$). Subgroup analyses revealed no significant interactions between dietary live microbe intake and gender, age, BMI, hypertension, or diabetes status.

Conclusion: Higher dietary live microbe intake group may be associated with a reduced risk of KSD. Further prospective studies are necessary to validate these findings and to elucidate the specific mechanisms and optimal intake levels of dietary microbes.

KEYWORDS

dietary live microbes, kidney stone disease, food-gut-health axis, NHANES, cross-sectional study

1 Introduction

Kidney stone disease (KSD) is one of the most common urological disorders, characterized by the deposition of inorganic substances (such as crystalline salts) and organic components (such as urinary macromolecules) in the renal parenchyma or pelvic system, leading to stone formation (1). Globally, the prevalence of KSD is continuously increasing (2). Epidemiological studies indicate that over 10% of the U.S. population is affected by kidney stones, with a high recurrence rate exceeding 50% (3, 4). Individuals with kidney stones are also at increased risk

for hypertension (5), chronic kidney disease (6), and renal cancer (7). The economic burden associated with KSD has also escalated, with costs in the U.S. rising from an estimated $2 billion in 2000 to over $10 billion in 2006 (1). These challenges underscore the urgent need for effective prevention strategies.

Given that KSD formation is commonly associated with diet, metabolic disorders, genetic factors, environmental influences, and underlying medical conditions, it is increasingly recognized as a chronic metabolic disease (8). The human microbiome, which encompasses all microorganisms living in and on the human host, is a key area of study, particularly the gut microbiome. Functionally, it interacts with host cells to perform various biological processes and regulate overall host metabolism (9). The gut microbiome is significantly influenced by diet, and differences in gut microbial communities contribute to the formation of kidney stones (10, 11).

Consuming safe, live microorganisms through daily diet may interact with the mucosal surfaces of the digestive tract, modulate the immune system, enhance gut function, and improve the body's ability to reduce susceptibility to chronic diseases (12). Marco et al. proposed a classification system using NHANES data to define and estimate dietary intake of live microbes. They suggested assessing existing evidence from dietary databases to quantify the health benefits of consuming live microbes, leveraging data from observational studies like NHANES (12, 13). Based on this classification, literature has explored the associations between dietary live microbes and conditions such as depression (14), cognitive function (15), sarcopenia (16), and cardiovascular diseases (17).

However, no studies have yet evaluated the relationship between dietary live microbe intake and kidney stones. Therefore, this study aims to explore the association between dietary live microbe intake and KSD using NHANES data from 2007 to 2016.

2 Materials and methods

2.1 Study population

The NHANES database comprises a series of studies designed to evaluate the health and nutritional status of individuals in the United States. To investigate the association between dietary live microbe intake and kidney stone disease (KSD), data from six NHANES cycles (2007–2016) were used to obtain an adequate sample size. Individuals were excluded if they lacked information on dietary live microbe intake or KSD status, were under 20 years old, or had incomplete data on key covariates. These covariates included age, gender, race, poverty income ratio (PIR), marital status, education level, smoking and drinking status, body mass index (BMI), waist circumference, diabetes, hypertension, total energy intake, total water intake, total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), serum creatinine (Scr), blood urea nitrogen (BUN), and uric acid (UA). Ultimately, 20,380 participants were included in the study (Figure 1).

2.2 Dietary live microbe data

Dietary intake data were obtained through face-to-face interviews conducted by trained professionals using the 24-h dietary recall method to collect information on all consumed foods and beverages. While this method is subject to recall bias due to participants' reliance on memory, NHANES employs rigorous quality control procedures to mitigate such bias. Interviewers are trained to assist participants in accurately recalling their dietary intake, using tools such as portion size models, detailed probing questions, and multiple pass methods to reduce underreporting or overreporting (18). Additionally, NHANES uses automated systems to minimize interviewer bias and standardize data collection (19).

The NHANES database includes 9,388 food codes categorized into 48 subgroups. A panel of four experts in the field estimated the live microbe content (per gram) for these food items. Foods were classified into three levels of live microbe content: low (<10^4 CFU/g), medium (10^4–10^7 CFU/g), and high (>10^7 CFU/g). The three participants groups of dietary live microbe were categorized as follows in this study: Low dietary live microbe group (all foods are Lo); Medium dietary live microbe group (any foods are Med but not Hi); High dietary live microbe group (any foods are Hi) (13, 14).

2.3 Covariates

The analysis considered the following covariates: sex, age, marital status, education level, race, PIR, diabetes, hypertension, total energy intake, total water intake, BMI, waist circumference, smoking status, and drinking status. Additionally, laboratory measurements provided information on TC, TG, HDL, Scr, BUN, and UA.

2.4 Definition of KSD

The primary outcome measure was whether participants had a history of kidney stone disease (KSD). Data related to KSD during the selected cycles were collected in participants' homes using the Computer-Assisted Personal Interview (CAPI) system, conducted by extensively trained interviewers. Participants were asked, "Have you ever had kidney stones?" If they responded affirmatively, they were classified as having KSD.

2.5 Statistical analysis

According to NHANES analysis guidelines, our analysis considered the complex sampling design and sample weights from the mobile examination centers. Data analysis was performed using the "survey" package in R to conduct weighted analyses, which account for the complex survey design of NHANES. Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables were expressed as numbers (percentages). All means and SDs for continuous variables and percentages for categorical variables were weighted. Baseline characteristics between those with and without KSD were compared using t-tests or Mann–Whitney tests for continuous variables and chi-square tests for categorical variables. Weighted univariate analyses were conducted to identify variables that differed significantly between subjects with and without KSD. Additionally, weighted multivariable regression analyses were performed to evaluate the association between dietary live microbe group and KSD, calculating odds ratios (OR) and 95% confidence

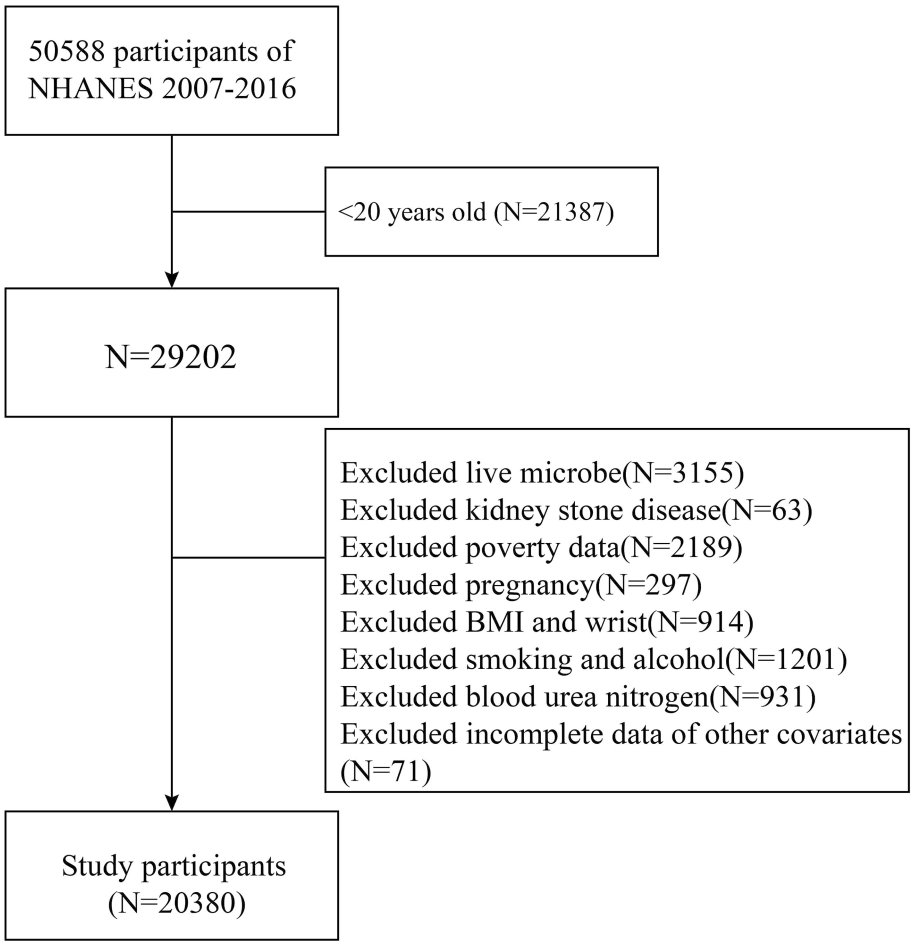

FIGURE 1
Participant flowchart.

intervals (CI) to describe these associations. To avoid bias due to the deletion of samples with missing covariates, we conducted multiple imputations as a sensitivity analysis. Stratified analyses and interaction tests were conducted for specific variables, including sex, age (<40 or ≥ 40 years), diabetes status (no or yes), hypertension status (no or yes), and BMI, to assess potential modifiers of the relationship between dietary live microbe group and KSD.

All analyses were conducted using R Statistical Software (The R Foundation).[1] A p-value <0.05 was considered statistically significant.

3 Results

3.1 Baseline characteristics of the included participants

Table 1 presents the weighted demographic and medical characteristics of participants stratified by the presence of kidney stone disease (KSD). Overall, the study included 20,380 participants with a weighted mean age of 47.4 years. The overall prevalence of KSD was 9.7%. The prevalence of KSD in the low, medium, and high dietary live microbe groups was 11.0, 9.7, and 8.6%, respectively. Significant differences ($p < 0.05$) were observed between individuals with and without KSD in terms of age, BMI, waist circumference, sex, race, marital status, drinking status, smoking status, hypertension, diabetes, dietary live microbe intake group, BUN, UA, Scr, TC, TG, and HDL levels. Participants with KSD exhibited higher values in the following characteristics: age (46.7 ± 16.7 vs. 53.2 ± 15.6 years, $p < 0.05$), BMI (28.75 ± 6.62 vs. 30.47 ± 7.00 kg/m^2, $p < 0.05$), and waist circumference (98.61 ± 16.19 vs. 104.57 ± 16.67 cm, $p < 0.05$). They also had higher rates of diabetes (30% vs. 45%, $p < 0.05$) and hypertension (8.3% vs. 18%, $p < 0.05$), as well as elevated levels of BUN, UA, Scr, TC, TG, and lower levels of HDL ($p < 0.05$ for all comparisons).

3.2 Univariate analysis

Table 2 lists the results of the univariate analysis. The study found that the risk of kidney stone disease (KSD) increased with higher age, BMI, waist circumference, BUN, Scr, and UA levels ($p < 0.05$). Additionally, the presence of hypertension and diabetes was associated with an increased risk of KSD ($p < 0.05$). Compared to females, males

1 http://www.R-project.org

TABLE 1 Basic characteristics of participants by kidney stones disease among US adults.

		KSD		
Characteristic	Total (*N* = 20,380)	No (*N* = 18,403)	Yes (*N* = 1977)	*p* Value
Age (years)	47.4 ± 16.7	46.7 ± 16.7	53.2 ± 15.6	<0.001
BMI(Kg/m2)	28.91 ± 6.68	28.75 ± 6.62	30.47 ± 7.00	<0.001
Waist, cm	99.19 ± 16.33	98.61 ± 16.19	104.57 ± 16.67	<0.001
Gender, n (%)				<0.001
Female	10,146 (51%)	9,297 (51%)	849 (43%)	
Male	10,234 (49%)	9,106 (49%)	1,128 (57%)	
Race, n (%)				<0.001
Mexican American	3,075 (15.1%)	2,813 (15.3%)	262 (13.3%)	
Non-Hispanic Black	4,025 (19.7%)	3,786 (20.6%)	239 (12.1%)	
Non-Hispanic White	9,244 (45.4%)	8,111 (44.1%)	1,133 (57.3%)	
Other Hispanic	2,074 (10.2%)	1,854 (10.1%)	220 (11.1%)	
Other Race	1,962 (9.6%)	1,839 (9.8%)	123 (6.2%)	
Education, n (%)				0.6
High school or equivalent	4,657 (22%)	4,214 (22%)	443 (23%)	
Less than high school	4,768 (15%)	4,270 (15%)	498 (16%)	
More than high school	10,955 (63%)	9,919 (63%)	1,036 (61%)	
PIR (%)	3.01 ± 1.66	3.01 ± 1.67	3.00 ± 1.61	0.8
Marital Status, n (%)				<0.001
Having a partner	12,212 (63%)	10,930 (62%)	1,282 (70%)	
No partner	4,454 (18%)	3,934 (18%)	520 (21%)	
Unmarried	3,714 (19%)	3,539 (20%)	175 (9.0%)	
Alcohol Status, n (%)				<0.001
Former drinking	3,596 (15%)	3,114 (14%)	482 (20%)	
Heavy drinking	4,139 (21%)	3,833 (22%)	306 (16%)	
Mild to moderate drinking	9,860 (53%)	8,925 (53%)	935 (54%)	
Never drinking	2,785 (11%)	2,531 (11%)	254 (11%)	
Smoking Status, n (%)				<0.001
Never Smoking	11,112 (55%)	10,167 (55%)	945 (48%)	
Smoking	9,268 (45%)	8,236 (45%)	1,032 (52%)	
Hypertension, n (%)	7,333 (32%)	6,340 (30%)	993 (45%)	<0.001
Diabetes, n (%)	2,538 (9.3%)	2,099 (8.3%)	439 (18%)	<0.001
Group, n (%)				0.027
Low dietary live microbe group	7,540 (33%)	6,772 (33%)	768 (37%)	
Medium dietary live microbe group	8,164 (39%)	7,375 (39%)	789 (39%)	
High dietary live microbe group	4,676 (28%)	4,256 (28%)	420 (25%)	
Total KCAL(Kcal)	2,161.45 ± 966.88	2,167.27 ± 972.64	2,107.39 ± 910.12	0.2
Total water(g)	3,096.70 ± 1,543.85	3,105.97 ± 1,553.61	3,010.62 ± 1,447.67	0.063
BUN(mg/dL)	13.46 ± 5.21	13.35 ± 5.10	14.53 ± 6.02	<0.001
Scr(mg/dL)	0.89 ± 0.32	0.88 ± 0.31	0.94 ± 0.38	<0.001
UA(mg/dL)	5.46 ± 1.39	5.44 ± 1.38	5.65 ± 1.48	<0.001
TG(mg/dL)	157.73 ± 133.49	155.70 ± 128.60	176.58 ± 171.24	<0.001
TC(mg/dL)	195.54 ± 41.90	195.81 ± 41.80	193.02 ± 42.76	0.013
HDL(mg/dL)	53.24 ± 16.72	53.63 ± 16.82	49.70 ± 15.34	<0.001

PIR, poverty income ratio; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; Scr, serum creatinine; BUN, blood urea nitrogen; UA, uric acid.

had a higher risk of developing KSD ($p < 0.05$). Non-Hispanic White individuals had an increased risk of KSD compared to Mexican American individuals, while Non-Hispanic Black individuals had a decreased risk ($p < 0.05$). Moreover, unmarried individuals had a lower risk of KSD compared to those with partners ($p < 0.05$). Smoking was also associated with an increased risk of KSD compared to non-smoking participants ($p < 0.05$). Notably, individuals in the high dietary live microbe intake group had a lower risk of KSD compared to those in the low intake group (OR = 0.78, 95% CI 0.65–0.93, $p < 0.05$).

3.3 Association between dietary live microbe intake with KSD

The results of the multivariable regression analysis are presented in Table 3. Overall, higher dietary live microbe intake group was associated with a lower incidence of kidney stone disease (KSD) across all models ($p < 0.05$). Compared to the low dietary live microbe intake group, the adjusted odds ratio (OR) for KSD in the high intake group was 0.78 (95% CI, 0.65–0.93) in the unadjusted model ($p < 0.05$). In Model 1, adjusted for age and sex, the adjusted OR for KSD in the high dietary live microbe group was 0.77 (95% CI, 0.64–0.93) compared to the low intake group ($p < 0.05$). In subsequent models (Models 2 to 4), further adjustments were made for race, education, family poverty income ratio, marital status, smoking status, alcohol status, BMI, waist circumference, diabetes, hypertension, total energy intake, total water intake, BUN, UA, Scr, TC, TG, and HDL. The association between higher dietary live microbe intake and reduced KSD risk remained statistically significant in these models (all trends $p < 0.05$). In Model 4, after adjusting for the aforementioned covariates, the adjusted OR for KSD in the high dietary live microbe group was 0.79 (95% CI, 0.64–0.98) compared to the low intake group ($p < 0.05$).

Based on Sanders' classification, unpasteurized fermented foods and probiotic supplementation fall under high dietary live microbe intake. This finding is consistent with recent studies proving that probiotic supplementation, such as with *Oxalobacter formigenes*, Lactobacillus, Bifidobacterium, and *Bacillus subtilis*, can reduce intestinal oxalate absorption, which may help prevent kidney stone formation (20, 21). Additionally, a cross-sectional study showed that a higher intake of fermented vinegar was statistically significantly associated with a lower risk of kidney stone formation (22).

3.4 Subgroup analyses

Subgroup analyses were conducted based on sex, age, BMI, hypertension, and diabetes status (Figure 2; Supplementary Table 2). The results indicated no significant interactions between dietary live microbe intake and these stratified variables regarding the risk of kidney stone disease (KSD; P for interaction >0.05). Specifically, male participants, those aged ≥60 years, and those with a BMI >25 exhibited a reduced risk of KSD in the medium and high dietary live microbe intake groups. In the hypertensive subgroup, the high dietary live microbe group had an OR (95% CI) of 0.81 (0.67–0.99) for KSD. Among non-diabetic participants, the ORs (95% CI) for KSD were 0.88 (0.78–1.00) for the medium dietary live microbe group and 0.83 (0.72–0.96) for the high dietary live microbe group, compared to the low intake group. However, the protective effect of high dietary live microbe intake on KSD was attenuated in the presence of diabetes (all $p < 0.05$).

3.5 Sensitivity analysis

We conducted multiple imputations to reduce sample bias caused by excluding covariates with missing data. The final results showed that, in the fully adjusted Model 4, the odds ratio (OR) for KSD in the high dietary live microbe group was 0.82 (95% CI, 0.68–0.99, $p < 0.05$) compared to the low intake group. The correlation results remained stable (Supplementary Table 3).

4 Discussion

Our study utilized a nationally representative sample of US adults and observed a decrease in the prevalence of kidney stone disease (KSD) among individuals with higher dietary live microbe intake group. The results of the multivariable regression analysis indicated that higher dietary live microbe intake group is associated with a lower risk of KSD. Furthermore, subgroup analyses suggested that the presence of diabetes might influence the relationship between dietary live microbe intake group and KSD. Specifically, our study found that the protective effect of higher dietary live microbe intake group on KSD was attenuated in individuals with diabetes. These findings suggest that higher dietary live microbe intake may be beneficial in preventing KSD.

The association between KSD and the gut microbiome has been well established (10, 21). A meta-analysis of eight studies indicated that patients with KSD have characteristic gut dysbiosis, with significant differences in the overall abundance of microbial communities between KSD patients and controls (23). Urinary oxalate is a key risk factor for KSD, and the gut plays a crucial role in oxalate balance and subsequent oxalate homeostasis (9, 24, 25). Current microbial research on KSD treatment has largely focused on the gut microbiota capable of degrading oxalate (26–30). The gut-kidney axis in KSD is not limited to oxalate-degrading gut microbiota (e.g., *Oxalobacter formigenes*) (31). In KSD patients, the functional activity of gut microbiota involved in oxalate degradation, lipid, carbohydrate, and energy metabolism, glycan synthesis, and amino acid biosynthesis is altered. These findings emphasize the complex interactions between the gut microbiome and KSD, suggesting that interventions targeting the gut microbiome could be a promising strategy for KSD prevention and treatment (32–34).

A metagenome-wide association study (MWAS) has shown that inter-individual differences in gut microbiome composition are influenced by various factors, including lifestyle, diet, disease, and medication, with diet being the most critical (35). The stone-promoting effects of high salt and high oxalate intake, and the preventive effects of increased fruit, vegetable, juice, and water intake, are mediated, at least in part, by changes in gut microbiome composition and metabolic function (36). According to Sanders' classification, foods that have been pasteurized or processed at high temperatures are considered low in live microbes. Fresh, unpeeled vegetables and fruits are classified as medium, while unpasteurized fermented foods and probiotic supplements are classified as high (13).

TABLE 2 Univariate analysis of variables with kidney stones disease.

Variable	OR (95% CI)	*p*-value
Gender		
Female	1.00 (Reference)	
male	1.37 (1.17–1.60)	<0.001
Age	1.02 (1.02–1.03)	<0.001
Race		
Mexican American	1.00 (Reference)	
Non-Hispanic Black	0.63 (0.50–0.78)	<0.001
Non-Hispanic White	1.52 (1.29–1.80)	<0.001
Other Hispanic	1.22 (0.94–1.59)	0.14
Other Race	0.91 (0.65–1.27)	0.57
Education		
High school or equivalent	1.00 (Reference)	
Less than high school	1.03 (0.86–1.23)	0.72
More than high school	0.95 (0.81–1.12)	0.58
Family PIR	1.0 (0.95–1.04)	0.80
Marital status		
Having a partner	1.00 (Reference)	
No partner	1.04 (0.88–1.24)	0.61
Unmarried	0.40 (0.31–0.52)	<0.001
Smoking status		
Never Smoking	1.00 (Reference)	
Smoking	1.34 (1.17–1.54)	<0.001
Alcohol status		
Never drinking	1.00 (Reference)	
Former drinking	1.45 (1.10–1.90)	0.009
Heavy drinking	0.70 (0.51–0.96)	0.026
Mild to moderate drinking	1.00 (0.77–1.31)	>0.99
BMI	1.04 (1.03–1.04)	<0.001
Waist	1.02 (1.02–1.02)	<0.001
Total energy	1.00 (1.00–1.00)	0.10
Total water	1.00 (1.00–1.00)	0.042
Group		
Low dietary live microbe group	1.00 (Reference)	
High dietary live microbe group	0.78 (0.65–0.93)	0.007
Medium dietary live microbe group	0.89 (0.76–1.04)	0.14
Hypertension		
No	1.00 (Reference)	
Yes	1.89 (1.66–2.16)	<0.001
Diabetes		
No	1.00 (Reference)	
Yes	2.50 (2.17–2.89)	<0.001
BUN	1.04 (1.03–1.05)	<0.001
Scr	1.44 (1.23–1.68)	<0.001
UA	1.11 (1.05–1.17)	<0.001
TG	1.00 (1.00–1.00)	<0.001
TC	1.00 (1.00–1.00)	0.017
HDL	0.98 (0.98–0.99)	<0.001

PIR, poverty income ratio; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; Scr, serum creatinine; BUN, blood urea nitrogen; UA, uric acid; OR, odds ratio; CI, confidence interval.

Increasing the intake of live microbes may be a crucial strategy for improving KSD outcomes. Several studies adopting Sanders' classification method have shown that diets rich in live microbes are associated with various positive health outcomes, including healthier metabolism, lower BMI (37), reduced cardiovascular disease risk (17), lower depression risk (14), and better cognitive function (15). Probiotic interventions, such as those with *Oxalobacter formigenes*, Lactobacillus paragasseri UBLG-36, and other Lactobacillus strains, have been shown to alter the gut microbiome, affecting the activity of oxalate-degrading bacteria and ultimately reducing oxalate levels and stone formation (26, 28, 38). In our study, high dietary live microbe intake was associated with a lower risk of KSD, while medium intake did not show a significant association in all analyses. The differences in microbial strains and inter-individual variations in the gut microbiome may explain these health outcome discrepancies. It is also essential to consider that, besides live microbes, other dietary components may contribute to health-related parameters. In our study, total energy and total water intake were included in the analysis to strengthen our results. Further evidence, especially in different disease states, is needed to inform dietary recommendations for live microbes. Additionally, Microbiome-targeted interventions, such as probiotic supplementation or dietary modifications aimed at enhancing beneficial microbial populations, represent promising strategies for kidney stone prevention. As research in this area advances, randomized controlled trials and mechanistic studies are needed to validate the efficacy of these interventions and identify the most effective microbial strains and dosages for reducing kidney stone risk.

The potential link between high dietary live microbe intake group and reduced KSD risk can be attributed to several factors. First, probiotics can reduce urinary oxalate levels by influencing the degradation, absorption, and transport of oxalate in the gut, thus maintaining oxalate homeostasis and reducing the risk of KSD (30, 39, 40). Second, safe live microbes can promote the production of metabolites such as short-chain fatty acids (SCFAs), which can downregulate the expression of SLC26A3, a transporter responsible for oxalate absorption in the ileum, cecum, and colon. This results in lower urinary oxalate levels and reduced renal calcium oxalate (CaOx) crystal deposition in rats, contributing to the prevention of kidney stones (41, 42). Overall, these mechanisms highlight the complex interactions between the gut microbiome and KSD. Further mechanistic studies are needed to elucidate the therapeutic advantages of dietary microbes in the prevention and management of KSD.

TABLE 3 Association between dietary live microbe intake group and kidney stones disease.

Model	Low dietary live microbe	Medium dietary live microbe OR (95% CI)	*p*-value	High dietary live microbe OR (95% CI)	*p*-value
Crude	1.00 (Reference)	0.89 (0.76–1.04)	0.14	0.78 (0.65–0.93)	0.007
Model 1	1.00 (Reference)	0.84 (0.71–0.99)	0.033	0.77 (0.64–0.93)	0.007
Model 2	1.00 (Reference)	0.82 (0.69–0.97)	0.022	0.73 (0.60–0.89)	0.003
Model 3	1.00 (Reference)	0.84 (0.71–1.00)	0.052	0.77 (0.63–0.94)	0.012
Model 4	1.00 (Reference)	0.86 (0.72–1.03)	0.1	0.79 (0.64–0.98)	0.032

Crude was model with no adjustment for covariates. Model 1 was adjusted for age and gender. Model 2 was adjusted for Model 1, and race, education, family poverty income ratio, marital status. Model 3 was adjusted for Model 2, and smoking Status, alcohol Status, bmi, waist, diabetes and hypertension. Model 4 was adjusted for Model 3, and total energy, total water, total cholesterol, blood urea nitrogen, triglyceride, uric acid, high-density lipoprotein cholesterol and serum creatinine. OR, odds ratio; CI, confidence interval.

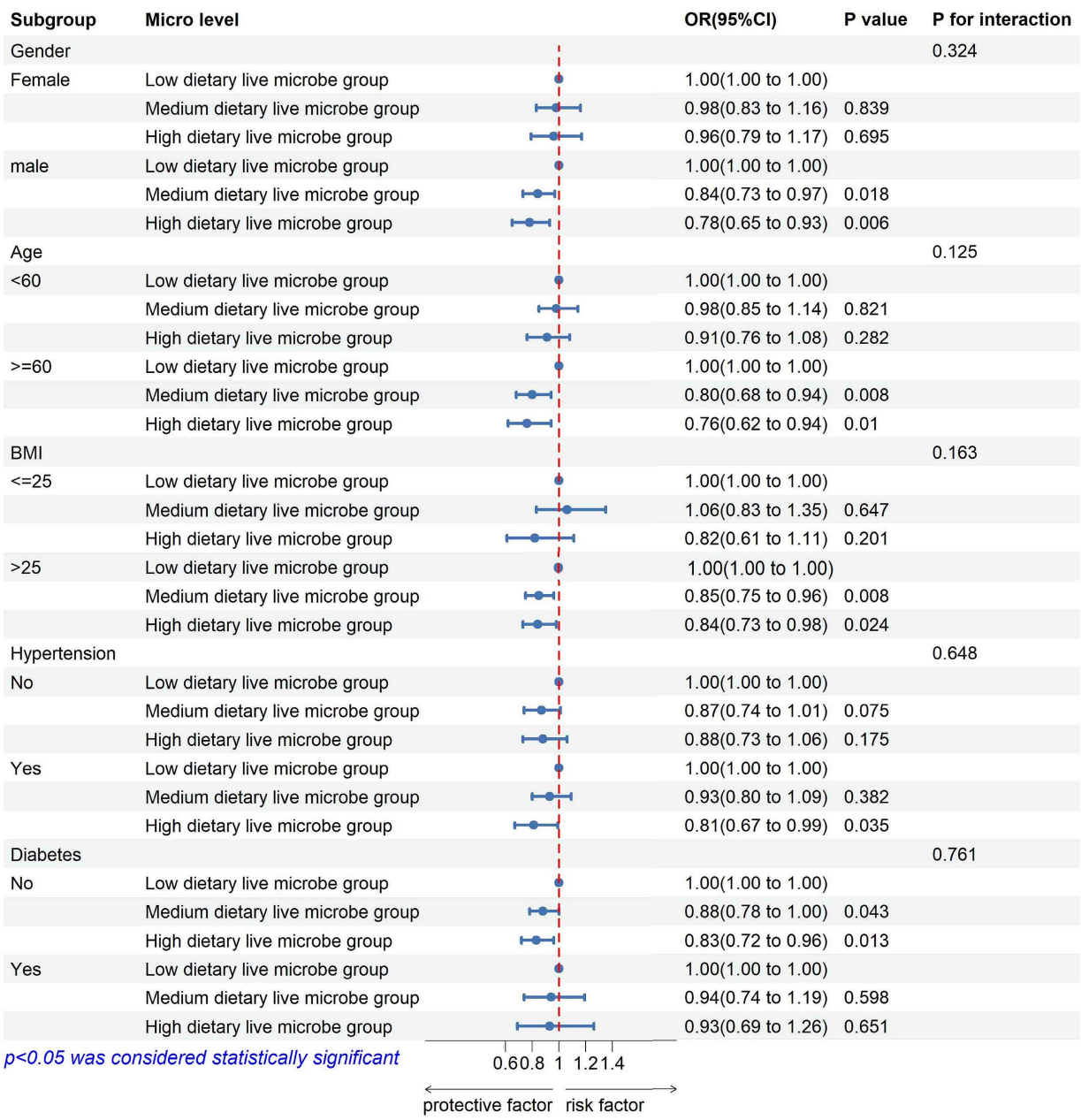

FIGURE 2
Subgroup analysis of the association between high dietary live microbe group and prevalence of kidney stones disease.

Our subgroup analysis revealed interesting differences in the association between dietary live microbe intake group and KSD risk. Notably, the protective effect of high dietary live microbe intake was attenuated in individuals with diabetes. The increased risk of KSD in type 2 diabetes patients is primarily attributed to insulin resistance, which is associated with altered renal ammonium secretion, increased urinary acidification, hypocitraturia, and hypercalciuria, all of which contribute to the formation of uric acid and calcium stones (43). The gut microbiome plays a crucial role in the development and progression of both KSD and diabetes (10, 44). Additionally, the use of medications for type 2 diabetes has been linked to changes in the gut microbiome (45, 46), which may partially influence the impact of live microbes on KSD.

NHANES's rigorous quality control procedures and complex sampling design enabled us to evaluate the association between dietary live microbe intake and KSD in a large, nationally representative sample of U.S. adults. However, several limitations must be acknowledged. First, it is important to recognize the limitation of our study's cross-sectional design. This design only allows us to examine associations at a single point in time, making it impossible to establish causality. While we observed significant associations between higher dietary live microbe intake and a lower risk of KSD, the temporal sequence of events cannot be determined, and we cannot infer causal relationships. Future longitudinal studies or randomized controlled trials are needed to confirm the causal relationships between dietary live microbe intake and KSD risk. Second, the method used for dietary live microbe classification was based on expert consensus, which may be less accurate than direct measurement methods. While this approach provides a useful framework for categorizing live microbe intake, the lack of microbiome data, particularly those related to dietary microbes, limits our ability to comprehensively understand how these microbes influence disease states. Third, our study population consisted of U.S. adults, limiting the generalizability of the findings to other populations. Another limitation of our study is the reliance on self-reported dietary data, which may introduce recall bias. Participants might inaccurately report their dietary intake, leading to potential misclassification of dietary exposure. While NHANES employs multiple methods to reduce recall bias, including trained interviewers and standardized recall tools, the potential for bias cannot be entirely eliminated. Recognizing these limitations, future research should aim to overcome these challenges, possibly through longitudinal designs, larger and more diverse population samples, and more precise microbial measurement techniques to determine the optimal dosage and types of live microbes for preventing kidney stones.

5 Conclusion

Our study indicates that high dietary live microbe intake group is associated with a lower risk of KSD. However, given the limitations of our findings, large prospective studies are necessary to further validate the association between dietary live microbe intake and KSD and to elucidate the underlying mechanisms.

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 National Center for Health Statistics (NCHS) Ethical Review Board. 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

ZZ: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. XC: Funding acquisition, Investigation, Supervision, Writing – review & editing.

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Conflict of interest

The author(s) 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/fnut.2024.1463352/full#supplementary-material

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Uncovering the mechanisms underlying the efficacy of probiotic strains in mitigating food allergies: an emphasis on gut microbiota and indoleacrylic acid

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Food allergies manifest as systemic or digestive allergic responses induced by food allergens, and their progression has been demonstrated to be intimately associated with the host's gut microbiota. Our preceding investigation has revealed that the probiotic strains *Lactiplantibacillus plantarum* CCFM1189 and *Limosilactobacillus reuteri* CCFM1190 possess the capability to mitigate the symptoms of food allergy in mice. However, the underlying mechanisms and material foundations through which these probiotic strains exert their effects remain enigmatic. Here, we initially compared the ameliorative effects of these two probiotic strains on food allergy mice subjected to antibiotic cocktail (ABX) treatment. It is indicated that ABX treatment was ineffective in alleviating weight loss, diarrhea, and allergic symptoms in mice, and it also inhibited the reduction of histamine and T helper cell 2 (Th2) cytokines mediated by effective strains, suggesting that effective strains must operate through the gut microbiota. Then, building upon the outcomes of prior non-targeted metabolomics studies, by quantifying the content of indoleacrylic acid (IA) in single-strain fermentation of probiotic strains and mouse feces, it was ascertained that effective strains do not synthesize IA themselves but can augment the concentration of IA in the gut by modulating the gut microbiota. Ultimately, we discovered that direct intervention with IA could mitigate diarrhea, allergic symptoms, and intestinal damage by modulating immunoglobulin E (IgE) levels, histamine, Th2 cytokines, and tight junction proteins, thereby corroborating that IA is a pivotal metabolite for the alleviation of food allergies. These observations underscore the significance of gut microbiota and metabolites like IA in the management of food allergies and hold potential implications for the development of novel therapeutic strategies.

KEYWORDS

food allergy, probiotic, antibiotic cocktail, gut microbiota, indoleacrylic acid

Introduction

Food allergies are immune system disorders triggered by protein antigens in food, resulting in a range of clinical symptoms that can affect the gastrointestinal tract, respiratory system, skin, and central nervous system (1). Currently, there is no complete cure for food allergies. The most common approach to managing these conditions is the strict avoidance of allergenic foods to prevent reactions (2). However, due to insufficient regulation of food labeling, identifying potential allergenic components in food products remains challenging. Consequently, there is an urgent need to explore new methods for treating food allergies. The gut microbiota has been acknowledged as a critical factor in the etiology and regulation of allergic diseases (3, 4). The gut microbiota engages in direct interactions with the intestinal mucosal surface, thereby modulating the host's immune response via diverse mechanisms, such as the regulation of immune cell populations (5), the synthesis of antimicrobial peptides (6), and the maintenance of intestinal barrier integrity (7). Researchers hypothesize that inadequate exposure to specific beneficial microbes during early life may contribute to the escalating prevalence of allergic conditions (8), underscoring the microbiota's indispensable role in the maturation and function of the immune system. Based on this, probiotic therapy is regarded as a promising strategy to mitigate allergic diseases by modulating the gut microbiota (9).

Recent studies have demonstrated that probiotics exert a significant impact on reducing specific immunoglobulin E (sIgE) concentrations, balancing the T helper cell 1 (Th1)/T helper cell 2 (Th2) immune axis, fine-tuning dendritic cell activity, suppressing mast cell activation, and augmenting intestinal barrier integrity in subjects afflicted with food allergies (10–12). These multifaceted effects are hypothesized to ameliorate food allergy manifestations by rectifying intestinal immune dysregulation, optimizing gut microbiota diversity and configuration, and harmonizing gut metabolic processes. However, there is a paucity of analysis on the mechanism and material basis of probiotics in alleviating food allergies, and research on the relationship between food allergies and intestinal metabolism remains limited. By introducing beneficial bacteria into the gut, probiotics are capable of altering the composition and metabolic activity of the resident microbiota (13, 14). Intestinal small molecule metabolites, which are byproducts of microbial metabolism, exert significant effects on the host's immune response and intestinal barrier function. These metabolites encompass short-chain fatty acids, bile acids, and tryptophan-indole derivatives, all of which are known for their anti-inflammatory properties and ability to strengthen the intestinal barrier (15). Thus, probiotic strains that foster the growth of bacteria capable of producing such beneficial metabolites may aid in regulating intestinal immune homeostasis and enhancing barrier function, potentially mitigating symptoms of food allergy in mice and possibly in humans as well.

In a prior study, we demonstrated that *Lactiplantibacillus plantarum* CCFM1189 and *Limosilactobacillus reuteri* CCFM1190 have the potential to mitigate food allergy symptoms in ovalbumin (OVA)-induced mice (16). Based on the findings from untargeted metabolomics analysis, it is speculated that the efficacy of these strains may be mediated by the Gut microbiota specific small molecule metabolite, indoleacrylic acid (IA). Therefore, in this study, we initially utilize an antibiotic cocktail (ABX)-treated mouse model to examine whether the two efficacious probiotic strains augment the fecal concentration of IA in mice by modulating the gut microbiota, thus restoring the Th1/Th2 immune balance in food-allergic mice and alleviating the pathological manifestations associated with food allergy. Furthermore, we directly administer IA to mice to validate its efficacy, thereby confirming whether IA is the active component responsible for the alleviation of food allergy.

Materials and methods

Cultivation of probiotic strains used in this study

The probiotic strains, *L. plantarum* CCFM1189 and *L. reuteri* CCFM1190, were obtained from the Culture Collection of Food Microorganisms at Jiangnan University (Wuxi, China). CCFM1189 was incubated under anaerobic conditions in de Man-Rogosa-Sharpe (MRS) liquid medium at 37°C for a duration of 16–24 h. CCFM1190 was cultivated in MRS liquid medium supplemented with 0.5 g·L^{-1} L-cysteine hydrochloride within an anaerobic chamber at 37°C for a period of 24–48 h. After strain amplification, centrifuge at 4°C and 8,000 × *g* for 10 min, discard the supernatant, wash twice with 0.9% sterile saline, resuspend the bacterial slurry in 5 mL of sucrose solution, count, and store in a − 80°C freezer. Before gavage, centrifuge at 4°C and 8,000 × *g* for 10 min, dilute the bacterial suspension to 5 × 10^{9} CFU·mL^{-1} with 0.9% sterile saline based on the counting results, and set aside.

Preparation of ABX solution and IA solution

The dosage regimen for the ABX solution in this study was adapted from Scott et al. (17): Ampicillin (9 mg·kg^{-1}), metronidazole (9 mg·kg^{-1}), neomycin sulfate (9 mg·kg^{-1}), and vancomycin (4.5 mg·kg^{-1}) were administered via intragastric route at a volume of 0.2 mL per day, commencing from the adaptation phase and continuing until the 30th day, which precedes the initiation of the stimulation protocol.

The IA was solubilized using a Dimethyl sulfoxide (DMSO) solution (18). The composition of the DMSO solution included DMSO, polyethylene glycol 300 (PEG300), tween-80, and saline solution in the ratio of 2:8:1:9, respectively. The IA was dissolved in this DMSO solution. The low-dose IA group (IA1) received 0.2 mL of the low-dose IA solution (4.8 mg·mL^{-1}), whereas the high-dose IA group (IA2) was administered 0.2 mL of the high-dose IA solution (48 mg·mL^{-1}).

Animals and experimental protocol

The mice used in this experiment were specific pathogen free (SPF) 4-week-old female BALB/c mice, which were purchased from Charles River Laboratory Animal Technology Co., Ltd. (Zhejiang, China), and raised in the barrier facilities of the Experimental Animal Center at Jiangnan University. The initial body weights of the mice ranged from 13 to 16 g. A one-week acclimatization period was observed prior to the commencement of the experiment. The environmental conditions for feeding, the dietary regimen, the

establishment of the food allergy model, and the protocols for execution and sampling were all in accordance with the methodologies detailed in our previous study (16), the schematic diagram is shown in Figure 1.

The mice were randomly allocated into 10 distinct groups: control, model, ABX, ABX + CCFM1189, ABX + CCFM1190, CCFM1189, CCFM1190, DMSO, IA1, and IA2. Each group comprised 6–7 mice. Mice in the ABX + CCFM1189, ABX + CCFM1190, CCFM1189, and CCFM1190 groups received a daily gavage of 0.2 mL (5×10^9 CFU·mL^{-1}) of probiotic suspension from day 0 to day 43. Conversely, mice in the control and model groups were administered an equivalent volume of sterile saline solution. For the ABX-treated groups (ABX, ABX + CCFM1189, ABX + CCFM1190), each mouse received a daily gavage of 0.2 mL of ABX solution from the adaptation period until the 30th day (the day preceding the initiation of stimulation), with the ABX being dissolved in saline solution. The DMSO group received intragastric administration of 0.2 mL of DMSO solution from day 0 to day 43. For the IA-treated groups, the IA1 group received 0.2 mL of low-dose IA solution (4.8 mg·mL^{-1}) per mouse from day 0 to day 43, while the IA2 group received 0.2 mL of high-dose IA solution (48 mg·mL^{-1}) per mouse over the same period. The mice were weighed at the same time each week from the adaptive period, with their weights expressed as percentages (weight% = current weight / initial weight (day 0) × 100%).

This investigation was conducted in full compliance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23 Rev. 985) and received formal approval from the Laboratory Animal Ethics Committee of Jiangnan University (Wuxi, Jiangsu, China; approval numbers JN. No. 20210915b1121201[300] and JN. No. 20211115b0581228[434]).

Assessment of food allergy symptom score and diarrhea index in mice

The calculation method of food allergy symptom score in mice detailed in our previous study (16). Feces of mice were collected for a period of time, and the diarrhea index was calculated (diarrhea index = the number of soft stools / the total amount of feces).

Determination of OVA-sIgE, interleukin (IL)-4, IL-5, IL-13, and histamine (HIS) contents

Serum and jejunal tissue samples were procured and subjected to analysis using mouse-specific OVA-sIgE, IL-4, IL-5, IL-13, and HIS enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Senbeijia Biological Technology Co., Ltd., Nanjing, China), in accordance with the manufacturer's guidelines. HIS, a biogenic amine present in the human body, is primarily recognized for its function as a mediator in allergic responses; however, it also serves as a crucial signaling molecule within the nervous system, gastrointestinal tract, integumentary system, and immune system. The absorbance was quantified at a wavelength of 450 nm utilizing a microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

Immunohistochemical analysis of tight junction proteins zonula occludens 1 (ZO-1) and occludin in jejunal tissue of mice

The jejunal tissues were preserved in a 4% paraformaldehyde solution. Subsequently, the tissues were processed for immunohistochemical sectioning, encompassing steps such as repair, dehydration, transparency, waxing, embedding, sectioning, dewaxing, antigen retrieval, sealing, incubation with primary and secondary antibodies, Diaminobenzidine coloration, termination of coloration, and redyeing and dewatering. The sections were digitized and examined at 400× magnification using a digital slide scanner (3DHISTECH Ltd., Hungary). The positive staining area and total area were quantified using Image J 1.8.0 (National Institutes of Health, United States).

In vitro fermentation assay of probiotic strains

The CCFM1189 and CCFM1190 strains were activated using MRS liquid medium over two generations. The second generation was harvested at the logarithmic phase, centrifuged at 4000 × *g* for 10 min, and the supernatant was subsequently discarded. An equivalent volume of the pre-reduced isotonic potassium phosphate buffer (PPS; 10 mM $MgSO_4$, 21.46 mM KH_2PO_4, 18.54 mM K_2HPO_4, pH 7.0) was prepared for the purpose of cleansing bacterial cells. The cells were thoroughly resuspended in the PPS, which was subsequently removed under the same centrifugation conditions as previously described. Following two cycles, an equivalent volume of PPS supplemented with 1 mM tryptophan was added and incubated in an anaerobic workstation for 24 h. Subsequently, the mixture was centrifuged at 8000 × *g* for 10 min, and the supernatant was filtered through a 0.22 μm filter membrane for subsequent detection.

Determination of IA content in feces of mice and bacterial culture supernatant

The collected fecal samples were subjected to lyophilization under conditions of −107°C and 0.2 mbar. The freeze-dried fecal samples were precisely weighed to 50 mg, and subsequently augmented with 800 μL of a methanol aqueous solution (methanol: water = 1: 1). The samples underwent vortex mixing for 15 s, followed by tissue crushing at a frequency of 65 Hz, each cycle lasting 30 s (repeated 5 times). The homogenized samples were incubated at 4°C overnight. Following centrifugation at 16000 × *g* for 10 min, 500 μL of the supernatant was aspirated and concentrated under vacuum at 45°C for 2–4 h. The supernatant was resuspended in 200 μL of a methanol solution (methanol: water = 1: 9), centrifuged at 14000 × *g* for 10 min, and subsequently filtered through a 0.22 μm filter membrane.

A stock solution of IA with a concentration of 1,000 ppm was prepared by diluting a methanol-aqueous solution (methanol: water = 1: 9) to yield concentrations of 50 ppm, 25 ppm, 10 ppm, 5 ppm, 1 ppm, 500 ppb, 250 ppb, 125 ppb, 100 ppb, 50 ppb, 25 ppb, 12.5 ppb, 6.25 ppb, 3.125 ppb, and 1.5625 ppb. These varying concentrations of IA were employed to construct a

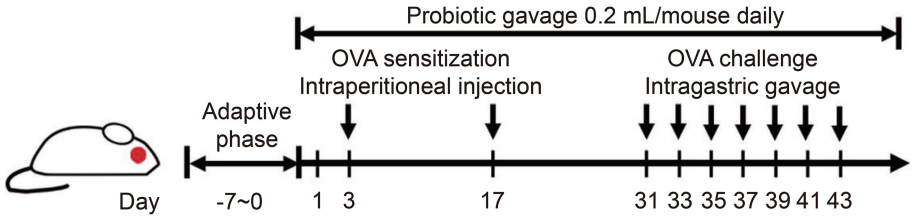

FIGURE 1
The procedure for modeling OVA-induced food allergy and intervention cycle in mice.

standard curve correlating peak area with concentration. IA was quantified using ultra-performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS; Thermo Fisher Scientific, Waltham, MA, USA; C18 UPLC Columns). The column temperature was maintained at 36.5°C, while the automatic sampler temperature was set at 4°C. Each sample was analyzed in positive ion mode with mobile phases comprising (A) acetonitrile and (B) 0.1% formic acid in aqueous solution. The injection volume was 2 μL, and the flow rate was 300 μL/min. The gradient elution program settings are detailed in Table 1.

TABLE 1 Gradient elution program settings.

Time (min)	Percentage of mobile phase A (%)	Percentage of mobile phase B (%)
0–3	5	95
3–9	30	90
9–15	100	0
15–16.5	100	0
16.5–20	5	95

High throughput sequencing of the gut microbiota

The FastDNA Spin Kit for Feces (MP Biomedicals, CAT. NO.6570200) was employed to extract DNA from the fecal samples of mice, adhering strictly to the manufacturer's protocol. The polymerase chain reaction (PCR) was utilized to amplify the DNA within the V3–V4 region. Post-PCR, the agarose gel was excised for purification purposes, utilizing the DNA Gel/PCR Purification Miniprep Kit (BW-DC3511, Beiwo Meditech Co., Ltd., Hangzhou, China), in strict accordance with the manufacturer's guidelines. The PCR primers (341F and 806R), sequencing platform (Illumina NovaSeq 6,000), quality control method of the raw data, and analysis platform [QIIME 2 (19)] were referred to Lu et al. (20). Conduct a differential genus analysis utilizing the integrated LEfSe (Linear Discriminant Analysis Effect Size) utility within OmicStudio (21), accompanied by visualization, and employing the subsequent filters: Kruskal-Wallis test threshold set at 0.05; Wilcoxon test threshold set at 0.05; LDA score threshold set at 3.

Statistical analysis

The allergy symptom scores, diarrhea index, ELISA results, and IA content were subjected to statistical analysis using IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, United States). The assessment of significant differences between the groups was conducted through one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. All data are presented as mean values ± the standard error of the mean (SEM). A p-value of <0.05 was deemed indicative of a statistically significant difference between the groups, corresponding to a confidence interval exceeding 95%.

Results

Oral administration of CCFM1189 and CCFM1190 cannot alleviate food allergy symptoms in mice treated with ABX

Initially, we performed exhaustive evaluations and statistical analyses on the food allergy-associated phenotypic indices across all experimental mice groups. In alignment with our prior research, CCFM1189 and CCFM1190 exhibited the outstanding capacity to alleviate weight loss, allergy scores, and diarrhea symptoms in normal food allergic mice (Figures 2A–C). Nevertheless, in contrast to these two groups, ABX treatment intensified the disease phenotypes in food-allergic mice, and the oral administration of CCFM1189 and CCFM1190 failed to significantly alleviate the symptoms in ABX-treated mice.

Subsequently, we determined the levels of serum OVA-sIgE across all mice groups. As shown in Figure 2D, administration of ABX resulted in a reduction of serum OVA-sIgE levels in mice when compared to the model group. In the case of CCFM1189 intervention, no significant difference was observed in the response to OVA-sIgE whether or not ABX was treated. Regarding the CCFM1190 group, it is exhibited a significant reduction in serum OVA-sIgE levels in mice ($p < 0.01$), whereas the ABX-treated CCFM1190 group did not demonstrate such a reduction. The results of jejunum HIS levels (a critical mediator in allergic responses; measured in supernatant) showed that effective bacterial strains have the potential to substantially mitigate the heightened HIS levels induced by food allergies; however, probiotic interventions in mice subjected to ABX treatment failed to diminish the HIS content within the jejunum (Figure 2E).

The levels of Th2 cytokines (IL-4, IL-5, and IL-13) in the jejunum supernatant of mice indicated that ABX treatment mitigated the inhibitory impact of the effective strains CCFM1189 and CCFM1190 on the Th2 immune response. In comparison to the model group, CCFM1189 and CCFM1190 notably diminished the IL-4 levels in the jejunum of mice, with the alleviating effect of the ABX-treated strain

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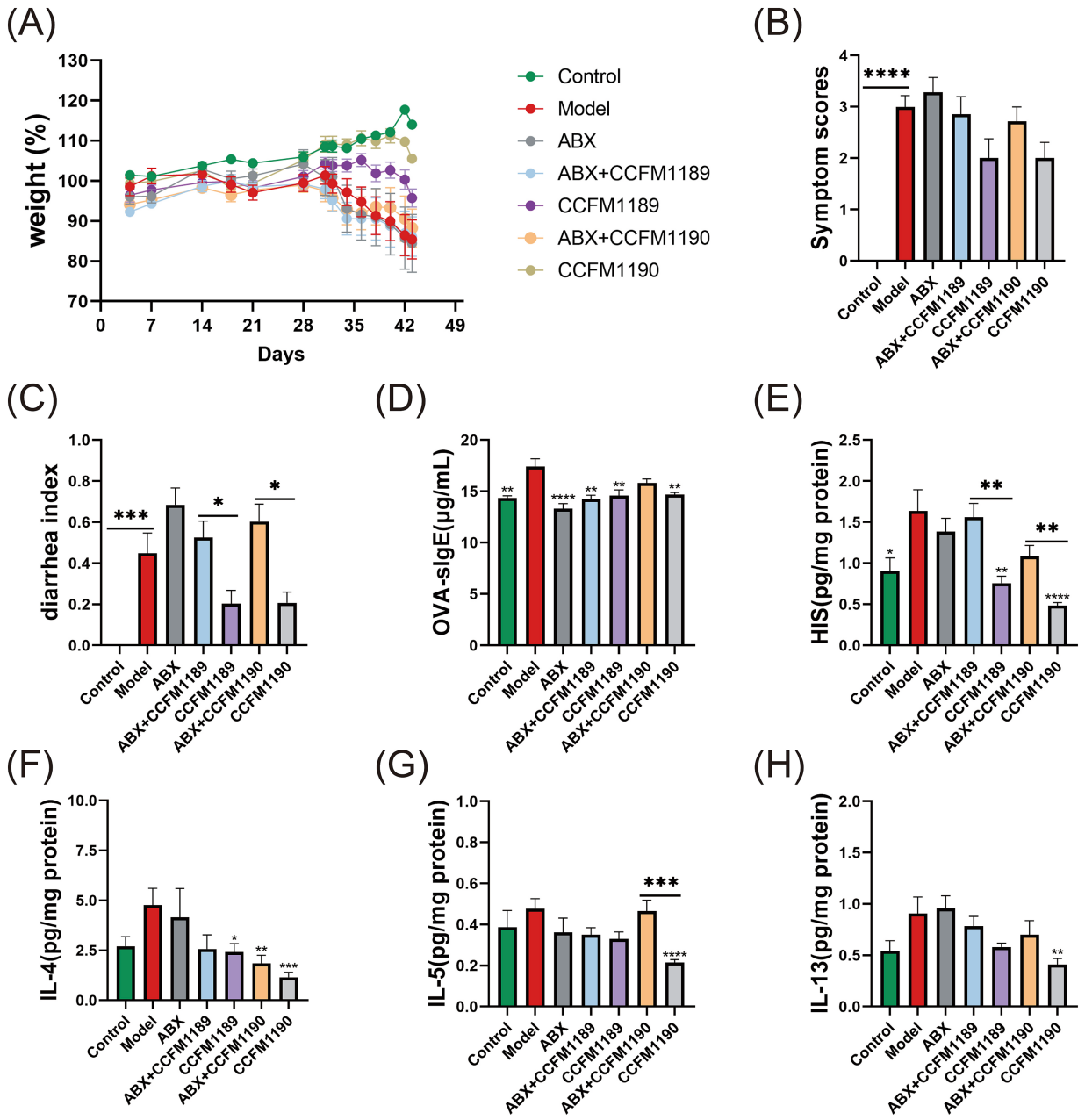

FIGURE 2
Alterations in allergy-related biomarkers and cytokine concentrations in both normal food allergic mice and food allergic mice subjected to ABX treatment, with the intervention of effective probiotic strains. **(A)** The weight (%) changes from day 0 to day 43. The symptom scores **(B)**, diarrheal indexes **(C)**, levels of serum OVA-sIgE **(D)**, levels of jejunal tissues' HIS **(E)**, IL-4 **(F)**, IL-5 **(G)**, and IL-13 **(H)** of food allergic mice on day 43. The result represents mean ± SEM. The *p*-value were measured using one-way ANOVA with Duncan's multiple range test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

being less pronounced (Figure 2F). Regarding IL-5 and IL-13, CCFM1189 decreased the levels of these cytokines; however, this reduction was not statistically significant when compared to the ABX + CCFM1189 group; CCFM1190, in the absence of ABX treatment, significantly lowered the levels of IL-5 (Figure 2G) and IL-13 (Figure 2H; $p < 0.01$). Collectively, the ABX + CCFM1189 and ABX + CCFM1190 groups exhibited a lesser inhibitory effect on the Th2-type immune response in food allergic mice compared to the CCFM1189 and CCFM1190 groups.

Moreover, the immunohistochemical analysis of tight junction proteins ZO-1 and Occludin in the jejunum of mice was conducted, with the proportion of positively stained regions being quantified (Figures 3A,B). The results indicated that ABX treatment led to a reduction in the expression levels of ZO-1 and Occludin in the jejunum of mice. The expression levels of ZO-1 and Occludin in the ABX + CCFM1189 and ABX + CCFM1190 groups were markedly lower compared to those in the CCFM1189 and CCFM1190 groups without ABX treatment ($p < 0.05$). After histopathological examination of the jejunum sections in mice, it is found that the small intestinal villi of the model group mice exhibited disorganization and abscission, while the lamina propria appeared disordered and lax. The intestinal villi of mice in the CCFM1189 and CCFM1190 groups were

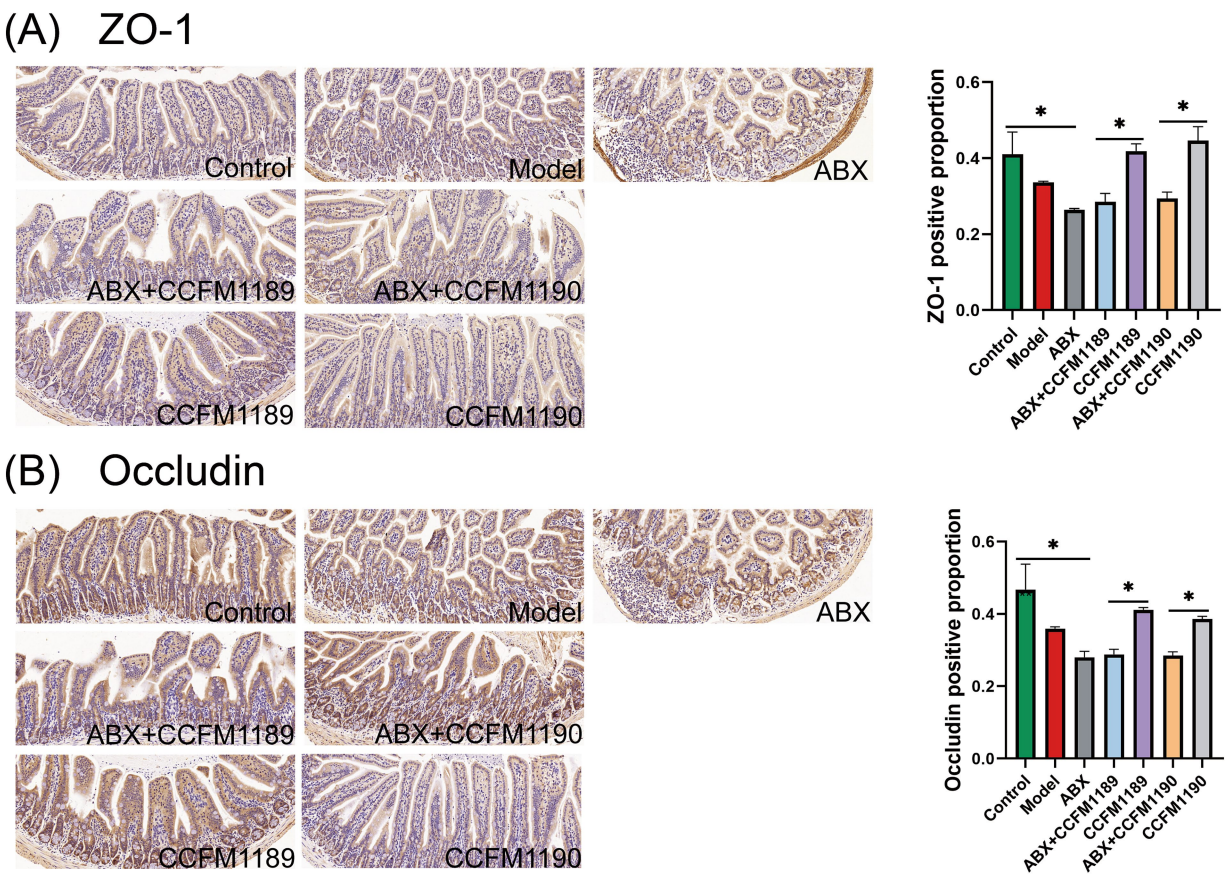

FIGURE 3
Alterations in intestinal barrier markers in both normal food allergic mice and food allergic mice subjected to ABX treatment, with the intervention of effective probiotic strains. **(A)** The pathological sections of the jejunum with immunohistochemistry (ZO-1, 20×) and the positive percentage of ZO-1. **(B)** The pathological sections of the jejunum with immunohistochemistry (Occludin, 20×) and the positive percentage of Occludin. The result represents mean ± SEM. The *p*-value were measured using one-way ANOVA with Duncan's multiple range test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

orderly arranged, with the lamina propria being intact and closely aligned, resembling the phenotype of the control group and thereby restoring the integrity of the intestinal barrier to a certain extent. In contrast, the ABX treatment groups (ABX, ABX + CCFM1189, and ABX + CCFM1190) displayed comparable phenotypes and elevated levels of inflammatory cell infiltration, akin to the model group.

The effective strains themselves do not produce IA, but can augment the intestinal concentration of IA by modulating the gut microbiota

In our prior research endeavors, we have ascertained that the probiotic strains CCFM1189 and CCFM1090 significantly mitigate food allergy in ovalbumin-induced mice, with IA being identified as a crucial microbial-derived metabolite. To elucidate the mechanisms responsible for the variations in IA levels within the gastrointestinal tract under probiotic intervention, we initially utilized an *in vitro* fermentation methodology. By incorporating tryptophan into the culture medium, we assessed the capacity of CCFM1189 and CCFM1190 to metabolize tryptophan and yield IA (Figure 4A). The results indicated that none of these strains were capable of producing IA through tryptophan metabolism during *in vitro* fermentation. Subsequently, to verify whether CCFM1189 and CCFM1190 augment the IA content in mouse feces by influencing other symbiotic bacteria within the intestinal tract, the IA content of effective strain groups treated with ABX (ABX + CCFM1189, ABX + CCFM1190) was compared with those not treated with ABX (CCFM1189, CCFM1190). The results demonstrated that the IA content in the feces of ABX-treated mice was lower than that of both the control and model groups. The fecal IA content of the CCFM1189 and CCFM1190 groups without ABX treatment exhibited an increase compared to the ABX-treated groups (Figure 4B). The aforementioned findings imply that CCFM1189 and CCFM1190 could potentially mitigate food allergy symptoms by augmenting the IA concentration in the fecal matter of mice, with the elevated IA levels possibly attributable to the probiotic strains' influence on the intestinal symbiotic microbiota of the mice.

Then, we conducted an analysis of the diversity and composition of the gut microbiota across various groups of mice. As depicted in Figure 4C, CCFM1189 and CCFM1190 exhibit the capability to reverse the reduction in microbial species diversity induced by food allergy models. Following treatment with ABX, a marked decrease in the diversity of the gut microbiota in mice was observed, and the intervention of probiotic strains failed to mitigate the dysbiosis induced by antibiotics (Figure 5A). The results pertaining to β

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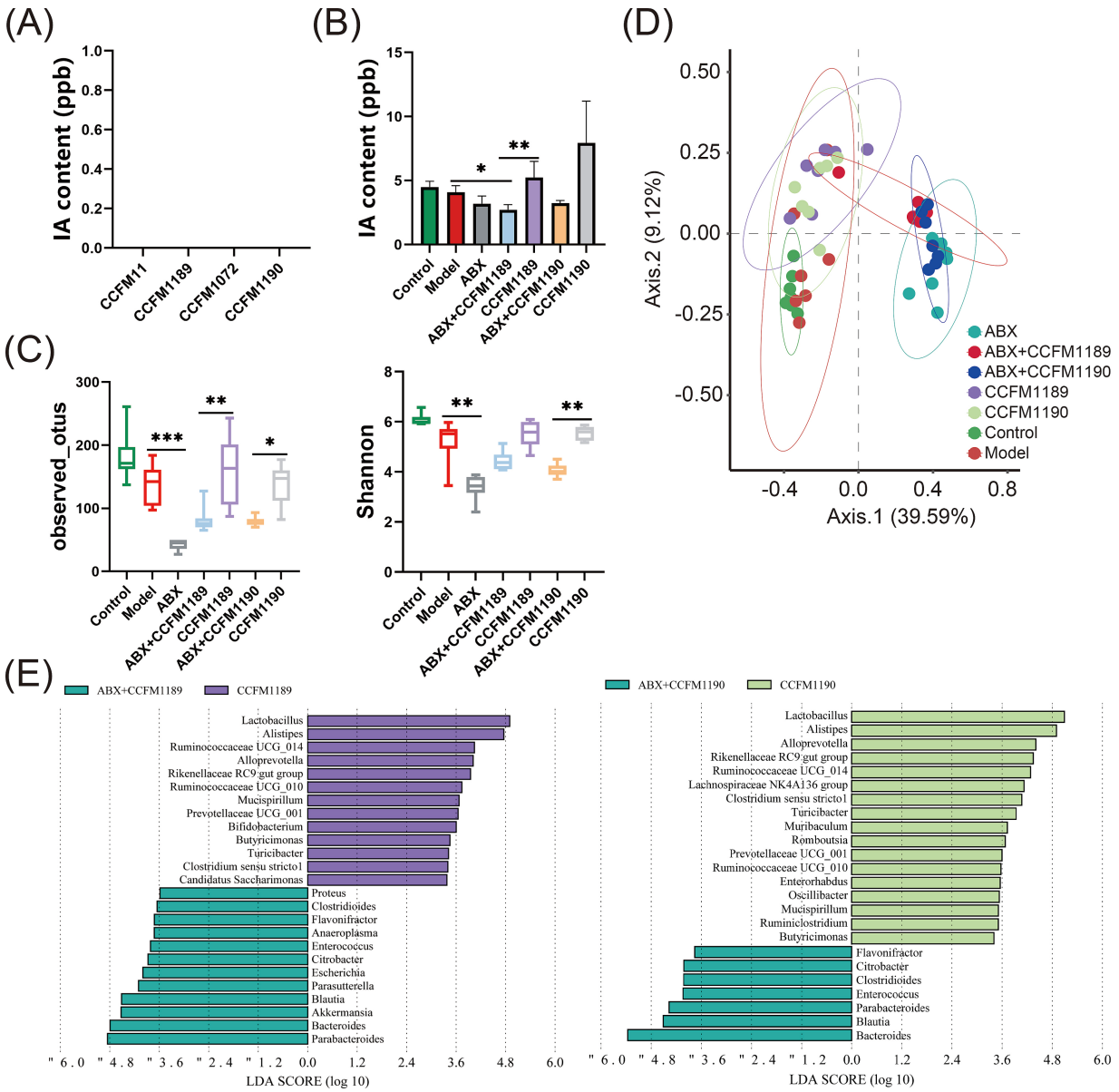

FIGURE 4
Alterations in intestinal IA levels and microbiota in both normal food allergic mice and food allergic mice subjected to ABX treatment, with the intervention of effective probiotic strains. **(A)** IA content *in vitro* fermentation. **(B)** The IA content in the feces of mice. **(C)** Changes in alpha diversity index. **(D)** Bray-Curtis principal coordinate analysis (PCoA) of beta diversity between the groups. **(E)** The differences in bacterial genera between the CCFM1189 group and ABX + CCFM1189 group, as well as between the CCFM1190 group and ABX + CCFM1190 group. The result represents mean ± SEM. The *p*-value were measured using one-way ANOVA with Duncan's multiple range test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

diversity indicated that the distributions of ABX treatment groups (ABX, ABX + CCFM1189, and ABX + CCFM1190) was more concentrated, while were dispersed in the control, model, CCFM1189, and CCFM1190 groups (Figure 4D).

Moreover, an analysis was conducted to identify the differential bacterial genera within the gut microbiota of mice between the probiotic intervention group and the group receiving both ABX treatment and probiotic intervention. As shown in Figure 4E, it was observed that within the CCFM1189 group, the dominant bacterial species included *Lactobacillus*, *Alistipes*, *Ruminococcaceae UCG-014*, *Alloprevotella*, *Rikenellaceae RC9 gut group*, *Ruminococcaceae UCG-010*, *Mucispirillum*, *Prevotellaceae UCG-001*, *Bifidobacterium*, *Butyricimonas*, *Turicibacter*, *Clostridium sensu stricto 1*, and *Candidaatus saccharimonas*. In the CCFM1190 group and the ABX + CCFM1190 group, *Lactobacillus*, *Alistipes*, *Alloprevotella*, *Rikenellaceae RC9 gut group*, *Ruminococcaceae UCG-014*, *Lachnospiraceae NK4A136 group*, *Clostridium sensu stricto 1*, *Turicibacter*, *Muribaculum*, *Romboutsia*, *Prevotellaceae UCG-001*, *Ruminococcaceae UCG-010*, *Enterorhabdus*, *Oscillibacter*, *Mucispirillum*, *Rumiclostridium*, and *Butyricimonas* were found to be enriched within the CCFM1190 group. Among the bacterial genera enriched under the intervention of effective probiotic strains, *Clostridium*, *Prevotella*, *Bifidobacterium*, and *Lachnospira* have been identified as bacterial genera capable of producing tryptophan-indole derivatives (22). Consequently, we assert that the ingestion of the effective strains CCFM1189 and

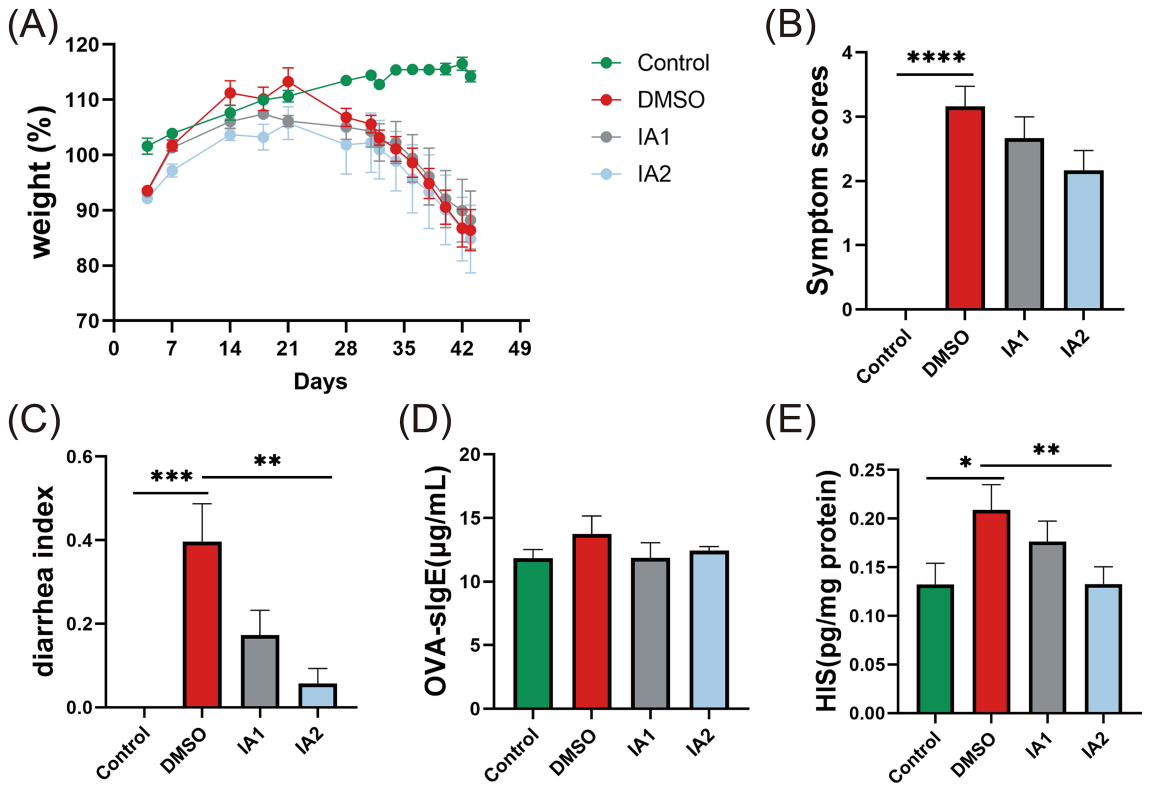

FIGURE 5
The impact of external IA intervention on food allergy related-indicators in mice. **(A)** The weight (%) changes from day 0 to day 43. **(B)** The symptom scores of food allergic mice on the day 43. **(C)** The diarrheal index of food allergic mice on the day 43. **(D)** The levels of serum OVA-sIgE of mice. **(E)** The levels of HIS in the supernatant of jejunal tissues. The result represents mean ± SEM. The *p*-value were measured using one-way ANOVA with Duncan's multiple range test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

CCFM1190 can modulate the gut microbiota, promoting the proliferation of bacteria that produce elevated levels of IA and resulting in an increased concentration of IA within the gut.

IA can directly relieve food allergic symptoms in mice

In order to further elucidate the mechanisms and underlying material basis through which probiotics effectively mitigate food allergies, we conducted direct interventions using low-dose and high-dose IA in murine models of food allergy. The results indicated that neither dosage of IA significantly influenced the weight loss observed in the mice (Figure 5A). However, in comparison to the DMSO group, the IA intervention exhibited remarkable efficacy in ameliorating the phenotypic manifestations (Figure 5B) and the diarrhea index (Figure 5C) in food allergy mice, with the high-dose IA demonstrating a more pronounced effect. Furthermore, neither dosage of IA produced a significant reduction in serum OVA-sIgE levels in food allergy mice (Figure 5D). Regarding the HIS levels in the jejunal tissue supernatant of the mice, both low-dose and high-dose IA were effective in decreasing HIS concentrations, with the high-dose IA showing a notably greater impact (Figure 5E).

IA has the potential to inhibit the Th2-type immune response to a certain degree. In comparison to the DMSO group, low-dose IA was found to significantly reduce the levels of IL-4 in the jejunal supernatant of mice (Figure 6A). Regarding IL-5 (Figure 6B) and IL-13 (Figure 6C), high-dose IA exhibited a decreasing effect. Furthermore, IA was observed to enhance the positive expression of ZO-1 and Occludin immunohistochemical staining. In the DMSO group, the expression levels of ZO-1 (Figure 6D) and Occludin (Figure 6E) in the jejunum of mice were diminished; however, IA administration resulted in an elevation of these expression levels. Notably, there was no significant difference in the regulatory effects of IA on tight junction proteins between the two dosage levels. In conclusion, we have confirmed that IA serves as a crucial metabolite in the mitigation of food allergies and have elucidated the mechanisms of action of the probiotic strains CCFM1189 and CCFM1190.

Discussion

Our prior investigation has revealed that the probiotic strains CCFM1189 and CCFM1190 mitigate OVA-induced food allergy in mice through the modulation of gut microbiota and the enhancement of IA levels in fecal specimens. Nevertheless, before that, it remained unclear whether CCFM1189 and CCFM1190 produce IA through their own fermentation processes or by regulating the intestinal symbiotic microbiota. Preceding research has documented those bacteria harboring the phenyllactate dehydrase gene cluster (fldAIBC) possess the capability to metabolize tryptophan, yielding IA and indole-3-proionic acid (23). With the exception of

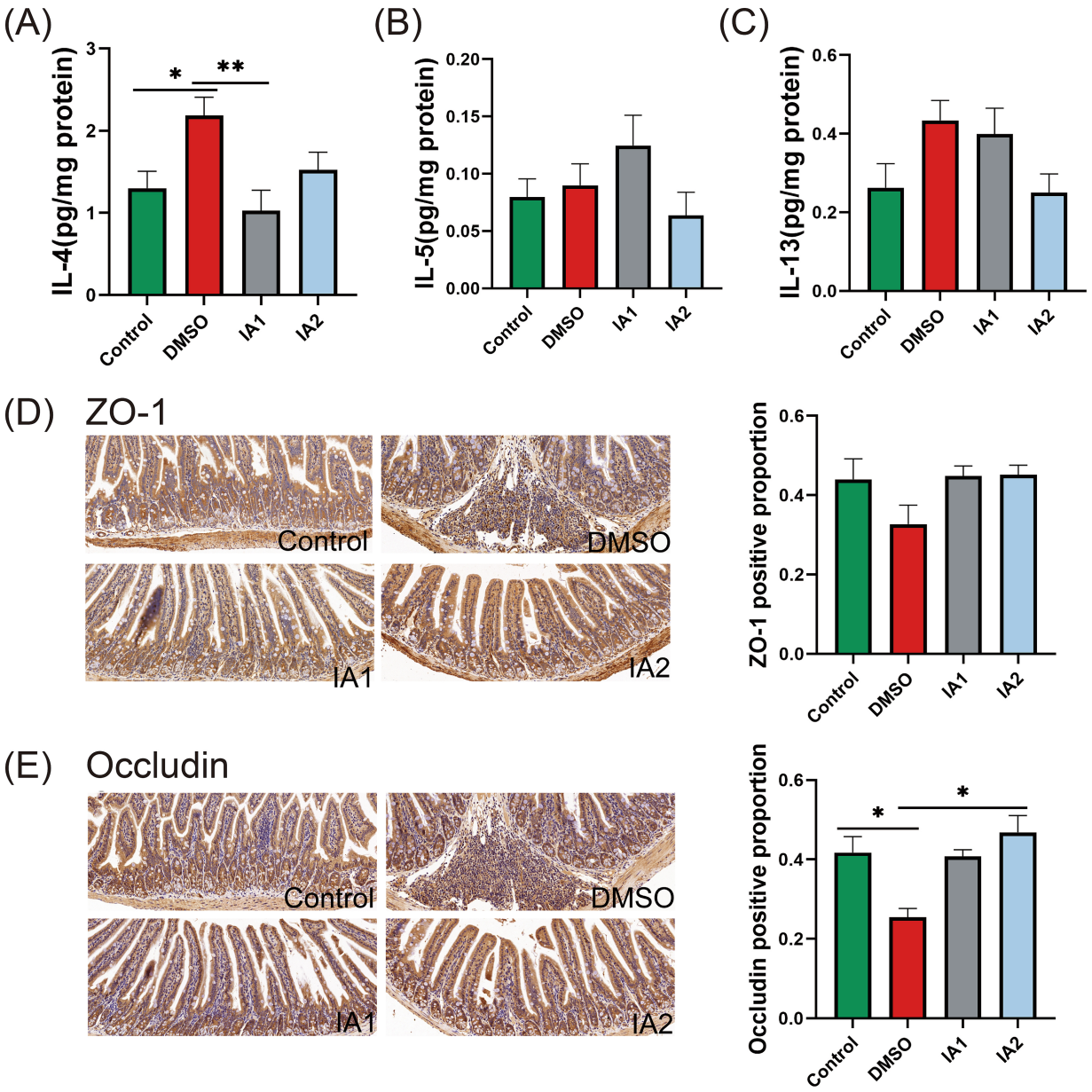

FIGURE 6
The impact of external IA intervention on cytokines and intestinal barrier in food allergic mice. **(A–C)** The levels of IL-4, IL-5, and IL-13 in the supernatant of jejunal tissues. **(D)** The pathological sections of the jejunum with immunohistochemistry (ZO-1, 20×) and the positive percentage of ZO-1. **(E)** The pathological sections of the jejunum with immunohistochemistry (Occludin, 20×) and the positive percentage of Occludin. The result represents mean ± SEM. The *p*-value were measured using one-way ANOVA with Duncan's multiple range test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Peptostreptococcus, neither *Lactobacillus* nor *Bifidobacterium* have been documented to harbor this gene cluster. The experimental outcomes of *in vitro* fermentation conducted in this study further corroborated that CCFM1189 and CCFM1190 are incapable of producing IA through the direct metabolism of tryptophan. In light of the aforementioned findings, it is plausible to infer that IA production is mediated by the regulation of gut microbiota.

Employing pseudo-germ-free mice as experimental subjects, we substantiated that these two probiotic strains capable of mitigating food allergies necessitate interaction with the gut's endogenous microbiota. Zhang et al. (24) noted exacerbated allergic manifestations, including scratching, convulsions, and diminished activity, in mice subjected to antibiotic treatment, a result congruent with our observations. Nevertheless, the serum concentrations of OVA-sIgE in OVA-sensitized mice treated with ABX were marginally elevated compared to those in untreated mice, a discrepancy that does not correlate with the phenotypic symptom exacerbation. This incongruity may be attributable to certain antibiotics, such as tetracycline, potentially suppressing IgE production (25). Furthermore, we discovered that ABX treatment attenuated the inhibitory impacts of CCFM1189 and CCFM1190 on Th2 immune responses. This may be because antibiotic treatment perturbs the composition of gut microbiota and their metabolites, thereby modulating the Th1/Th2 balance (26). Zhang et al. (27) demonstrated that *Lactobacillus*

acidophilus ST218 intervention could repress the elevation of Th2 cytokines IL-4 and IL-5 induced by airway inflammation in antibiotic-treated mice, a result consistent with our findings.

Alterations in tryptophan (Trp) metabolism have been documented to be intimately linked with a multitude of diseases (28). Multiple pro-inflammatory mediators and deleterious T cell activities are modulated by Trp and its metabolites, functioning as an adaptive regulatory mechanism to curtail excessive acute immune responses within tissues (29). Roughly 4–6% of tryptophan transport is conveyed to the large intestine, where it undergoes degradation by the gut microbiota. In the context of microbiome influence, tryptophan can be transformed into indoles and their derivatives, such as indole, tryptamine, indole-3-acetamide, indole-3-lactic acid, indole-3-propionic acid, indole-3-acetaldehyde, indole acetic acid, indole-3-aldehyde, and IA (30). These indole derivatives have been extensively identified to exhibit properties that suppress intestinal inflammation (17, 31), augment gut barrier function (32–34), boost host antioxidant capacity (35, 36), and regulate host immune responses (37, 38).

Our research has, for the first time, demonstrated that IA exerts a direct ameliorative effect on food allergy in mice. Despite the lack of an alleviative effect of IA intervention on weight loss in mice, it effectively mitigated allergic and diarrhea symptoms, as well as OVA-sIgE, HIS, and Th2-type cytokine levels in the jejunum, and enhanced the expression of tight junction proteins ZO-1 and Occludin, thereby alleviating intestinal injury induced by food allergy. As a metabolite of tryptophan, IA is capable of activating the AhR signaling pathway (39). The activation of this pathway subsequently inhibits Th2-type cytokines and antigen-specific antibodies generated by B cells, thereby modulating the Th2-mediated immune response (40). Tight junction proteins constitute a barrier at the apex of the adjacent epithelial cell membrane, thereby inhibiting paracellular molecular transport between cells. In conjunction with adherent connexins, these proteins assemble into an apical connexin complex, which plays a pivotal role in maintaining intestinal barrier integrity (41, 42). It has been reported that other small molecular tryptophan metabolites exert regulatory effects on the expression of intestinal tight junction proteins, thereby modulating intestinal barrier integrity (17, 23). For example, indole-3-propionic acid has been shown to upregulate mRNA encoding tight junction proteins and augment the expression of Claudin and Occludin (32). Additionally, indole-3-propionic acid can enhance the secretion of TFF3 and RELMβ by MUC2, MUC4, and goblet cells, thereby reinforcing the mucus barrier (34). Our findings suggest that IA enhances the expression of ZO-1 and Occludin in the jejunum tissue of food allergic mice. Consequently, these results imply that tryptophan indole metabolites possess a general capacity to augment intestinal barrier integrity.

Conclusion

In summary, our investigation has elucidated the underlying mechanisms and material foundations through which probiotic strains *L. plantarum* CCFM1189 and *L. reuteri* CCFM1190 mitigate food allergies. Mice afflicted with food allergies and subjected to antibiotic treatment failed to exhibit symptom alleviation upon probiotic supplementation, thereby substantiating those efficacious strains must exert their effects via the gut microbiota. In addition, we also elucidate the pivotal role of IA in mitigating food allergies, explicate the underlying mechanism through which probiotics that do not produce IA manifest their effects, and endeavor to furnish scientific substantiation for prospective food allergy prophylactic and therapeutic approaches predicated on gut microbiota.

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/, PRJNA1182655.

Ethics statement

The animal study was approved by This investigation was conducted in full compliance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23 Rev. 985) and received formal approval from the Laboratory Animal Ethics Committee of Jiangnan University (Wuxi, Jiangsu, China; approval numbers JN. No. 20210915b1121201[300] and JN. No. 20211115b0581228[434]). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZP: Writing – original draft, Data curation, Formal analysis, Resources. LQ: Writing – original draft. TM: Writing – review & editing. HW: Supervision, Writing – review & editing. WL: Funding acquisition, Writing – review & editing. YC: Conceptualization, Methodology, Supervision, Writing – review & editing. QZ: Conceptualization, Validation, 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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Therapeutic potential of short-chain fatty acids for acute lung injury: a systematic review and meta-analysis of preclinical animal studies

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Background: Short-chain fatty acids (SCFAs), derived from the fermentation of dietary fiber by intestinal commensal bacteria, have demonstrated protective effects against acute lung injury (ALI) in animal models. However, the findings have shown variability across different studies. It is necessary to conduct a comprehensive evaluation of the efficacy of these treatments and their consistency.

Objective: This systematic review and meta-analysis aimed to explore the effects of SCFAs on ALI based on preclinical research evidence, in order to provide new treatment strategies for ALI.

Methods: We included studies that tested the effects of SCFAs on ALI in animal models. This study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A comprehensive search for relevant studies was conducted in the PubMed, Embase, Web of Science, Cochrane Library, and China National Knowledge Infrastructure (CNKI) databases up to February 2024. The data were extracted in accordance with the established selection criteria, and the risk of bias was evaluated for each study.

Results: A total of 16 articles were finally included in the meta-analysis. The results indicated that the SCFAs significantly reduced lung wet-to-dry weight (SMD = −2.75, 95% CI = −3.46 to −2.03, $p < 0.00001$), lung injury scores (SMD = −5.07, 95% CI = −6.25 to −3.89, $p < 0.00001$), myeloperoxidase (SMD = −3.37, 95% CI = −4.05 to −2.70, $p < 0.00001$), tumor necrosis factor-alpha (SMD = −3.31, 95% CI = −4.45 to −2.16, $p < 0.00001$) and malondialdehyde (SMD = −3.91, 95% CI = −5.37 to −2.44, $p < 0.00001$) levels in animal models of ALI. The results of the subgroup analysis indicated that the efficacy of SCFAs varies significantly with dosage and duration of treatment.

Conclusion: SCFAs can reduce inflammation and oxidative stress in animal models of ALI. The clinical efficacy of SCFAs for ALI deserves further in-depth research.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=584008, CRD42024584008.

KEYWORDS

acute lung injury, meta-analysis, preclinical evidence, short-chain fatty acids, the gut-lung axis

1 Introduction

In recent years, with the outbreak of novel epidemic respiratory viruses, the deaths caused by the acute lung injury (ALI) has significantly increased, posing a substantial threat to human health (1, 2). ALI is a serious disease characterized by excessive inflammatory responses, usually triggered by various direct or indirect factors, including pneumonia, sepsis, trauma, and blood transfusions (3, 4). During the acute exudative phase of ALI, a large number of immune cells accumulate at the site of injury, initiating a series of inflammatory signaling pathways and releasing a range of pro-inflammatory cytokines (5). Subsequently, the inflammation disrupts the barrier function of the alveolar epithelium and endothelium, leading to increased permeability of the alveolar-capillary membrane (6). As a consequence of this persistent acute inflammatory process, the majority of patients with ALI will rapidly experience a deterioration in their respiratory function, which will eventually progress to the more severe form of and acute respiratory distress syndrome (ARDS) (7). Despite some advancements in the scientific understanding of the pathophysiological mechanisms underlying ALI, current treatment approaches still mainly depend on supportive therapies, including mechanical ventilation (8). ALI/ARDS remains one of the most fatal clinical syndromes in intensive care (4, 9). In light of these developments, researchers engaged in both clinical and preclinical studies are directing their attention toward the creation of novel treatments and medications, with the objective of providing patients with ALI/ARDS more efficacious therapeutic alternatives.

The emerging theory of the gut-lung axis elucidates a robust interconnection between the gut and the lungs (10). The gut microbiota and their metabolites have been demonstrated to play a role in the pathogenesis of various lung diseases, including asthma and respiratory infections, by regulating both innate and adaptive immunity (11). A substantial body of research indicates that the release of short-chain fatty acids (SCFAs) represents a pivotal mechanism through which the gut microbiota maintains host health and gut homeostasis (12). SCFAs are a type of fatty acid produced by beneficial bacteria in the gut through the fermentation of dietary fiber (13). In the intestines, the primary SCFAs include acetate, propionate, and butyrate (12). SCFAs can be absorbed and utilized by intestinal epithelial cells, with a portion serving as an energy source for cellular metabolic activities, while another portion enters the peripheral circulation to function as signaling molecules that regulate the host's biological responses (14). They play an important role in regulating gut microbiota balance, maintaining intestinal barrier function, suppressing inflammation, and improving immune system function (15). The relationship between elevated levels of SCFAs in the gut and improved lung health is becoming increasingly clear (16). In patients with impaired immune function, an elevated concentration of SCFAs in their feces is associated with a markedly reduced risk of developing lung infections (14, 17). Currently, researchers have begun to explore the effects of exogenous supplementation of SCFAs in the treatment of ALI in animal experiments. For example, some studies have found that supplementing with SCFAs can significantly reduce the lung injury scores, lung wet-to-dry (W/D) ratio, myeloperoxidase (MPO), tumor necrosis factor-alpha (TNF-α), and malondialdehyde (MDA) levels in animal models of ALI (18–20). However, other studies have reached contradictory conclusions (21, 22). Therefore, it is necessary to summarize the results of the relevant publications to more comprehensively assess the impact of SCFAs on ALI.

Although there is currently an absence of clinical evidence regarding the application of SCFAs in patients with ALI/ARDS, the results of preclinical studies in animal experiments are invaluable for clinical practice and provide a crucial foundation for in-depth research into disease pathology and mechanisms (23). Our study aimed to evaluate the efficacy and potential mechanisms of SCFAs in ALI/ARDS animal models through a systematic review and meta-analysis. The findings could provide robust support for future experimental design and clinical application.

2 Materials and methods

2.1 Search strategy

A search in databases including PubMed, Cochrane Library, Embase, Web of Science, and China National Knowledge Infrastructure was performed from inception to February 2024.

The keywords of the search were as follows: "actue lung injury," "acute respiratory distress syndrome," "ALI," "ARDS," "short chain fatty acid," "SCFA," "acetate," "propionate," "butyrate." After manual screening, additional studies were identified from relevant reviews and citations (the detailed search strategy is shown in Supplementary Table S1).

2.2 Inclusion and exclusion criteria

Studies included in this review must meet the following criteria: (1) the participants were rat or mouse animal models of ALI/ARDS; (2) the intervention drugs were solutions of SCFAs such as sodium acetate, sodium propionate or sodium butyrate, while the control group could be either a blank control or an equal volume of PBS or normal saline; and (3) the literature was published in Chinese and English. The exclusion criteria were as follows: (1) reviews, clinical trials, case reports, and protocols; (2) articles for which the full text was not available.

2.3 Data extraction

Two researchers independently reviewed the literature, extracted data and cross-checked the data, and the third researcher negotiated

and adjudicated in case of disagreement. The main content of the data extraction included: first author's name, year of publication, sample size of each group, animal species, modeling methods, duration, types and dose of SCFAs, and outcome-related indicators. For articles that only reported data in the form of images, we used Origin 2022 software to extract relevant data from the images. When administering different doses or durations of SCFAs, we recorded all data meticulously for subsequent subgroup analysis. The primary outcome measure was the lung W/D ratio and lung injury scores, while the secondary outcome measures included MPO, TNF-α, and MDA.

2.4 Quality assessment

The SYRCLE's risk of bias tool was used to assess the risk of bias in the included studies (24). It was developed on the basis of the Cochrane risk of bias tool and consisted of 10 entries. The results were assessed using "yes," "no" and "unclear" to represent low, high and unclear risk of bias, respectively (25).

2.5 Statistical analyses

The analysis was conducted using RevMan 5.2 (Cochrane Collaboration, Oxford, United Kingdom) and Stata 14.0 (StataCorp, TX, United States) software. Considering that the results of the outcome indicators were all continuous variables, the standardized mean difference (SMD) was calculated with 95% confidence interval (CI) as the overall effects. Heterogeneity was assessed using I^2, and a value exceeding 50% was considered to indicate high heterogeneity. For each outcome measure, specified a random-effects model of analysis. Subgroup and sensitivity analyses were performed to explore potential heterogeneity between studies and to identify different sources of confounding. The presence of publication bias was identified through the use of funnel plots and Egger's test. Finally, we employed trim-and-fill methods to detect potential asymmetry and to assess the robustness of the conclusions.

3 Results

3.1 Selection and characteristics of included studies

As shown in Figure 1, we initially obtained 868 articles, of which 316 were identified as duplicates and subsequently excluded. In addition, seven articles were identified through citation searches. According to the predetermined screening criteria, 18 eligible articles were finally included (18–21, 26–39). All studies described the species of experimental animals, with 10 articles using mice and the remainder using SD rats. The most frequently utilized modeling methods in all articles were intratracheal injection of lipopolysaccharide (LPS) and cecal ligation and puncture (CLP) to induce ALI. A total of 11 articles used sodium butyrate as the intervention, while three articles employed acetate, two articles used a solution of mixed SCFAs, one used propionate, and one used both acetate and propionate simultaneously. A detailed account of the specific details and characteristics of each included study can be found in Table 1 and Supplementary Table S2.

3.2 Risk of bias assessment

The overall result is shown in Figure 2 and Supplementary Table S3. Of the 18 articles included, only three clearly reported the use of a random number table for animal randomization (20, 33, 36), while the remaining articles merely mentioned "randomization" without specifying the method and were therefore rated as "unclear." Based on the descriptions of animal housing conditions in 14 articles (18–21, 26, 28–30, 33, 35–39), it could be concluded that animals were randomly housed during the experiments. In the integrity report entries, we found that two articles did not provide accurate sample size data, thereby potentially posing a higher risk of bias (28, 37). To ensure the reliability of the analysis results, we only recorded the characteristics of these two studies and did not include their experimental data. In the end, a total of 16 articles were included in this meta-analysis.

3.3 Meta-analysis of primary outcomes

3.3.1 Lung W/D ratio

Lung W/D ratio is a direct indicator of pulmonary edema and an important measure for assessing the severity of ALI (40). A total of 11 articles (18–21, 27, 29, 31, 33, 35, 36, 38), including 22 studies, reported the levels of W/D ratios. The results indicated that the SCFAs intervention significantly reduced the lung W/D ratio ($I^2 = 70\%$; SMD = −2.88, 95% CI = −3.63 to −2.13, $p < 0.00001$) in comparison to the control group (Figure 3).

3.3.2 Lung injury scores

A total of 10 articles (19, 21, 27, 29, 30, 33–36, 39), which include 16 studies, reported changes in lung injury scores. The results indicate that SCFAs intervention can effectively reduce lung injury scores ($I^2 = 74\%$; SMD = −5.07, 95% CI = −6.25 to −3.89, $p < 0.00001$) compared to the control group (Figure 4).

3.4 Meta-analysis of secondary outcomes

3.4.1 MPO

MPO serves as a marker of neutrophil activation and is significantly increased in tissue injury and various associated inflammatory diseases (41). A total of 8 articles (20, 29–32, 34, 35, 38), including 16 studies, compared lung tissue MPO activities between the SCFAs and control groups. The SCFAs intervention groups showed lower MPO activities ($I^2 = 44\%$; SMD = −3.37, 95% CI = −4.05 to −2.70, $p < 0.00001$) than the control group (Figure 5).

3.4.2 TNF-α

TNF-α is a pleiotropic cytokine that leads to the development of inflammatory responses in ALI (42). A total of 7 articles (18, 21, 27, 29, 31, 33, 35), including 15 studies, reported changes in TNF-α levels in the bronchoalveolar lavage fluid (BALF). Compared to the control group, the SCFAs intervention could significantly reduce the TNF-α

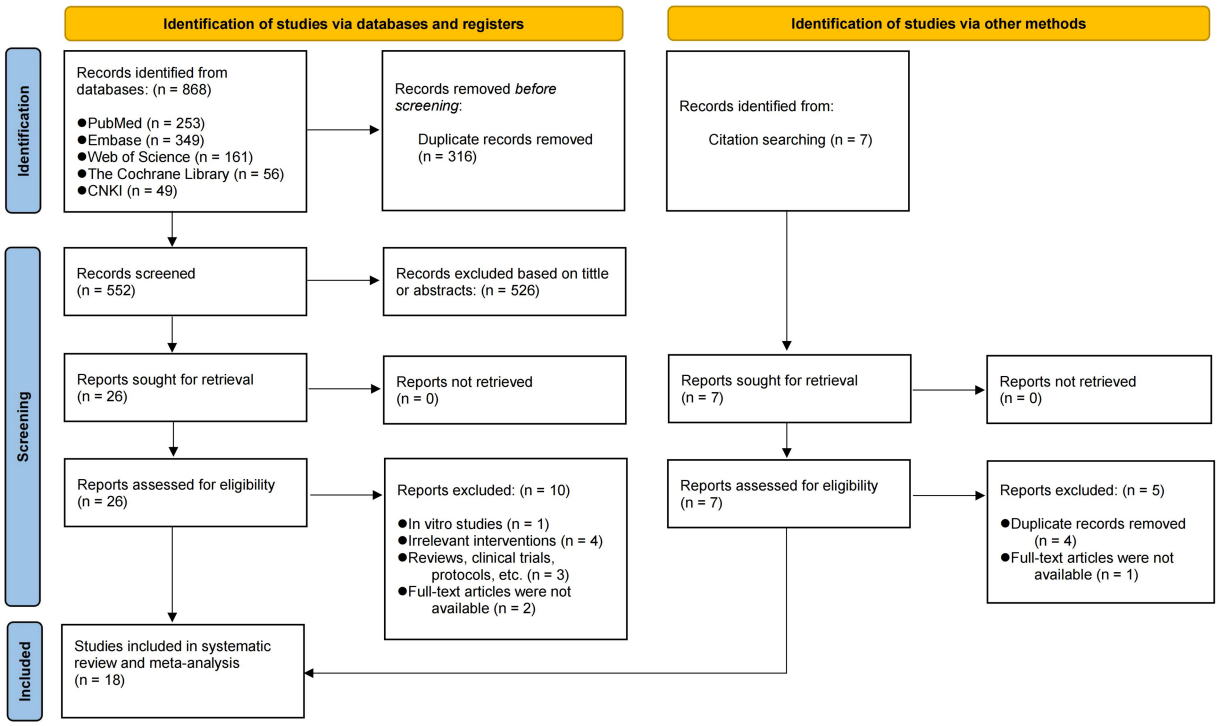

FIGURE 1
PRISMA flow diagram of the study selection process.

levels (I^2 = 66%; SMD = −3.31, 95% CI = −4.45 to −2.16, $p < 0.00001$; Figure 6).

3.4.3 MDA

MDA is one of the most frequently examined biomarkers of oxidative stress (43). A total of 7 articles (20, 21, 27, 29, 32, 35, 39), including 12 studies, compared lung tissue MDA levels between SCFAs and control groups. The SCFAs intervention significantly alleviated MDA expression (I^2 = 87%; SMD = −3.91, 95% CI = −5.37 to −2.44, $p < 0.00001$) compared to the control group (Figure 7).

3.5 Subgroup analysis of primary outcome indicators

We conducted subgroup analyses basing on the following variables: the modeling methods, types of SCFAs, dosage, duration, animal species, and administration route. For the dosage subgroup analysis, we excluded one study because it did not define the drug dosage (39).

3.5.1 Lung W/D ratio

The results of the subgroup analysis showed that the 400 mg/kg/d dosage group may have a more significant effect in reducing W/D levels (I^2 = 88%; SMD = −8.31, 95% CI = −13.02 to −3.59, $p = 0.0006$) compared to other dosages. Additionally, intervention duration exceeding 24 h may provide better results (I^2 = 24%; SMD = −3.81, 95% CI = −4.91 to −2.70, $p < 0.00001$) compared to shorter treatment durations. The analysis also indicated that SCFAs showed better efficacy in rat models (I^2 = 82%; SMD = −3.90, 95% CI = −5.28 to −2.52, $p < 0.00001$). There were no statistically significant differences between the subgroups concerning the modeling method, type of SCFAs, and administration route ($p > 0.05$). The results of all subgroups were consistent with the overall results (Table 2; Supplementary Figures S1–S6).

3.5.2 Lung injury scores

In 15 studies involving drug dosage, we found that a dosage of 400 mg/kg/d was more effective in reducing lung injury scores (I^2 = 69%; SMD = −7.46, 95% CI = −10.48 to −4.45, $p < 0.00001$) compared to other dosage groups. Additionally, a treatment duration of 24 h to 7 days may yield more pronounced effects (I^2 = 64%; SMD = −6.87, 95% CI = −9.55 to −4.18, $p < 0.00001$). No statistically significant differences were found among the subgroups regarding the modeling method, type of SCFAs, animal species, and administration route ($p > 0.05$; Table 3; Supplementary Figures S7–S12).

3.6 Sensitivity analysis

In order to explore the stability of the results, a sensitivity analysis was conducted on five meta-analyses. Eliminating any single study did not reverse the overall effects of SCFAs on the ALI (Supplementary Figure S13). It suggested that the meta-analyses results were relatively stable.

3.7 Publication bias

The funnel plots of the five meta-analyses exhibited asymmetry (Supplementary Figure S14), and the results of Egger's test were

TABLE 1 Main characteristics of the 18 included studies.

Author (Year)	Animal species (Gender)	Model	Sample size (EG/CG)	Intervention			
				Type of SCFAs	Method of intervention	Medication time	Duration
Chu (18)	C57BL/6 J mice (Male)	Hyperoxia-induced ALI	6/6	Sodium acetate (200 mM)	Oral administration	Pre-treatment	3 weeks
Cui (19)	SD rats (Male)	Acute Pancreatitis-induced ALI	10/10	Sodium butyrate (5 mg/kg/d)	Intravenous injection	Post-treatment	12 h
Ding (20)	SD rats (Male)	LPS-induced ALI	10/10	Sodium butyrate (1 g/kg/d)	Intravenous injection	Post-treatment	6 h
Hildebrand (2021) (26)	C57BL/6 mice (Male)	LPS-induced ALI	12/12	Sodium acetate, propionate, butyrate (50 mM each)	Oral administration	Pre-treatment	2 weeks
Hung (27)	SD rats (Male)	I/R-Induced ALI	6/6	Sodium acetate (100 mg/kg/d, 200 mg/kg/d, 400 mg/kg/d)	Pulmonary artery perfusion	Post-treatment	60 min
Li (28)	BALB/c mice (Male)	LPS-induced ALI	10/10	Sodium butyrate (500 mg/kg)	Intraperitoneal injection	Pre-treatment	24 h
Liang (29)	SD rats (Female)	Burn-induced ALI	8/8	Sodium butyrate (400 mg/kg/d)	Intraperitoneal injection	Post-treatment	12 h, 24 h, 48 h
Liu (31)	ICR mice (Female)	LPS-induced ALI	10/10	Sodium butyrate (25 mg/kg/d)	Intragastric administration	Pre-treatment	12 h
Ni (30)	BALB/c mice (Male)	LPS-induced ALI	3/3	Sodium butyrate (10 mg/kg/d)	Intragastric administration	Pre-treatment	1 h, 3 h, 6 h, 12 h, 24 h
Tang (32)	SD rats (Male)	Intestinal I/R-induced ALI	10/10	Sodium butyrate (400 mg/kg/d)	Subcutaneous injection	Post-treatment	1 h, 4 h
Xiang (2022) (33)	SD rats (Male)	LPS-induced ALI	5/5	Sodium acetate, propionate, butyrate (300 mg/kg/d, 100 mg/kg/d, 100 mg/kg/d; 600 mg/kg/d, 200 mg/kg/d, 200 mg/kg/d)	Intragastric administration	Pre-treatment	7 days
Xiong (34)	BALB/c mice (Male)	Acute Pancreatitis-induced ALI	8/8	Sodium butyrate (200 mg/kg/d, 500 mg/kg/d)	Intragastric administration	Pre-treatment	7 days
Xu (35)	C57BL/6 J mice (Male)	LPS-induced ALI	10/10	Sodium acetate (4 mmol/kg/d)	Intraperitoneal injection	Post-treatment	6 h
Yang (36)	C57BL/6 J mice (Male)	CLP-induced ALI	6/6	Sodium butyrate (25 mg/kg/d)	Intragastric administration	Pre-treatment, Post-treatment	24 h
Ying (37)	C57BL/6 J mice (Male)	I/R-Induced ALI	NA	Sodium butyrate (5 mg/kg/d)	NA	Pre-treatment	1 week
Zhang L (38)	C57BL/6 J mice (Male)	CLP-induced ALI	6/6	Sodium butyrate (200 mg/kg/d)	Intraperitoneal injection	Pre-treatment	18 h
Zhang TT (21)	SD rats (Male)	LPS-induced ALI	5/5	sodium propionate (300 mg/kg/d, 500 mg/kg/d)	Intragastric administration	Pre-treatment	7 days
Zhang YD (39)	C57BL/6 mice (Male)	ZnONPs-induced ALI	5/5	Sodium acetate (100 mM), Sodium propionate (100 mM)	Oral administration	Pre-treatment	21 days

EG, experimental group; CG, control group; LPS, lipopolysaccharides; CLP, cecal ligation and puncture; I/R, ischemia/reperfusion; NA, not applicable.

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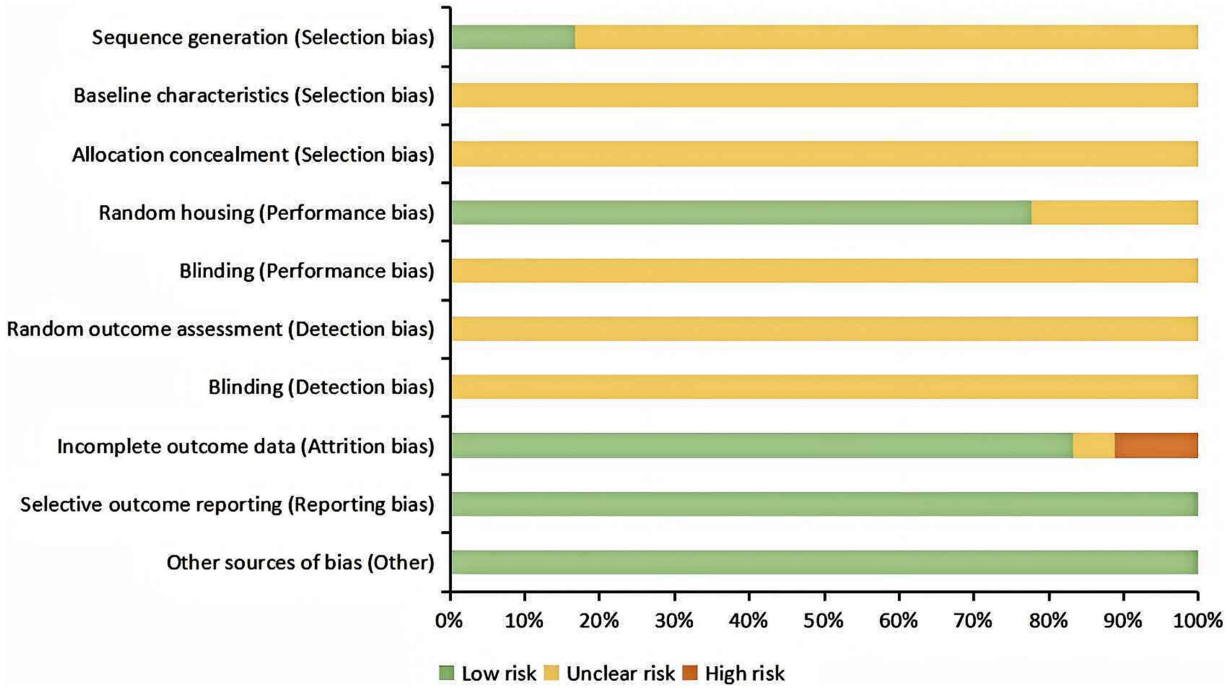

FIGURE 2
Risk of bias assessment results.

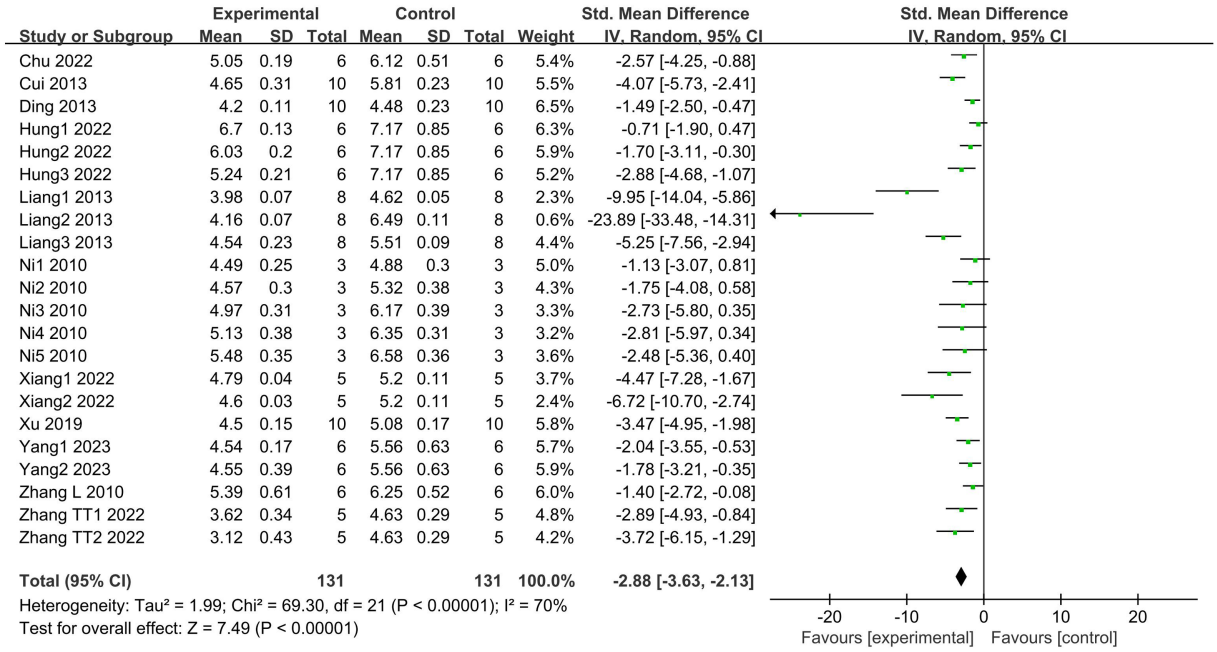

FIGURE 3
Forest plot of the effects of SCFAs intervention on the lung W/D ratio.

statistically significant ($p < 0.05$). While these findings suggested a certain degree of publication bias, the trim-and-fill analyses indicated that publication bias did not affect the overall estimate (Supplementary Figure S15; no trimming was performed and the data remained unchanged). Consequently, the conclusions remained robust.

4 Discussion

As the role of gut microbiota and their metabolites in regulating immunity and inflammatory responses becoming increasingly clear, their relationship with lung disease (also known as gut-lung axis) have

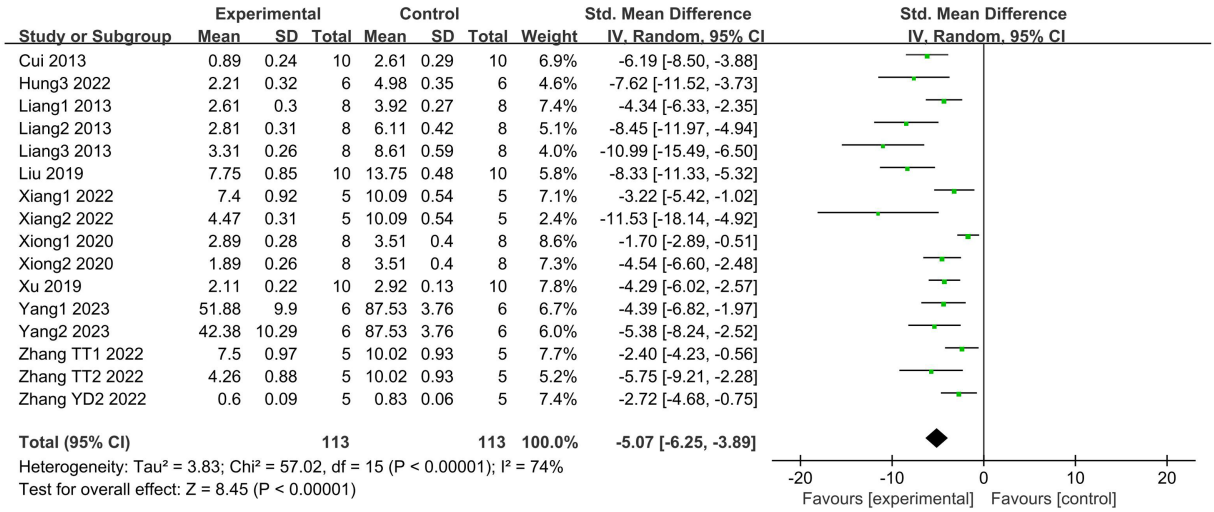

FIGURE 4
Forest plot of the effects of SCFAs intervention on the lung injury scores.

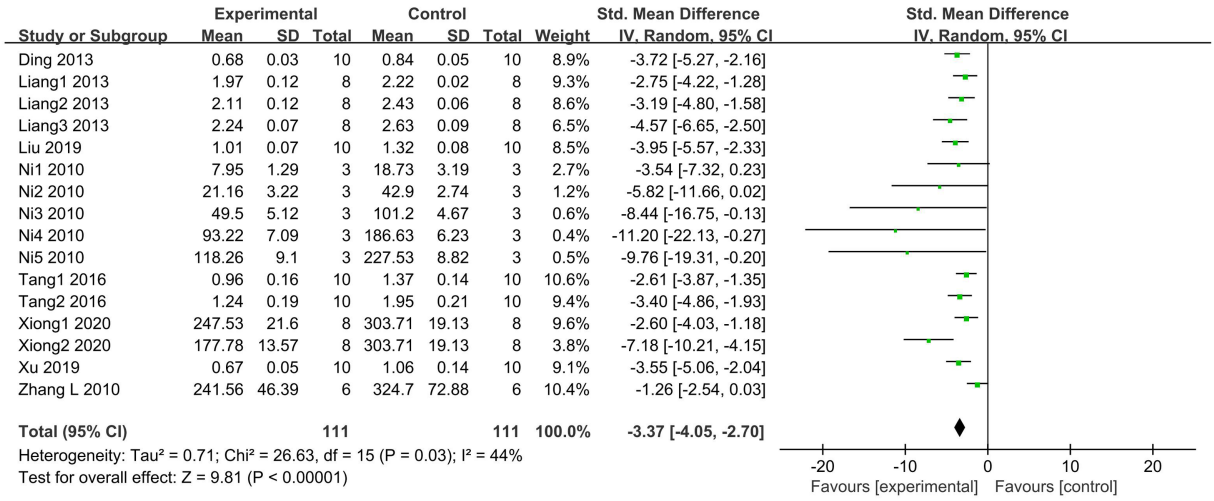

FIGURE 5
Forest plot of the effects of SCFAs intervention on MPO activity in lung tissue.

attracted significant attention (10). SCFAs, produced by gut microbiota fermentation, are regarded as key molecules in the gut-lung axis, providing a novel therapy to alleviate even reverse the ALI deterioration (44, 45). To the best of our knowledge, this is the first systematic review and meta-analysis exploring the effects of SCFAs supplementation on ALI in animal research. The subgroup analysis results indicated that the therapeutic effects of SCFAs may be closely related to the dosage and duration of treatment, while being less influenced by the modeling method and the type of SCFAs. In particular, when the dosage reached 400 mg/kg/d and was administered continuously for 24 h to 7 days, the SCFAs showed a better efficacy. Despite the high heterogeneity and publication bias, SCFAs still significantly improved the inflammation levels and lung injury severity in ALI animals based on the overall results. Additionally, we obtained similar results through sensitivity analysis and trim-and-fill analysis, which enhanced the robustness of our findings.

The homeostasis of the gut actually impacts lung health (45). How is this effect mediated by SCFAs? Some studies have explored this in depth.

In fact, there is an immune mechanism regulated by the microbiota that exists between the respiratory and gastrointestinal tracts. The term "gut microbiota" refers to the complex ecological community composed of symbiotic and pathogenic microorganisms residing in the gut, encompassing thousands of species of bacteria, archaea, viruses, fungi, and other microbes (46, 47). These microorganisms maintain a dynamic balance and protect the gut through multifaceted mechanisms, such as synthesizing metabolites and toxins, providing nutrients, regulating the immune system, and preserving the function of the intestinal barrier (48, 49). Once the

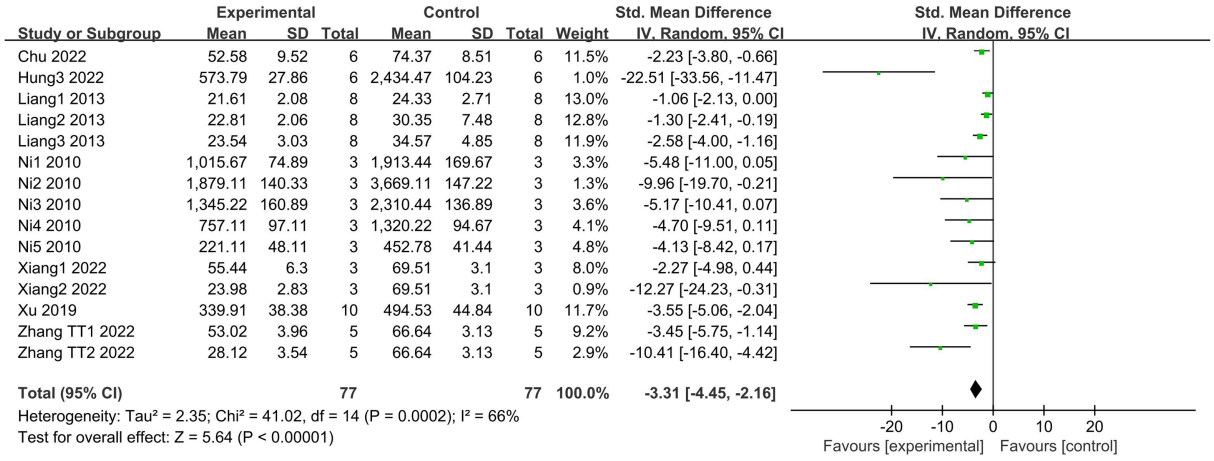

FIGURE 6
Forest plot of the effects of SCFAs intervention on TNF-α in BALF.

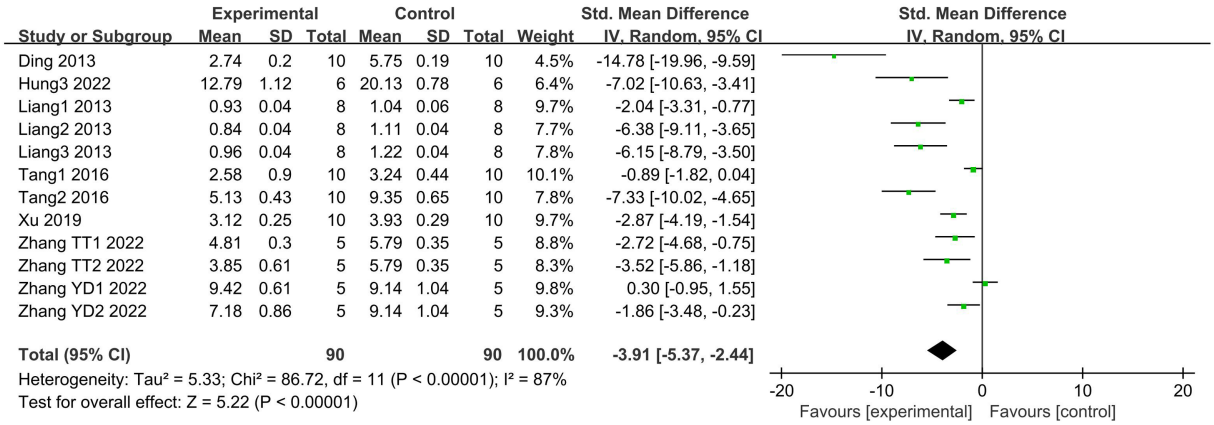

FIGURE 7
Forest plot of the effects of SCFAs intervention on MDA level in lung tissue.

balance is disrupted, abnormal microorganisms may breach the intestinal barrier, enter the bloodstream, or migrate to the lungs, leading to systemic infections (sepsis) and lung damage (50). Among the various factors influencing gut microbiota, diet is considered one of the most effective interventions (51). Research shows that a high-fiber diet promotes the production of abeneficial metabolites, especially SCFAs (52). SCFAs construct and sustain the host's intestinal defense system through various mechanisms, including stimulating the expression of intestinal mucins, enhancing the function of tight junction proteins (TJPs), and regulating the survival of intestinal neurons (53–55). In addition, SCFAs can promote the development of B cells and the differentiation and expansion of regulatory T cells (Tregs) (56–58). Subsequently, these lymphocytes migrate from the gut mucosa through the bloodstream and lymphatic system to reach distant effector sites, ultimately eliciting similar immune responses in mucosal areas throughout the body, including the respiratory mucosa (49, 59, 60).

Exceeding 90% of SCFAs are absorbed in the intestines, with a fraction dedicated to sustaining the metabolic functions of intestinal epithelial cells, while the remainder is disseminated through the circulatory system to a variety of organs (61). It has been demonstrated that SCFAs are present in the human respiratory tract, with their levels being closely correlated with the functional integrity of the gut microbiota (44). The capacity of SCFAs to regulate local immune responses is primarily dependent on two mechanisms: firstly, by directly inhibiting histone deacetylases (HDACs) to modulate gene expression, and secondly, by activating G protein-coupled receptors (GPCRs) to transmit signals. The GPCRs that can be activated by SCFAs are GPR41 (also known as FFAR3), GPR43 (also known as FFAR2), and GPR109A (62). These three receptors are widely distributed in various respiratory epithelial cells and immune cells, where they are capable of inhibiting LPS-induced pulmonary inflammatory signaling (63). As a result, they reduce alveolar edema and decrease the production of TNF-α in the ALI model (58, 64). However, different SCFA receptors exhibit varying affinities for different SCFAs. GPR41 displays a greater affinity for butyrate and propionate than

TABLE 2 Subgroup analysis for lung W/D ratio.

Variables	Studies	SMD (95% CI)	I^2	*p* for overall effect	*p* for subgroup differences
Total	22	−2.88 (−3.63, −2.13)	70%	<0.00001	
Modeling method					
LPS-induced ALI	11	−2.67 (−3.48, −1.86)	34%	<0.00001	0.04
CLP-induced ALI	3	−1.71 (−2.53, −0.90)	0%	<0.0001	
Others	8	−4.12 (−6.01, −2.24)	86%	<0.0001	
Type of SCFAs					
Sodium acetate	5	−2.18 (−3.22, −1.14)	59%	<0.0001	0.11
Sodium propionate	2	−3.23 (−4.80, −1.67)	0%	<0.0001	
Sodium butyrate	13	−3.01 (−4.17, −1.86)	76%	<0.00001	
Mixed SCFAs	2	−5.22 (−7.52, −2.93)	0%	<0.00001	
Dosage					
≤100 mg/kg/d	9	−1.99 (−2.76, −1.22)	33%	<0.00001	0.05
200-350 mg/kg/d	5	−2.51 (−3.54, −1.48)	46%	<0.00001	
400 mg/kg/d	4	−8.31 (−13.02, −3.59)	88%	0.0006	
≥500 mg/kg/d	3	−3.45 (−6.16, −0.73)	76%	0.01	
Duration					
<12 h	8	−1.84 (−2.54, −1.15)	34%	<0.00001	0.005
12-24 h	8	−3.75 (−5.57, −1.93)	82%	<0.0001	
>24 h	6	−3.81 (−4.91, −2.70)	24%	<0.00001	
Species					
Rats	12	−3.90 (−5.28, −2.52)	82%	<0.00001	0.02
Mice	10	−2.12 (−2.69, −1.55)	0%	<0.00001	
Administration route					
Intragastric administration	11	−2.46 (−3.16, −1.76)	9%	<0.00001	0.08
Intraperitoneal injection	5	−6.23 (−9.52, −2.94)	90%	0.0002	
Intravenous injection	2	−2.69 (−5.22, −0.17)	85%	0.04	
Others	4	−1.81 (−2.80, −0.82)	44%	0.0004	

for acetate, whereas GPR43 exhibits a higher preference for acetate over butyrate and propionate. GPR109A, on the other hand, is primarily activated by butyrate (44). Furthermore, propionate and butyrate are widely recognized as HDAC inhibitors. HDACs are enzymes that remove acetyl groups from histones, leading to the formation of compact chromatin structures that inhibit transcription (65). Thus, the inhibition of HDAC activity can promote gene transcription by increasing histone acetylation. In a mouse model of lung injury induced by polycyclic aromatic hydrocarbons, the restoration of butyrate levels in the gut has been demonstrated to reduce the HDAC level in the lung tissues, enhancing the expression of the Foxp3 gene and regulating Th17/Treg cell differentiation (66). In addition, other studies have shown that butyrate and propionate may reverse lung inflammation and oxidative stress by inhibiting HDAC activity, blocking the nuclear factor (NF)-kappaB signaling pathway, and suppressing the release of MDA in lung tissue (26, 31, 67). A summary of the role of SCFAs in regulating systemic and pulmonary inflammation is provided in Figure 8.

Despite variations in receptor selectivity among different SCFAs that could influence their mechanisms, our findings showed that there are no significant differences in their effectiveness in alleviating ALI. We hypothesized that short-chain fatty acids (SCFAs) may provide a lung-protective effect by activating and integrating various biological signaling pathways, rather than depending solely on the selective activation of a specific receptor. This multi-pathway, cross-receptor mechanism elucidates the synergistic effects of different SCFAs in the body, emphasizing their potential as novel therapeutic strategies for ALI. Nevertheless, further in-depth research is still needed to fully elucidate the mechanisms of action of SCFAs and establish clear causal relationships.

Subgroup analysis indicated that higher dosages of SCFAs and longer treatment durations appeared to be more effective in alleviating pulmonary edema in ALI animal models. SCFAs, as HDAC inhibitors, can silence the transcription of specific inflammatory genes. Previous study reported that SCFAs can only effectively reduce HDAC activity at higher millimolar concentrations (68). High concentrations of butyrate (0.5 mM) directly inhibited the activation, proliferation, and production of cytokines (IFNγ, IL-17) in CD4 T cells by increasing histone acetylation, while low concentrations (0.065 mM) did not

TABLE 3 Subgroup analysis for lung injury scores.

Variables	Studies	SMD (95% CI)	I^2	*p* for overall effect	*p* for subgroup differences
Total	16	−5.07 (−5.25, −3.89)	74%	<0.00001	
Modeling method					
LPS-induced ALI	6	−5.03 (−7.01, −3.05)	71%	<0.00001	0.93
CLP-induced ALI	2	−4.81 (−6.66, −2.96)	0%	<0.00001	
Others	8	−5.30 (−7.21, −3.40)	82%	<0.00001	
Type of SCFAs					
Sodium acetate	2	−5.48 (−8.61, −2.35)	58%	0.0006	0.15
Sodium propionate	3	−3.10 (−4.67, −1.53)	31%	0.0001	
Sodium butyrate	9	−5.65 (−7.45, −3.84)	81%	<0.00001	
Mixed SCFAs	2	−6.77 (−14.82, 1.29)	82%	0.1	
Dosage					
≤100 mg/kg/d	4	−5.94 (−7.49, −4.40)	28%	<0.00001	0.001
200-350 mg/kg/d	4	−2.79 (−4.01, −1.57)	52%	<0.00001	
400 mg/kg/d	4	−7.46 (−10.48, −4.45)	69%	<0.00001	
≥500 mg/kg/d	3	−6.01 (−8.93, −3.09)	5%	<0.0001	
Duration					
≤12 h	5	−5.72 (−7.25, −4.20)	50%	<0.00001	0.02
24-48 h	4	−6.87 (−9.55, −4.18)	64%	<0.00001	
≥7 days	7	−3.42 (−4.77, −2.07)	62%	<0.0001	
Species					
Rats	9	−5.97 (−7.78, −4.16)	71%	<0.00001	0.14
Mice	7	−4.22 (−5.73, −3.89)	84%	<0.00001	
Administration route					
Intragastric administration	9	−4.51 (−6.06, −2.96)	74%	<0.00001	0.48
Intraperitoneal injection	4	−6.36 (−8.94, −3.79)	74%	<0.00001	
Others	3	−5.07 (−6.25, −2,29)	74%	0.0005	

exhibit these effects. In comparison, acetate and propionate required higher concentrations (10 mM and 1 mM, respectively) to achieve similar inhibitory effects (22). Similarly, propionate could reshape the metabolic stress and immune function of alveolar macrophages following LPS exposure, contingent on its concentration and timing of administration (44). These findings were consistent with our previous observations and further supported the hypothesis that SCFAs may have a dose-dependent effect. However, the specific dose–response relationship has not yet been clearly defined, which provides important evidence and direction for future studies on the optimal administration of SCFAs.

From the perspective of microbial ecology, SCFAs are primarily produced by the fermentation of dietary fiber and complex carbohydrates by gut bacteria, with Firmicutes and Bacteroidetes playing particularly important roles in this process (69). The two studies by Hildebrand (26) and Xiong (34) collectively revealed key changes in the gut microbiome during lung injury: significant decreases in Lactobacillus, Bacteroides, and Bifidobacterium, which were positively correlated with SCFAs concentrations, providing potential microbiological markers for ALI. It is noteworthy that the interactions between different microorganisms (including bacteria, archaea, and fungi) and their metabolic products also influence the production and diversity of SCFAs (70). For instance, lactic acid can help create an acidic intestinal environment suitable for butyrate-producing bacteria, while pentanoate in feces may originate from symbiotic relationships between archaea and other intestinal bacteria (71, 72). Moreover, SCFAs themselves can feedback-regulate the microbial community, promoting the growth of beneficial bacteria while inhibiting the virulence of intestinal pathogens (73). The interaction between the gut microbiome and SCFAs is a multilayered and complex system. Its mechanisms extend beyond nutrition and metabolism to include immune regulation, interactions among microbial lineages, and ultimately influence gut barrier function and the homeostasis of the gut-lung axis (74–76). However, there is currently no research that can provide a systematic and comprehensive elucidation of the specific processes and interrelationships of these mechanisms. Therefore, it is still necessary to explore strategies for targeting the regulation of the gut microbiome and SCFAs as potential treatments for ALI.

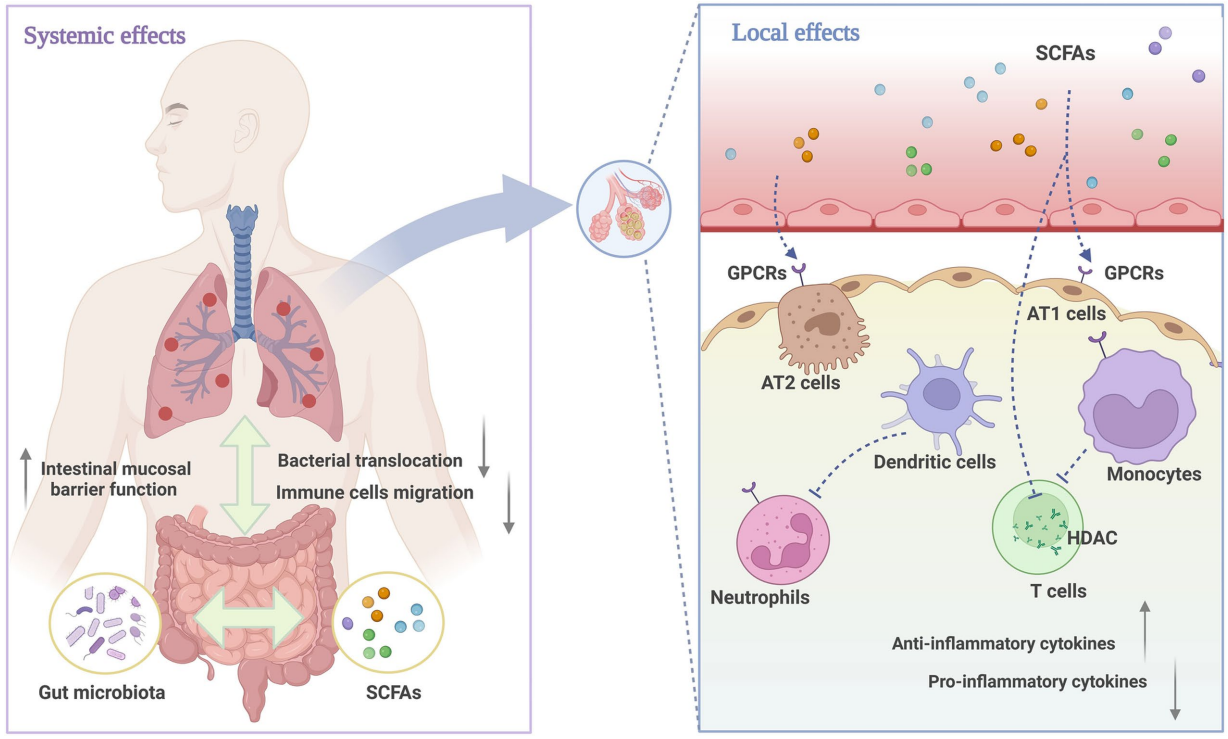

FIGURE 8
The role of SCFAs in systemic and pulmonary inflammation. The immune mechanisms connecting the respiratory and gastrointestinal tracts are regulated by microbiota, with beneficial metabolites like SCFAs enhancing immune defenses in both systems. Locally, SCFAs act as anti-inflammatory mediators that effectively reduce inflammatory damage. The specific mechanisms include activating GPCRs on alveolar cells, neutrophils, and monocytes, inhibiting HDAC activity to regulate the function of Treg cells, and ultimately affecting the production and release of regulatory cytokines. Created with BioRender.com.

5 Limitations

This study still has some limitations that warrant attention in future research. First, the quality scores of the included articles were relatively low, which may have resulted in selection bias, implementation bias, and measurement bias. Although we have taken measures to control these biases, they may still affect the reliability of the study results to some extent. Secondly, following the subgroup analysis, there was no significant improvement in heterogeneity within each subgroup. This may be related to the limited number of studies and insufficient variable information in the literature, which could have led to the omission of some potential factors. To more accurately determine the optimal treatment strategy, we recommend conducting more high-quality, multidimensional studies in the future to extensively investigate relevant variables and thoroughly analyze the dose-dependent efficacy of SCFAs. Additionally, we expect subsequent research to employ more standardized experimental designs and evaluation indicators to enhance the overall quality of animal experiments, thereby providing reliable scientific evidence for clinical applications.

6 Conclusion

This meta-analysis highlights the therapeutic potential of SCFAs in animal models of ALI. The overall evidence supports that SCFAs can alleviate the severity of ALI, suppress inflammatory responses, and reduce oxidative stress levels. Subgroup analyses further suggest that SCFAs may have a dose-dependent effect. These findings will provide important directions for further research and clinical applications in this field.

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.

Author contributions

LX: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. LW: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. YL: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. MY: Methodology, Software, Supervision, Validation, Writing – review & editing. TM: Data curation, Investigation, Writing – review & editing. RF: Funding acquisition, Writing – review & editing. YH: Conceptualization, Funding acquisition, Supervision, Visualization, Writing – review & editing. GZ: Conceptualization, Funding acquisition, Supervision, 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.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1528200/full#supplementary-material

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Association between the dietary index for gut microbiota and diabetes: the mediating role of phenotypic age and body mass index

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Objectives: The global prevalence of diabetes is continuously rising, and the gut microbiota is closely associated with it. The Dietary Index for Gut Microbiota (DI-GM) assesses the impact of diet on the microbiota, but its association with diabetes risk remains unclear. This study aims to investigate the association between DI-GM and the risk of diabetes and analyze the mediating roles of phenotypic age and body mass index (BMI).

Methods: Utilizing data from the National Health and nutrition examination survey (NHANES) 1999–2018, we included 17,444 adults aged 20 years and older. DI-GM (score range: 0–13) was calculated based on dietary recall. Diabetes was diagnosed based on laboratory results and self-reported information. Multivariable logistic regression was used to analyze the association between DI-GM and diabetes, adjusting for relevant covariates. Mediation analysis evaluated the roles of phenotypic age and BMI.

Results: After adjusting for confounders, higher DI-GM scores were significantly associated with a lower risk of diabetes (OR = 0.93, 95% CI = 0.90–0.96, $p < 0.001$). Compared to the group with DI-GM scores of 0–3, those with scores of 5 (OR = 0.76, 95% CI = 0.67–0.86) and ≥ 6 (OR = 0.77, 95% CI = 0.68–0.88) had significantly reduced diabetes risk. Phenotypic age and BMI accounted for 41.02 and 25.57% of the association between DI-GM and diabetes, respectively.

Conclusion: Higher DI-GM scores are associated with a lower risk of diabetes, partially mediated through reduced phenotypic age and BMI.

KEYWORDS

dietary index for gut microbiota, diabetes, phenotypic age, body mass index, mediation analysis, NHANES

1 Introduction

Diabetes is a metabolic disease characterized by hyperglycemia, primarily classified into type 1 and type 2 diabetes, with type 2 diabetes mellitus (T2DM) accounting for the majority (1). According to the Global Burden of Disease study, the incidence and prevalence of diabetes have significantly increased over the past decades, becoming a major global public health challenge (2). Diabetes not only reduces patients' quality of life but also increases the risk of

complications such as cardiovascular disease, nephropathy, and retinopathy, imposing a heavy economic burden on individuals and society (3) Therefore, exploring effective prevention and management strategies to address the diabetes epidemic is urgently needed.

In recent years, the role of the gut microbiota in metabolic diseases has received widespread attention. Studies have shown that dysbiosis of the gut microbiota is closely associated with insulin resistance, chronic inflammation, and glucose metabolism disorders (4, 5). Therefore, maintaining a healthy gut microbiota may be a potential avenue for preventing and managing diabetes. Diet is a key factor influencing the composition and function of the gut microbiota. Different dietary patterns can significantly alter the diversity and metabolic products of the gut microbiota, thereby affecting the host's metabolic health (6). Accordingly, Kase et al., based on a review of 106 articles on adult diet and gut microbiota relationships, proposed a new dietary index—the Dietary Index for Gut Microbiota (DI-GM)—to assess the impact of diet on the gut microbiota (7). DI-GM includes 14 dietary components that are beneficial or detrimental to the gut microbiota and effectively reflects the association between dietary quality and gut microbiota diversity.

Moreover, biological age and obesity are important factors influencing diabetes risk. Phenotypic age is an aging indicator based on biomarkers, reflecting an individual's health status and disease risk (8, 9). Obesity, usually measured by Body Mass Index (BMI), is one of the main risk factors for diabetes (10). Previous studies have shown that dysbiosis of the gut microbiota can accelerate biological aging processes and promote inflammation in adipose tissue, increasing the risk of metabolic diseases (11, 12). Therefore, exploring the association between DI-GM and diabetes, as well as the mediating roles of phenotypic age and BMI, is of significant research value.

However, current research on the relationship between DI-GM and diabetes risk remains limited. To fill this research gap, this study utilized a large representative sample from the National Health and Nutrition Examination Survey (NHANES) to investigate the association between DI-GM and the risk of diabetes. Additionally, we analyzed the mediating roles of phenotypic age and BMI in this association. Our study contributes to a deeper understanding of the complex relationships among diet, gut microbiota, biological age, and diabetes, providing new scientific evidence for the prevention and intervention of diabetes.

2 Methods

2.1 Data source

This study used data from the NHANES 1999–2018. NHANES is a continuous cross-sectional survey based on the non-institutionalized population in the United States, collecting participants' health, nutrition, and demographic data through a multistage probability sampling method. The data used were derived from public files and were approved by the National Center for Health Statistics Ethics Review Board, with all participants providing written informed consent. This study follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

2.2 Study design and population

Participants were adults aged 20 years and older who took part in NHANES from 1999 to 2018. During initial screening, individuals lacking diabetes diagnosis data, components of the DI-GM, phenotypic age, BMI data, and covariates were excluded. A total of 17,444 eligible participants were included in the analysis, of whom 3,334 were diagnosed with diabetes (Figure 1).

2.3 Definition of diabetes

Diabetes was diagnosed based on laboratory test results and self-reported information provided in NHANES. Diagnostic criteria included any of the following: physician diagnosis of diabetes, glycated hemoglobin (HbA1c) level $\geq$ 6.5%, fasting blood glucose level $\geq$ 7.0 mmol/L, random or 2-h oral glucose tolerance test (OGTT) blood glucose level $\geq$ 11.1 mmol/L, or use of diabetes medication/insulin. Participants meeting any of these criteria were classified as having diabetes (13).

2.4 Assessment of the dietary index for gut microbiota

The DI-GM is a novel dietary quality assessment index based on the relationship between diet and gut microbiota, aiming to reflect the potential impact of dietary patterns on gut microbiota diversity and identify dietary characteristics that help maintain a healthy gut microbiota (7). The dietary data from NHANES were obtained through a 24-h recall method (Automated Multiple-Pass Method, AMPM) developed by the United States Department of Agriculture (USDA) (14). This standardized interview procedure, administered by professionally trained interviewers, captures all foods and beverages consumed within the previous 24 h. During data collection, NHANES implemented uniform training for interviewers and employed standardized protocols and tools, thereby minimizing interviewer bias and recall bias from participants (15). For the calculation of DI-GM and the analysis of other diet-related variables, we took the average of two independent 24-h dietary recall interviews for each participant. DI-GM consists of 14 food or nutrient components, including 10 considered beneficial for gut microbiota diversity—avocado, broccoli, chickpeas, coffee, cranberries, fermented dairy products, fiber, green tea (this component may be omitted in some analyses due to NHANES not specifically recording green tea consumption), soy, and whole grains—and 4 components considered detrimental to gut microbiota diversity—red meat, processed meats, refined grains, and high-fat diets (≥40% of total energy from fat). Scoring is based on sex-specific median intake levels: for beneficial components, a score of 1 is assigned if intake is above the median and 0 if below; for detrimental components, a score of 1 is assigned if intake is below the median and 0 if above (for high-fat diets, a score of 1 is assigned if fat intake is less than 40% of total energy). The components along with scoring criteria for the DI-GM can be found in Supplementary Table 1. The total DI-GM score ranges from 0 to 13, with beneficial components contributing 0 to 9 points and detrimental components contributing 0 to 4 points. Higher scores indicate greater potential dietary benefits to the gut microbiota. Based on previous research, DI-GM scores were

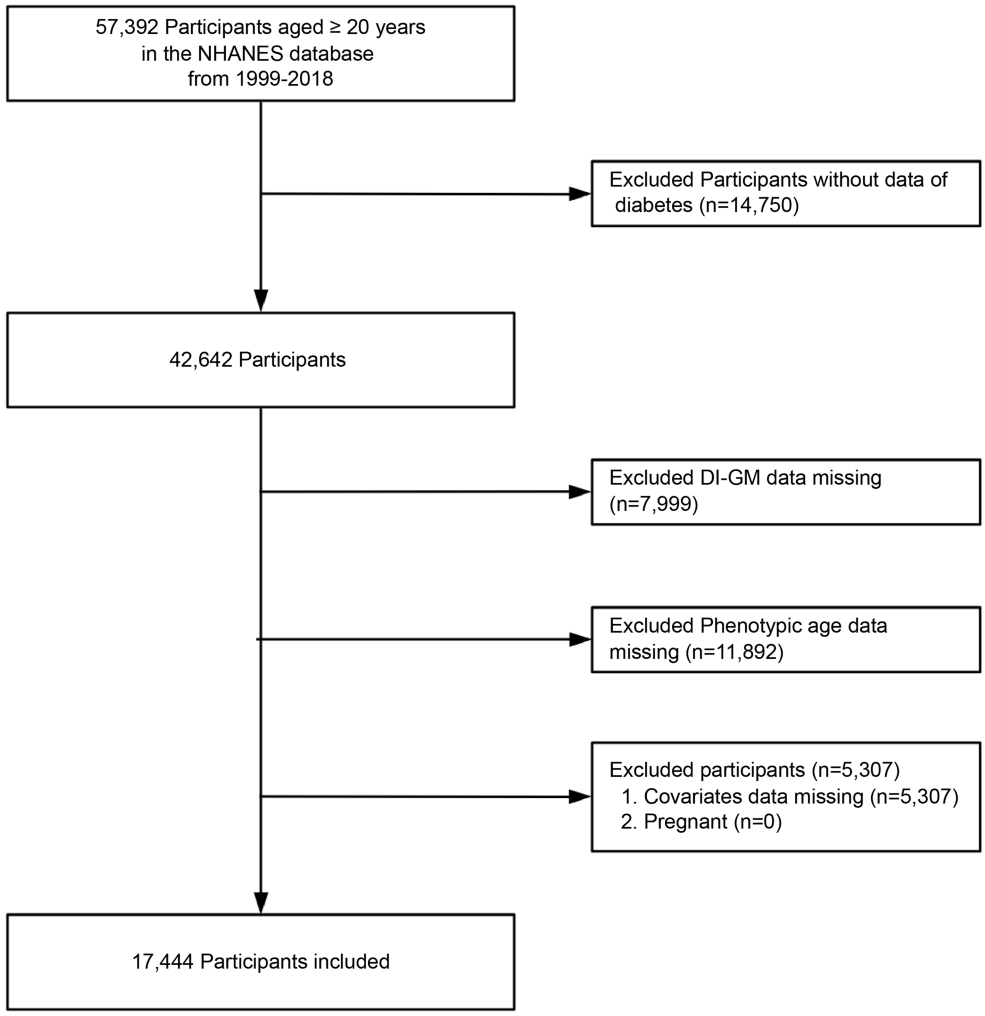

FIGURE 1
Flow chart of the screening of the NHANES 1999–2018 participants.

divided into four categories based on quartiles: 0–3, 4, 5, and ≥ 6 points (16).

2.5 Definition of phenotypic age and BMI

Phenotypic age was calculated based on an algorithm involving 10 clinical biomarkers, including chronological age (CA), albumin, creatinine, blood glucose, C-reactive protein, lymphocyte percentage, mean corpuscular volume, red cell distribution width, alkaline phosphatase, and white blood cell count (17). BMI was calculated by dividing weight in kilograms by height in meters squared.

2.6 Covariates

Based on previous research and clinical judgment, multiple potential confounding variables were considered, including age, gender, race, marital status, education level, poverty income ratio (PIR), physical activity, smoking status, alcohol intake, cardiovascular disease (CVD), hypertension, and hyperlipidemia (18, 19). Specific definitions are as follows: Age was treated as a continuous variable, recording participants' actual age. Gender recorded participants' gender. Race was categorized as non-Hispanic White, others (non-Hispanic Black, Mexican American, other Hispanic, and other races). Marital status was categorized as married/living with a partner and unmarried/other (including widowed, divorced, or separated). PIR was divided into three categories: 1–1.3, 1.31–3.50, and > 3.50 (20). Education level was classified as less than high school, high school or equivalent, and more than high school. Smoking status was categorized as never smokers (smoked less than 100 cigarettes in their lifetime), former smokers (smoked more than 100 cigarettes but are currently non-smokers), and current smokers (smoked more than 100 cigarettes and currently smoke occasionally or daily) (21). Participants were categorized according to alcohol intake as never (< 12 drinks in their lifetime), former (≥ 12 drinks in 1 year and did not drink last year or did not drink last year but drank ≥12 drinks in their lifetime), or current drinkers (including heavy alcohol use [≥3 drinks per day for females, ≥4 drinks per day for males, or binge drinking ≥4 drinks on the same occasion for females or ≥ 5 drinks for males on 5 or more days per month], moderate alcohol use [≥2 drinks per day for females, ≥3 drinks per day for males, or binge drinking ≥2 days per month], and mild alcohol use [≤1 drink per day

for females, ≤2 drinks per day for males]) (22). Physical activity time was a continuous variable indicating the time spent in walking, cycling, work, and recreational activities per week, categorized into three levels: inactive (0 MET-min/week), insufficiently active (1–599 MET-min/week), and sufficiently active (≥600 MET-min/week) (23). CVD: Self-reported diagnosis of coronary heart disease, angina, stroke, myocardial infarction, or congestive heart failure. Hypertension was defined by an average systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, self-reported diagnosis, or use of antihypertensive medications (21); hyperlipidemia was defined as meeting any one of the following criteria: (1) use of lipid-lowering medications; (2) hypertriglyceridemia (≥150 mg/dL); (3) hypercholesterolemia (total cholesterol ≥200 mg/dL, or LDL ≥130 mg/dL, or HDL <40 mg/dL).

2.7 Statistical analysis

All statistical analyses were performed using R statistical software and Free Statistics software. Statistical significance was defined as a two-sided p-value < 0.05. Continuous variables were described using means and standard deviations (SD), and categorical variables were expressed as percentages. Group differences were compared using chi-square tests, two-sample independent t-tests, and Mann–Whitney U tests.

To evaluate the association between DI-GM and the risk of diabetes, multivariable logistic regression models were constructed to calculate odds ratios (OR) and their 95% confidence intervals (CI). The models included: Model 1: Crude model without adjustment for any covariates. Model 2: Fully adjusted model, adjusting for all potential confounders listed above. Additionally, DI-GM was divided into four categories (0–3, 4, 5, ≥6) to explore the effect of DI-GM grouping on diabetes risk. Furthermore, we performed an analysis for each individual components of DI-GM to assess their independent associations with diabetes risk. Restricted cubic spline (RCS) analysis was employed to assess the potential nonlinear relationship between DI-GM and diabetes, setting four knots at the 5th, 35th, 65th, and 95th percentiles of DI-GM scores. Subgroup analyses were conducted based on variables such as age, gender, physical activity, smoking status, alcohol intake, CVD, hypertension, and hyperlipidemia to assess the consistency of the association across different populations.

Sensitivity analyses included: (1) Multiple Imputation: Missing data were handled using the multiple imputation method with chained equations (MICE), generating five imputed datasets. Logistic regression analyses were repeated on these datasets. (2) Propensity Score Matching (PSM): A 1: 1 PSM was conducted using "diabetes status" (presence or absence of diabetes) as the primary matching variable to address potential confounding. Logistic regression analyses were then conducted on the matched sample. (3) Following the NHANES analytical guidelines, we accounted for the complex sampling design and incorporated mobile examination center (MEC) sample weights into our analysis to address batch effects, including variations in data collection time periods and geographic distribution (24). This adjustment ensures that our findings are representative of the U.S. population and accounts for potential biases arising from differences in sampling methods across survey cycles. To evaluate the association between DI-GM and the risk of diabetes, we constructed multivariable logistic regression models to calculate ORs with their corresponding 95% CIs. Detailed information regarding the weighted analysis can be found in the Supplementary materials. Mediation analyses explored the roles of phenotypic age and BMI using the Sobel test and bootstrap method with 1,000 simulations to calculate 95% CIs of the mediation effect. The mediation effect was expressed as the proportion mediated.

3 Results

3.1 Participant characteristics

As shown in Table 1, the study included 17,444 participants from NHANES 1999–2018, of whom 3,334 were diagnosed with diabetes and 14,110 did not have diabetes. The average age was 50.62 years (SD = 17.59). Compared to non-diabetic individuals, participants with diabetes were older (61.26 vs. 48.11 years, $p < 0.001$) and had a higher proportion of males (51.83% vs. 48.86%, $p = 0.002$). Significant differences were also observed in race, income ratio, education level, smoking and alcohol status, physical activity level, and comorbidities such as CVD, hypertension, and hyperlipidemia ($p < 0.05$).

3.2 Association between DI-GM and diabetes

As shown in Table 2, multivariable logistic regression indicated that higher DI-GM scores were significantly associated with a lower risk of diabetes. In the crude model, each unit increase in DI-GM was associated with a 3% decrease in the odds of having diabetes (OR = 0.97, 95% CI = 0.94–0.99, $p = 0.01$); in the adjusted model, the association was more pronounced (OR = 0.93, 95% CI = 0.90–0.96, $p < 0.001$). Compared to the group with DI-GM scores of 0–3, those with scores of 5 (OR = 0.76, 95% CI = 0.67–0.86, $p < 0.001$) and ≥ 6 (OR = 0.77, 95% CI = 0.68–0.88, $p < 0.001$) were significantly associated with lower odds of having diabetes.

Further analysis showed that higher scores for detrimental dietary components were significantly associated with increased diabetes risk (OR = 0.85, 95% CI = 0.82–0.89, $p < 0.001$), whereas scores for beneficial components were not significantly associated ($p = 0.794$). Analysis of individual components of DI-GM revealed the following results: Among the beneficial components, whole grains (OR = 1.12, 95% CI = 1.02–1.22, $p = 0.015$) were associated with a higher risk of diabetes, while coffee (OR = 0.89, 95% CI = 0.82–0.98, $p = 0.012$) was linked to a lower risk in the adjusted model. Other beneficial components, including fiber, fermented dairy, avocados, and soybeans, did not demonstrate significant associations after adjustment for confounders (Supplementary Table 2). RCS analysis (Figure 2) indicated a linear association between DI-GM and diabetes risk ($p = 0.556$). Subgroup analyses demonstrated that the negative association between DI-GM and diabetes was significant in most subgroups, indicating the applicability and robustness of DI-GM across different populations (Figure 3).

3.3 Sensitivity analysis

Multiple imputation results showed that the negative association between DI-GM and diabetes remained significant in the adjusted model (OR = 0.93, 95% CI = 0.89–0.96, $p < 0.001$). PSM analysis

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TABLE 1 Characteristics of the NHANES 1999–2018 participants.

Variables	Total (*n* = 17,444)	Without diabetes	Diabetes	*p-value*
Number of participants	17,444	14,110	3,334	
Age, Mean ± SD	50.62 ± 17.59	48.11 ± 17.52	61.26 ± 13.41	< 0.001
Gender, n (%)				0.002
Male	8,622 (49.43)	6,894 (48.86)	1728 (51.83)	
Female	8,822 (50.57)	7,216 (51.14)	1,606 (48.17)	
Race, n (%)				< 0.001
Non-Hispanic White	8,298 (47.57)	6,945 (49.22)	1,353 (40.58)	
Others	9,146 (52.43)	7,165 (50.78)	1981 (59.42)	
Marital status, n (%)				0.313
Married/ Living with partner	10,765 (61.71)	8,733 (61.89)	2032 (60.95)	
Never married/Other	6,679 (38.29)	5,377 (38.11)	1,302 (39.05)	
PIR group, n (%)				< 0.001
1–1.3	4,963 (28.45)	3,904 (27.67)	1,059 (31.76)	
1.31–3.50	6,911 (39.62)	5,493 (38.93)	1,418 (42.53)	
>3.50	5,570 (31.93)	4,713 (33.40)	857 (25.70)	
Education level, n (%)				< 0.001
Less than high school	4,032 (23.11)	2,988 (21.18)	1,044 (31.31)	
High school or equivalent	4,144 (23.76)	3,312 (23.74)	832 (24.96)	
Above high school	9,268 (53.13)	7,810 (55.35)	1,458 (43.73)	
Smoking status, n (%)				< 0.001
Never	9,374 (53.74)	7,716 (54.68)	1,658 (49.73)	
Former	4,567 (26.18)	3,416 (24.21)	1,151 (34.52)	
Current	3,503 (20.08)	2,978 (21.11)	525 (15.75)	
Alcohol intake, n (%)				< 0.001
Never	2,196 (12.59)	1,679 (11.90)	517 (15.51)	
Former	3,431 (19.67)	2,417 (17.13)	1,014 (30.41)	
Current	11,817 (67.74)	10,014 (70.97)	1803 (54.08)	
Physical activity, n (%)				< 0.001
Inactive	4,432 (25.41)	3,156 (22.37)	1,276 (38.27)	
Insufficiently active	3,335 (19.12)	2,713 (19.23)	622 (18.66)	
Sufficiently active	9,677 (55.47)	8,241 (58.41)	1,436 (43.07)	
CVD, n (%)				< 0.001
No	15,404 (88.31)	12,926 (91.61)	2,478 (74.33)	
Yes	2040 (11.69)	1,184 (8.39)	856 (25.67)	
Hypertension, n (%)				< 0.001
No	9,807 (56.22)	8,891 (63.01)	916 (27.47)	
Yes	7,637 (43.78)	5,219 (36.99)	2,418 (72.53)	
Hyperlipidemia, n (%)				< 0.001
No	4,716 (27.04)	4,295 (30.44)	421 (12.63)	
Yes	12,728 (72.96)	9,815 (69.56)	2,913 (87.37)	
DI-GM score, Mean ± SD	4.52 ± 1.52	4.54 ± 1.52	4.46 ± 1.52	0.01
DI-GM group, n (%)				0.234
0–3	4,446 (25.49)	3,553 (25.18)	893 (26.78)	
4	4,363 (25.01)	3,529 (25.01)	834 (25.01)	

(Continued)

TABLE 1 (Continued)

Variables	Total (n = 17,444)	Without diabetes	Diabetes	p-value
5	4,201 (24.08)	3,426 (24.28)	775 (23.25)	
≥6	4,434 (25.42)	3,602 (25.53)	832 (24.96)	
Beneficial to gut microbiota	2.00 (1.00, 3.00)	2.00 (1.00, 3.00)	2.00 (1.00, 3.00)	0.778
Unfavorable to gut microbiota	2.31 ± 1.03	2.32 ± 1.02	2.27 ± 1.04	0.005
BMI (kg/m^2), Mean ± SD	29.42 ± 6.88	28.69 ± 6.52	32.54 ± 7.46	< 0.001
Phenotypic age, Mean ± SD	50.13 ± 21.0	45.86 ± 19.40	68.18 ± 17.64	< 0.001

DI-GM, dietary index for gut microbiota; BMI, Body Mass Index; PIR, poverty income ratio; CVD, cardiovascular disease.

TABLE 2 Association between DI-GM and diabetes.

Characteristics	Diabetes			
	Crude model		Adjusted model	
	OR (95% CI)	p-value	OR (95% CI)	p-value
DI-GM	0.97 (0.94–0.99)	0.01	0.93 (0.90–0.96)	<0.001
DI-GM group				
0–3	Ref		Ref	
4	0.94 (0.85–1.04)	0.252	0.86 (0.77–0.97)	0.014
5	0.90 (0.81–1.00)	0.054	0.76 (0.67–0.86)	<0.001
≥6	0.92 (0.83–1.02)	0.116	0.77 (0.68–0.88)	<0.001
Trend test		0.081		<0.001
Beneficial to gut microbiota	0.99 (0.96–1.02)	0.397	1.00 (0.97–1.04)	0.794
Unfavorable to gut microbiota	0.95 (0.91–0.98)	0.005	0.85 (0.82–0.89)	<0.001

DI-GM, dietary index for gut microbiota; PIR, poverty income ratio; CVD, cardiovascular disease; CI, Confidence interval; OR, Odd Ratio.

results were consistent with multiple imputation, further supporting the robustness of the main findings (Table 3). In Supplementary Table 3, the weighted analysis confirmed the stability of the association between DI-GM and diabetes risk.

3.4 Mediation analysis

As shown in Figure 4, mediation analysis explored the mediating roles of BMI and phenotypic age. Higher DI-GM scores were associated with lower BMI ($\beta = -0.21$, 95% CI = −0.28 to −0.13, $p < 0.001$) and lower phenotypic age ($\beta = -0.26$, 95% CI = −0.36 to −0.16, $p < 0.001$). Increases in BMI (OR = 1.06, 95% CI = 1.05–1.06, $p < 0.001$) and phenotypic age (OR = 1.09, 95% CI = 1.08–1.10, $p < 0.001$) significantly increased diabetes risk. BMI accounted for 25.57% (95% CI = 12.12–95.80%, $p = 0.012$) and phenotypic age for 41.02% (95% CI = 23.01–99.82%, $p = 0.002$) of the association between DI-GM and diabetes.

4 Discussion

This study systematically evaluated the association between DI-GM and diabetes risk and explored the mediating roles of phenotypic age and BMI. Our results showed that higher DI-GM scores were significantly associated with a lower risk of diabetes, partially mediated through inverse in phenotypic age and BMI. This finding underscores the complex relationships among dietary patterns, gut microbiota, biological aging, weight control, and diabetes.

Previous studies have shown that dysbiosis of the gut microbiota is closely associated with insulin resistance, chronic inflammation, and glucose metabolism disorders (25). Diet is a key factor influencing the gut microbiota; different dietary patterns can lead to significant changes in gut microbiota diversity and metabolic products (6). Our findings support these views, indicating that optimizing diet to promote a healthy gut microbiota can reduce the risk of diabetes. Notably, we found that higher scores for dietary components detrimental to the gut microbiota (such as red meat, processed meats, refined grains, and high-fat diets) were significantly associated with increased diabetes risk. This is consistent with previous studies; excessive consumption of these foods has been shown to reduce gut microbiota diversity, promote the proliferation of harmful bacteria, and induce inflammatory responses and metabolic disorders (26, 27). For example, high-fat diets can decrease the proportion of Bacteroidetes and increase the proportion of Firmicutes, affecting energy metabolism (28). In contrast, foods rich in dietary fiber and phytochemicals (such as whole grains, legumes, and fruits) help increase beneficial gut bacteria like *Bifidobacterium* and *Lactobacillus*, produce short-chain fatty acids, and improve insulin sensitivity (29).

However, scores for beneficial dietary components, except for coffee, were not significantly associated with a lower risk of diabetes.

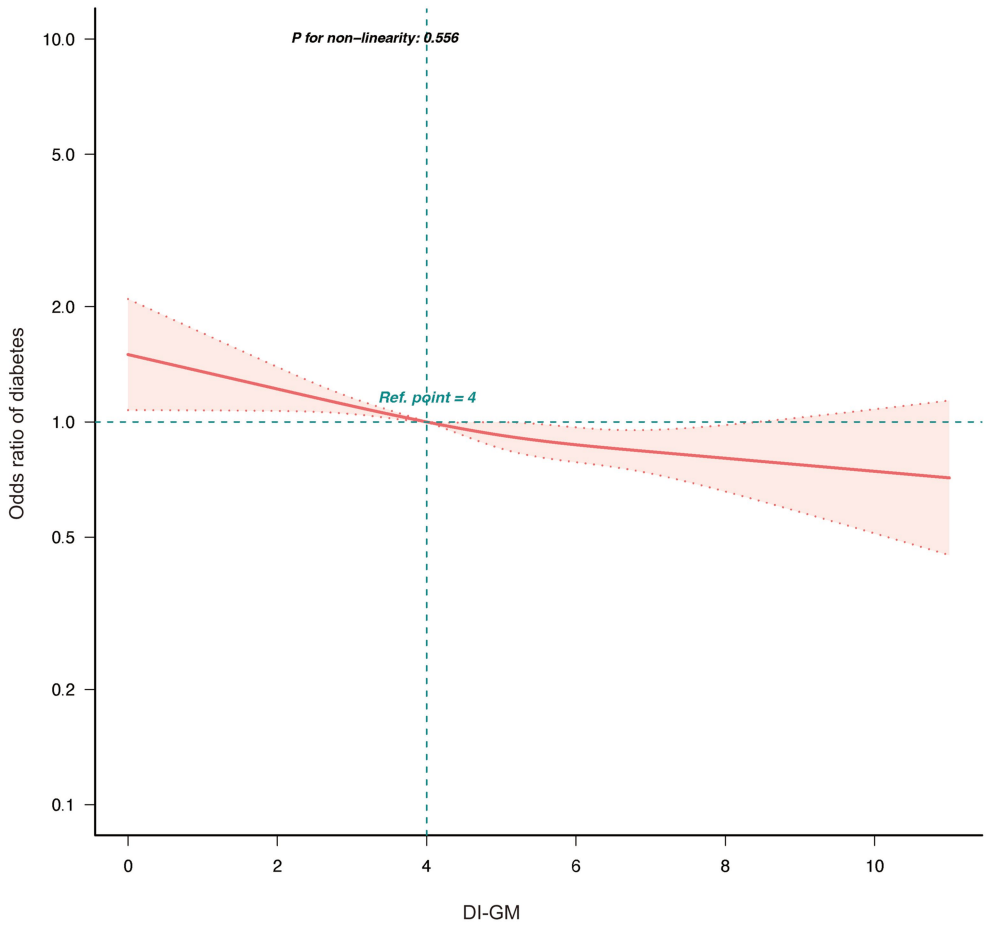

FIGURE 2
Association between DI-GM and diabetes in NHANES 1999–2018 participants by RCS. DI-GM, dietary index for gut microbiota; PIR, poverty income ratio; CVD, cardiovascular disease; RCS, restricted cubic spline. The model was adjusted for age, gender, race, marital status, education level, PIR, physical activity, smoking status, Alcohol intake, CVD, Hypertension, and Hyperlipidemia.

This may be due to various factors. First, the impact of diet on the gut microbiota is complex and individualized; dietary habits, genetic background, and lifestyle factors may influence results (30). Second, beneficial effects may require a longer duration to manifest, which our cross-sectional design could not capture (31). Additionally, dietary intake data based on 24-h recalls may not fully reflect long-term dietary patterns.

The inclusion of phenotypic age and BMI in the mediation analysis in this study was primarily based on an exploratory perspective, aiming to identify potential mediating pathways between the DI-GM and diabetes. This approach also seeks to provide theoretical support and directions for future longitudinal studies or interventional trials. Specifically, phenotypic age, as a metric that quantifies overall health status and the degree of biological aging, was selected because accelerated biological aging is commonly observed in patients with diabetes (32). Moreover, biological aging is closely associated with alterations in gut microbiota composition and changes in dietary behaviors (33). BMI, a widely used indicator of obesity, was included due to its established role as a critical risk factor for diabetes (10). Poor dietary habits and gut microbiota dysbiosis may contribute to obesity and insulin resistance, thereby increasing the risk of developing diabetes (34). Thus, phenotypic age and BMI are important mediators that may play a key role in the "diet-gut microbiota" pathway and its association with diabetes. The mediation analysis showed that phenotypic age and BMI had significant mediating effects in the association between DI-GM and diabetes. This implies that higher DI-GM scores may reduce diabetes risk by lowering biological age and controlling weight. Phenotypic age reflects biological aging, and accelerated aging is associated with increased risks of diabetes and other chronic diseases (35). The gut microbiota can influence aging processes by regulating inflammatory responses, oxidative stress, and metabolic functions (36, 37). For instance, dietary fiber enhances the growth of butyrate-producing bacteria such as *Faecalibacterium* and *Roseburia*, which produce SCFAs that reduce inflammation and oxidative stress, thereby delaying aging (38). Furthermore, the gut microbiota is closely related to obesity, affecting energy intake and fat storage, thereby influencing BMI (39, 40). Improving diet to promote a healthy gut microbiota may help delay aging, control weight, and reduce diabetes risk.

Strengths of this study include the use of the large, nationally representative NHANES dataset, enhancing external validity. We adjusted for multiple potential confounders to reduce bias and

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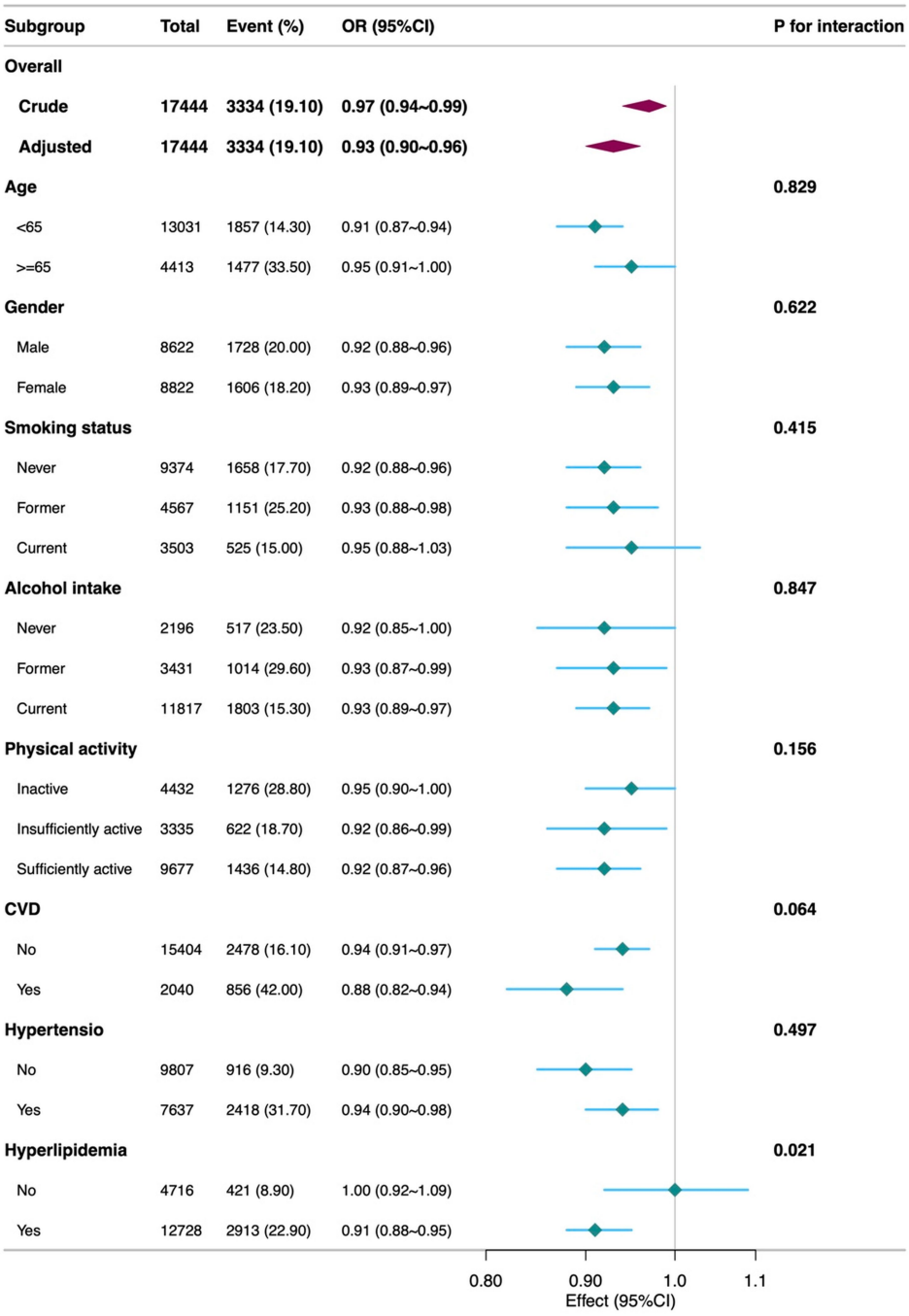

FIGURE 3
Subgroup analyses of the Association between DI-GM and diabetes among participants. DI-GM, dietary index for gut microbiota; PIR, poverty income ratio; CVD, cardiovascular disease; CI, Confidence interval; OR, Odd Ratio. The model was adjusted for age, gender, race, marital status, education level, PIR, physical activity, smoking status, Alcohol intake, CVD, Hypertension, and Hyperlipidemia.

validated the robustness of results through sensitivity analyses and PSM. However, limitations exist. First, the cross-sectional design cannot establish causality. Longitudinal studies and randomized controlled trials are needed to confirm causal associations. Second, dietary intake data were self-reported, possibly introducing recall bias. Future studies may use more objective dietary assessment methods. Third, unmeasured confounders, such as genetic factors and direct gut microbiota sequencing data, may influence results. Additionally, due to NHANES limitations, we could not account for green tea consumption, potentially underestimating DI-GM scores.

Future research should explore mechanistic relationships among diet, gut microbiota, biological age, and metabolic diseases. Understanding how specific dietary components affect the gut microbiota can aid in developing personalized dietary interventions. Considering individual variability, studies should focus on specific effects in different populations.

TABLE 3 Sensitivity analyses.

	Diabetes			
	Crude model		Adjusted model	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Multiple imputations of missing data	0.94 (0.91–0.97)	<0.001	0.93 (0.89–0.96)	<0.001
Propensity score matching	0.98 (0.96–1.00)	0.046	0.93 (0.90–0.96)	<0.001

DI-GM, dietary index for gut microbiota; PIR, poverty income ratio; CVD, cardiovascular disease; CI, Confidence interval; OR, Odd Ratio.

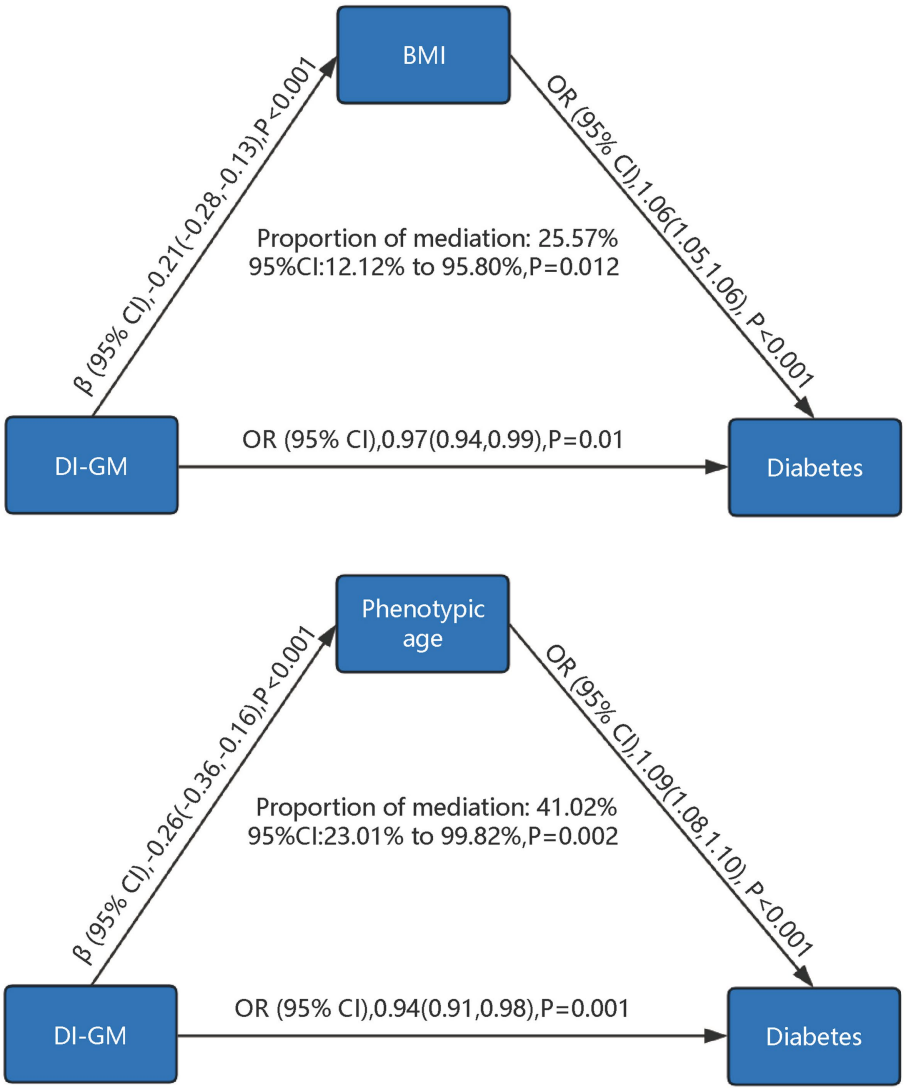

FIGURE 4
Mediation analysis of phenotypic age and body mass index in the association between DI-GM and diabetes. DI-GM, dietary index for gut microbiota; BMI, Body Mass Index; PIR, poverty income ratio; CVD, cardiovascular disease; CI, Confidence interval; OR, Odd Ratio. The models were adjusted for age, gender, race, marital status, education level, poverty-to-income ratio (PIR), physical activity, smoking status, alcohol intake, cardiovascular disease (CVD), hypertension, hyperlipidemia, as well as BMI and phenotypic age, with mutual adjustment performed between BMI and phenotypic age to account for their potential interdependence in the analysis.

5 Conclusion

In summary, this study found that a higher DI-GM score was associated with a lower prevalence of diabetes, partly mediated by reductions in phenotypic age and BMI. Although we did not directly measure changes in the gut microbiome, these findings highlight the importance of dietary patterns in metabolic health. Future research and interventions leveraging the DI-GM may help inform strategies to reduce the burden of diabetes.

Data availability statement

Publicly available datasets were analyzed in this study. All data entered into the analysis were from NHANES, which is publicly accessible to all.

Ethics statement

The studies involving humans were approved by NCHS Ethics Review Board (ERB) Approval. 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

YxH: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. XbL: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. CL: Formal analysis, Writing – original draft. XC: Formal analysis, Writing – original draft. YL: Formal analysis, Writing – original draft. YsH: Software, Writing – original draft. YW: Supervision, Writing – review & editing. XqL: 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/fnut.2025.1519346/full#supplementary-material

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The intricate interplay between dietary habits and cognitive function: insights from the gut-brain axis

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The intricate relationship between dietary habits and cognitive function is gaining increasing attention, with a focus on the gut-brain axis as a modifiable target for intervention. This review synthesizes evidence on the impact of dietary patterns, particularly the Mediterranean diet, plant-based diets, and low-carbohydrate diets, on cognitive health. These diets, rich in antioxidants, anti-inflammatory compounds, and neuroprotective nutrients, are suggested to slow cognitive decline and reduce the risk of neurodegenerative disorders through mechanisms such as reduced inflammation and oxidative stress, and enhanced neurogenesis. The Mediterranean diet has been associated with improved cognitive performance and a delay in cognitive decline in elderly populations. However, challenges in dietary intervention implementation, including adherence and individual variability, remain. Future research must adopt a multidisciplinary approach, incorporating long-term, large-scale, multicenter randomized controlled trials to assess the enduring impacts of various dietary patterns on cognitive function, considering socioeconomic and cultural factors. This review underscores the potential of dietary interventions to prevent and mitigate cognitive impairment, ultimately aiming to improve quality of life.

KEYWORDS

cognitive function, gut-brain axis, Mediterranean diet, plant-based diet, low-carbohydrate diet, neuroprotective nutrients

1 Introduction

Cognitive health serves as a fundamental component of overall well-being and is increasingly recognized as a critical factor influencing quality of life, particularly among aging populations (1). The World Health Organization (WHO) projects that by 2050, the global demographic of individuals aged 60 and above will reach 2.2 billion, constituting 22% of the world's population (2). As the demographic shifts toward older age groups, we are witnessing a rise in cognitive decline and the incidence of dementia, which present significant challenges for healthcare systems and society at large on a global scale. Notably, Alzheimer's disease (AD) and various forms of dementia have emerged as pressing public health concerns worldwide. The 2019 Global Dementia Report by Alzheimer's Disease International indicates that the number of individuals living with dementia is anticipated to escalate from 50 million in 2019 to 152 million by 2050 (3, 4). This surge not only profoundly affects patients and their families but also exerts substantial strain on healthcare resources and the economy. Consequently, the identification of modifiable risk factors and the development of effective interventions to enhance cognitive health have become paramount public health objectives.

GRAPHICAL ABSTRACT

The gut-brain axis, a bidirectional communication system linking the central nervous system with the enteric nervous system, it can use the microorganisms in the intestine to influence the signal transmission of the enteric nervous system, thereby directing brain activity and behavior, and play a pivotal role in cognitive function (5). An imbalance in the gut microbiota, a key component of this axis, has been associated with an increased risk of cognitive impairment. Studies have shown that intestinal microorganisms can affect the regulation of the gut-brain axis through the nervous system, endocrine system, immune system, and metabolic pathways (6). The nervous system transmits information to the brain, the endocrine system is responsible for growth and metabolism, the immune system protects the body from harm, and glands secrete hormones into the blood for communication. The gut microbiota influences cognitive processes through the production of neurotransmitters, modulation of inflammation, and regulation of the blood–brain barrier (7–9). Therefore, understanding the interplay between the gut-brain axis and cognitive health is essential for developing novel therapeutic strategies.

Dietary patterns have long been hypothesized to influence cognitive function, with a growing body of evidence supporting this relationship. The Mediterranean diet (MeDi), plant-based diets, and low-carbohydrate diets (LCDs) have garnered attention for their potential neuroprotective effects (10–12). MD was initially proposed by Keys et al. with the goal of promoting healthy aging and reducing the risk of disease through dietary modifications (13). Today, it is widely recognized as one of the healthiest dietary patterns globally. Plant-based diets generally refer to dietary patterns centered around plant-derived foods while including minimal or no animal-based products. However, different researchers hold varying definitions of plant-based diets. Notably, Satija et al. introduced the plant-based diet index (PDI), which has become a widely accepted criterion for evaluating adherence to plant-based diets (14). LCDs focus on significantly reducing carbohydrate intake while increasing the proportion of protein and fat. The LCD scoring system is currently regarded as the standard metric for assessing adherence to such diets (15). These dietary patterns are characterized by high consumption of fruits, vegetables, whole grains, nuts, and healthy fats, which are rich in bioactive compounds with antioxidant and anti-inflammatory properties. Conversely, excessive intake of saturated fats, sugars, and processed foods has been associated with an increased risk of cognitive impairment. The purpose of this review is to synthesize the latest evidence on the impact of dietary interventions on cognitive health, with a focus on the Mediterranean diet, plant-based diets, and low-carbohydrate diets. We aim to critically evaluate the mechanisms by which these dietary patterns may confer cognitive benefits, including their effects on inflammation, oxidative stress, and neurogenesis.

Furthermore, we will examine the role of specific nutrients, such as polyunsaturated fatty acids, B vitamins, polyphenols, and vitamin D, in cognitive protection (16, 17). The aim of this review is to offer an exhaustive overview of the existing research, which is targeted at guiding clinical practice and public health strategies for preventing cognitive decline and improving the quality of life of people prone to cognitive impairment. Moreover, it intends to further explore the potential role of dietary interventions along the gut-brain axis in promoting cognitive function and alleviating symptoms of

neurodegenerative diseases. It presents a comprehensive management strategy for cognitive health and aging.

2 Analysis of the correlation between diet and cognitive dysfunction

2.1 Mediterranean diet

The "Mediterranean Diet" has its roots in the "Seven Countries Study" initiated by Ancel Keys in the early 1960s, and it was formally recognized at the International Mediterranean Diet Conference in 1993 (18). The MeDi is characterized by a plant-based dietary pattern with a significant emphasis on extra virgin (cold-pressed) olive oil, a variety of vegetables—especially leafy greens—fruits, whole grains, nuts, and legumes. This dietary framework also includes a moderate intake of fish, other meats, dairy products, and red wine, while limiting the consumption of eggs and sugars. Studies had shown that the MeDi could reduce the *Firmicutes*-to-*Bacteroidetes* ratio and increase the levels of SCFAs in feces (19).

It is widely recognized that the MeDi constitutes a beneficial dietary framework. An epidemiological investigation revealed that a diet incorporating a variety of nutrients is more efficacious in mitigating the risk of Alzheimer's disease (AD) and enhancing cognitive function compared to an emphasis on singular nutrients (20). The study indicates that the health advantages associated with the MeDi may arise from elevated consumption of monounsaturated fats and polyphenols found in olive oil, polyunsaturated fats sourced from fish, as well as the antioxidant characteristics present in vegetables, fruits, and wine (21). In comparison to alternative dietary frameworks, the MeDi demonstrates superior scalability. A study conducted by GUADALUPE revealed that interventions based on the MeDi were linked to a decrease in age-related cognitive decline in Chile, a nation characterized by a climate akin to that of the Mediterranean region (22). A longitudinal investigation conducted by CINTA found that among local elderly cohorts in the U.S., France, Spain, and Greece, adherence to the Mediterranean diet, particularly when enhanced with olive oil or nuts, was associated with improved cognitive performance (23).

Research has established a positive correlation between the MeDi and cognitive performance in the elderly. The diet can delay the progression of cognitive decline associated with AD and vascular dementia, providing significant preventative measures even before clinical onset (24). A fundamental aspect of the MeDi is the endorsement of extra virgin olive oil (EVOO) as the principal source of fat. The nutritional properties of EVOO are vital in mitigating age-associated cognitive deterioration and cognitive deficits. A study conducted by OLIVERAS et al. revealed that individuals participating in the MeDi intervention who incorporated high-polyphenol EVOO into their daily regimen exhibited enhanced antioxidant levels, which contribute to the prevention of additional cognitive decline in patients with Alzheimer's disease (25). Comparable findings were also corroborated by a prospective cohort study carried out by Tsolaki et al. (26). Moreover, moderate wine intake is a significant characteristic of the MeDi. A randomized controlled trial conducted by RESTANI revealed that the wine suggested in MeDi frequently outperforms other alcoholic drinks in improving cognitive function (27). Furthermore, a double-blind controlled study conducted by Lee et al. revealed that moderate wine intake, when incorporated into a standard diet, provides protective benefits against recognized pathological metabolic deterioration in the early stages of Alzheimer's disease (28). Subsequently, dairy products represent another key component of the MeDi pattern. The beneficial outcomes associated with dairy interventions may stem from alterations in the ratio of omega-3 to omega-6 fatty acids, which can diminish the synthesis of inflammatory mediators, consequently affecting age-related cognitive decline and the risk of AD. A study conducted by Talaei et al. indicates that dairy consumption during middle adulthood may confer protective benefits against cognitive deterioration (29). Vegetables and fruits are fundamental elements of the MeDi and are vital for maintaining cognitive health. The compounds that enhance cognitive function in these foods are probably not isolated entities; instead, they stem from the synergistic effects of a diverse array of antioxidant nutrients and non-nutritive bioactive compounds found within them.

A cohort study conducted by Wu demonstrated that greater adherence to the MeDi correlates with a more significant delay in cognitive decline and a reduced risk of AD when compared to lower adherence levels (30). Mediterranean Diet interventions play a crucial role in enhancing cognitive function in AD and in delaying its onset. These interventions offer numerous benefits, such as cost-effectiveness, ease of implementation, and minimal observable side effects and contraindications. Consequently, the Mediterranean Diet should be regarded as a fundamental approach in the prevention and management of metabolic syndrome (MetS).

2.2 Plant-based diets

In recent years, motivated by the imperative of animal welfare, ecological sustainability, and the enhancement of human health, the academic community has advocated for a decrease in the intake of animal-derived foods within human diets to facilitate a shift toward more healthful dietary practices (31). Plant-based foods, including vegetables, fruits, whole grains, legumes, nuts, and seeds, are essential elements of nutrition, with plant-based diets that minimize or restrict animal-derived products such as meat, eggs, and dairy gaining traction as a prominent dietary trend in affluent nations (32, 33). Plant-based dietary approaches encompass pescatarian, lacto-ovo-vegetarian, vegan, and various other plant-centric eating patterns, which are recognized as primary strategies for preventing or possibly postponing cognitive decline (34, 35). Emerging research indicates that diets rich in plant-based foods contribute positively to neurological well-being (36, 37). Wu found that a plant-based diet during middle age is associated with a reduced risk of cognitive impairment in later years (38). A substantial cohort study conducted in Taiwan, involving 12,062 participants, revealed that vegetarians exhibited a 38% reduced risk of developing dementia in comparison to their non-vegetarian counterparts. Conversely, a cohort study comprising 13,588 healthy adults in the United States found no significant correlation between high meat consumption (OR = 1.06, [95% CI: 0.92, 1.22], $p = 0.88$) or elevated fruit and vegetable intake (OR = 0.99, [95% CI: 0.88, 1.12], $p = 0.34$) and the incidence of dementia after a 20-year follow-up period, which may be closely associated with the duration of the follow-up (39, 40). Furthermore, studies suggest that plant-based diets can influence depressive symptoms among the elderly population. A higher intake of nutritious

plant-based foods is associated with a decreased risk of depression, whereas an increased consumption of less healthy plant-based elements correlates with an elevated risk (41). Subsequent research indicates that suboptimal plant-based dietary patterns exert a more pronounced effect on depressive symptoms among elderly individuals exhibiting central obesity, in contrast to their counterparts without such conditions (42). Consequently, dietary patterns centered around plant-based foods may have a beneficial impact on cognitive function.

The pathways through which plant-based dietary patterns influence cognitive health encompass the following: (1) Mitigating the risk of cardiometabolic disorders: Following a nutritious diet and maintaining optimal weight management can substantially lower the risk of cardiometabolic conditions, which serve as precursors to cognitive decline (43, 44). Extensive research has demonstrated that vegetables, fruits, and plant-based oils are abundant in essential nutrients, including polyphenols, unsaturated fatty acids (such as Omega-3 and Omega-6), and dietary fiber. These nutrients have been shown to mitigate inflammation and oxidative stress, thereby impacting the development of neurodegenerative disorders (45–50). Flavonoid modulation of inflammation and oxidative stress: Dietary flavonoids have the capacity to modulate systemic inflammation and oxidative stress, thereby affecting metabolites associated with the gut-microbiota-brain axis. Most of the research indicates a consistent inverse correlation between flavonoid consumption and cognitive performance. Furthermore, unsaturated fatty acids play a crucial role in the regulation of metabolism, immune responses, and inflammatory mechanisms within the central nervous system (51, 52). (2) Supporting the gut microbiota: Plant-based diets may benefit the gut microbiota, directly affecting neurotransmitters and acting as part of the gut-brain axis. Consuming tryptophan-rich foods can elevate 5-HT (serotonin) levels in the body, contributing to improved mood regulation. Additionally, the probiotic strain *Bifidobacterium longum infantis 35,624* has been shown to modulate the 5-HT pathway, thereby reducing depressive-like behaviors (53, 54). The gut microbiota plays a significant role in enhancing the breakdown of fiber and polyphenols while suppressing the breakdown of bile acids, choline, L-carnitine, and amino acids. These mechanisms may have implications for the central nervous system (55–57).

Recent findings indicate that plant-based diets offer protective benefits for cognitive health and related risk factors. It is crucial to select nutritious plant-based foods to maximize these advantages. Thus, dietary recommendations should contemplate integrating wholesome plant-based dietary patterns as a viable approach to address and prevent cognitive health issues.

2.3 Low-carbohydrate diets

Low-carbohydrate diets are characterized by limiting carbohydrates to less than 20% of total caloric intake (58). This involves reducing high-carbohydrate foods like sugars, bread, and pasta, while increasing the intake of fats and proteins such as meat, poultry, fish, eggs, cheese, nuts, and seeds (59).

LCDs are a dietary strategy aimed at reducing food consumption without causing malnutrition (60). Research in rodent models has demonstrated that cutting caloric intake by 20–40% can extend lifespan and improve cognitive function, making LCDs one of the most effective nutritional approaches for enhancing cognition in rodents (61). Studies dating back to the mid-1930s have consistently shown the cognitive benefits of LCDs in mouse models, with further research in humans indicating that promoting calorie restriction can help combat age-related cognitive decline and cognitive impairments associated with obesity and type 2 diabetes (62–69).

The mechanisms by which LCDs benefits cognitive health include the following: (1) Reduction of Oxidative Stress: Research indicates that oxidative stress contributes to the high metabolic activity of the brain, particularly affecting regions involved in cognitive functions such as the frontal cortex, amygdala, and hippocampus. LCD's antioxidant properties play a critical role in protecting neurons by regulating ROS production and maintaining internal balance (70–73). (2) Promotion of Anti-inflammatory Responses: LCDs is linked to anti-inflammatory effects that are crucial for cognitive health. Inflammation, associated with cellular aging, can impact neuron survival and connectivity. LCDs has been shown to reduce inflammation by suppressing the activation of astrocytes and enhancing neuroprotective factors (73–75). (3) Enhancement of Neurogenesis and Synaptic Plasticity: Neurogenesis, the generation of new cells from neural progenitor cells, is essential for learning, memory consolidation, and tissue repair. BDNF, a key neurotrophic factor, influences neurogenesis and synaptic plasticity, thereby affecting cognitive performance. LCDs has the potential to increase BDNF levels in the hippocampus and prefrontal cortex, enhancing spatial and working memory (76–80).

In conclusion, LCDs can positively influence cognition through various pathways. Further research is needed to understand the intricate interactions of these mechanisms within the complex structure and functions of the brain. These studies are crucial for developing targeted dietary interventions for specific populations.

2.4 Ketogenic diet

The ketogenic diet (KD) is defined by its low carbohydrate and high fat content, facilitating energy production through the enhancement of ketone body synthesis. Research conducted on animal models has demonstrated that this dietary approach can diminish microglial activation and neuroinflammation, leading to improvements in pathological alterations and cognitive performance in mice with AD (81). For older adults experiencing cognitive decline, following a ketogenic diet can be quite challenging. BRANDT carried out a 12-week investigation into the Modified Atkins Diet (MAD) among individuals with mild to moderate AD. The findings indicated that the generation of ketone bodies within the body may improve episodic memory and self-reported cognitive vitality in patients with early-stage AD (82). Nevertheless, a higher consumption of dietary fats could elevate concentrations of low-density lipoprotein (LDL) cholesterol and triglycerides in the bloodstream, potentially harming the cardiovascular and cerebrovascular systems while hastening cognitive deterioration (83). This appears to be at odds with the cognitive advantages associated with ketone bodies in the ketogenic diet, which serve as an alternative energy substrate for the brain. OLSON suggested that the ketogenic diet could worsen cognitive deficits caused by intermittent hypoxia in murine models (84). Human studies indicated that this dietary pattern may negatively impact the gut microbiome, leading to a decrease in its overall diversity. In children with epilepsy, the ketogenic diet has been shown to reduce

the relative abundance of *Bifidobacterium*, *Eubacterium rectale*, and Dialister, while increasing the relative abundance of *Actinobacteria* and *Escherichia coli* (19).

Consequently, future investigations should prioritize the safety and tolerability of the ketogenic diet among elderly patients to ensure that its implementation does not impose an excessive burden. Considering the potential adverse effects associated with the ketogenic diet, some researchers have suggested a modified Mediterranean ketogenic diet, which integrates essential components of both the Mediterranean and ketogenic dietary frameworks. A randomized trial conducted in 2021 demonstrated that this hybrid diet can enhance patients' daily functioning and overall quality of life; however, additional trials are necessary to further substantiate its efficacy (85).

2.5 Other dietary patterns

An increasing body of research indicates that both the Dietary Approaches to Stop Hypertension (DASH) and ketogenic diets positively influence cognitive health. The DASH diet is acknowledged as an effective non-pharmacological intervention for hypertension. Its nutritional framework prioritizes a well-rounded diet abundant in fruits, vegetables, whole grains, low-fat dairy, and lean proteins. Research has demonstrated that among older adults, sustained adherence to the DASH diet correlates with enhanced cognitive function (86). The unsaturated fatty acids, antioxidants, and polyphenols present in the DASH diet have the potential to mitigate neuroinflammation in the brain that is associated with cognitive decline (87).

Current research findings, however, exhibit a lack of consistency. For instance, a randomized trial investigating cognitive impairment in adults indicated that the DASH diet alone did not yield cognitive advantages (88). Conversely, another study found no correlation between adherence to the DASH diet and improved cognitive health within a European demographic (89). Notably, a longitudinal study that analyzed 12 years of follow-up data determined that adherence to the Mediterranean-DASH Intervention for Neurodegenerative Delay (MIND) diet could potentially lower the risk of cognitive decline by 53% (90). In summary, the variability in study outcomes may be attributed to factors such as trial design, the assessment metrics employed to evaluate adherence to the DASH diet, and the variations in food selection across the studies.

Vegetarian diet, primarily composed of vegetables and fruits, excludes all types of meat, fish, and seafood (91). This dietary pattern has been shown to reduce the loss of dopaminergic neurons and improve negative emotional states and constipation symptoms in Parkinson's disease (PD) patients (92). Research indicates a positive correlation between the abundance of Enterobacteriaceae and PD severity. PD patients exhibit increased levels of Akkermansia, *Lactobacillus*, and *Bifidobacterium*, along with reduced *Prevotellaceae* abundance (93, 94). Interestingly, vegetarians experience lower levels of stress and anxiety, exhibit better emotional well-being, and can effectively alleviate anxiety and depression in PD patients compared to meat-eaters, and vegetarians had higher abundance of certain *Bacteroidetes* in their guts, especially *Prevotella* (95). Fecal short-chain fatty acid levels were positively correlated with fruit, vegetable, and legume intake (96). A German case–control study comparing gut microbiota composition in PD patients and healthy individuals found that lacto-ovo vegetarian dietary interventions altered gut microbiota composition in PD patients (97). Increasing evidence supports the hypothesis that inflammatory processes play a crucial role in PD and may represent a key mechanism within the gut-brain axis. Changes in the gut microbial metabolome may have direct or indirect effects on brain health and disease progression. As such, dietary interventions that shift gut microbiota composition hold promise as potential therapeutic strategies to influence disease progression and alleviate symptoms in neurodegenerative disorders like PD.

The Western diet, commonly observed in developed Western countries, is characterized by a low intake of grains and a high consumption of animal-based foods and added sugars, often lacking in fiber, vitamins, and minerals (98). High-calorie intake associated with this dietary pattern can harm central dopaminergic neurons through neurotoxic effects. Additionally, the heme iron abundant in red meat, a staple of the Western diet, contributes to oxidative stress and increases the risk of PD (99). A systematic review has revealed that ultra-processed foods (a hallmark of the Western diet) disrupt gut microbiota, damage the nervous system, and promote the development and progression of neurodegenerative diseases, including PD (100). Although traditional Chinese dietary habits differ from the Western diet, western influences have led to increased consumption of ultra-processed and high-sugar foods in recent years. This shift has correlated with rising rates of PD, anxiety, and depression in the population, highlighting the need for greater attention to dietary habits and their long-term impacts on health. In conclusion, we present a summary of the dietary pattern in Figure 1.

3 Effects of nutrients on cognitive function

Numerous studies suggested that various dietary patterns can be effective in preventing or treating cognitive health issues. However, considering the synergistic or antagonistic interactions among different nutrients, one limitation of altering entire dietary approaches is identifying which specific nutrients are associated with cognitive improvement. Consequently, increasing attention is being directed toward the effects of individual nutrients on cognitive function. In this regard, we will discuss their impacts on cognitive improvement from the perspectives of polyunsaturated fatty acids, vitamins, polyphenols, and dietary fiber.

3.1 Polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids (LC-PUFAs) encompass both ω-6 and ω-3 fatty acids, with the latter being the focus of more comprehensive research concerning cognitive function. The principal ω-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). G-protein coupled receptors (GPCRs), situated on neuronal membranes within the brain, function as receptors for neurotransmitters such as dopamine and serotonin (101). The role of ω-3 fatty acids is intricately linked to GPCR functionality, underscoring their importance for the proper operation of neural tissues. The primary source of ω-3 fatty acids for the human body is through dietary intake. Prolonged dietary insufficiencies of ω-3 fatty acids can disrupt normal cognitive

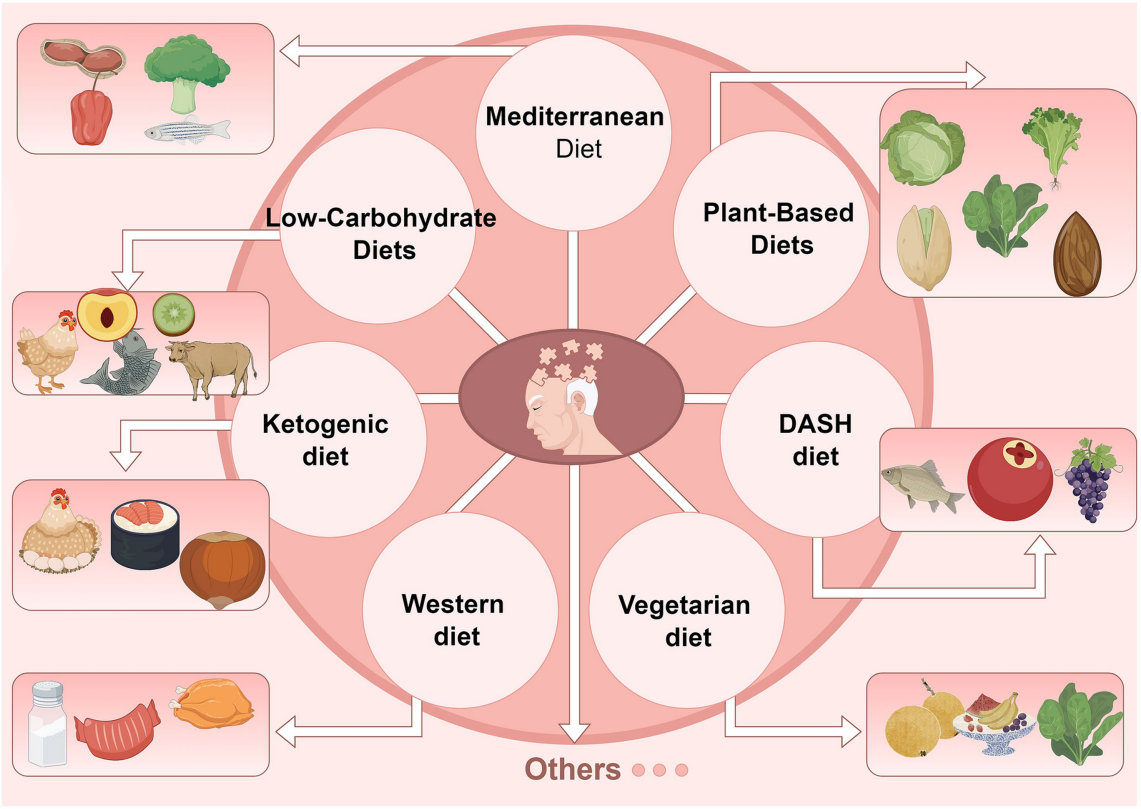

FIGURE 1
Different dietary patterns affect cognitive function.

processes, resulting in cognitive deterioration, memory impairments, and compromised spatial navigation abilities. Furthermore, such deficiencies may correlate with various neurological conditions, including mood disorders and dementia. The World Health Organization (WHO) advises a daily consumption of 500 mg of EPA and DHA, which corresponds to the quantity present in approximately 50 grams of salmon (102).

The findings indicated that a decreased consumption of ω-3 and ω-6 fatty acids could be linked to cognitive decline; however, no significant correlation was observed between variations in the ratio of these two fatty acids and cognitive function (103). A comprehensive review examined the association between ω-3 fatty acids and cognitive function in older adults with differing initial cognitive conditions (104). The findings revealed that in 10 of the 14 randomized controlled trials (RCTs) analyzed, supplementation with ω-3 fatty acids enhanced specific cognitive domains in the elderly, including working memory, executive function, verbal memory, short-term memory, and perceptual speed. This indicates that ω-3 fatty acid supplementation exerts a beneficial influence on cognitive functions in older adults. Furthermore, the cognitive effects of ω-3 supplementation may be associated with the initial cognitive status of the elderly population. Clinical trials evaluating the impact of LC-PUFA on cognitive performance may be affected by variables including the specific type and origin of fatty acids, as well as the initial cognitive condition of the participants (105). Consequently, further investigation is essential to gain a deeper insight into the impact of LC-PUFA on cognitive deficits associated with Alzheimer's disease.

The connection between the consumption of dietary *ω*-3 fatty acids and cognitive health remains incompletely elucidated; however, various mechanisms have been suggested: (1) Antioxidant and Anti-inflammatory Effects: ω-3 polyunsaturated fatty acids, especially DHA and EPA, demonstrate significant antioxidant and anti-inflammatory effects. These compounds can impede lipid peroxidation in the brain and neuronal apoptosis, thereby playing a role in cognitive protection (106–108). (2) Immune Response Modulation: DHA and EPA contribute to immune responses by suppressing genes associated with inflammation. This modulation may aid in diminishing inflammatory processes within the brain that could hinder cognitive function. (3) Cell Membrane Composition: By substituting ω-6 polyunsaturated fatty acids and cholesterol, ω-3 PUFAs modify the composition of cell membranes, resulting in alterations in lipid raft clustering and influencing cellular signaling mechanisms. (4) Vascular Regulation: Certain derivatives of DHA and EPA function as vasoactive agents that assist in the regulation of cerebral perfusion, thereby potentially improving blood circulation to the brain. (5) Specialized Pro-resolving Mediators (SPMs): ω-3 polyunsaturated fatty acids are metabolized into SPMs, which exhibit anti-inflammatory and pro-resolving characteristics. SPMs facilitate the restoration of homeostasis by effectively orchestrating inflammatory responses, downregulating pro-inflammatory cytokines, and enhancing the expression of anti-inflammatory cytokines. They encourage the phagocytosis of cellular debris and apoptotic cells without inducing immune suppression, while also competing with pro-inflammatory eicosanoids derived from ω-6 fatty acids (106, 108–110). (6) Neurotransmitter Modulation:

Recent studies suggest that the acute administration of EPA improves γ-aminobutyric acid (GABA) transmission through the modulation of serotonin 6 receptors, which may play a role in addressing learning and memory deficits observed in both adult and adolescent mice (111, 112).

Although ω-3 polyunsaturated fatty acids may mitigate cognitive decline via their anti-inflammatory properties, certain research indicates that dietary interventions may not lead to substantial enhancements in cognitive deficits (113). Furthermore, a study conducted by Pinelopi et al. suggests that administering high doses of ω-3 and ω-6 fatty acid supplements, alongside antioxidant vitamins, may enhance cognitive performance in older adults experiencing mild cognitive impairment (114). Nevertheless, the pathways through which ω-6 fatty acids exert their effects are not yet fully understood, underscoring the necessity for additional investigations to elucidate the connection between ω-6 fatty acid consumption and cognitive function.

3.2 Vitamins

3.2.1 Vitamin B

B vitamins play a crucial role in numerous vital physiological functions, including the synthesis and repair of DNA, RNA, proteins, and phospholipids, as well as the methylation cycle, nutrient metabolism, cellular metabolism and repair, and energy generation. Some studies indicate that B vitamin supplementation may offer significant neuroprotective benefits. Vitamin B1 is a critical nutrient for brain metabolism, cellular function, and the production of neurotransmitters, such as acetylcholine. A deficiency in this vitamin can result in disruptions in oxidative metabolism, neuroinflammation, endoplasmic reticulum stress, autophagy, and neurodegenerative processes (115). Numerous studies indicate that elevated levels of vitamin B1 and its analogs may mitigate Alzheimer's disease-associated pathological alterations; however, further clinical evidence is required to substantiate this perspective (116). Furthermore, vitamins B2 and B5 play a role in oxidative processes; however, the debate continues regarding their potential to mitigate age-related cognitive decline through the reduction of oxidative damage. Vitamin B3 serves as a precursor to the coenzyme nicotinamide adenine dinucleotide (NAD). Preliminary research indicates that NAD and its precursors may contribute to the preservation of normal cognitive function across different pathological conditions (117). Research findings show that vitamin B3 interacts with the specific receptor hydroxycarboxylic acid receptor 2 (HCAR2), leading to a decrease in amyloid plaque load and neuritic dystrophy in a mouse model of Alzheimer's disease (5x FAD mice) (118). Choline, known as vitamin B4, plays a crucial role in the management of patients with Alzheimer's disease, with cholinesterase inhibitors being a primary pharmacological intervention employed in the treatment of AD (119).

Folic acid (vitamin B9), vitamin B6, and vitamin B12 are essential cofactors crucial in the one-carbon metabolism pathway, responsible for producing and transporting organic groups with a single carbon atom. The impact of these B vitamins on cognitive impairment associated with Alzheimer's disease is linked to their function in the methionine cycle. Insufficient levels of these vitamins may result in the buildup of homocysteine (Hcy), leading to reduced S-adenosyl methionine (SAM) levels. A clinical trial demonstrated that daily intake of 0.8 mg folic acid, 0.5 mg vitamin B12, and 20 mg vitamin B6 could effectively reduce Hcy levels and decelerate the progression of mild cognitive impairment (MCI) in patients (120). Elevated levels of Hcy have been linked to an increased risk of AD in older individuals (121). Nevertheless, a study conducted by Kwok et al. revealed that providing daily doses of 0.5 mg vitamin B12 and 0.4 mg folic acid to patients with MCI and high serum Hcy levels (≥10.0 mmol/L) did not result in enhanced cognitive function. Instead, the supplementation only temporarily alleviated depressive symptoms in MCI patients (122). Additionally, the researchers identified a potential interaction between aspirin and B vitamins.

3.2.2 Vitamin D

Vitamin D is a crucial nutrient for the human body and is a fat-soluble vitamin primarily acquired through skin synthesis and dietary consumption. Within the body, vitamin D is present in two primary forms, vitamin D2 and vitamin D3, with vitamin D3 constituting the majority (90–95%) of the total vitamin D content. Both forms of vitamin D are biologically inert and require hydroxylation in the liver and kidneys to be transformed into their active forms, 1,25-dihydroxyvitamin D2 and 1,25-dihydroxyvitamin D3, which then exert their physiological effects (123). Vitamin D is crucial not only for calcium and phosphorus metabolism, immune regulation, and anti-inflammatory processes, but also for various brain functions such as neuroimmune regulation, neurotrophic factor modulation, neuroprotection, neuroplasticity, and brain development (124–127). Recent studies have increasingly connected vitamin D to neuropsychiatric conditions, with research indicating a link between vitamin D deficiency and disorders like tic disorders and cognitive impairment (128–132).

Numerous studies have identified a link between inadequate vitamin D levels and depression. In a prospective investigation by Ronaldson and colleagues, involving 127, 244 middle-aged participants from the UK Biobank, it was observed that individuals with insufficient or deficient vitamin D levels exhibited a higher susceptibility to developing depression (133). Briggs conducted a study involving 3,965 individuals aged 50 and above residing in the community, revealing that those with insufficient levels of vitamin D (<30 nmol/L) exhibited a notably increased susceptibility to depression onset (134). The research involving 186 individuals with gout revealed that 32 of them (17.2%) were found to have depression. Interestingly, it was observed that these patients exhibited notably lower levels of 25-hydroxyvitamin D in comparison to those who did not experience depression (135). Research has indicated a correlation between vitamin D levels and cognitive function among individuals suffering from depression. Those with depression and lacking sufficient vitamin D are at a higher risk of experiencing cognitive deficits, which can impact their overall well-being. Moreover, studies have shown a potential association between vitamin D levels and the development of AD, with severe vitamin D deficiency (<10 ng/mL) significantly increasing the likelihood of dementia and AD onset (136, 137). Attention deficit and hyperactivity disorder (ADHD), a prevalent chronic neurodevelopmental disorder affecting approximately 7.2% of children globally, has been the focus of research by Thomas et al. (138) and Li et al. (139). A case–control study conducted by the researchers revealed that 52.4% of children diagnosed with ADHD had a vitamin D deficiency (defined as <20 μg/L). The study also highlighted a negative correlation between vitamin D levels and the

overall ADHD screening scale score, particularly in the inattention subscale. Furthermore, a randomized double-blind controlled trial illustrated that vitamin D supplementation led to a significant improvement in impulsive behavior among children with ADHD (140). These results underscore the potential link between vitamin D levels and ADHD development, suggesting that vitamin D supplementation could serve as an innovative approach to ameliorate clinical symptoms in affected children.

3.2.3 Antioxidant vitamins

Common antioxidant vitamins such as vitamin A, vitamin C, vitamin E, and beta-carotene are believed to play a crucial role in protecting the brain from oxidative damage. Some researchers suggest that oxidative stress or insufficient antioxidant defense might contribute to the development and progression of dementia. An 8-year vitamin supplementation trial demonstrated sustained cognitive benefits, including improved episodic and verbal memory even 6 years after the trial's conclusion, particularly evident in individuals with lower baseline antioxidant levels (141). However, a separate randomized controlled trial involving 2,824 women with cardiovascular disease or at risk of cardiovascular disease showed that supplementation with vitamins A, C, and E did not halt cognitive decline in these women, highlighting uncertainty regarding the impact of vitamin supplementation on cognitive function (142). Furthermore, various cross-sectional studies and intervention trials have provided evidence supporting the beneficial effects of dietary intake or supplementation of antioxidant vitamins on cognitive function (143, 144).

3.3 Polyphenols

Polyphenols constitute a vast category of phytochemicals prevalent throughout the plant kingdom, encompassing around 8,000 distinct structures. These compounds are identified by the presence of one or more aromatic rings with hydroxyl groups and are frequently present in fruits, vegetables, whole grains, olive oil, and green tea (145). Recent studies indicate that flavonoids could regulate signaling pathways associated with cognitive and neuroprotective functions, including the inhibition of acetylcholinesterase, butyrylcholinesterase, tau protein aggregation, and β-secretase. This modulation contributes to the deceleration of Alzheimer's disease progression. Furthermore, dietary polyphenols are essential for their anti-inflammatory properties, ability to diminish oxidative stress, safeguard endogenous compounds from oxidative harm, regulate metabolism, and enhance endothelial and platelet function (146). Several research studies have indicated that certain refined polyphenols such as curcumin, EGCG from green tea, cyanidin, resveratrol, and tannic acid have the potential to alleviate age-related cognitive decline (147–149). SHISHTAR conducted a study that examined the correlation between long-term dietary flavonoid intake and cognitive impairment in humans, suggesting that a diet high in flavonoids could lower the risk of developing Alzheimer's disease (150). Additionally, a prospective placebo-controlled trial demonstrated that supplementation with freeze-dried grape powder rich in polyphenols could help reduce brain pathology in individuals with mild cognitive impairment and offer protection against cognitive decline23. Kaplan and colleagues also found that a diet rich in polyphenols offers neuroprotective benefits against age-related brain atrophy (151). The cognitive advantages associated with polyphenols are believed to stem from the combined effects of diverse phenolic compounds thanks to their potent antioxidant properties. Studies have confirmed that natural antioxidants, such as polyphenols, can modulate gut microbiota, opening new avenues for their application in patients with mild cognitive impairment (MCI), the prodromal stage of Alzheimer's disease (152).

3.4 Dietary fiber

Dietary fiber (DF) refers to a class of carbohydrate polymers with a degree of polymerization ≥3 that cannot be digested or absorbed in the small intestine. Naturally occurring DF is diverse, with cereals providing DF primarily from bran or rice husk, including arabinoxylan, β-glucan, resistant starch, hemicellulose/lignin, among others. DF directly supplies energy and nutrients to gut microbiota, increasing the diversity and abundance of beneficial gut microbes, enhancing gut immune metabolism, and ameliorating cognitive impairment (153). Research indicates that specific dietary fibers and other components can improve gut health and function by increasing concentrations of beneficial metabolites like short-chain fatty acids (SCFAs). These metabolites exert anti-inflammatory effects, mitigating neuroinflammation (154, 155). Furthermore, DF improves cognitive impairment by promoting anti-inflammatory and beneficial gut bacteria, such as Bifidobacterium and Lactobacillus, which modulate neurotransmitter levels and the immune system, directly influencing brain function and emotional states (153). Shi et al. found that a chronic dietary fiber deficiency (FD) diet induces gut dysbiosis (Bacteroides and increased Proteobacteria), leading to neuroinflammation and synaptic engulfment by microglia in the hippocampus, which subsequently causes systemic neuroinflammation and cognitive deficits (156). Therefore, DF intake is closely associated with gut barrier integrity, metabolic regulation, and immune function, playing a significant role in mitigating neuro-metabolic disorders, and preventing neurodegenerative diseases such as depression and Alzheimer's disease. In addition, there are other nutrients that also have a significant impact on cognitive function, which we summarize in Table 1.

4 Dietary intervention in the gut-brain axis improves cognitive function

The gut-brain axis serves as a two-way communication network that links the central nervous system (CNS) with the enteric nervous system (ENS) (157). The gut microbiota is essential for the synthesis of vitamins, amino acids, and peptides from undigested food in the gastrointestinal tract. They generate a range of secretions and microbial byproducts that boost immune responses, stimulate the release of cytokines, and regulate inflammatory processes. Moreover, the gut microbiota prevents protein decay and the proliferation of harmful microbes by metabolizing compounds like nitrosamines, hydrogen sulfide, and lactic acid (158, 159). An imbalance in the diversity and composition of gut microbiota is a significant contributor to the deterioration of gut integrity and functionality, resulting in heightened gut permeability, inflammation, and an altered gut

TABLE 1 The effects of nutrients on cognitive function.

Nutrients	Influence	Mechanisms and effects
Vitamin B	Improves memory and cognition	Participate in neurotransmitter synthesis, promote energy metabolism, and support neuronal function
Vitamin C	Improve cognitive function	It has antioxidant effects, protects neurons, and reduces oxidative stress
Vitamin E	Reduces the risk of cognitive impairment	Antioxidant, reduce Aβ protein deposition, improve neurological health
Omega-3 fatty acids	Improves memory and learning	Promote neuron growth, strengthen neural connections, and improve brain function
Iron	Maintain brain energy metabolism	Involved in oxygen transport and neurotransmitter synthesis, a deficiency can affect cognitive abilities
Magnesium	Improves learning and memory	Participate in nerve signal transmission, regulate nerve excitability
Zinc	Promote neuroprotection	Involved in neurotransmitter synthesis, support neuron growth and repair
Complex carbohydrates	Improve cognitive ability	Stabilizes blood sugar levels, provides sustained energy, and supports brain function
Saturated fatty acid	May reduce cognitive function	Excessive consumption is associated with cognitive decline

environment. These alterations interfere with the communication pathways linking the gut and brain through neural connections, the hypothalamus-pituitary–adrenal axis, and the immune system, thereby triggering neurological dysfunction. Notably, cognitive function is particularly affected by these changes (160, 161). Dysregulation of the gastrointestinal system plays a role in the development and advancement of various diseases, such as neurological conditions (162). Research has identified a robust correlation between the gut-brain axis and cognitive performance. Empirical evidence shows that experimental mice deprived of gut microbiota display marked cognitive impairments (163).

Dietary interventions play a crucial role in enhancing neurodegenerative conditions and are strongly correlated with relevant risk factors. Research studies indicate that consuming excessive saturated fatty acids can worsen neurodegeneration in AD and PD by heightening oxidative stress and lipid peroxidation. Furthermore, high-calorie diets have been linked to the early onset of Huntington's disease (HD) (164–168). Increased consumption of saturated fats can lead to inflammatory responses, allowing immune cells to enter the central nervous system (169). Diets high in fat may disrupt the balance of gut microbiota, potentially causing neuropsychiatric issues (170). These diets can cause oxidative stress in the gut, leading to neuronal cell death and cognitive decline through the inactivation of the Nrf2 pathway. Furthermore, high-fat diets can increase the deposition of amyloid-beta, raising the risk of Alzheimer's disease (171). Nutrients and their byproducts in the diet play a role in regulating neuroinflammation and improving brain function. Dietary changes can help manage metabolic and functional problems associated with neurodegenerative diseases. In contrast, the intake of DHA has been linked to a reduced risk of Alzheimer's and Parkinson's diseases. DHA not only influences gut microbiota and gene expression but also enhances communication between the gut and brain (172). Animal research has shown that enriching diets with ω-3 polyunsaturated fatty acids containing DHA can influence the composition of gut microbiota, leading to enhanced cognitive function and reduced anxiety-related behaviors in mice (173). Notably, the effects of DHA supplementation differ according to gender, with male mice experiencing more significant benefits compared to females. Furthermore, the KD, characterized by high fat and low carbohydrate intake, has been found to impact gut microbiota. In newborns, a week of KD has been shown to decrease harmful bacteria and increase beneficial microbes compared to infants following a typical diet (174). In children with epilepsy, long-term adherence to KD results in decreased gut microbiota diversity, lower levels of *Firmicutes*, and higher levels of *Bacteroidetes* (175). The ketogenic diet demonstrates neuroprotective properties through its influence on gut microbiota. Studies on animals reveal that KD promotes the growth of beneficial gut bacteria, enhances blood flow to the brain, and supports neurogenesis by activating endothelial nitric oxide synthase (eNOS) and inhibiting mTOR signaling (176). Moreover, the production of (D)-3-hydroxybutyrate ketone bodies induced by KD may facilitate communication between the gut and the brain via G protein-coupled receptor (GPCR) signaling pathways and the epigenetic regulation of relevant genes (Figure 2).

The diversity and integrity of gut microbiota play a crucial role in maintaining health, closely tied to normal bodily functions and changes related to disease, we summarize the main classifications and functions of the human gut microbiota in Table 2. Gut microbiota have a significant impact on advanced brain activities like cognition and emotion, and their imbalance can be a key factor in the development of neurodegenerative conditions. The complex gut environment is influenced by various internal and external factors, making gut microbiota both a source of stress and a potential target for intervention. Imbalance in gut bacteria can impair cognitive abilities and contribute to neurodegenerative diseases. Dietary habits influence the gut microbiota composition, subsequently impacting the interaction between the gut and the brain. This interaction boosts cognitive functions and mitigates symptoms associated with neurodegenerative conditions, as shown as Table 3. However, extended consumption of high-fat or high-sugar diets may harm cognitive function and worsen neurological conditions. Although the exact relationship between gut microbiota, cognitive function, and neurodegenerative diseases is not fully established, forthcoming research is expected to provide more insights into how personalized nutrition can leverage the gut-brain connection to support cognitive well-being and prevent neurodegenerative ailments, we summarized the relationship between intestinal flora and cognitive dysfunction in Table 4.

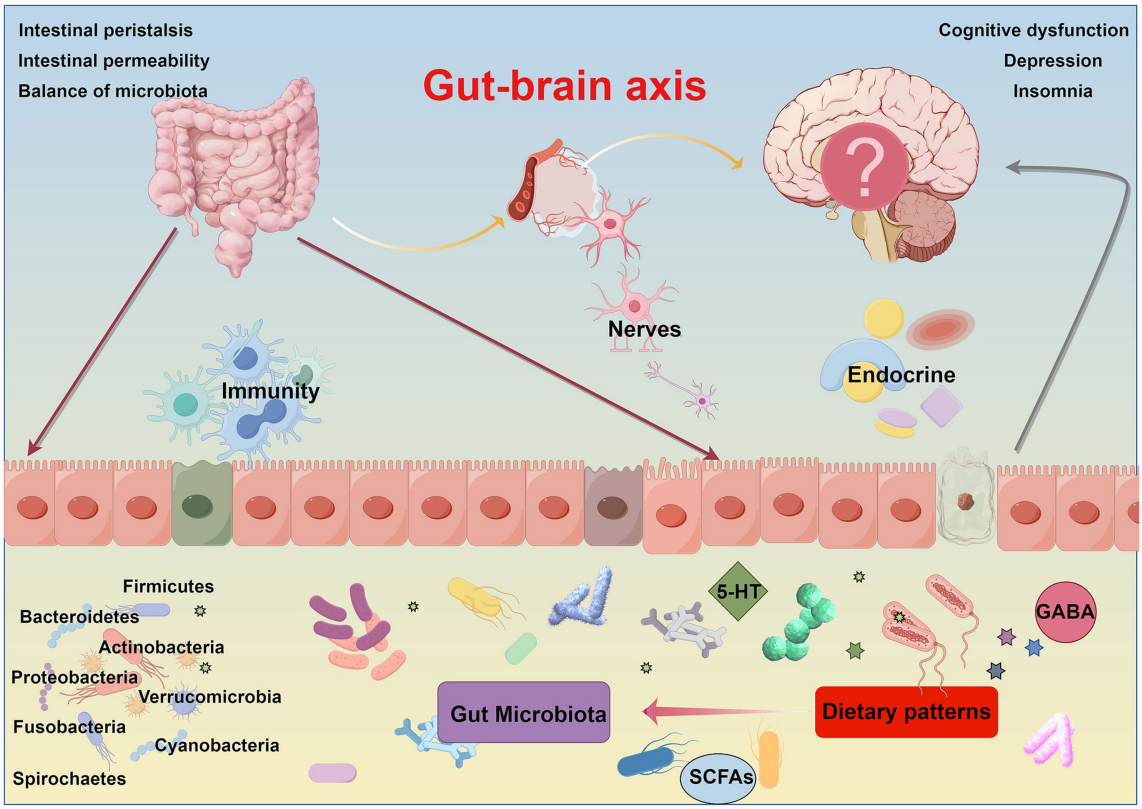

FIGURE 2
Dietary patterns affect cognitive function through the gut-brain axis.

TABLE 2 Main classification and function of human gut microbiota.

Types of microorganisms	Key members	Main functions
Bacteria	Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia	Produce short-chain fatty acids, regulate the immune system, inhibit pathogen growth; synthesize vitamins, maintain intestinal barrier function, etc.
Fungi	*Candida albicans*, Aspergillus	Participate in the fermentation of carbohydrates; competing with bacteria for niche; Modulating immune response
Virus	Bacteriophages, Eukaryotic viruses	Regulates bacterial population dynamics, influences the evolution of bacterial genomes, and interacts with the host immune system
Protist	Amoebae, Flagellates	Involved in the ecological balance of the gut, interacting with bacteria and the host immune system
Archaea	Methanobrevibacter	It is involved in methane production and affects the intestinal fermentation process

5 Dietary recommendations for gut-brain axis treatment of cognitive dysfunction

Numerous epidemiological studies indicate a correlation between diet and cognitive function, with healthy dietary habits serving as a protective factor for cognitive well-being. Hence, improving the quality of life for elderly individuals with cognitive impairments necessitates the development of a tailored dietary health management plan that aligns with the lifestyle practices of Chinese residents. This discussion will delve into two key components: dietary patterns and essential nutrients.

Among various dietary schemes, the Mediterranean diet, plant-based diet, and low-carbohydrate diet exhibit protective properties against cognitive decline and mild cognitive impairments in the elderly. However, it is crucial to acknowledge the dual nature of the ketogenic diet in relation to the potential development of eating disorders. Moreover, findings from different studies present inconsistencies, possibly influenced by research methodologies, assessment tools, and participant demographics. Therefore, future investigations should prioritize long-term, large-scale, multi-center randomized controlled trials to comprehensively assess the impact of diverse nutrients and dietary patterns on cognitive function.

TABLE 3 Effects of different dietary patterns on gut microbiota and cognition health.

Dietary patterns	Main features	Effects on the gut microbiome	Effects on the cognition health
Mediterranean diet	Rich in fruits, vegetables, whole grains, nuts, olive oil, and fish	Enhances diversity of gut microbiota; increases beneficial bacteria like Bifidobacteria and Lactobacilli; reduces inflammation	Improves cognitive function, reduces AD risk. Studies show benefits in Chile and among U.S., French, Spanish, and Greek elderly populations. EVOO and moderate wine intake linked to cognitive benefits. Dairy improves omega-3:omega-6 ratio, reducing inflammation
Plant-based diets	Rich in Plant-based foods and minimize or restrict animal-derived products	Increases fiber-degrading bacteria; promotes diversity and beneficial metabolites; reduces inflammation	Reduces dementia risk in Taiwanese vegetarians; inconsistent effects in U.S. cohort studies. Helps mitigate depressive symptoms in obese elderly and improves gut microbiota diversity
Low-carbohydrate diets	High in fat, moderate in protein and very low in carbohydrates	Reduces carbohydrate-fermenting bacteria; alters microbiota diversity; may favor growth of ketone-utilizing microbes	Improves cognitive function in obesity and diabetes-related impairments. Increases BDNF in hippocampus and prefrontal cortex
Ketogenic diet	Rich in low carbohydrate and high fat content	Decreases diversity; increases Akkermansia, which has anti-inflammatory properties; may disrupt long-term microbiota balance	Improves cognition in AD mice; Modified Atkins Diet showed memory benefits in early-stage AD patients. Suggested hybrid Mediterranean-Ketogenic Diet offers cognitive and functional benefits
Dietary approaches to stop hypertension (DASH) diets	High in fruits, vegetables, whole grains, low-fat dairy	Supports gut microbiota diversity; increases bacteria associated with fiber fermentation and short-chain fatty acid production	Enhances cognitive health in elderly. MIND diet, a hybrid of DASH and Mediterranean diets, reduces cognitive decline risk by up to 53%. However, inconsistent effects across studies
Vegetarian diet	Free of meat and animal products and rich in fruits, vegetables, legumes, nuts and whole grains	Promotes beneficial bacteria like Lactobacillus and Bifidobacterium; decreases Prevotellaceae, linked to inflammation	Alleviates anxiety, depression, and PD symptoms. Alters gut microbiota composition in PD patients, supporting gut-brain axis modulation
Western diet	High in fat, sugar and salt	Reduces beneficial bacteria; increases pro-inflammatory bacteria; disrupts gut barrier integrity	Associated with PD progression, gut microbiota disruption, and neuroinflammation. Linked to increased PD, anxiety, and depression in populations adopting Western dietary habits

TABLE 4 The relationship between gut microbiota and cognitive dysfunction.

Name of disease	Changes in gut microbiota
Alzheimer's disease	Gut microbial diversity decreased significantly, with an increase in Bacteroidetes, a decrease in Firmicutes, a decrease in beneficial bacteria (such as Lactobacillus and bifidobacterium), and an increase in bacteria associated with inflammation (such as Clostridium)
Parkinson's disease	Gut microbiota composition differed from healthy controls, with a decrease in beneficial bacteria such as Lactobacillus and Bifidobacterium, an increase in bacteria associated with inflammation and neurodegeneration (such as Proteobacteria), and a significant decrease in Prevotellaceae
Autism spectrum disorder	The diversity of gut microbes has decreased, with a decrease in the genera Prevotella and Faecalibacterium, an increase in the genera Clostridium and Desulfovibrio, and an abnormal proportion of bacteria related to neurotransmitter metabolism
Mild cognitive impairment	Intestinal microbial diversity decreased, Bacteroidetes increased, Firmicutes decreased, short-chain fatty acid (SCFAs) producing bacteria decreased, and bacteria associated with inflammation increased
Multiple sclerosis	Intestinal microbial diversity decreased, with an increase in Bacteroidetes, a decrease in Firmicutes, a decrease in bacteria associated with immune regulation (such as Ackermannia), and an increase in bacteria associated with inflammation (such as Proteobacteria)
Depression	Gut microbial diversity decreased, bacteria associated with inflammation (such as clostridium) increased, beneficial bacteria such as Lactobacillus and Bifidobacterium decreased, and short-chain fatty acid (SCFAs) producing bacteria decreased
Anxiety disorder	The composition of the gut microbiome is abnormal, with a decrease in bacteria associated with neurotransmitter metabolism (such as lactobacillus) and an increase in bacteria associated with inflammation

Numerous animal experiments randomized controlled trials involving human subjects as participants, and the analysis of essential nutrients in humans have collectively revealed that ω-3 polyunsaturated fatty acids, B vitamins, polyphenolic compounds, and vitamin D are notably associated with cognitive protection. Specifically, ω-3 polyunsaturated fatty acids are known for their anti-inflammatory, immunomodulatory, and neuroprotective properties. The extensive body of evidence supports the notion that consuming adequate levels of ω-3 polyunsaturated fatty acids can enhance cognitive function significantly (177, 178). Recent research has indicated a strong correlation between the consumption of B vitamins and cognitive performance in individuals diagnosed with Alzheimer's disease and those experiencing mild cognitive impairment (179). B vitamins exhibit promising therapeutic benefits in addressing vascular cognitive dysfunction and cognitive issues in individuals with diabetes and cerebral infarction when compared to conventional treatments. Additionally, the long-term consumption of polyphenolic compounds can aid in preventing cognitive decline among older individuals. Notably, research indicates that elderly individuals in India who incorporate curcumin-rich diets have a significantly lower dementia rate, around 73%, in comparison to their counterparts in the United States. Furthermore, Vitamin D, a fat-soluble vitamin, plays a crucial role in enhancing calcium and phosphorus absorption in the small intestine while also supporting skin cell growth, immune function regulation, and differentiation. Food sources rich in Vitamin D include fatty marine fish, animal liver, egg yolk, butter, and cod liver oil. It is important to note that while single nutrient supplementation or multi-component supplements may yield conflicting outcomes, the interplay of various food components in a balanced diet is key to promoting health. The synergy between phytochemicals from fruits and vegetables combined with fatty acids from fish, as well as the mutual absorption of different nutrients when consumed together, underscores the importance of considering the overall dietary pattern rather than focusing solely on individual nutrients.

Current research, both domestic and international, has made significant progress in understanding the dietary needs of elderly individuals with cognitive impairment. There is a substantial body of evidence indicating that modifications in diet and nutrition play a crucial role in preserving cognitive function, emotional well-being, immune response, and vascular health. While early dietary adjustments tend to yield more favorable outcomes, adopting health-promoting behaviors at any stage of life can enhance longevity and overall well-being (180). We generally recommend adopting a well-rounded and moderate diet, adjusting the intake of carbohydrates and fats, and ensuring adequate consumption of vitamins and minerals as a potential optimal approach to preventing or enhancing cognitive function.

6 Discussion

Recent studies have extensively investigated the effects of dietary interventions on cognitive well-being. Notable diets such as the

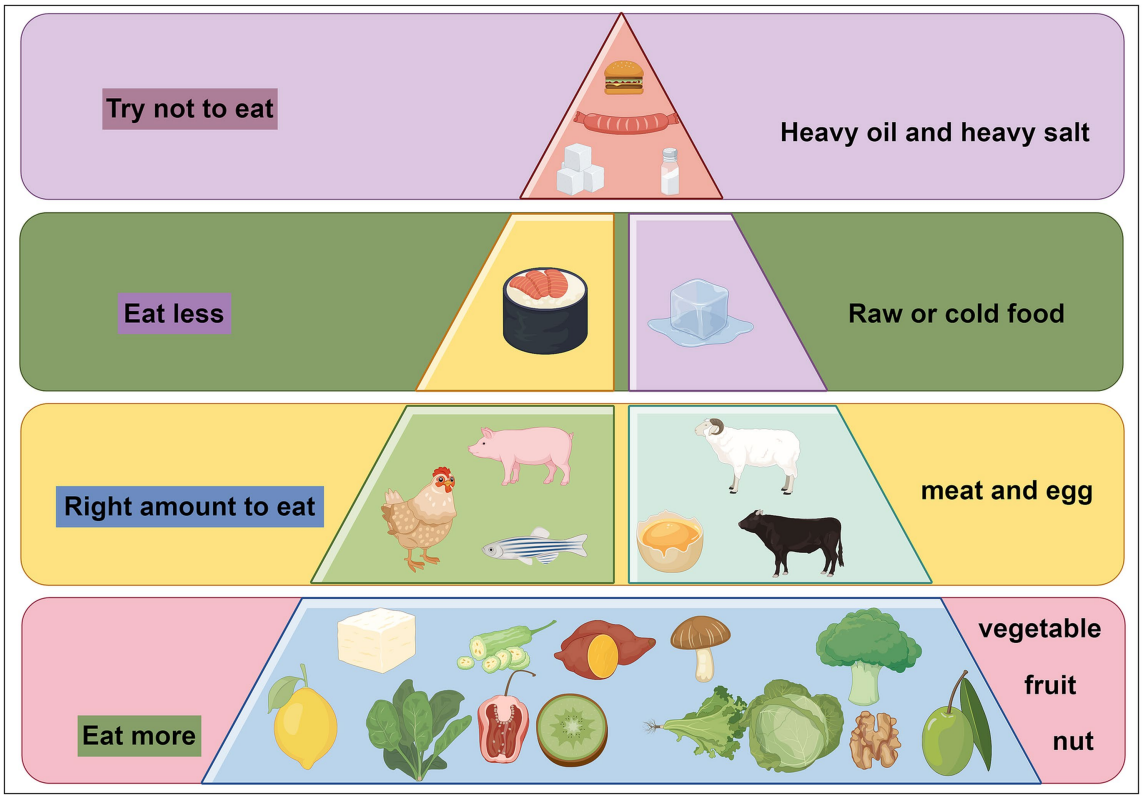

FIGURE 3
Dietary recommendations for cognitive dysfunction. We recommend eating more fresh fruits and vegetables, eating a moderate amount of meat, eating less raw and cold food, and trying not to eat foods high in salt, sugar, and fat.

Mediterranean diet, plant-based diets, and low-carbohydrate diets have shown protective advantages for cognitive function. These dietary patterns are rich in antioxidants, anti-inflammatory agents, and neuroprotective nutrients like polyunsaturated fatty acids, B vitamins, polyphenols, and vitamin D. Research suggests that these nutrients may decelerate cognitive decline and reduce the risk of neurodegenerative conditions by addressing inflammation, lessening oxidative stress, and promoting neurogenesis, among other mechanisms.

However, despite the potential benefits of dietary interventions for cognitive health, several obstacles impede their successful implementation. Challenges include difficulties in maintaining adherence to specific dietary patterns over time and variations in individual responses to dietary changes across different populations, necessitating personalized dietary guidance. Moreover, factors such as socioeconomics, cultural influences, and resource availability also impact the effectiveness of dietary interventions.

Drawing from existing literature, we recommend various dietary strategies to improve cognitive health. These include emphasizing the consumption of fruits, vegetables, whole grains, nuts, and healthy fats such as olive oil, while moderating fish and meat intake. Increasing the intake of vegetables, fruits, whole grains, legumes, nuts, and seeds while reducing animal product consumption is also beneficial. Additionally, limiting high-carbohydrate foods like sugar, bread, and pasta while increasing fat and protein intake is advised. Supplementing with polyunsaturated fatty acids, B vitamins, polyphenols, vitamin D, and essential nutrients is also suggested, as shown as Figure 3.

To enhance our comprehension of how dietary interventions impact cognitive health, future research should adopt a multidisciplinary approach involving nutrition, neuroscience, psychology, and sociology to comprehensively evaluate the effects of dietary modifications. Long-term, large-scale, multi-center randomized controlled trials are essential to explore the sustained impacts of diverse dietary patterns on cognitive performance. Analyzing existing dietary frameworks can aid in developing personalized dietary recommendations tailored to individual variances, thereby enhancing adherence and efficacy of interventions. Furthermore, examining the influence of socioeconomic and cultural factors on dietary strategy implementation is crucial for establishing more effective public health initiatives. Through these holistic endeavors, we can optimize dietary interventions to prevent and alleviate cognitive impairment, ultimately enriching individuals' quality of life.

7 Conclusion

With the advancement of human gut microbiota analysis technologies, the critical role of microorganisms has become increasingly evident. The brain and gut interact through the microbiota-gut-brain axis, regulating brain functions and the progression of cognitive impairments. The gut microbiota, as a dynamic ecosystem, undergoes continuous changes influenced by internal and external environmental factors. Dysbiosis of the gut microbiota may serve as both a stressor and an intervention target for neurodegenerative diseases, as it can induce cognitive dysfunction and contribute to these conditions. Dietary modifications and the broad intake of nutrients can mediate gut-brain communication by regulating the gut microbiota, thereby improving cognitive function, and alleviating neurodegenerative diseases. However, the causal relationship between dietary habits, gut microbiota, and the pathology of cognitive functions has yet to be fully established. More extensive and in-depth research is needed to clarify how different dietary patterns influence the gut microbiota and their impact on cognition and neurodegenerative diseases. Importantly, improving dietary structures and adjusting the quantity, types, and distribution of gut microbiota to address cognitive dysfunction may become a novel therapeutic strategy in the future.

Author contributions

RZ: Writing – original draft, Funding acquisition. MZ: Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Project administration. PW: 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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Granola consumption with multiple prebiotics in Japanese participants increases *Bifidobacterium* abundance and improves stress and subjective sleepiness

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Background: Sleep is essential for physical and mental health. However, stress-related sleep disorders are common in Japan, and the gut–brain axis may play a role in sleep and stress management. This study investigated whether the consumption of granola containing multiple prebiotic ingredients could alleviate stress and improve insomnia in adults with stress-related sleep problems, regardless of individual differences in the gut microbiota. Additionally, we aimed to investigate the relationship between changes in gut microbiota and the observed improvements.

Method: A single-arm uncontrolled trial was conducted with 27 adults with high stress levels and sleep disturbance. The participants consumed 50 g of prebiotics-containing granola daily for 8 weeks. Subjective sleep quality was assessed using the Athens Insomnia Scale, Epworth Sleep Scale, and Oguri-Shirakawa-Azumi Sleep Inventory-Middle-aged and Aged version (OSA-MA). Stress levels were assessed by administering the Brief Job Stress Questionnaire and Profile of Mood States 2nd edition (POMS2). Gut microbiota composition was analyzed using 16S rDNA sequencing.

Results: After 8 weeks, subjective insomnia scores and sleep onset and maintenance improved significantly, whereas the stress and mood disturbance scores decreased significantly. Gut microbiota analysis showed that the relative abundance of *Bifidobacterium* increased, whereas that of *Bacteroides* decreased. Correlation analysis suggested a significant association between increased *Bifidobacterium* level and reduced stress ($r = -0.39$, $p = 0.0035$) and insomnia levels ($r = -0.3$, $p = 0.026$).

Conclusion: Prebiotics-containing granola improved subjective sleep quality and reduced stress in adults with stress-related sleep disturbances, which may be attributed to alterations in gut microbiota, particularly the increase in *Bifidobacterium* abundance.

KEYWORDS

gut–brain axis, stress, sleep disorders, microbiota, prebiotics, granola

1 Introduction

Sleep is essential for maintaining physiological homeostasis and cognitive functions. It regulates the autonomic nervous, cardiovascular, immune, and metabolic systems (1–4). Additionally, sleep plays a crucial role in cognitive performance, memory consolidation, and emotional regulation (5–7). However, sleep deprivation has become a widespread concern, particularly in Japan, where a significant proportion of the population reports insufficient sleep (8). Chronic sleep deprivation is associated with an increased risk of cardiovascular disease, diabetes, metabolic syndrome, and depression (9–11).

Among the various factors influencing sleep, stress is one of the most significant. Stress triggers physiological responses that increase alertness, potentially leading to primary or secondary sleep disorders (12–14). Acute and chronic stress-related insomnia affects a substantial proportion of the population, emphasizing the need for interventions that alleviate stress to improve sleep quality (15–18).

Recent evidence suggests a strong connection between the gut microbiota and brain function, known as the gut–brain axis (19). Stress alters gut microbiota composition, as demonstrated in animal models where restraint stress reduced *Bifidobacterium,* Ruminiclostridium and Lachnoclostridium and increased *Akkermansia* and *Faecalibaculum* levels (20). Conversely, gut microbiota may modulate stress responses. Germ-free mice, which lack gut microbiota, exhibit an exaggerated stress response, whereas colonization with normal gut microbiota restores stress resilience (21–23). Human studies also support this interaction; consumption of probiotic beverages has been shown to reduce perceived stress and lower salivary cortisol levels (24). One human study demonstrated that probiotic supplementation improved sleep quality and stress resilience. Medical students, who are generally assumed to be under chronic stress, were administered probiotic tablets containing *Bifidobacterium*. The intervention resulted in a reduction in subjective stress levels, improved Pittsburgh Sleep Quality Index (PSQI) scores, and decreased sleep latency (25). In addition, probiotic consumption has been shown to alter gut microbiota composition, reduce PSQI scores, increase total sleep time, and enhance mental well-being (26, 27). Additionally, prebiotic intake enhances short-chain fatty acid production and mitigates stress-induced hormonal responses (28). Prebiotics are non-digestible food components that selectively stimulate the growth and activity of beneficial gut bacteria, particularly *Bifidobacterium* and *Lactobacillus* (29). These compounds promote the production of short-chain fatty acids, such as butyrate, which have been linked to anti-inflammatory effects, stress regulation, sleep patterns and improved gut–brain communication (30, 31). Studies have shown that dietary interventions incorporating prebiotic-rich, indigestible food ingredients, formulated by a dietitian, lead to significant reductions in stress questionnaire scores and improvements in sleep quality (32). Given these insights, prebiotic intake may provide a novel approach to improving sleep and reducing stress-related insomnia. In this study, we formulated a granola containing multiple prebiotic compounds to evaluate their effects on gut microbiota composition, stress, and sleep quality. We aimed to determine whether prebiotic-containing granola consumption could alleviate stress and enhance sleep quality in individuals experiencing stress-related insomnia.

2 Materials and methods

2.1 Participants

Participants were recruited to this study through public calls in the form of posters, web pages, and emails from Clinical Creative (Sapporo, Japan). In total, 116 men and women aged 24–58 years participated in this study. We conducted a survey using the Epworth Sleep Scale, Athens Insomnia Scale, and Brief Job Stress Questionnaire. We prescreened 50 people using the following selection criteria: (1) those with an Epworth Sleepiness Scale score of ≥11 were excluded. (2) those judged to be under high stress based on the Brief Job Stress Questionnaire were included (score ≥ 77 in area B or total score ≥ 76 in areas A and C and ≥ 63 in area B). (3) If the total number of candidates who qualified after step (2) were > 50, the participants were selected in order of their score in area B. In case the scores were equal for area B, the participants were selected in the order of their scores on the Athens Insomnia Scale.

Next, the 50 pre-screened individuals were instructed to use an activity meter (Fitbit Inspire HR; Fitbit, San Francisco, CA, United States) to measure their sleep quality and sleep habits over a 7-day period. Additionally, they were required to complete the Profile of Mood States 2nd edition (POMS2), which is a questionnaire on defecation frequency (number of times of defecation/week), and a food frequency questionnaire (FFQ). We selected 27 volunteers (male, 12; female, 15) based on the following exclusion criteria: (1) had taken or had planned to take antibiotics, anti-allergy medications, sleeping pills, or sleeping aids in the month preceding the test date; (2) regular intake of supplements at a rate of more than three times/week (such as probiotic preparations and prebiotic supplements) that may affect the study outcome within the month preceding the test date; (3) history of serious diseases or current illnesses of the heart, liver, nervous system, digestive system, etc.; (4) incidence of chronic or acute serious infections; (5) scheduled to receive vaccinations during the study period; (6) pregnant or planning to become pregnant or breastfeeding; (7) habitual drinking of alcohol more than three times/week; (8) irregular eating habits; (9) body mass index (BMI) ≥ 30 (10) plans to significantly alter their lifestyle during the study period, such as traveling overseas to a different time zone (11); primary caretaker for persons requiring nursing care or an infant or may have their sleep disturbed by other external factors; (12) food allergies; (13) participation in a clinical study for other medicines or health foods or planning to participate in another clinical study less than 1 month after the end of this study or after providing consent to participate in the relevant study; (14) not engaged in full-time employment; (15) working in shifts or late night work; or irregular sleeping and waking times (with a difference of >5 h at bedtime); or extremely short or irregular sleep times (sleep for <4 h; as confirmed using Fitbit measurements); (16) non-possession of a smartphone; (17) unable to install the Fitbit applications on their smartphones; and (18) less than three bowel movements/week. Participants with diagnosed psychiatric disorders, such as schizophrenia, bipolar disorder, anxiety, or depression, were not explicitly excluded. However, we confirmed that none of the participants were taking medications commonly prescribed for these mental illnesses. Based on this criterion, we considered the study population to be free of psychiatric disorders. After the test, participants received an incentive. The sample size was determined based on the following. As it is known that the

composition of the gut microbiota, a secondary evaluation item in this study, changes due to the intake of cereal (33), the effect size of this study was calculated using G*Power, assuming a large effect size (effect size 0.80). At a significance level of 0.05, the sample size required to achieve 95% power was $n = 23$. Furthermore, taking into account those who withdrew, were lost to follow-up, or were excluded from the analysis, we set the sample size at 27 participants per group. From the recruitment of participants to the completion of testing for all participants, this trial took place from November 2023 to June 2024. This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of Sapporo Yuri no Kai Hospital (approval number: 029). All participants provided informed consent before enrolment in the study. This clinical trial was registered with the Institutional Ethics Committee of the Japanese University Hospital Medical Information Network (clinical trial reference number: UMIN 000053189).

2.2 Study design and procedure

The clinical trial was conducted in Hokkaido between April and June 2024 after the participants were selected for the single-arm uncontrolled single-blind before-and-after comparative study. Participants were informed that they would be consuming a cereal product as a test meal but were not aware of whether it contained prebiotics. This approach was implemented to minimize expectancy bias regarding the potential effects of prebiotics on sleep and stress. All participants were instructed to consume the test meals every day for 8 weeks, and record their meals including the consumption of the test meals in a food diary to ensure compliance with the intervention. Participants were allowed to consume granola once per day, and while breakfast was the recommended time, no specific instructions were given. All participants were required to complete a 7-day sleep measurement and answer the OSA-MA questionnaire at weeks 0 (1 week before the start of the study), 4 (4 weeks after the start of the study), and 8 (8 weeks after the start of the study). Additionally, the participants were instructed to use a Metabolokeeper® (Techno Suruga Labo Co., Ltd., Shizuoka, Japan) to collect their feces on 1 day of the following weeks: 0, 4, and 8. At the start of the study and on the last days of weeks 4 and 8, the participants were instructed to complete the questionnaires on defecation (number of times of defecation in a week and BSS for stool consistency), Athens Insomnia Scale, Epworth Sleep Scale, and POMS2. Additionally, the participants had to complete the Brief Job Stress Questionnaire and FFQ at the end of the study. Defecation frequency and stool consistency were recorded as an additional parameter to evaluate the effects of prebiotic intake on gut microbiota and gastrointestinal function. Since stress is known to affect gut function (34), we also examined whether changes in defecation frequency were associated with improvements in stress and sleep quality. All participants completed the questionnaires anonymously to ensure honest responses. The administration and data collection of the questionnaires were conducted by a third-party organization, ensuring impartiality. Before completing the questionnaires, participants received standardized instructions explaining the purpose of each item and how to respond appropriately. These measures were implemented to maintain the reliability and authenticity of the responses. The details of the study schedule are shown in Figure 1. The flow diagram of the participants in this study is shown in Supplementary Figure 1. There were no withdrawals among the participants who consumed the test meals, and all participants were used in the analysis. In this study, outcome measures were categorized into primary and secondary outcomes as follows. Primary Outcomes: The primary outcome measures included EEG-based sleep parameters, which served as objective indicators of sleep architecture and quality. These measures were selected as the primary indicators for evaluating the intervention's impact on sleep. Secondary Outcomes: The secondary outcome measures included the OSA-MA questionnaire, the Athens Insomnia Scale, the Epworth Sleepiness Scale, POMS2, the Brief Job Stress Questionnaire, the Food Frequency Questionnaire (FFQ), questionnaires on defecation, and gut microbiota analysis. These measures were incorporated to assess subjective sleep quality and provide additional insights into various factors influencing sleep, such as insomnia severity, daytime sleepiness, mood states, occupational stress, dietary habits, and gut microbiota composition.

2.3 Test meals

The test meal comprised granola containing six types of prebiotic compounds (inulin, resistant starch, fructooligosaccharide, galactooligosaccharide, cacao mass, and barley). The consumption of each of these prebiotics alone increases short-chain fatty acid production (35–44). The granola contains approximately 1.4 g of each of the six prebiotics. The participants consumed 50 g of granola and 200 mL of milk daily. The brand of milk was specified to minimize the variation in the nutrient content of milk. The nutritional content of the test meal per serving (50 g) was 194.6 kcal; protein: 3.5 g; fat: 3.5 g; and carbohydrates: 41.2 g. This granola is already on the market, so the participants can eat it safely.

2.4 Sleep electroencephalography (InSomnograf®) measurement

S'UIMIN's InSomnograf® is a device that measures the sleep stage based on EEG data obtained using five electrodes. These results show an 86.9% correlation with polysomnography (PSG) (45). Parameters related to sleep quality and quantity such as total sleep time (s), sleep onset latency (s), wakefulness after sleep onset (WASO; s), N1 total time (s), N2 total time (s), N3 total time (s), REM total time (s), and REM sleep rate (%) were calculated from the results of the sleep stage assessment (hypnogram) and used in the analysis. The participants wore the device immediately before bedtime to obtain EEG measurements. The average value was calculated for each of the 7 days of every week to determine the participant EEG parameter for the analysis.

2.5 16S rDNA gene sequencing and analysis

DNA was extracted from the collected feces using a revised version of a previously described method (46). The fecal sample (100 mg) was suspended in 4 M guanidine thiocyanate, 100 mM Tris–HCl (pH 9.0), and 40 mM EDTA, followed by bead-milling using a Precellys Evolution system (Bertin Instruments, FRA). DNA was

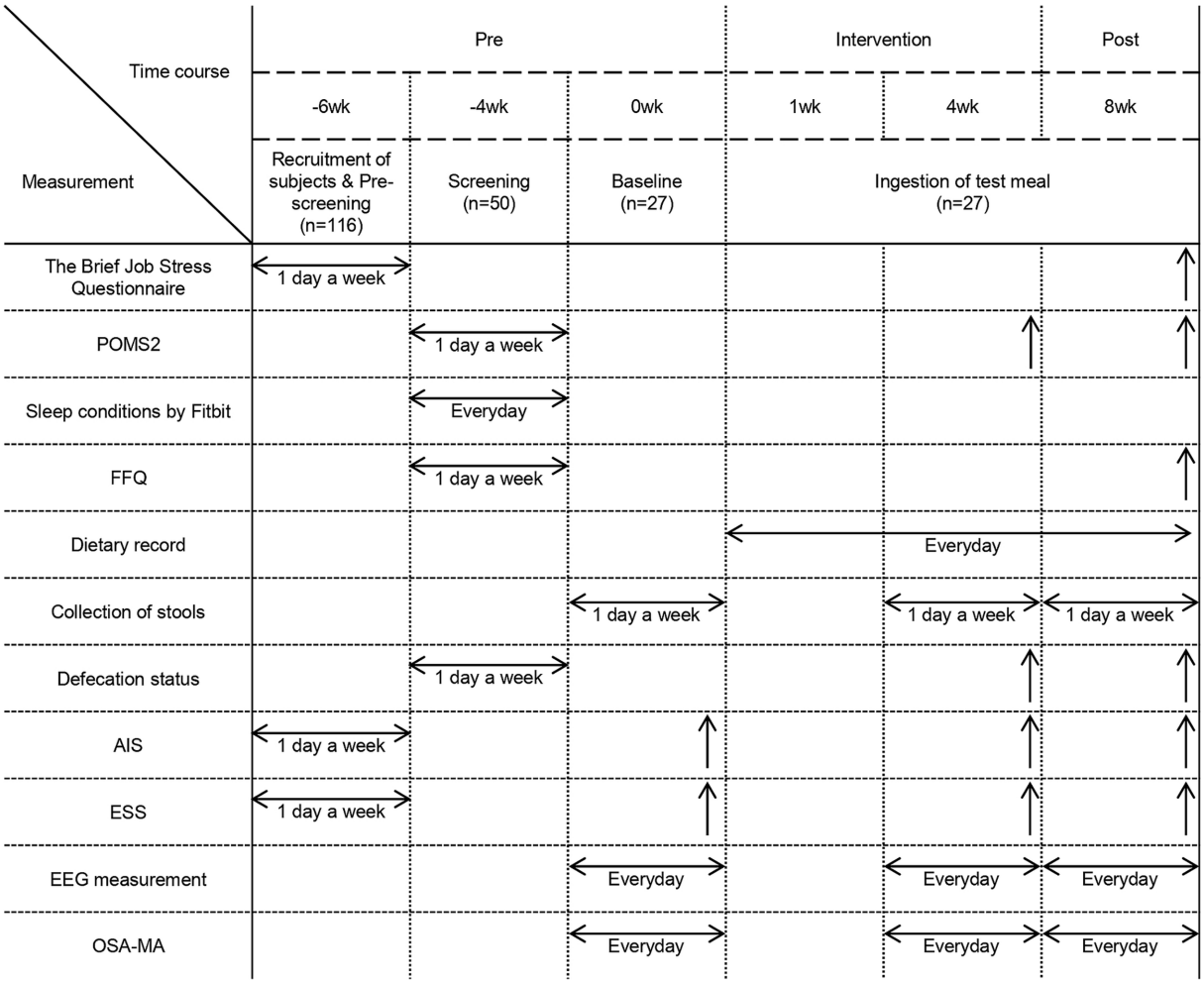

FIGURE 1
Experimental schedule. Experimental schedule and parameters measured during each period. POMS2: Profile of Mood States, 2nd Edition. FFQ, Food Frequency Questionnaire. AIS, Athens Insomnia Scale. ESS, Epworth Sleepiness Scale. EEG measurement, Electroencephalographic measurements. OSA-MA, Oguri-Shirakawa-Azumi Sleep Inventory, Middle-aged and Aged version. Measurements conducted over a period of time are represented by horizontal arrows, while those conducted on a specific day are represented by upward arrows, such as on the final day of the intervention.

extracted from the bead-treated suspension using the PI-480 and NR-201 systems (Kurabo Industries, Japan). The concentration and purity of the extracted DNA were measured using a spectrophotometer with the NanoDrop ND8000 instrument (Thermo Fisher Scientific, United States). The final DNA sample concentration was adjusted to 10 ng/μl.

The V3–V4 region of the 16S rDNA was amplified using the Pro341F/Pro805R primer set for prokaryotes. Primers were designed to complement the index primers provided by Illumina. The following primer sequences were used: Forward primer, 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGAGGCAGCAGCCTACGGGNBGCASCAG-3′; Reverse primer, 5'-CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACNVGGGTATCTAATCC-3′.

The touchdown PCR method for thermal cycling was used with a Rotor-Gene Q quantitative thermal cycler (Qiagen, Germany) to reduce the formation of spurious byproducts during the amplification process. The reaction mixture (25 μL) contained 10 ng of genomic DNA, MightyAmp for Real Time (SYBR Plus; Takara, Japan), and 0.25 μM of each primer. The PCR reaction conditions for DNA amplification were as follows: initial denaturation at 98°C for 2 min, followed by 35 cycles of annealing from 65°C to 55°C for 15 s, and extension at 68°C for 30 s. The annealing temperature was reduced by 1°C/cycle until the temperature was 55°C, which was maintained for the remaining cycles. The PCR products were purified using a MultiScreen PCRu96 filter plate (Merck Millipore, United States) and analyzed using a Bioanalyzer DNA 1000 Chip Kit (Agilent Technologies, United States) to detect primer dimers and determine the average molecular weight of each product. The purified products were quantified using real-time quantitative PCR (q-PCR) on a Rotor-Gene Q quantitative thermal cycler using MightyAmp for Real Time (SYBR Plus), 0.2 μM of each primer, which were derived from Illumina adapters, and serially diluted PhiX control library (Illumina, United States) as the standard. The PCR reaction conditions for quantification of each PCR product were as follows: initial denaturation at 98°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and extension at 68°C for 30 s. A quantification step was performed to determine the

concentration of the amplified libraries and confirm the presence of suitable primers for Illumina sequencing.

Sequencing was performed using MiSeq (Illumina, USA) and MiSeq Reagent Kit v3 (600 cycles). The sequenced 16S rDNA were processed using the quantitative Insights into microbiological Ecology 2 (QIIME2) pipeline (version 2024.2). The DADA2 algorithm was used to remove noise, perform quality filtering, and generate amplicon sequence variants (ASVs). The ASVs were assigned taxonomic groups from the phylum to genus levels using the Silva SSU Ref Nr 99 (version 132) classifier. Additionally, principal coordinate analysis (PCoA) was performed to determine alpha diversity, such as the Simpson diversity index, and beta diversity using weighted UniFrac distance.

2.6 Statistical analysis

2.6.1 One-way repeated ANOVA or Friedman test

All statistical analyses were performed using GraphPad Prism version 9.0.2 (GraphPad Software Inc., San Diego, CA, United States). Data are expressed as mean ± SEM. The D'Agostino–Pearson test was used to assess the normality of the distributed data, whereas Bartlett's test was used to examine whether the variation was equal or skewed. If the data showed a normal distribution and equal variation, statistical significance was determined using one-way repeated ANOVA with Tukey's *post-hoc* test. If the data showed a non-normal distribution or biased variation, statistical significance was determined using Friedman's and Dunn's *post-hoc* tests. Statistical significance was set at $p < 0.05$.

2.6.2 Correlation analysis

The D'Agostino–Pearson test was used to check for normal data distribution. Pearson's correlation coefficient was calculated as a parametric test to check for correlation between normally distributed data. Spearman's correlation coefficient was calculated as a non-parametric test to check for correlation between non-normally distributed data. We calculated the *p*-value for each test. A correlation coefficient of $r < -0.2$ or $r > 0.2$ was considered a significant correlation if $p < 0.05$.

2.6.3 Multiple regression analysis

The value of each item was standardized using the STANDARIZE function in Excel, and analysis was performed using these standardized values. Additionally, the values for physical characteristics, such as age, BMI, sex, diastolic blood pressure, and systolic blood pressure, were calculation as adjustment variables. Furthermore, the VIF was calculation as a measure of multicollinearity, and it was confirmed to be ≤5.

3 Results

3.1 Survey of participant physical characteristics and food frequency questionnaire

The physical characteristics of the participants during the test period are listed in Table 1. The average age of the participants was 44.1 ± 1.50 years (mean ± SEM), and their average height was 167.1 ± 1.69 cm. The diastolic blood pressure reduced significantly after 8 weeks of consumption of granola containing several prebiotics. The quantity of nutrients consumed was calculated using the FFQ and compared between the pre- and post-tests; however, no significant difference was observed. This implies that the frequency of daily food intake did not change significantly during the test period (Figure 2).

3.2 Consuming granola containing prebiotics improves participant sleepiness and insomnia

We performed an EEG to assess objective sleep status and administered the Athens Insomnia Scale, Epworth Sleepiness Scale, and OSA-MA questionnaires to assess subjective sleep status. None of the sleep-state parameters obtained from EEG measurements showed significant differences (Figure 3). In contrast, the Athens Insomnia Scale for the subjective sleep parameters showed significant lower scores at both weeks 4 and 8 after the consumption of prebiotic granola compared with that at week 0 (Figure 4). Additionally, the score for Factor 1 (sleepiness on rising) on the OSA-MA showed an increasing trend on week 4, and the score for Factor 2 (initiation and maintenance of sleep) increased significantly (Figures 4C,D). These results suggest that the consumption of prebiotics-containing granola improves subjective sleepiness and insomnia. Here, we examined the correlation between an objective sleep indicator, electroencephalogram (EEG) measurements, and subjective sleep questionnaire evaluations. The correlation analysis showed a positive correlation between subjective sleep time and objective sleep time [Factor 5-TST ($r = 0.27$)] and a negative correlation between subjective sleep onset and sleep maintenance and objective middle of the night awakening and sleep latency [Factor 2-WASO ($r = -0.2$), Factor 2-SOL ($r = -0.27$)]. Although subjective and objective ratings showed a correlation, subjective sleepiness (AIS) and objective indicators did not show any correlation, and inconsistent results were obtained (Supplementary Figure 2).

TABLE 1 Physical characteristics of all participants.

	0 week	4 week	8 week	*p*-value
Weight (kg)	64.7 ± 2.74	64.7 ± 2.78	64.7 ± 2.71	0.9533
Body mass index	22.9 ± 0.630	22.9 ± 0.653	22.9 ± 0.619	0.9269
Systolic blood pressure (mmHg)	122.8 ± 1.98	119.0 ± 2.21	118.6 ± 2.47	0.0543
Diastolic blood pressure (mmHg)	79.5 ± 1.39	75.4 ± 1.47**	76.7 ± 1.73*	0.0079

All values are shown as mean ± SEM ($n = 27$; male $n = 12$, female $n = 15$). The *p*-value in the table indicate the *p*-value of the one-way repeated ANOVA test. **$p < 0.01$, *$p < 0.05$, evaluated using the one-way repeated ANOVA test with Tukey's *post-hoc* test (vs. 0 week).

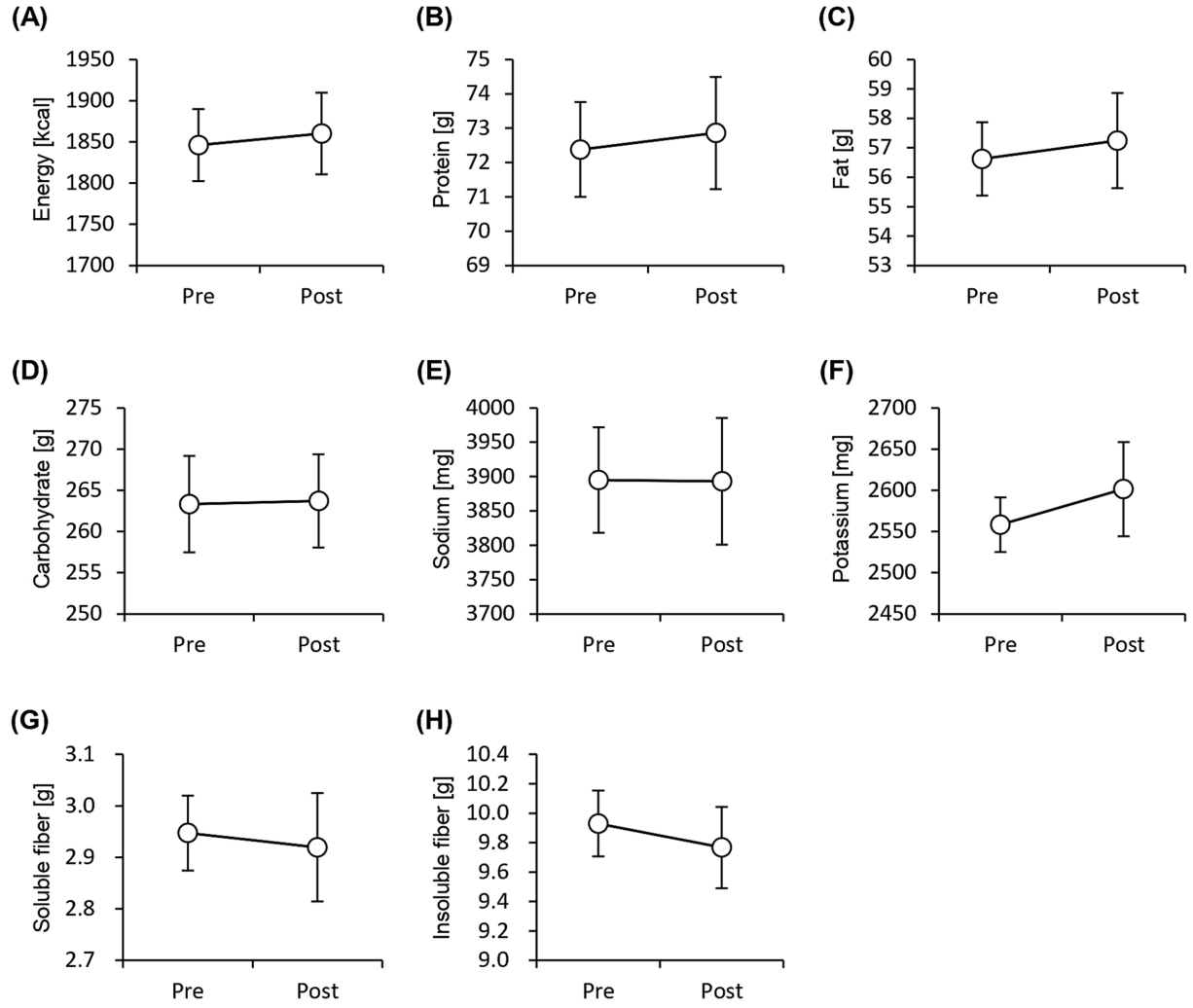

FIGURE 2
Frequency of nutrient intake does not change significantly before and after the study period. Daily consumption of nutrients was estimated using pre-period and post-period Food Frequency Questionnaires. **(A)** energy, **(B)** protein, **(C)** fat, **(D)** carbohydrate, **(E)** sodium, **(F)** potassium, **(G)** soluble dietary fiber, and **(H)** insoluble dietary fiber levels. All values are represented as mean ± SEM (n = 27; male n = 12, female n = 15).

3.3 Consumption of prebiotics-containing granola improves stress and total mood disturbance

We administered the Brief Job Stress Questionnaire and the POMS2 to examine the stress and psychological mood states of the participants. The scores for all three areas (area A: job stressors, area B: psychological and physical stress reactions, and area C: social support at work) were significantly low after 8 weeks of consuming the prebiotics-containing granola (Figures 5A–C). The POMS2 scores on the DD (depression–dejection) and FI (fatigue–inertia) scales decreased significantly after 8 weeks of consuming prebiotics-containing granola. Additionally, the total mood disturbance (TMD) score calculated based on the six POMS2 scales decreased significantly (Figures 5D–K). These results suggest that the consumption of prebiotics-containing granola improves stress and reduces feelings of depression, fatigue, and lethargy, thereby reducing psychological mood disturbances. Additionally, we examined the number of bowel movements and stool characteristics; however, no significant differences were observed (Supplementary Figure 3).

3.4 Consumption of prebiotics-containing granola increases the relative abundance of *Bifidobacterium* and decreases that of *Bacteroides*

We investigated the changes in the gut microbiota associated with the consumption of prebiotics-containing granola. The alpha diversity of the gut microbiota reduced significantly, as indicated by the Shannon and Simpson diversity indices (Figures 6A–D). However, the Chao1 diversity index and Observed_features, which strongly reflects the number of bacterial species, did not differ significantly. Thus, the results suggest that the decline in gut microbiota diversity may be attributed to a decrease in the evenness of the microbiota rather than a decrease in the number of species.

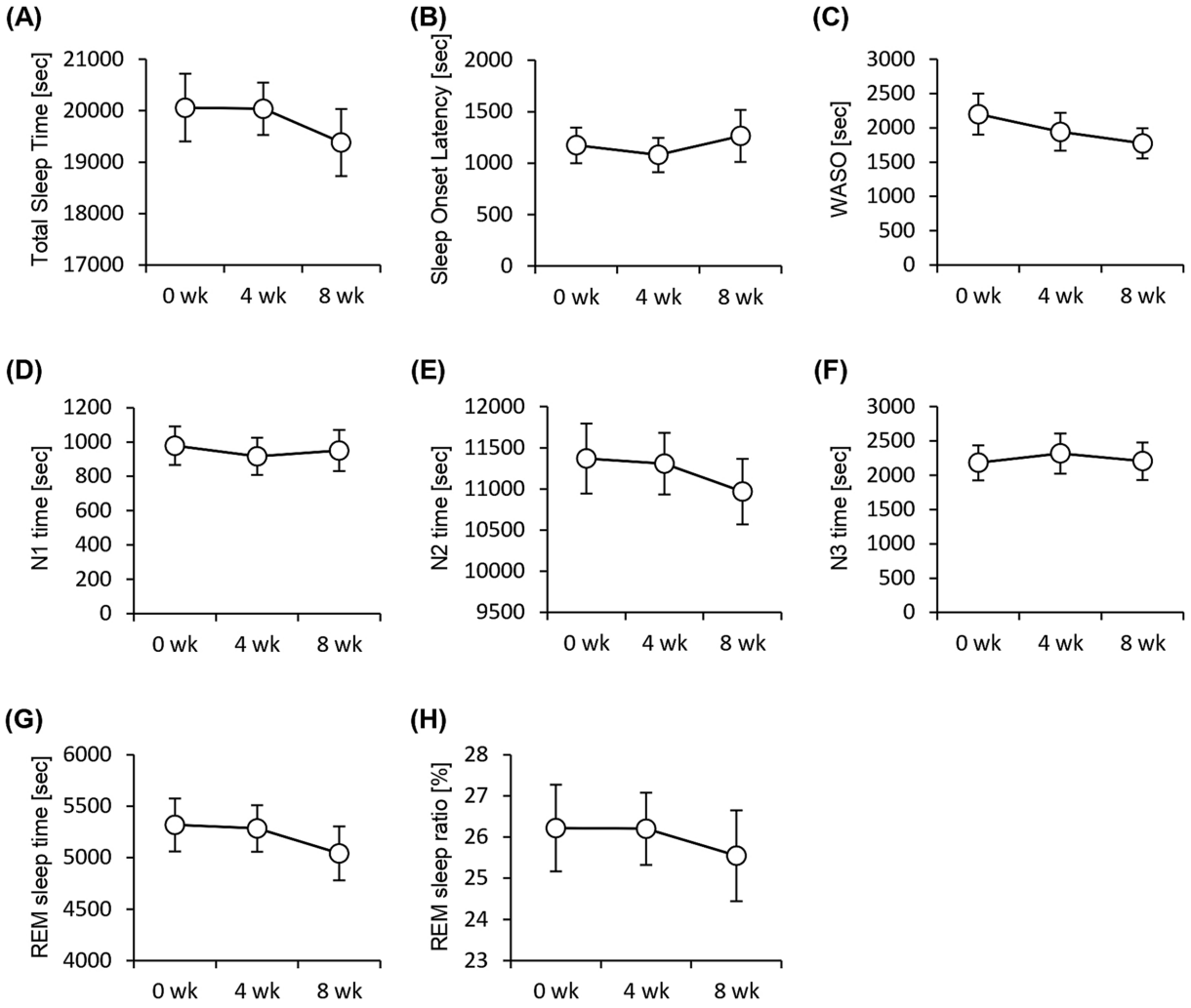

FIGURE 3
EEG measurement shows no significant difference in the sleep parameters. Summary of sleep parameters obtained from the EEG measurements. **(A)** Total Sleep Time. **(B)** Sleep onset latency, **(C)** WASO, wake time after sleep onset, **(D)** N1, non-REM sleep stage 1, **(E)** N2, non-REM sleep stage 2, **(F)** N3, non-REM sleep stage 3, **(G)** REM sleep time, and **(H)** REM sleep ratio. All values are represented as mean ± SEM (n = 27; male n = 12, female n = 15).

The composition of the gut microbiota was measured using beta diversity. The results were compared weekly; however, no significant difference was observed between each week ($p = 0.74$), and the composition of the gut microbiota did not change significantly even when the prebiotics-containing granola was consumed. However, comparison of the gut microbiota between individuals showed significant differences ($p = 0.001$), implying that the difference in gut microbiota composition was more because of variation between individuals than because of the consumption of prebiotics-containing granola (Figures 6E,F).

We examined the relative abundance of gut bacteria at the phylum (Figure 7A) and genus levels (Figure 7F). At the phylum level, the relative abundance of Bacteroidota significantly decreased, whereas that of Actinobacteriota significantly increased after 8 weeks of consumption of prebiotics-containing granola (Figures 7B–E). At the genus level, the relative abundance of *Bifidobacterium* increased, whereas that of *Bacteroides* significantly decreased (Figures 7G,H).

3.5 Increasing the relative abundance of *Bifidobacterium* may improve mental and physical stress response

We conducted a correlation analysis to explore the relationship between the gut microbiota and subjective sleep indicators, as measured by various questionnaires (Figure 8). This analysis included the Athens Insomnia Scale; Factors 2 of the OSA-MA; Areas A, B, and C of the Brief Job Stress Questionnaire; DD scale; FI scale; TMD, *Bifidobacterium*, and *Bacteroides* of the POMS2. A correlation matrix was created. The values obtained from the pre-period and week 8 (post) were used in the correlation analysis, whereas the values obtained from week 0 in the pre-period were used for AIS and ESS. Age, BMI, diastolic blood pressure, and systolic blood pressure were also included as physical characteristics in the correlation matrix. We focused on relationships where the absolute value of the correlation coefficient was >0.2. *Bifidobacterium* showed a significant negative correlation with AIS, areas A and B,

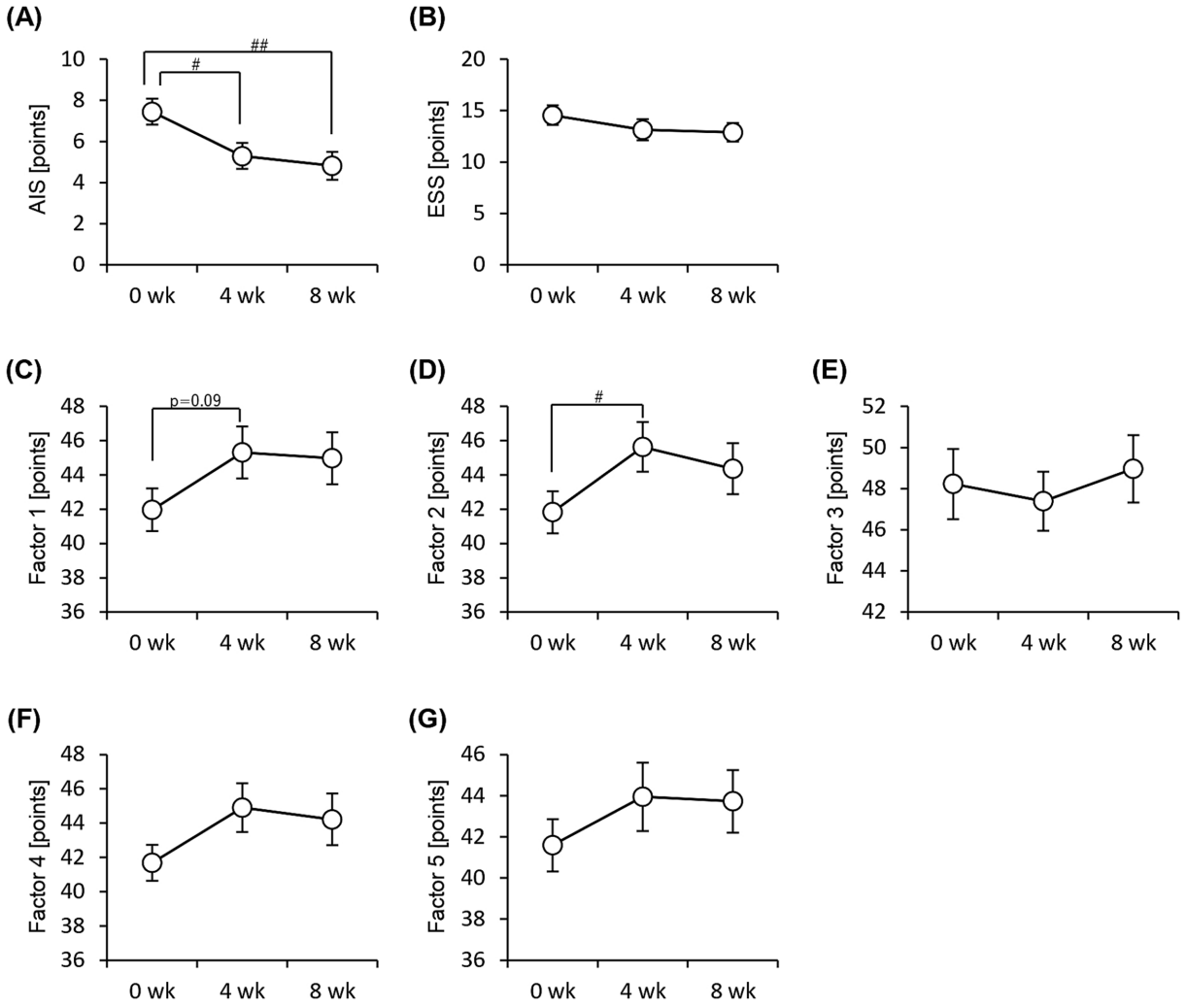

FIGURE 4
Subjective sleep quality improves with consumption of granola containing prebiotics. Summary of scores obtained from answers to each questionnaire. **(A)** AIS, Athens Insomnia Scale. **(B)** ESS, Epworth Sleepiness Scale. **(C–G)** OSA-MA, Oguri-Shirakawa-Azumi Sleep Inventory, Middle-age and Aged version [**(C)** Factor 1: sleepiness on rising, **(D)** Factor 2: initiation and maintenance of sleep, **(E)** Factor 3: frequent dreaming, **(F)** Factor 4: refreshing, **(G)** Factor 5: sleep length]. All values are represented as mean ± SEM (n = 27; male n = 12, female n = 15). ##p < 0.01, #p < 0.05, evaluated using the Friedman test with Dunn's *post-hoc* test.

and TMD. Additionally, we detected a significant negative correlation between *Bacteroides* and Factor 2 (Figure 8). As positive correlation was observed between AIS and BMI and negative correlation between *Bifidobacterium* and systolic blood pressure, the influence of physical characteristics was considered, and multiple regression analysis was performed to investigate in detail the relationship between gut bacteria and subjective sleep indicators. For the multiple regression analysis, AIS, Areas A and B, and TMD were used as objective variables, *Bifidobacterium* as the explanatory variable, and physical characteristics as the adjustment factor. *Bifidobacterium* showed a negative association with AIS and TMD, and a significant negative association with areas A and B (Table 2). However, no significant association was observed between *Bacteroides* and Factor 2 (Table 3). These results suggest that the consumption of prebiotics-containing granola may increase the relative abundance of *Bifidobacterium* and contribute to reductions in mental and physical stress. While not statistically significant, this increase also showed a tendency to improve subjective insomnia and mood states.

4 Discussion

In this study, although the subjective sleep index improved, none of the objective EEG measures showed significant changes. We examined the correlation between subjective and objective sleep indicators. While a strong relationship was observed in some cases, no correlation was found in others, resulting in inconsistent findings. Poor subjective sleep quality is often closely associated with short total sleep time and frequent middle-of-the-night awakening (measured objectively) (47–50). A study compared the Athens Insomnia Scale scores of healthy adult men and women who were sorted into good, moderate, and poor sleep quality groups. The sleep quality was identified using cluster analysis of the proportion of each

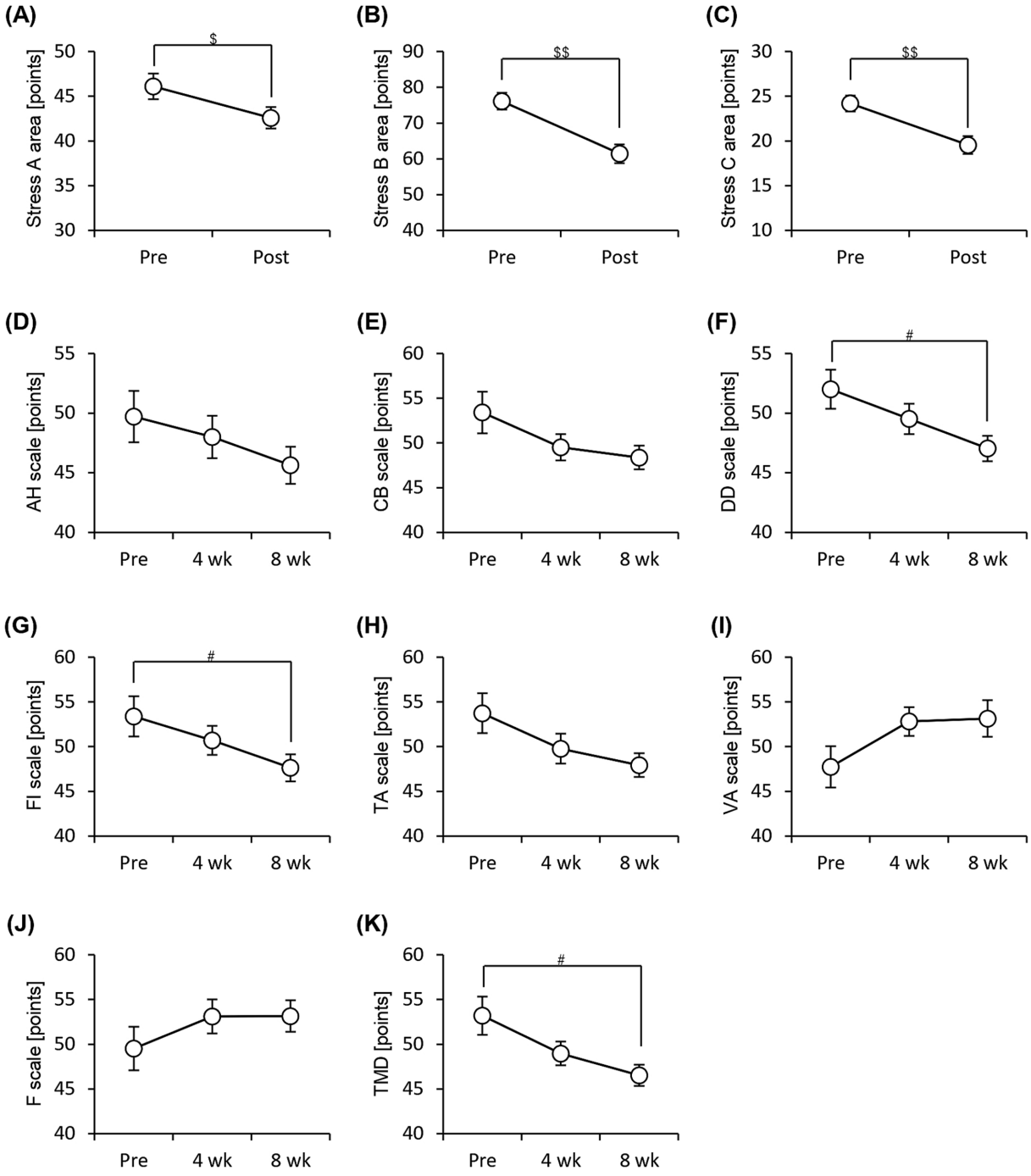

FIGURE 5
Subjective stress and mood states improve with consumption of granola containing prebiotics. Summary of scores obtained from answers to each questionnaire. **(A–C)** Brief Job Stress Questionnaire [**(A)** Stress area A, job stressors, **(B)** Stress area B, psychological and physical stress reactions, and **(C)** Stress area C, buffering factors such as social support at work], **(D–K)** POMS2, Profile of Mood States 2nd edition [**(D)** AH, anger–hostility scale; **(E)** CB, confusion–bewilderment scale; **(F)** DD, depression–dejection scale; **(G)** FI, fatigue–inertia scale; **(H)** TA, tension–anxiety scale; **(I)** VA, vigor–activity scale, **(J)** F, friendliness scale, **(K)** TMD, total mood disturbance]. All values are represented as mean ± SEM (n = 27; male n = 12, female n = 15). ##p < 0.01, #p < 0.05, evaluated using the Friedman test with Dunn's *post-hoc* test. $$p < 0.01, $p < 0.05, evaluated using Wilcoxon signed-rank test.

sleep stage determined based on sleep EEG, sleep latency, midnight awakenings, total sleep time, and subjective insomnia disturbance. No significant differences were observed between the groups (51). Systolic blood pressure was examined and found to be significantly high in the poor sleep group. Although the objective sleep indicators showed a correlation with systolic blood pressure, no correlation was observed with the Athens Insomnia Scale, which is a subjective sleep indicator (51). Moreover, comparing the depression symptom scores using the Athens Insomnia Scale between patients with major depression, schizophrenia, bipolar disorder, and anxiety disorder who were sorted into insomnia and non-insomnia groups showed that the scores were significantly high in the insomnia group;

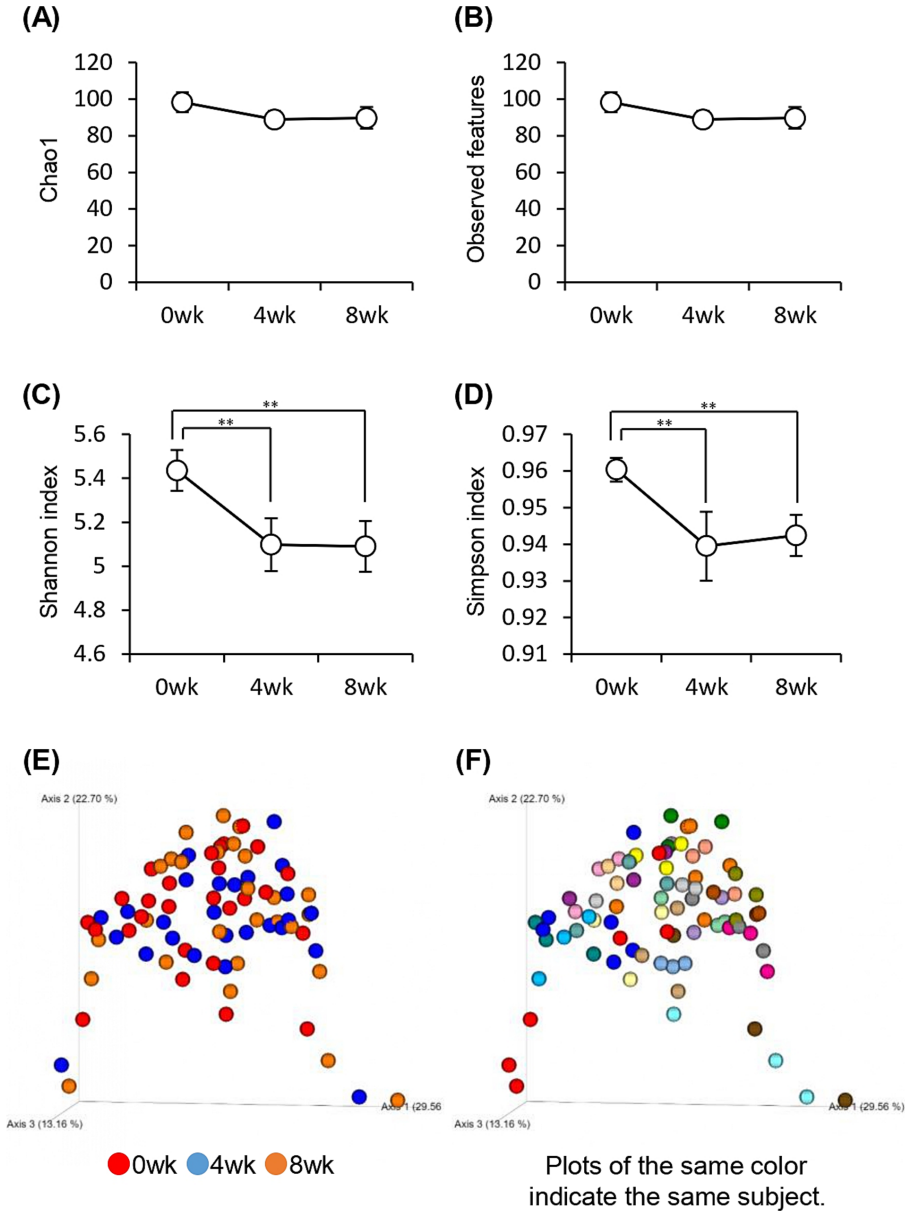

FIGURE 6
Consuming prebiotics-containing granola reduced intestinal microbiota diversity. **(A–D)** Bacterial alpha diversity; comparison of **(A)** Chao1, **(B)** Observed features, and **(C)** Shannon index. **(D)** Simpson index-based estimation of the 16S rDNA gene libraries at 99% similarity to sequencing analysis. **(E–F)** Bacterial beta diversity comparison by **(E)** week or **(F)** individual. All values are represented as mean ± SEM (n = 27; male n = 12, female n = 15). $^{**}p < 0.01$, evaluated using the one-way repeated ANOVA with Tukey's *post-hoc* test.

however, when divided into insomnia and non-insomnia groups based on sleep EEG, no differences were observed in the depression symptom scores (52). These studies suggest that objective and subjective sleep indicators may quite possibly diverge without correlation. Furthermore, the items of objective and subjective sleep indicators show high variability; hence, no consistent objective indicator is available for judging subjective sleep quality (53). Therefore, subjective measures depend on an individual's cognitive and psychological states, whereas EEG reflects physiological processes. These differences in cognitive and physiological responses are thought to exert an effect. Of course, since this study employed a single-group pre-post comparison design, the possibility of a placebo effect or psychological expectancy cannot be ruled out. Participants' awareness of the intervention may have influenced their perception, leading them to subjectively report improved sleep quality. In addition, an intervention study using electronic noise-masking earbuds for medical professionals with sleep disorders reported a greater improvement in subjective questionnaire responses than in objective EEG measures (54). In other words, objective sleep evaluation may be more sensitive to intervention effects than subjective sleep evaluation. To assess the sensitivity of the EEG measurements, a *post-hoc* power analysis was conducted using G*Power. The analysis revealed that, given our sample size (N = 27) and the assumed effect size (d = 0.3), the achieved statistical power

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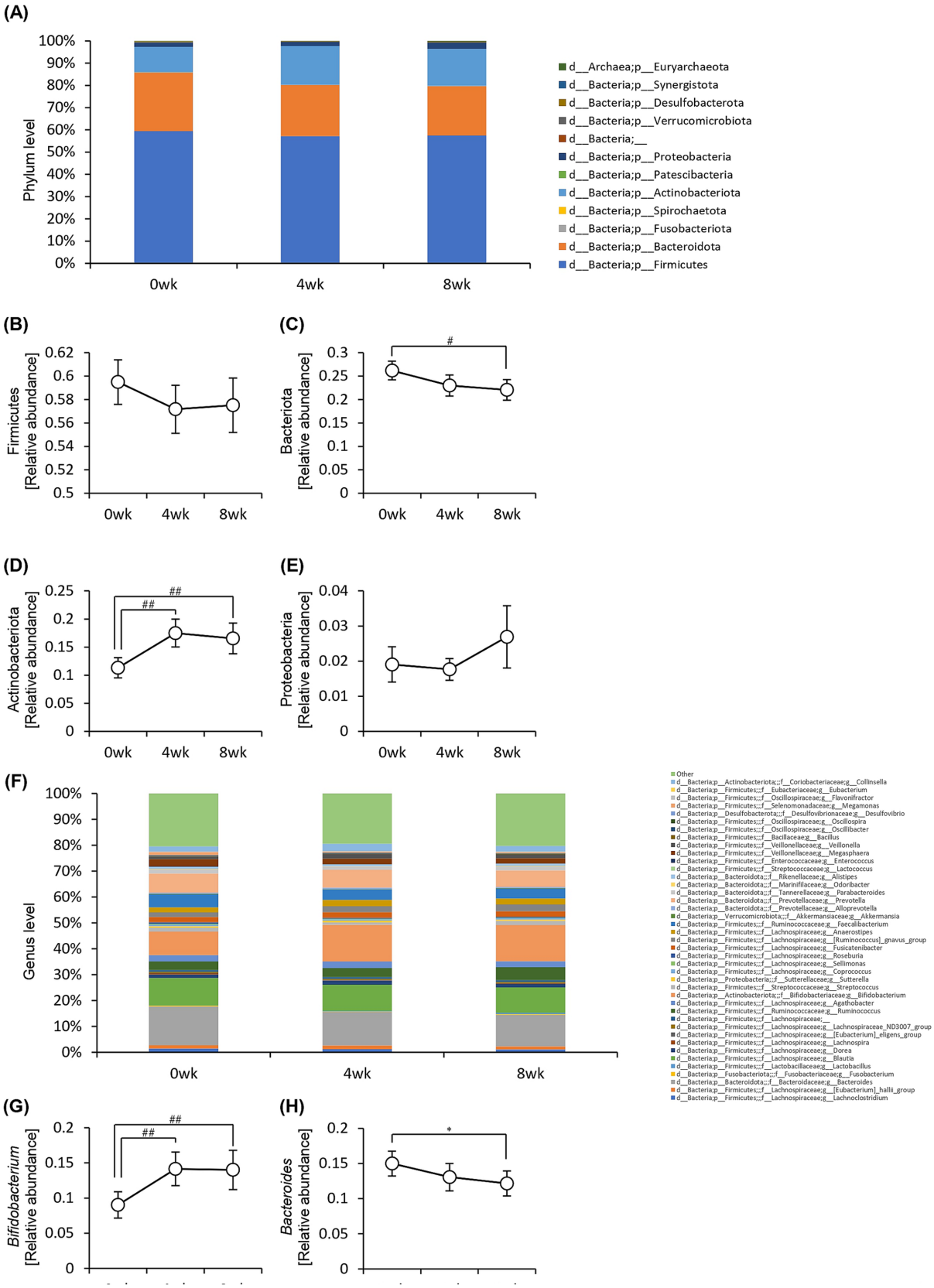

FIGURE 7
Consumption of prebiotics-containing granola increased the relative abundance of *Bifidobacterium* and reduced that of *Bacteroides*. **(A)** Composition of intestinal microbiota at phylum level. **(B–E)** Relative abundance of microbes at phylum level [**(B)** Firmicutes, **(C)** Bacteriota, **(D)** Actinobacteriota,

(Continued)

FIGURE 7 (Continued)
(E) Proteobacteria]. **(F)** Composition of the intestinal microbiota at genus level. (G–H) Relative abundance of microbes at genus level [**(G)** *Bifidobacterium*, **(H)** *Bacteroides*]. All values are represented as mean ± SEM (n = 27; male n = 12, female n = 15). *p < 0.05, evaluated using the one-way repeated ANOVA with Tukey's *post-hoc* test. ##p < 0.01, #p < 0.05, evaluated using the Friedman test with Dunn's *post-hoc* test.

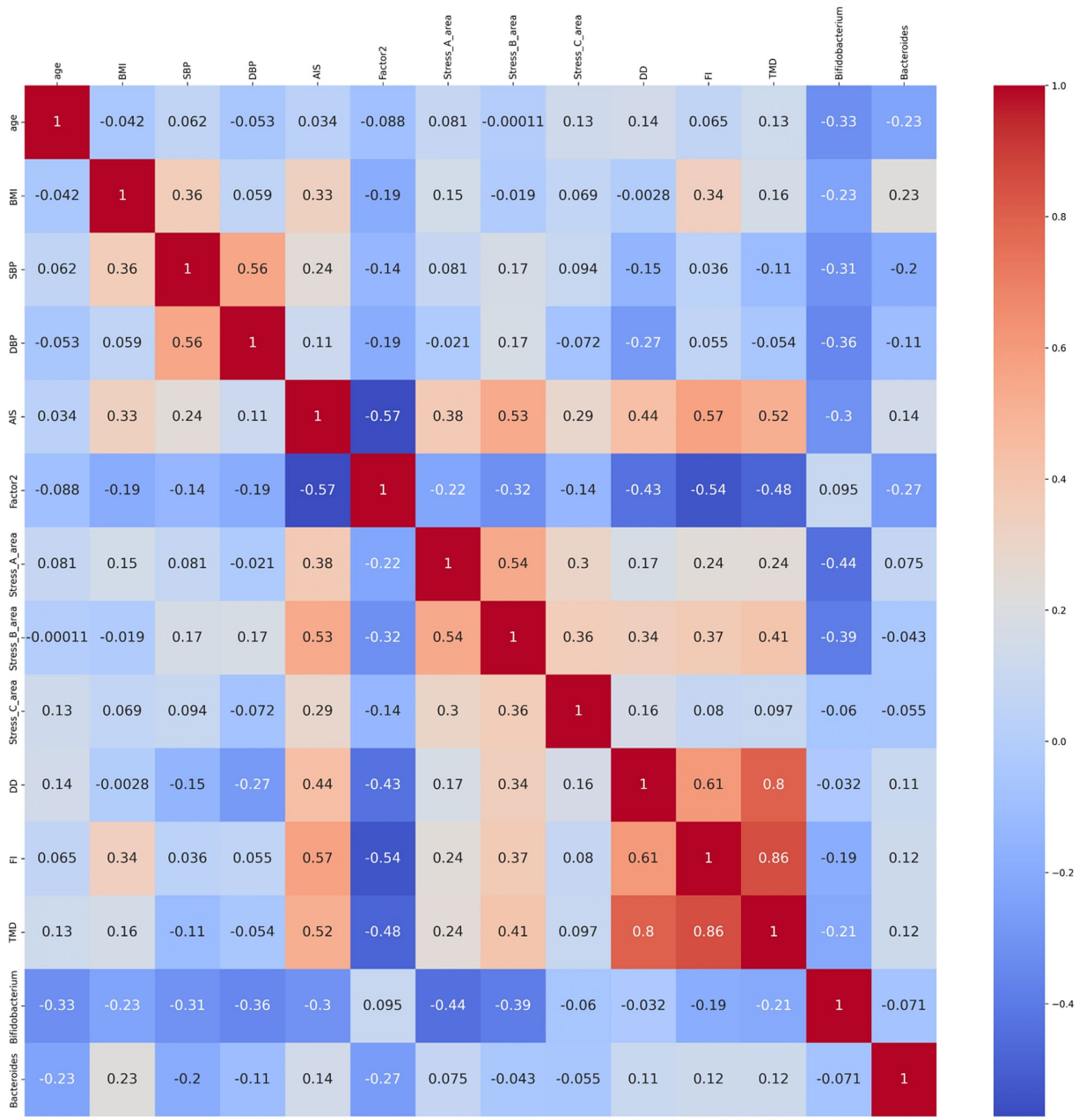

FIGURE 8
Bifidobacterium showed correlation with sleep and mood states. Heatmap of the correlation between age; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; AIS, Athens Insomnia Scale; ESS, Epworth Sleepiness Scale; OSA-MA (Factor 2; initiation and maintenance of sleep); Brief Job Stress Questionnaire (stress area A: job stressors, stress area B: psychological and physical stress reactions, and stress area C: buffering factors such as social support at work); and POMS2 (DD: depression–dejection scale, FI: fatigue–inertia scale; TMD: total mood disturbance), *Bifidobacterium*, and *Bacteroides*. The numbers in the diagram represent the correlation coefficients, which were calculated using the Spearman's correlation coefficient (n = 27; male n = 12, female n = 15).

was 0.92. This indicates that our EEG analysis had sufficient sensitivity to detect potential changes in sleep parameters. Therefore, the absence of significant changes in EEG metrics suggests that the intervention may not have induced measurable alterations in objective sleep architecture, rather than the results being limited by insufficient statistical power.

TABLE 2 Multiple regression analysis using *Bifidobacterium* as explanatory variable.

	Objective value			
	AIS	A_area	B_area	TMD
R^2-value	0.0943	0.0669	0.0527	0.0169
Bifidobacterium	−0.270 [−0.584–0.045] (0.061)	−0.418* [−0.737–−0.099] (0.011)	−0.399* [−0.720–−0.077] (0.0162)	−0.285 [−0.612–0.043] (0.066)
Age	0.0016 [−0.310–0.313] (0.568)	−0.011 [−0.326–0.305] (0.946)	−0.181 [−0.499–0.138] (0.260)	0.215 [−0.225–0.423] (0.541)
Sex_id	−0.252 [−0.995–0.492] (0.499)	−0.306 [−1.061–0.449] (0.419)	0.252 [−0.508–1.013] (0.507)	−0.386 [−1.161–0.389] (0.321)
BMI	0.095 [−0.250–0.440] (0.582)	−0.018 [−0.368–0.331] (0.916)	−0.149 [−0.502–0.203] (0.399)	0.073 [−0.286–0.432] (0.684)
SBP	0.226 [−0.195–0.650] (0.286)	0.114 [−0.314–0.542] (0.595)	0.228 [−0.203–0.660] (0.292)	−0.214 [−0.653–0.225] (0.332)
DBP	−0.084 [−0.471–0.304] (0.665)	−0.189 [−0.582–0.204] (0.339)	−0.085 [−0.481–0.312] (0.669)	−0.0061 [−0.410–0.398] (0.976)

The numbers are partial regression coefficients, the numbers in brackets are 95% confidence intervals, and the numbers in parentheses are *p*-values, $^{**}p < 0.01$, $^{*}p < 0.05$ ($n = 27$; male $n = 12$, female $n = 15$). AIS, Athens Insomnia Scale. A_area, Area A in the Brief Job Stress Questionnaire (job stressors). B_area, Area B in the Brief Job Stress Questionnaire (psychological and physical stress reactions). TMD, Total mood disturbance. BMI, Body mass index. SBP, Systolic blood pressure. DBP, Diastolic blood pressure.

TABLE 3 Multiple regression analysis using *Bacteroides* as explanatory variable.

	Objective value
	Factor 2
R^2-value	0.0106
Bacteroides	−0.209 [−0.547–0.130] (0.221)
Age	−0.192 [−0.526–0.141] (0.251)
Sex_id	−0.020 [−0.880–0.841] (0.964)
BMI	−0.069 [−0.470–0.332] (0.730)
SBP	−0.082 [−0.522–0.358] (0.710)
DBP	−0.146 [−0.527–0.236] (0.447)

The numbers are partial regression coefficients, the numbers in brackets are 95% confidence intervals, and the numbers in parentheses are *p*-values, $^{**}p < 0.01$, $^{*}p < 0.05$ ($n = 27$; male $n = 12$, female $n = 15$). Factor 2: Factor 2 on the OSA-MA (initiation and maintenance of sleep). BMI, Body mass index. SBP, Systolic blood pressure. DBP, Diastolic blood pressure.

In this study, the consumption of prebiotics-containing granola improved stress response and total mood disturbance. As mentioned previously, both subjective and objective stress indicators exist. The two major methods for objectively assessing stress are biochemical methods that analyze the components of blood, urine, and saliva and physiological methods that analyze biological signals such as heart rate variability and respiratory activity (55). However, cortisol in the blood and urine, which is used as a stress marker, responds to acute stress (55) and would probably be unsuitable for evaluating stress fluctuations over an 8-week period, as was set in this study. Moreover, salivary stress markers, including cortisol, are affected by circadian rhythms; therefore, collecting samples at regular intervals is vital (56). Previous studies have reported a positive correlation between heart rate variability and subjective stress indicators, even after adjusting for confounding factors such as sex, age, physical activity, and body fat percentage. However, subjective stress and physical activity were correlated, and a strong relationship was reported between heart rate variability and age; therefore, heart rate variability may be influenced by other factors (57). Although subjective and objective indicators of stress do not always correlate, measuring several objective indicators may allow us to further investigate the relationship between stress, gut microbiota, and sleep. Previous studies have reported mixed findings on the relationship between objective and subjective stress indicators. For example, while some studies have identified a positive correlation between heart rate variability and perceived stress levels, others have found no significant association after adjusting for confounding factors such as sex, age, physical activity, and autonomic nervous system function (57, 58). One possible explanation for these inconsistencies is that objective stress indicators, such as heart rate variability and cortisol levels, are influenced by multiple physiological and environmental factors. Heart rate variability is highly sensitive to acute stressors but may not always reflect chronic stress fluctuations over extended periods. Similarly, cortisol levels exhibit circadian variation and can be affected by factors such as sleep patterns, physical activity, and dietary intake (55, 56). Additionally, individual differences in autonomic nervous system regulation and stress resilience may contribute to variations in physiological stress responses (59). In this study, we did not measure objective stress indicators such as heart rate variability or cortisol levels; therefore, we cannot directly assess their relationship with subjective stress measures. Future research incorporating both subjective and objective stress indicators will be essential to further clarify their interrelationship and the potential impact of prebiotic interventions on physiological stress responses.

In this study, when prebiotic-containing granola was consumed for 8 weeks, diastolic blood pressure began to decrease from the fourth week. Previous studies have reported that granola consumption significantly reduces both diastolic and systolic blood pressure in hemodialysis patients (60). In particular, β-glucan, a component of granola, has been shown to improve cardiovascular risk factors, including blood pressure (61). One proposed mechanism by which dietary fiber intake lowers blood pressure involves short-chain fatty acids (SCFAs). SCFAs produced by gut bacteria are thought to activate GPR41, which in turn lowers blood pressure by reducing sympathetic nerve activity (62). Alternatively, SCFAs may act on vascular

endothelial cells, activating endothelial nitric oxide synthase (eNOS) to produce nitric oxide, which induces vasodilation and lowers blood pressure (63, 64). Although these pathways are believed to play a role, the precise reason why only diastolic blood pressure decreased significantly in this study remains unclear. One possibility is that different types of stress influence diastolic and systolic blood pressure differently (65, 66). For example, one study reported that under calculation task-induced stress, depressive symptoms were associated with changes in systolic but not diastolic blood pressure (67). This suggests that the responses of diastolic and systolic blood pressure vary depending on stress conditions. In this study, many participants exhibited a tendency toward higher diastolic blood pressure, which may explain why significant reductions in diastolic blood pressure were observed following granola consumption.

In this study, consuming prebiotics-containing granola for 8 weeks significantly reduced some indicators of alpha diversity in the gut microbiota, resulting in lower gut microbiota diversity. Despite this reduction in alpha diversity, subjective feelings of insomnia and mental and physical stress responses improved. The use of antibiotics and unbalanced diet reduce the diversity of the gut microbiota, and a reduction in gut microbiota diversity is associated with several diseases, including obesity, type 2 diabetes, nonalcoholic liver disease, and heart disease (68–70). Additionally, reduced alpha diversity has been reported in patients with depression and insomnia (71–73). However, some studies suggest that alpha diversity is higher in people with depression (74), whereas others suggest no link between alpha diversity and depression (75). The correlation among the Pittsburgh Sleep Quality Index, sleep apnea score, and alpha diversity was analyzed in patients with chronic insomnia, but no significant correlation was detected (76). This implies that no consistent association exists between gut microbiota diversity and disorders such as sleep and stress responses, and perhaps decrease in alpha diversity is not associated with improvements in insomnia or stress responses. Although some studies have reported that feeding prebiotics and probiotics increased the Alpha Diversity Index, others have reported that it decreased it; thus, the results are inconsistent (77–80). Various factors such as the sex and presence of absence of diseases may have contributed to this inconsistency. In the current study, the composition of the gut microbiota may have shown a trend toward a certain convergence direction as a result of the consumption of prebiotics-containing granola, which is not normally eaten for breakfast for 8 weeks; furthermore, and the diversity index may have decreased as a result. However, consuming probiotics has been shown to maintain alpha diversity in response to stress (24); therefore, further studies are warranted to clarify the relationship between changes in gut microbiota diversity due to prebiotic consumption and stress responses and sleep.

In this study, the relative abundance of *Bifidobacterium* increased after 8 weeks of consuming prebiotics-containing granola, and a significant association was observed between *Bifidobacterium* and stress responses and subjective insomnia. In this study, although many elements of Area A in the Brief Job Stress Questionnaire cannot be directly modified, stress perception and experience may still vary within a fixed occupational environment, potentially leading to a decrease in Area A scores. In humans, emotional stress causes short- and long-term decrease in *Bifidobacterium* abundance (81). Patients with bipolar disorder showed significant negative correlation between *Bifidobacterium* counts and cortisol levels, which implies that *Bifidobacterium* may be involved in stress response (82). Among Japanese individuals, middle-aged and older women with functional constipation reported longer WASO times and lower *Bifidobacterium* population (83). Moreover, *Lactobacillus gasseri* CP2305 administration to Japanese medical students taking the National Medical Practitioners Qualifying Examination significantly reduced anxiety and sleep disturbances and suppressed stress-induced *Bifidobacterium* reduction (25). *Bifidobacterium adolescentis* SBT2786 administration increased the duration of sleep time in healthy Japanese men and women between the ages of 30 and 59 who were dissatisfied with their quality of sleep; however, it increased the duration of light sleep and did not improve their subjective sleep quality. In contrast, it improved their mood. Additionally, a subgroup analysis of participants with high stress levels showed increase in sleep time and improvement in sleepiness on rising (84). *Bifidobacterium breve* M-16 V administration reduced heart rate under stress and improved mood and sleep scores in participants with high anxiety levels (85). Thus, increasing *Bifidobacterium* abundance may be beneficial for mental and physical stress symptoms, sleep quality, and mood. Several studies have focused on prebiotics rather than probiotics, suggesting that galacto-oligosaccharides and inulin may reduce stress responses (86, 87) and that yeast mannan may increase N3 sleep duration (88). However, none of these studies have reported an increase in *Bifidobacterium* following prebiotic consumption. Additionally, most studies examining the relationship between sleep and gut microbiota have focused on probiotics, with relatively few investigating prebiotics (89). Therefore, future research is needed to determine whether prebiotic consumption increases *Bifidobacterium* and influences sleep and stress.

Several studies have reported that probiotic supplementation reduces subjective stress, improves subjective sleep quality, and reduces anxiety and depression-like behavior (90–92). The possible pathways through which gut bacteria regulate stress and sleep include changes in gut hormones, gut-associated peptides, and the vagus nervous system (93, 94). According to this study, prebiotics-mediated regulation of these pathways may be specifically related to the production of short-chain fatty acids by the gut microbiota. Short-chain fatty acids such as butyric acid and propionic acid affect the afferent vagus nerve system, which transmits information to the brain (95–97). Stimuli transmitted to the brain via the vagus nerve are relayed to the limbic system and hypothalamus through the solitary nucleus (98, 99). This process may help alleviate stress responses by influencing the secretion of serotonin and dopamine (100). This reduces stress responses and affects sleep quality. Furthermore, short-chain fatty acids stimulate the secretion of the gastrointestinal hormones GLP-1 and PYY (101, 102). The secreted GLP-1 is believed to cross the blood–brain barrier and act on GLP-1 receptors, with the stimulation of these receptors being processed through the amygdala and influencing emotional behavior (103). On the other hand, PYY is believed to influence the brain through indirect signaling via the vagus nerve rather than by crossing the blood–brain barrier (104). As *Bifidobacterium* promotes the production of acetic and butyric acids (105, 106), an increase in its relative abundance may increase the quantity of short-chain fatty acids in the intestinal tract. Additionally, *Bifidobacterium adolescentis* possesses a particularly high gamma-aminobutyric acid (GABA) producing capacity among the *Bifidobacterium* genus (107). GABA produced in the gut affects GABA receptors, the vagus nerve system alters the quantity of GABA

receptors produced in the brain. This possibly increases the GABA production capacity (108). In addition to short-chain fatty acids and GABA, another potential mechanism linking prebiotics to stress and sleep regulation involves the serotonin pathway. Serotonin, a key neurotransmitter in mood regulation and sleep, is primarily synthesized from tryptophan, an essential amino acid. Approximately 90% of serotonin is produced in the gut, and its synthesis is influenced by gut microbiota composition (109, 110). Certain gut bacteria, including *Bifidobacterium* species, have been shown to modulate tryptophan metabolism, leading to increased serotonin availability in the periphery and potentially affecting central nervous system function (111). In other words, increasing *Bifidobacterium* through prebiotic consumption may be associated with the activation of the serotonin pathway (89). Nevertheless, the gut microbiota likely regulates the brain through multiple complex pathways. However, this study did not measure the metabolites produced by the gut bacteria. Hence, the primary pathways underlying microbial activity may be identified in future studies by measuring the metabolites in the feces and blood and analyzing the correlation between them.

This study has several limitations. First, the diets of the participants were not completely controlled. In this study, we implemented measures such as excluding the individuals who consumed an excessive amount of alcohol or probiotic supplements to mitigate the effects of their usual diets to the highest possible extent; however, their diets were not completely standardized. Furthermore, although the exclusion criteria ruled out individuals taking probiotic preparations or prebiotic supplements, it did not exclude those taking tryptophan or serotonin supplements. Therefore, their usual diet could have affected the study outcomes. Additionally, we administered a food frequency questionnaire to check for major changes in diet before and after the test. However, these surveys were self-reported; hence, the possibility of errors or self-efficacy in the meal data cannot be discounted. Furthermore, because adherence to the test meals was verified solely through self-reported food diaries, the possibility of errors in the dietary data cannot be entirely ruled out. Although the participants were instructed to maintain consistent eating times to the best of their abilities, we were unable to control it. The circadian rhythm of the gut microbiota is linked to the timing of the eating and fasting periods (112, 113). Thus, changes in eating times, such as eating dinner later than usual or shortening the time period between late dinner and breakfast, may possibility exert an effect on the gut microbiota. Furthermore, since there were no specific guidelines regarding the timing of granola consumption, the timing of intake may have had a considerable impact. Second, the time course for the pre-period was increased to 6 weeks because the careful selection of participants took longer than anticipated owing to a large number of volunteers and limited number of participants with Fitbits. Hence, we decided to extend the screening period. Consequently, sleep and stress levels may have varied during these 6 weeks. In fact, several people scored <11 on the Epworth Sleepiness Scale at the pre-screening stage but scored ≥11 at the start of the experiment. Additionally, seasonal variations occur in sleep time and cortisol levels (114, 115). Hence, similar studies must be conducted during other seasons to validate these results. Third, this study was designed as a single-group before-and-after comparison study; therefore, no control group was established. Hence, we could not completely eliminate the possible placebo effects or psychological expectations. A future randomized double-blind study is warranted to confirm the reliability of the results. Finally, unmeasured and uncontrolled confounding factors need to be considered. Social background factors such as economic and marital status could be confounding factors. Additionally, the study participants showed a certain inclination toward a healthy lifestyle. Hence, they may have implemented certain approaches to sleep or stress to improve their lives, and these may have acted as potential biases. Furthermore, mental disorders were not directly assessed through clinical diagnoses or structured interviews. Although we confirmed that none of the participants were taking medications typically prescribed for mental disorders, the possibility that some participants had undiagnosed conditions, such as anxiety or depression, cannot be ruled out. These conditions may have influenced their sleep patterns and responses to the intervention. Therefore, the results of this study should be interpreted within this social context and with considerations made for potential biases.

In summary, we administered the Brief Job Stress Questionnaire to select participants who were experiencing high levels of stress and insomnia for this study requiring the consumption of prebiotics-containing granola for 8 weeks. The results showed improvement in subjective insomnia, initiation and maintenance of sleep, and stress responses. Additionally, the relative abundance of Actinobacteria and *Bifidobacterium* increased, while Bacteriota and *Bacteroides* decreased. Based on these results and multiple regression analysis result, we suggest that increase in *Bifidobacterium* abundance may be associated with improved sleep conditions and stress responses.

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/s.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Sapporo Yuri no Kai Hospital (approval number: 029). 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

HS: Formal analysis, Investigation, Visualization, Writing – original draft. HM: Conceptualization, Methodology, Project administration, Validation, Writing – review & editing. SN: Project administration, Validation, Writing – review & editing. CT: Formal analysis, Investigation, Writing – review & editing. YC: Formal analysis, Investigation, Validation, Writing – review & editing. KI: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. MY: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. TK: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

HS, HM, and KI were employed by the company Calbee, Inc. MY is a founder and CEO, TK is a CSO, and SN, CT and YC were employed by the company Sleep is the Ultimate Intelligent Mechanism In Nature (S'UIMIN) Inc.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2025.1551313/full#supplementary-material and https://dx.doi.org/10.6084/m9.figshare.28561178

SUPPLEMENTARY FIGURE 1
Flow diagram of the participants. A flow diagram showing the flow of the participants in this study and the criteria for each step.

SUPPLEMENTARY FIGURE 2
Heatmap of the correlation between sleep parameters obtained from EEG measurement and subjective sleep quality from the answers to each questionnaire. Heatmap of the correlation between N1, non-REM sleep stage 1 time; N2, non-REM sleep stage 2 time; N3, non-REM sleep stage 3 time; REM, REM sleep time; TST, Total sleep time; WASO, Wake time after sleep onset; REM ratio, REM sleep ratio; SOL, Sleep onset Latency; AIS, Athens Insomnia Scale; ESS, Epworth Sleepness Scale; and OSA-MA (Factor 1, sleepiness on rising; Factor 2, initiation and maintenance of sleep; Factor 3, frequent dreaming; Factor 4, refreshing; Factor 5, sleep length). The numbers in the diagram represent the correlation coefficient, which was calculated using Spearman's correlation coefficient (n = 27; male n = 12; female n = 15).

SUPPLEMENTARY FIGURE 3
There is no significant difference in the defecation frequency and the Bristol stool form. **(A)** Defection frequency per week, **(B)** Bristol stool form. The form of the stools is expressed by the following scores from type 1 to 7 (Type 1, separate hard lumps; Type 2, Lumpy and sausage like; Type 3, A sausage shape with cracks in the surface; Type 4, Like a smooth, soft sausage or snake; Type 5, Soft blobs with clear-cut edges; Type 6, Mushy consistency with ragged edges; Type 7, Liquid consistency with no solid pieces). All value are represented as mean ± SEM (n = 27; male n = 12, female n = 15).

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Unlocking the power of probiotics, postbiotics: targeting apoptosis for the treatment and prevention of digestive diseases

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Digestive diseases are becoming an increasingly serious health burden, creating an urgent need to develop more effective treatment strategies. Probiotics and postbiotics have been extensively studied for their potential to prevent and treat digestive diseases. Growing evidence suggests that programmed cell death, especially apoptosis, is a critical mechanism influencing the molecular and biological aspects of digestive diseases, contributing to disease progression. Understanding the mechanisms and signaling pathways by which probiotics and postbiotics regulate apoptosis could reveal new therapeutic targets for treating digestive diseases. This review focuses on the beneficial effects of probiotics and postbiotics in regulating apoptosis across a range of liver diseases, including non-alcoholic fatty liver disease, liver injury, cirrhosis, and liver cancer. It also explores their effects on gastrointestinal diseases, such as colorectal cancer, colitis, gastrointestinal injury, and infectious diarrhea. Furthermore, some probiotics help balance the gut microbiota, enhance intestinal barrier function, and regulate the immune system, all of which are closely associated with apoptosis. Moreover, emerging technologies, such as encapsulation methods, have been developed to stabilize probiotics, primarily based on experimental findings from rodent and human studies.

KEYWORDS

probiotics, postbiotics, apoptosis, gut microbiota, digestive system diseases

1 Introduction

The age-standardized incidence of digestive diseases worldwide is 95,582 per 100,000, representing over one-third of the total prevalence of all diseases (1). Digestive disorders result in millions of medical visits and billions of dollars in economic costs annually in the United States (2). The prevalence of digestive diseases is rising globally, placing a significant burden on public health (3, 4). Despite significant advancements in medical devices and healthcare, many patients still experience poor quality of life and prognosis, highlighting the need for more treatment options. Recent studies have shown that both probiotics and postbiotics have gained significant attention for their potential to improve various digestive diseases by regulating host immune function, maintaining intestinal barrier integrity, and modulating the gut microbiota (5), as shown in Figure 1.

Apoptosis, or programmed cell death (6), is a crucial process that maintains tissue homeostasis by removing damaged or dysfunctional cells (7). However, its dysregulation is a critical factor in the development of digestive diseases (8–10). In inflammatory bowel

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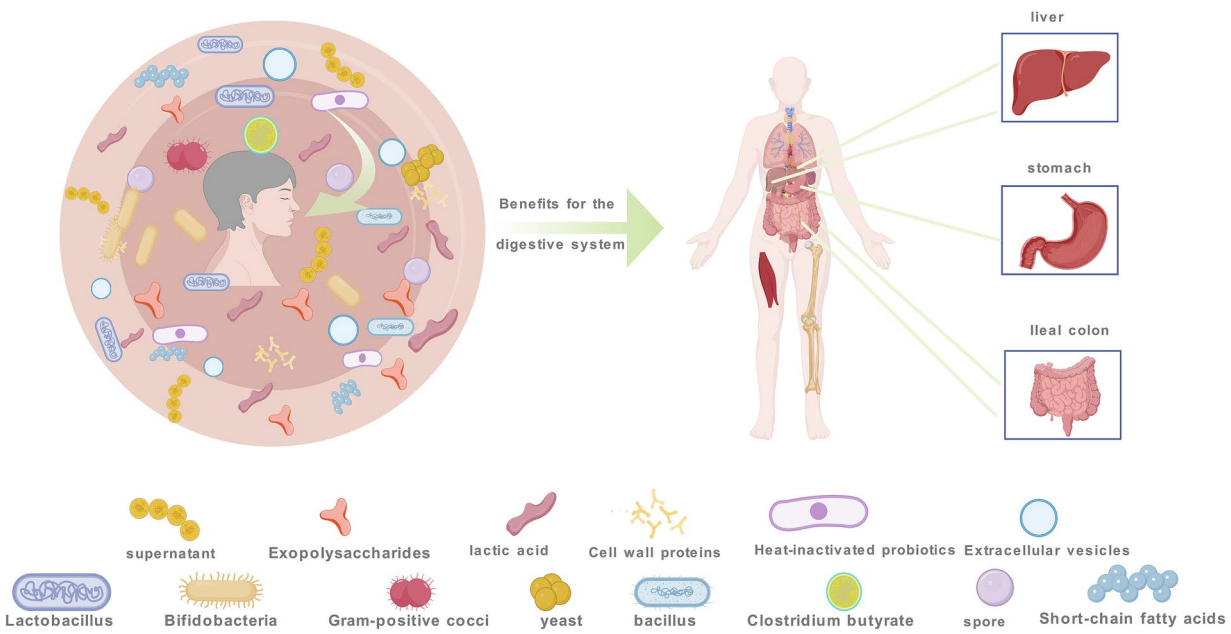

FIGURE 1
Probiotic and postbiotics aimed at improving gastrointestinal disorders by modulating apoptosis encompasses. These include probiotics like *Lactobacillus* and *Bifidobacterium*, *Gram-positive cocci, yeast*, *Bacillus*, *Clostridium butyricum*, postbiotics such as extracellular vesicles and lactic acid, spores and extracellular polysaccharides.

disease, excessive apoptosis of intestinal epithelial cells disrupts the mucosal barrier, worsening inflammation and microbial translocation, while impaired apoptosis of immune cells prolongs chronic inflammation (11). In colorectal cancer, the promotion of apoptosis in cancer cells is driven by key genes such as P53, K-ras, and Bcl, which are associated with intrinsic apoptosis pathways. These mechanisms contribute to inhibiting tumor growth and reducing chemotherapy resistance (9, 12). In gastrointestinal infections, rotavirus enters mucosal epithelial cells via virulence factors such as sialic acid and histo-blood group antigens, triggering apoptosis. This process results in mucosal damage and delayed healing (13). These findings highlight the importance of targeting apoptotic pathways in developing therapeutic strategies for digestive diseases, with probiotics and postbiotics emerging as promising modulators.

Probiotics (it refers to active microorganisms that confer benefits to the host), either directly or through the secretion of metabolites or the regulation of host signaling pathways such as Bcl-2/Bax, caspase cascades, and key anticancer pathways, can inhibit excessive apoptosis or induce apoptosis in abnormal cells while protecting normal cells. These mechanisms contribute to alleviating pathological conditions, as seen with *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and others (9, 14, 15). Postbiotics (it is a collective term for the metabolic components of probiotics after processing), as functional metabolites of probiotics, can directly or indirectly target key nodes in the apoptosis process, including short-chain fatty acids, heat-killed probiotics, etc. (15, 16). How do probiotics and postbiotics regulate apoptosis in different types of gastrointestinal diseases?

2 Probiotics, postbiotics, and apoptosis

Apoptosis is a crucial mechanism in the growth and development of multicellular organisms (7). The exogenous pathway, mediated by membrane receptors and regulated by Bcl-2 family proteins, and the endogenous pathway, mediated by mitochondria, are both closely linked to caspase regulation (17). When a cell detects internal abnormalities, it activates an intrinsic apoptosis program, initiating endogenous apoptosis. This process involves Bcl family proteins (pro-apoptotic members such as Bax, Bak, Bim, Bid, and PUMA, and anti-apoptotic members such as Bcl-2, Bcl-xl, Bcl-w, and MCL1) which alter the permeability of the mitochondrial outer membrane (18–22). This results in the release of cytochrome c, formation of the apoptosome with APAF1, and activation of caspase-9, which in turn activates apoptotic executor proteins caspase-3, -6, and -7, ultimately leading to apoptosis (23). The regulation of Bcl-2 protein transcription and phosphorylation in apoptosis involves CDK and p53 (24). The ERK1/2 and MAPK1 pathways promote cell survival partly by phosphorylating BIM, leading to its proteasomal degradation and inhibition of apoptosis (25). Probiotics are live, non-pathogenic microorganisms (26). This paper typically contains one or more microbial strains, with the main components including *Lactobacillus*

Abbreviations: EPS, Extracellular polysaccharides; EVs, Extracellular vesicles; SCFAs, Short-chain fatty acids; HKY, Heat-killed yeast; NAFLD, Non-alcoholic fatty liver disease; L., Lactobacillus; Lc., Lactobacillus casei; LGGs, Lactobacillus rhamnosus supernatant; DSS, Dextran sulfate sodium salt; B. bifidum, Bifidobacterium; S., Saccharomyces.

spp., *Bifidobacterium* spp., Gram-positive cocci spp., yeast spp., *Bacillus* spp., and *Clostridium butyricum* spp. This paper highlights that most probiotics regulate apoptosis through intrinsic pathways, influencing the expression of Bcl family proteins, caspases, cytochrome c, mitochondria, and key anticancer pathways, such as the EGFR/PI3K/AKT signaling cascade, mTOR pathway, P38/JNK pathway, TLR4/JNK/NF-κB pathway, TLR4/MAPK pathway, Wnt/β-catenin pathway, and cAMP-dependent signaling pathway, as shown in Figure 2. When a cell receives an external death signal, exogenous apoptosis is triggered through a cascade of reactions. This process involves the binding of ligands (FASL, TNF-a, TRAIL) (27–31) to their corresponding receptors (FAS, TNFRs, TRAILRS) (23, 32–34) followed by a cascade of reactions that activate the cleavage of caspase-8 and -10. Subsequently, the cleavage of apoptosis executor proteins caspase-3, -6, -7 and BID is triggered, ultimately leading to apoptosis (35). BID serves as a link between the exogenous apoptosis pathway and the mitochondrial pathway (36), while the activation of promoter caspases is negatively regulated by c-FLIP (37). Probiotics observed in liver and colon cancer have been found to induce apoptosis through core apoptotic pathways similar to antitumor drugs, involving AKT, RAS, Raf, MEK, ERK, and mTOR kinase signaling pathways (38–42), and inhibiting growth factor receptors such as EGFR, Her2/Neu, other ERBB family members, c-Met, and NTRK (43–48), as depicted in Figure 2. Some probiotics regulate apoptosis through both endogenous and exogenous pathways, such as *L. plantarum C88*, including the toll-like receptor signaling pathway. Postbiotics refer to "inanimate microorganisms and/or their components that are beneficial to host health" (49), including heat-killed bacteria, extracts of extracellular polysaccharides (EPS) (50), extracellular vesicles (EVs) (51), cell wall protein components (52), spores (53), short-chain fatty acids (SCFAs) (9), lactic acid (54) and others, as depicted in Figure 1. They regulate multiple core signaling pathways associated with growth and development, control the production and function of apoptosis factors, reduce apoptosis in healthy cells, selectively promote apoptosis in cancer cells, and contribute to overall body health. Compared with active probiotics, postbiotics, with their microbial composition similar to pharmacological molecules, offer advantages in absorption, metabolism, and excretion (55), making them more reliable dietary supplements, such as heat-killed yeast (HKY) (56), and EVs of *L. rhamnosus PTCC1637* (51). The regulation of apoptosis may be intricately linked to certain bacterial components. Advancements in science and technology have facilitated the mass production of probiotics, such as the engineering of bacteria (e.g., *butyrate* synthesized by *E. coli Nissle 1917*). Furthermore, nanomaterials and microgels significantly enhance the precision of anti-cancer targeting of probiotics (57). As shown in Figure 2, probiotic and postbiotics

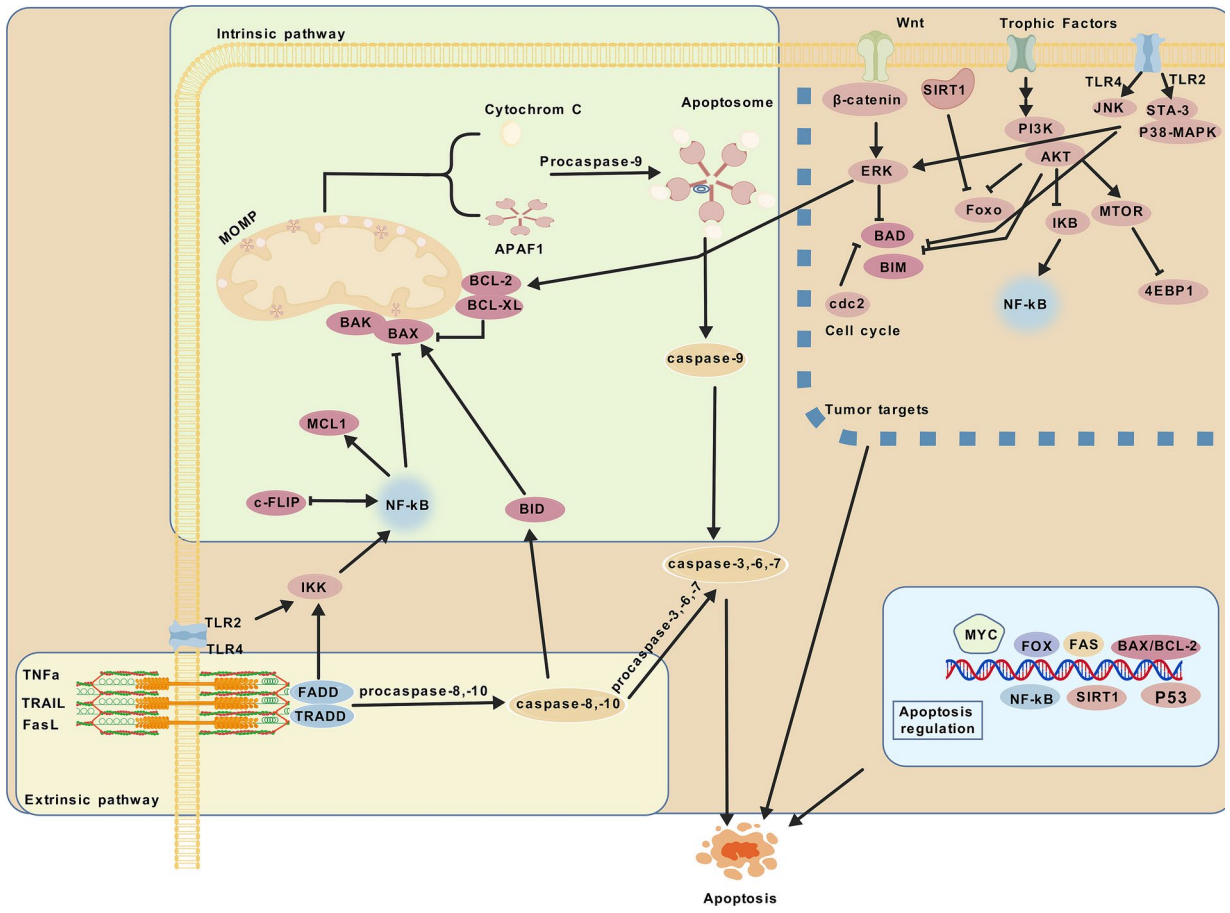

FIGURE 2
Modulation of apoptosis. Probiotic and postbiotics modulate apoptosis through extrinsic and intrinsic pathways, effectively influencing digestive diseases and targeting key pathways in tumors to promote cancer cell apoptosis.

regulate apoptosis to alleviate digestive diseases through both intrinsic and extrinsic pathways, promoting cancer cell apoptosis by targeting tumor-associated signaling pathways and key molecular targets.

3 Probiotics, postbiotics, and liver disease

3.1 Non-alcoholic fatty liver disease and liver injury

Non-alcoholic fatty liver disease (NAFLD) is a metabolic liver disorder unrelated to excessive alcohol consumption and is the most common chronic liver disease in Western countries (58). Recent studies have emphasized the important role of probiotics in treating and preventing liver diseases by modulating gut microbiota, strengthening the intestinal barrier, and interacting with the gut-liver axis (the gut-liver axis refers to the complex interplay between the gut and the liver, where gut microbiota-derived metabolites and immune-digestive interactions influence liver function, while liver-derived metabolites, in turn, regulate the gut microbiota and enhance intestinal barrier function) (59, 60). Apoptosis plays a crucial role (61). In a mouse model of NAFLD, *L. johnsonii BS15* was found to prevent the disease by regulating gut microbiota and modulating immunity. The anti-apoptotic effect was linked to reduced cytochrome c content and increased uncoupling protein-2 levels in mice (62). *Lc. paracasei HY7207* significantly alleviated symptoms and improved serum markers in mice with NAFLD. Additionally, the expression of Bcl-2 and Bax genes and their ratios were reduced, thereby decreasing apoptosis in human hepatocytes (8). As shown in Table 1, *Lactobacillus* spp. as dietary supplements, hold great potential for reducing endogenous apoptosis in the prevention and treatment of NAFLD. However, further studies are needed to determine whether they have similar effects in alcoholic fatty liver disease.

The liver, a vital metabolic organ, can be damaged by various factors, including infection, chemical toxicity, immune dysfunction, poor nutrition, circulatory issues, and genetic abnormalities (63). Supplementing with specific probiotics can alleviate the outcomes of autoimmune diseases by modulating immune responses and gut microbiota composition. In a liver injury model in lupus-prone mice, *L. paracasei GMNL-32*, *L. reuteri GMNL-89*, and *L. reuteri GMNL-263* were found to downregulate liver cell apoptosis and inflammation-related marker expression. This is closely linked to the inhibition of the MAPK and NF-kB signaling pathways, with the inhibition of the IKK/NF-κB pathway downregulating caspase-3 expression to reduce liver cell apoptosis, providing a basis for these preparations to serve as alternative drugs for liver diseases in systemic lupus erythematosus (64). Furthermore, the culture supernatant of *L. reuteri ZJ617* attenuates lipopolysaccharide (LPS)-induced acute liver injury by strengthening intestinal barrier integrity, modulating inflammatory responses, suppressing the hepatic TLR4/MAPK/NF-κB signaling pathway, and facilitating Beclin1-dependent autophagy. The anti-apoptotic effect, linked to downregulation of caspase-3 and Bax proteins, may result from suppression of the TLR4/MAPK signaling pathway (65). This suggests that different probiotics and postbiotics may alleviate liver injury through intersecting signaling pathways. However, further research is needed to determine whether the specific components involved are identical.

The NLRP3 inflammasome is a key component of the innate immune system (66). *Bacillus amyloliquefaciens SC06* significantly

TABLE 1 Molecular mechanism of *Lactobacillus* spp. regulated apoptosis and improving liver injury.

Probiotic-based preparation	Category	Digestive diseases	The specific molecular mechanism of apoptosis	References
Lc. paracasei HY7207	Probiotic	Non-alcoholic fatty liver disease	Human: Regulated Bcl-2 and Bax-related genes and alter the Bax/Bcl-2 ratio	Kim et al. (8)
L. johnsonii BS15	Probiotic	Non-alcoholic fatty liver disease	Animal: Mitigated the reduction in cytochrome c levels and the increase in uncoupling protein-2 levels, preserving mitochondrial function	Xin et al. (62)
L. paracasei GMNL-32, L. reuteri GMNL- 89 and L. reuteri GMNL-263	Probiotics	Systemic lupus erythematosus liver injury	Animal: Inhibited the IKK/NF-κB signaling pathway downregulates the expression of caspase-3	Hsu et al. (64)
L. reuteri ZJ617 culture supernatant	Postbiotic	Acute liver injury	Animals: Inhibited the TLR4/MAPK signaling pathway downregulates the protein expression of caspase-3 and Bax	Cui et al. (65)
Bacillus amyloliquefaciens SC06	Probiotic	Acute liver injury	Animal: The downregulation of Bax, Caspase-3, Caspase-9, and p53 gene expression may be associated with the inhibition of the NLRP3 inflammasome	Wang et al. (67)
Bacillus amyloliquefaciens B10	Probiotic	Acute liver injury	Animals: Inhibited the expression of Bax, Bcl-2, and Caspase-3 gene and protein	Li et al. (68)
L. plantarum C88	Probiotic	Acute liver injury	Animals: Inhibiting the TLR2/NF-κB and TLR4/NF-κB signaling pathways can modulate the cell death receptor and mitochondrial pathways by downregulating the expression of Fas, FADD, TRADD, and Caspase-8, decreasing Bax and caspase-3 expression in liver cells while enhancing Bcl-2 expression	Huang et al. (14)

inhibits the NLRP3 inflammasome and markedly reduces hepatocyte apoptosis (67). *Bacillus amyloliquefaciens B10* significantly reverses the gene and protein expression of Bax, Bcl-2, and Caspase-3 induced by aflatoxin B1, but lacks exploration of the underlying molecular mechanisms (68). *L. plantarum C88* has been shown to possess beneficial properties such as improving intestinal barrier function and inhibiting inflammation. It inhibited inflammation and excessive cell apoptosis mediated by the TLR2/NF-κB and TLR4/NF-κB signaling pathways, involving regulation of cell death receptors and mitochondrial pathways (14). These studies suggest a close correlation between using live *Lactobacillus* spp. or their supernatants to treat liver injury, enhance intestinal barrier function, and inhibit inflammation. Bacillus species present new insights into the mechanism of alleviating acute liver injury, particularly regarding apoptosis.

As shown in Table 1, live *Lactobacillus* spp. or their supernatants primarily affect the NF-κB and MAPK signaling pathways, reducing both endogenous and exogenous hepatocyte apoptosis. Further research is needed to explore the characteristics of other probiotics used for liver injury and their potential effects on apoptosis inhibition.

3.2 Liver cirrhosis and liver cancer

Globally, liver disease causes 40,000 deaths annually, representing 1% of global mortality (2,019 out of every 25 deaths worldwide). Hepatitis, alcoholic and non-alcoholic fatty liver disease, and chronic liver damage are closely associated with cirrhosis development. Further progression can lead to liver cancer, significantly burdening patients' lives (69). The efficacy of chemotherapy should not overshadow its harmful effects on healthy cells. Probiotics and postbiotics have recently emerged as promising interventions for preventing and treating cirrhosis and liver cancer, and mitigating disease complications. In treating *Schistosoma mansoni*-induced cirrhosis, oral administration of probiotics such as *L. acidophilus ATCC 4356* and *L. delbrueckii subsp. bulgaricus DSM 20080*, or their fermented yogurt, significantly reduce worm burden, egg production, and granuloma size and number in mouse liver tissue. Significant improvements in oxidative stress and liver fibrosis were observed, along with downregulation of caspase-3 and Bax/Bcl-2 expression (70). However, oral administration of EPS derived from *L. acidophilus ATCC 4356* in hepatocellular carcinoma rats induced by diethylnitrosamine and gamma radiation exhibited potent immunomodulatory effects. This effect is attributed to the inhibition of the TLR2/STAT-3/P38-MAPK pathway related to inflammation; However, the specific mechanism of apoptosis regulation remains unclear (50). Initial findings suggest that probiotics modulate intestinal flora, reduce toxic metabolite accumulation, and influence the progression of metabolic disorders, including diabetes and insulin resistance. Recent studies demonstrate that adding an emulsion containing *L. rhamnosus NCIMB 8010* and *Pediococcus acidilactici NCIMB 8018* to liver cancer cells mitigates insulin resistance induced by free fatty acid accumulation, enhances cell viability, and regulates the Bax/Bcl-2 and caspase axes to improve mitochondrial dysfunction. The mechanism may involve inhibition of the fetal protein/TLR4/JNK/NF-κB pathway (71). *In vitro* experiments with the human liver cancer cell line HepG2 showed that EVs derived from *L. rhamnosus PTCC 1637* prevent liver cancer and significantly increase the apoptosis index (Bax/Bcl-2 ratio), inducing cancer cell death (51). Further research is required to elucidate the mechanisms underlying extracellular vesicle and probiotic-host interactions. These findings demonstrate that *Lactobacillus* and its components, such as extracellular vesicles and polysaccharides, primarily regulate endogenous apoptosis through the Bax/Bcl-2 and caspase axis. These components are promising alternative therapies for preventing and treating liver cancer, as shown in Table 2. As a critical step in the progression of liver cancer, the significance of cirrhosis should not be overlooked while exploring probiotic strains and molecular mechanisms involved in liver cancer.

4 Probiotics, postbiotics, and colitis

The global prevalence of ulcerative colitis is rising, significantly affecting quality of life [not only affects the digestive system but also influences periodontitis (72)]. Effective treatment aims to induce and maintain remission (73). Current treatments include aminosalicylates, corticosteroids, antibiotics, adjunctive medications, and immunosuppressive agents; however, their efficacy is often suboptimal and tolerability limited. Exploring alternative treatments is essential (74). The ability of probiotics to

TABLE 2 Active probiotics and postbiotic regulate apoptosis in the treatment of liver cirrhosis and liver cancer.

Probiotic-based preparation	Category	Probiotic type	Digestive diseases	The specific molecular mechanism of apoptosis	References
EVs derived from *L. rhamnosus PTCC1637*	Postbiotic	*Lactobacillus*	Liver cancer	Human: Increased the apoptotic index (Bax/Bcl-2 expression ratio)	Behzadi et al. (51)
L. rhamnosus NCIMB8010 and *Pediococcus acidilactici NCIMB8018*	Probiotics	*Lactobacillus* Gram-positive coccus	Liver cancer	Human: Inhibited of fetoglobulin /TLR4/JNK/NF-κB axis, regulation of Bax/Bcl-2, caspase axis, improved mitochondrial function	Mularczyk et al. (71)
L. acidophilus ATCC4356 and *L. delbrueckii subsp. bulgaricus DSM20080*	Probiotics	*Lactobacillus*	Schistosomiasis infectious cirrhosis	Animals: Regulated expression levels of caspase-3 and Bax/Bcl-2 in liver tissue	El-Khadragy et al. (70)
EPS derived from *L. acidophilus ATCC 4356*	Postbiotic	*Lactobacillus*	Liver cancer	Animals: Suppressed the TLR2/STAT-3/P38-MAPK path	Khedr et al. (50)

regulate gut microflora, enhance the intestinal mucosal barrier, and improve immune function has positioned them as potential biological agents for treating colitis (75). In a Dextran Sulfate Sodium Salt (DSS)-induced mouse colitis model, the supernatant of *Lactobacillus GG-fermented* milk inhibited intestinal epithelial cell apoptosis, potentially by activating the PI3K/Akt pathway, which upregulates the anti-apoptotic protein Bad and inhibits FOXO transcription factors. This effect is linked to the unique p40 and p75 proteins in the probiotic-fermented milk supernatant (76). A pectin/zein hydrogel bead system improves protein delivery stability (77). Preliminary studies show that tumor necrosis factor-α (TNF-α) mediates inflammatory responses in inflammatory bowel disease. Its apoptotic role makes it a key target for destroying intestinal epithelial cells and a prime focus of pharmacotherapies. Oral administration of *L. BB12* and *L. plantarum LB-9* downregulates TNF-α expression and modulates caspase-8-mediated extrinsic apoptosis (78, 79). Secreted factors from *B. bifidum infantum 15697* reduce infections in a mouse model of necrotizing enterocolitis, prevent weight loss, and mitigate apoptosis caused by caspase-3 and caspase-7 activation, likely through NF-κB pathway inhibition (80). Combining probiotics with nanomaterials provides additional benefits, such as prolonged circulation and intestinal immunity regulation (81); *Bacillus amyloliquefaciens*-loaded nanoparticles enhance stability and improve endogenous apoptosis compared to free Bacillus. Specifically, They downregulate caspase-3 and cytochrome c expression while upregulating Bcl-2 and Bax, suggesting nanotechnology offers promising avenues for the food industry (82), suggesting that nanotechnology offers promising avenues for the food industry. These studies show that modulating the PI3K/Akt signaling pathway, regulating the TNF-α-mediated death receptor pathway, and inhibiting apoptosis through the Bcl-2 family and CytC-mediated mitochondrial pathway are key to the preventive and therapeutic effects of certain probiotics in colitis. Exploring novel probiotics and technologies that combine them with specific substances has further enhanced their potential benefits. As shown in Table 3, probiotics may serve as promising targeted therapeutic supplements. However, most studies still require further exploration of these novel formulations and more in-depth investigations into their molecular mechanisms, such as Limosilactobacillus reuteri FN041 (83).

5 Probiotics, postbiotics, and colorectal cancer

The prevalence of colon cancer has significantly increased in recent years due to poor dietary choices and unhealthy lifestyles, making it the second leading cause of cancer-related mortality worldwide (84). Recent studies suggest that probiotic preparations (it refers to probiotics and postbiotics) offer advantages over traditional drugs in certain cancer treatments and their side effects. When used as adjuvant therapy, probiotics can mitigate toxic side effects, optimize therapeutic outcomes, and enhance gut microflora, intestinal barrier function, and immune response (85). Inducing apoptosis in cancer cells is a key goal in advancing cancer treatments (86). A study using *S. cerevisiae* in a mouse model of colorectal tumors to inhibit cancer progression suggests that the downregulation of the Akt/NF-κB and Akt/mTOR signaling pathways may be linked to apoptosis in cancer cells. It also suggests a potential increase in beneficial gut microbiota and immune function regulation (87). Identifying specific *S. cerevisiae* strains with anti-colon cancer properties is crucial, as different strains have distinct therapeutic benefits (88). *The L. paracasei K5* strain adheres to human intestinal cancer cells, potentially inducing apoptosis by regulating Bcl-2 family proteins (89). Administration of *L. casei ATCC393* suppresses tumor growth and enhances apoptosis in mouse (CT26) and human (HT29) colon cancer cells by upregulating TRAIL expression and downregulating Survivin (90). The link between cell cycle regulation and apoptosis induction is well established (91). *L. paracasei subsp. paracasei X12* inhibits the mTOR/4EBP1 signaling pathway, induces G1 phase arrest in HT-29 cells, suppresses cyclin E1 expression, upregulates p27, and modulates apoptosis (92). The cell wall protein component of *L. paracasei ATCC25598* has been shown to mitigate apoptosis in the human intestinal Caco-2 cell line (52). This highlights the specificity of strains in regulating apoptosis mechanisms. Long-term retention of probiotics in the gut may improve disease prognosis, including cancer. *L. plantaris*, *L. rhamnosus*, *L. breve*, and *L. luciferi* extracted from human feces have anti-cancer effects by activating the Wnt/β-catenin pathway, antimicrobial peptides, and metabolites. These metabolites disrupt mitochondrial membrane integrity and trigger late apoptosis in tumors following colonization (93). Promoting the long-term presence of probiotics in the gut may improve disease outcomes, such as cancer prognosis. Similarly, a comparable study found that

TABLE 3 Probiotics, postbiotic regulate cell apoptosis and alleviate colitis.

Probiotic-based preparation	Probiotic type	Digestive diseases	The specific molecular mechanism of apoptosis	References
The supernatant of LGG-fermented milk	*Lactobacillus*	Colitis	Animal: Activated the EGFR/PI3K/Akt signaling pathway	Yoda et al. (76)
Lactis Strain BB12	*Lactobacillus*	Colitis	Animal: Down-regulated TNF-α expression suppressed caspase-8-mediated exogenous apoptosis	Chae et al. (78)
L. plantarum LB-9	*Lactobacillus*	Colitis	Animal: The down-regulation of TNF-α expression suppressed caspase-8-mediated exogenous apoptosis	Chae et al. (79)
The secreted factors of *B. bifidum infantum 15697*	*Bifidobacterium*	Necrotizing enterocolitis	Animal: Down-regulation of caspase-3 and caspase-7 activation	Weng et al. (80)
Bacillus amyloliquefaciens	*Bacillus*	Inflammatory bowel disease	Animal: The reduced levels of caspase-3 and cytochrome c, coupled with elevated Bcl-2 and Bax expression, led to increased endogenous cell apoptosis	Alkushi et al. (82)

L. salivarius CGMCC3606 inhibits both early and late tumor formation in mice. Notably, its metabolites effectively suppress the AKT signaling pathway by inhibiting phosphorylation of AKT, cyclin D1, and COX-2, leading to apoptosis (94). But further studies are needed to understand how *Saccharomyces burra* metabolites promote cell apoptosis (95). These findings suggest that a single active *Lactobacillus* and its metabolites can induce apoptosis in cancer cells through various signaling pathways, offering promising prospects for clinical applications. Cancer development and progression are closely linked to chronic inflammation, a major contributing factor (96). *L. helveticus NS8* significantly reduces tumor number and proliferation in colitis-associated colorectal cancer mice, while promoting the increase of beneficial microbiota. Upregulating caspase-3 to promote apoptosis may involve inhibiting NF-κB activation and modulating inflammatory factors (97). Recently, there has been increasing focus on the immune function and molecular mechanism of non-viable probiotics rendered inactive by physical or chemical methods, such as heat and ultraviolet radiation, in relation to diseases (49). Notably, it possesses greater stability compared to live probiotics, making it highly advantageous as a food additive (98). Compared to 5-FU, heat-inactivated *S. cerevisiae PTCC5052* downregulates p-Akt1, Bcl-XL, Rel A, pro-caspase-3, and − 9, and enhances Bax and caspase-3 expression to induce cell apoptosis. The former has a more pronounced effect on Bax regulation through the Akt/NF-κB signaling pathway (56). Heat-induced apoptosis of human colorectal adenocarcinoma HT-29 cells depends on factors like time, dose, and specific strains, such as *L. brevis IBRC_M1078* and *L. paracasei IBRC_M1079*. These probiotic strains promote apoptosis by enhancing the expression of pro-apoptotic genes like Bax, caspase-3, and caspase-8, while suppressing the anti-apoptotic gene Bcl-2 (99). The purification of probiotics and the use of their cell-free supernatant should not be overlooked (16). The cell wall protein component of *L. paracasei ATCC25598*, for example, induces apoptosis in intestinal Caco-2 cells and may serve as an anticarcinogenic agent (52). The effect of *Lactobacillus* cell-free supernatant (LCFS) on apoptosis induction in human colon cancer cells was observed in a 3D colorectal cancer model, where it inhibited NF-κB activation and downregulated PARP1 and Bcl-XL expression (100). This offers a novel approach to investigating the anticancer properties of probiotics across various cancer types. With many chemotherapy drugs used in tumor treatment, drug resistance has emerged as a major factor contributing to the decline in drug efficacy. Recently, the use of probiotic components and metabolites has been found to effectively mitigate this issue. For example, *L. plantarum CCARM0067*, which produces γ-aminobutyric acid (GABA), shows anti-cancer effects on 5-fluorouracil-resistant human colorectal adenocarcinoma cells by inducing apoptosis through cIAP2 regulation and inhibition of the cAMP-dependent signaling pathway (15). The cell-free culture supernatant of this strain boosts the cancer-inhibiting effects of SMCT1/butyric acid in colorectal cancer cells, making it a potential chemotherapy enhancer for HCT116 cells resistant to 5-Fluorouracil and butyric acid. It is also closely linked to the activity pattern of caspase-3 (101). These findings offer a novel approach to chemoprevention and treatment of colorectal cancer-related diseases.

Various probiotic mixtures regulate apoptosis to improve disease outcomes. *L. plantarum AdF10* and *L. rhamnosus ATCC53103* enhance oxidative stress resistance and normalize p53-mediated apoptosis gene expression, potentially safeguarding against stress-induced excessive apoptosis. This may improve cellular health and reduce diseases associated with uncontrolled apoptosis (102). Celecoxib suppresses the AKT pathway, leading to decreased CD133 expression in colon cancer (103). The combination of *L. acidophilus NCDC15* and *L. rhamnosus GG MTCC1408*, along with celecoxib, was observed to decrease tumor heterogeneity and enhance immune function and gut health. It upregulates P53 expression, downregulates K-ras proto-oncogene expression, and modulates Bax and Bcl-2 levels, potentially inhibiting tumor growth and promoting overall health (12). This suggests that the molecular mechanism of apoptosis underlying the effects of chemotherapy may be altered when combined with probiotics. This combined approach may help alleviate the severity and burden of diseases in highly susceptible individuals. However, clinical validation is required.

Recent research shows that SCFAs, such as acetate, propionate, and butyrate, act as metabolites for gut bacteria to metabolize dietary fiber. These metabolites play crucial roles in inflammation, immunity, lipid metabolism, apoptosis mechanisms, and the regulation of key targets related to disease prevention and outcomes (104). To mitigate rapid clearance and enhance bioavailability, novel short-chain fatty acid analogs (105) and probiotics combined with nanomaterials (106) have been developed. The application of engineered bacteria is highly promising. Engineered *E. coli Nissle 1917* with synthetic butyrate reduced tumor volume by 70% in mice and induced apoptosis in human colorectal cancer cells through the mitochondrial pathway, independent of P53. This represents a novel approach to targeted bacterial cancer therapy (107). The latest study on microcapsules enables probiotics to exert targeted tumor therapy (108). Microencapsulated *L. plantarum LAB12* significantly reduces tumor volume and weight, inhibiting angiogenesis. The anti-apoptotic effect is partially linked to upregulation of p53 and caspase-3 expression (109). In recent years, *L. reuteri* has shown great promise in the treatment of various digestive diseases (110). The use of *L. reuteri* delivered *via* microgel technology in colorectal cancer enhances beneficial bacterial flora, increases butyrate production, and modulates the caspase and Bcl pathways to induce apoptosis in human colorectal cancer cells (57). This technology also enhances the gastrointestinal tolerance of *L. reuteri*. *Lactobacillus paracasei strain CMU-Pb-L5* and *L. reuteri* promote cancer cell apoptosis through similar mechanisms. Future research should focus on a more detailed investigation of the specific bacterial components involved in tumor growth inhibition (111). Acetyl-ethyl extract from *L. plantarum* ATCC14917 and *L. rhamnosus* ATCC7469 exhibits targeted anti-colon cancer cell activity by inducing the intrinsic apoptosis pathway, downregulating the expression of anti-apoptotic genes Bcl-2 and Bcl-XL, and upregulating the expression of pro-apoptotic genes Bak, Bad, and Bax (9). It is a potential candidate for aiding in the fight against cancer from a nutritional perspective. The studies indicate similarities in the targeted anti-cancer mechanisms of short-chain fatty acids derived from *Lactobacillus*, particularly in their ability to predominantly activate the intrinsic pathway to induce apoptosis in cancer cells. Compared with healthy individuals, intestinal conjugated linoleic acid (CLA) is significantly reduced in CRC patients. Besides fecal microbiota transplantation (FMT) (112), exogenous supplementation with CLA-producing *Bifidobacterium breve CCFM683* and *Bifidobacterium pseudocatenulatum MY40C* significantly inhibits tumor progression, which is closely associated with CLA and the bbi gene responsible for its production. *CCFM683*

enhances intestinal barrier function by suppressing the NF-κB signaling pathway and promotes tumor cell apoptosis through the CLA-PPAR-γ axis (113), a mechanism consistent with the tumor-suppressive effects previously observed in *L. plantarum CCFM8661* (114).

Recent studies have found that new probiotics are effective in colorectal cancer. The novel probiotic strains *Streptococcus salivarius CP163* and *S. salivarius CP208*, originating from human colostrum, demonstrate multifaceted anti-cancer properties. These include directly adhering to cancer cells, secreting short-chain fatty acids, inducing cancer cell DNA fragmentation and morphological alterations, modulating caspase-2 activity, and triggering apoptosis. This study unveils an innovative biological strategy for using functional foods in colon cancer prevention (115). Additionally, *in vitro* experiments have shown that *Ligilactobacillus salivarius LZZAY01* promotes cancer cell apoptosis (116). In summary, as shown in Table 4, probiotics or postbiotic play a crucial role in exerting anti-colorectal cancer effects through various signaling pathways related to tumorigenesis or by directly promoting the expression of proteins involved in cell apoptosis. Compared to conventional chemotherapeutic agents, microbial preparations have minimal or absent toxic side effects, providing them with a significant advantage in biological applications. The development of novel technologies, such as microencapsulation and nanomaterials, has further enhanced the stability and tumor specificity of these microbial preparations, which are used as nutritional dietary supplements for colorectal cancer prevention and treatment. Therefore, due to their excellent gastrointestinal tolerance and biological safety profiles, these microbial preparations are expected to have broader clinical applications.

6 Probiotics, postbiotic, and gastrointestinal damage

Encountering harmful substances and microorganisms is inevitable in daily life. When the immune system weakens, direct or indirect contact with the gastrointestinal damage system can the intestinal barrier, leading to microbiota imbalance, intestinal damage, and diarrhea. Historically, gastrointestinal injuries were mainly treated with pharmaceuticals and surgery; recent studies have shown that probiotics can offer protection. Maintaining the intestinal barrier function is crucial for gastrointestinal health, as shown in various studies (117). An *in vitro* study showed that *Clostridium tyrobutyricum* protects porcine epithelial cells from lipopolysaccharide-induced injury by preserving intestinal barrier function and inhibiting the P38/JNK signaling pathway. Downstream genes, including AP-1, ELK-1, ATF-2, and p53, are downregulated, while Stat3 activates anti-apoptotic Bcl-2 and downregulates pro-apoptotic Bax and caspase-3/-8, reducing intestinal cell apoptosis (118). Ochratoxin A, a significant toxin for humans and animals, has been detected in *in vitro* models of Ochratoxin A-induced cell injury. *Bacillus subtilis CW14* upregulated DNA repair genes and downregulated death receptor pathway genes to reduce apoptosis. These effects may be mediated by activation of toll-like receptor signaling pathways (119). The specific mechanism requires further investigation. Cadmium, a heavy metal, can damage various bodily systems through long-term environmental exposure, with the gastrointestinal tract being the primary target organ (120, 121); Administration of multi-strain probiotics (*L. rhamnosus IRBC_M10783*, *L. helveticus TG_34*, *Lactobacillus casei IRBC_M10782*) significantly reduces intestinal tissue damage in mice compared to untreated controls. Probiotics modulate immune function by upregulating p53, Bax, and caspase-3, while downregulating Bcl-2 to reduce apoptosis (122). Similarly, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Lactobacillus brevis* inhibit indomethacin-induced mucosal cell apoptosis. Future research should focus on a more detailed investigation of the molecular mechanisms underlying the probiotic effects (123).

Research has shown that lactic acid, a *Lactobacillus* metabolite, mitigates ethanol-induced gastric mucosal damage by reducing local inflammation. It protects the stomach by inducing apoptosis through downregulation of IL-1β, TNF-α, and IL-6, as well as Bax and caspase-3, and by upregulating genes maintaining gastric mucosal integrity (10). However, the underlying mechanism remains unclear, and further research is needed to explore additional applications of probiotics in this field (54). Radiotherapy is a common and effective treatment for gastrointestinal tumors, but it inevitably damages adjacent healthy tissue (124). Restoring gut microbiota can partially alleviate this damage through fecal transplantation and probiotic therapy. The key is finding ways to mitigate the impact of stomach acid and ionizing radiation on the microbiota (125, 126). A recent study on probiotic spore layers (spore ghosts) showed that oral administration of three clinically approved probiotics (*Bacillus coagulans*, *Bacillus subtilis*, and *Bacillus licheniformis*) significantly enhanced the population of beneficial intestinal flora in mice. This effect was attributed to their exceptional stomach acid tolerance and biocompatibility. They also improved intestinal barrier function and reduced radiation-induced apoptosis in intestinal epithelial cells (53). These studies collectively indicate that apoptosis is a key mechanism through which various probiotics and their metabolites to prevent and treat gastrointestinal injury. Certain forms, such as spores, enhance gastrointestinal tolerance and play a crucial role in maintaining the integrity of the gastrointestinal barrier. Consequently, they hold promising potential as candidates for addressing gastrointestinal injury, as illustrated in Table 5. However, further research is needed to explore the use of additional probiotics, particularly through clinical trials.

7 Probiotics, postbiotic, and infectious diarrhea

In the United States, acute diarrheal diseases cause approximately 179 million outpatient visits annually (127). Probiotics and postbiotics, along with rehydration, medication, and improved hygiene, offer a promising approach for controlling early-stage gastrointestinal infections, providing a significant solution to this global health issue (128, 129). They can significantly modulate gut microbiota, enhance intestinal barrier function, and regulate immune responses, thereby exerting probiotic properties to alleviate diarrhea. Rotavirus is widely recognized as the leading cause of severe gastrointestinal infections in infants and children (130). In an experimental study with viral intervention in weaning piglets, *LGG* was found to significantly alleviate diarrhea caused by viral infection, upregulate the Bcl-2 gene, and downregulate the Bax gene, reducing apoptosis of jejunal mucosal cells (131). A recent *in vitro* study using a human model showed that both *LGG* and its conditioned medium (*mLGG*) reduced elevated

TABLE 4 Probiotic and postbiotic promote apoptosis to alleviate colon cancer.

Probiotic-based preparation	Category	Probiotic type	The specific molecular mechanism of apoptosis	References
L. paracasei K5	Probiotic	*Lactobacillus*	Human: Potentially inducing apoptosis by regulating Bcl-2 family proteins	Chondrou et al. (89)
L. casei ATCC393	Probiotic	*Lactobacillus*	Human, animal: Induced up-regulation of TRAIL and down-regulation of survivin	Nychas et al. (90)
L. paracasei subp. Paracasei X12	Probiotic	*Lactobacillus*	Human: The blockade of the mTOR/4EBP1 pathway causes HT-29 cancer cells to pause in the G1 phase, reduce cyclin E1 expression, elevate p27 levels, and initiate apoptosis	Huang et al. (92)
The cell wall protein component of *L. paracasei ATCC25598*	Postbiotic	*Lactobacillus*	Human: The specific mechanism underlying the augmentation of cancer cell apoptosis remains unclear	Nozari et al. (52)
Heat-inactivated *S. cerevisiae PTCC5052*	Postbiotic	*Saccharomyces*	Human: Down-regulates p-Akt1, Bcl-XL, Rel A, and pro-caspase-3, -9, and enhance Bax and caspase-3 expression	Shamekhi et al. (56)
heat-killed *L. brevis IBRC_M1078* and *L. paracasei IBRC_M1079*	Postbiotic	*Lactobacillus*	Human: Pro-apoptotic genes, including Bax, caspase-3, and caspase-8, are upregulated, whereas the anti-apoptotic gene Bcl-2 is downregulated	Karimi et al. (99)
L. genera (L. plantarum, L. rhamnosus, L. brevis and L. Lui) and their metabolites	Probiotics Postbiotic	*Lactobacillus*	Human, animal: Activation the Wnt/β-catenin pathway, AMPs and metabolites, which are continuously generated in tumors following colonization, disrupt the mitochondrial membrane integrity and trigger late apoptosis	Ghanavati et al. (93)
S. burra metabolites	Postbiotic	*Saccharomyces*	Human: Promote apoptosis of cancer cells	Pakbin et al. (95)
LCFS	Postbiotic	*Lactobacillus*	Human: The activation of NF-κB was suppressed, while the expression of PARP1 and Bcl-XL was downregulated	Yoo et al. (100)
Gaba-producing *L. plantarum CCARM0067*	Postbiotic	*Lactobacillus*	Human: Regulation of cIAP2 expression and inhibition of the cAMP-dependent signaling pathway	An et al. (15)
Cell-free culture supernatant of *L. plantarum CCARM0067*	Postbiotic	*Lactobacillus*	Human: Regulated caspase-3 activity	Kim et al. (101)
Butyrate synthesized by engineered colibacillus Nissle 1917	Postbiotic	*E. coli*	Human: The induction of mitochondrial apoptosis pathway is P53-independent, resulting in up-regulation of cytochrome C, Bax, and PARP-1 protein expression. Additionally, activation of caspase-3 and caspase-9 occurs	Chiang et al. (107)
L. reuteri	Probiotic	*Lactobacillus*	Human: The expression of Bcl-2 was downregulated, while the expression of Caspase-3 and Bax was upregulated	Li et al. (57)
(L. p. CMU-Pb-L5)	Probiotic	*Lactobacillus*	Animal: The protein expression of Bcl-2 was downregulated, while the expression of Caspase-3 and Bax was upregulated	Chang et al. (111)
CLA-producing *Bifidobacterium breve CCFM683* and *Bifidobacterium pseudocatenulatum MY40C, L. plantarum CCFM8661*	Probiotics Postbiotic	*Bifidobacterium Lactobacillus*	Animal: Increased the concentration of the pro-apoptotic protein Bax and reduced the anti-apoptotic protein Bcl-2 through the CLA-PPAR-γ axis	Chen et al. (113, 114)
The supernatant from fermenting Musa paradisiaca with *L. casei NCDC17 and B. bifidum NCDC255* is rich in SCFA	Postbiotic	*Lactobacillus Bifidobacterium*	Human: Lowering mitochondrial membrane potential and ATP synthesis induces mitochondrial pathway-mediated cell apoptosis: release of cytochrome C, activation of BAX, increased expression of caspase-3 and PARP, without affecting BCL-2 expression	Nie et al. (145)
Acetyl-ethyl extract from *L. plantarum ATCC14,917* and *L. rhamnosus ATCC7469*	Postbiotic	*Lactobacillus*	Human: Downregulating the expression of anti-apoptotic genes Bcl-2 and Bcl-xl, and upregulating the expression of pro-apoptotic genes Bak, Bad, and Bax	Amin et al. (9)
S. salivarius CP163 and *S. salivarius CP208,*	Probiotics	Gram-positive coccus	Human: Inducing cancer cell DNA fragmentation and morphological alterations, modulating caspase-2 activity, and triggering apoptosis	Srikham et al. (115)

(Continued)

TABLE 4 (Continued)

Probiotic-based preparation	Category	Probiotic type	The specific molecular mechanism of apoptosis	References
Ligilactobacillus salivarius LZZAY01	Probiotic	*Lactobacillus*	Human: The protein expression of Bcl-2 was downregulated, while the expression of Bax was upregulated	Wenhong Yang et al. (116)
S. cerevisiae	Probiotic	*Saccharomyces*	Animal: Down-regulation of caspase-3 and caspase-7 may be linked to the down-regulation of the Akt/NF-κB and Akt/mTOR signaling pathways	Li et al. (87)
L. helveticus NS8	Probiotic	*Lactobacillus*	Animal: The upregulation of caspase-3 expression may be associated with the inhibition of NF-κB pathway activation	Rong et al. (97)
L. salivarius CGMCC3606 and its metabolites	Probiotic Postbiotic	*Lactobacillus*	Animal: The AKT signaling pathway was inhibited, resulting in down-regulation of AKT phosphorylation and decreased expression of cyclin D1 and COX-2	Dong et al. (94)
L. plantarum AdF10 and L. rhamnosus ATCC53103	Probiotics	*Lactobacillus*	Animal: Restoring normal levels of p53, p21, and apoptotic genes (Bax, Bcl-2, caspase-3, and caspase-9) can suppress apoptosis via the p53 pathway	Walia et al. (102)
(*L. acidophilus NCDC15* and *L. rhamnosus MTCC1408*) along with cecxib	Probiotics	*Lactobacillus*	Animal: Upregulation P53 expression, downregulation K-ras proto-oncogene expression, and modulation Bax and Bcl-2 levels	Sharaf et al. (12)
L. plantarum LAB12	Probiotic	*Lactobacillus*	Animal: Upregulation of p53 and caspase-3 expression	Peng et al. (109)

TABLE 5 Probiotics and postbiotic regulate endogenous and exogenous cell apoptosis to alleviate gastrointestinal injury.

Probiotic-based preparation	Category	Probiotic type	The specific molecular mechanism of apoptosis	References
Bacillus subtilis CW14	Probiotic	*Bacillus*	Human: By activating the Toll-like receptor signaling pathway, upregulating DNA repair genes, and downregulating genes related to the death receptor pathway	Peng et al. (119)
Clostridium tyrobutyricum	Probiotic	*Clostridium butyricum*	Animal: Inhibition the P38/JNK signaling pathway, downregulation downstream genes (including AP-1, ELK-1, ATF-2, and p53), activation Stat3 expression, thereby regulating the Bcl and caspase families	Xiao et al. (118)
L. rhamnosus IRBC_M10783, L. helveticus TG_34, L. casei IRBC_M10782	Probiotics	*Lactobacillus*	Animal: The endogenous pathway of upregulating the expression of p53, Bax, and caspase-3 genes while downregulating Bcl-2 gene expression contributes to the reduction of cellular apoptosis	Dashtbanei et al. (122)
Lactobacillus metabolites: lactic acid	Postbiotic	*Lactobacillus*	Animal: By alleviating cell apoptosis induced by inflammatory cytokines and downregulating the expressions of Bax and caspase-3	Huang et al. (54)
L. rhamnosus, L. fermentum, and *L. brevis*	Probiotics	*Lactobacillus*	Animal: The up-regulation of Bcl-2 expression and the down-regulation of Bax expression	Gelen et al. (123)
Spores of *Bacillus coagulans, Bacillus subtilis and Bacillus licheniformis*	Probiotics	*Bacillus*	Animal: The apoptosis rate can be decreased	Zheng et al. (53)

caspase-3 activity, potentially through inhibition of the NF-κB signaling pathway. Their benefits differ: live *LGG* primarily suppresses enterotoxic and cytotoxic effects, while *mLGG* exerts postbiotic effects mainly by inhibiting chloride ion secretion pathways (129). The findings suggest that the mechanism through which *LGG* inhibits apoptosis and alleviates virus-induced diarrhea may be species-specific. Additionally, the addition of *Bacillus clausii* mixed strains (*O/C, T, SIN, N/R*) and their supernatant reduced intestinal cell apoptosis rates. This mechanism may involve activation of the cellular TLR3 pathway and suppression of NF-κB1, TRAF6, and MyD88. These findings encourage further exploration of the effects of *Bacillus clausii* on gastrointestinal infections caused by other pathogens in future studies (132). The 3D8 single-chain variable region protein shows potential antiviral activity by penetrating cells and degrading nucleic acids. Oral administration of *L. paracasei ATCC334*, which produces recombinant 3D8 single-chain variable region protein,

reduces norovirus coat protein VP1 expression and increases the expression of the anti-apoptotic protein survivin in mice (133). *Salmonella typhimurium* is a prevalent foodborne pathogen, while *Bacillus subtilis Gbi-30*, *Bacillus indium ATCC6633*, and *Bacillus coagulans IBRC-M10981* inhibit its growth in spore and heat-inactivated forms without toxic effects on intestinal cells. These strains are recommended for the prevention and treatment of disease (134). These studies show that probiotics and postbiotics can reduce gastrointestinal cell apoptosis induced by viral or bacterial infections, as shown in Table 6. This effect is likely due to their anti-inflammatory properties, maintenance of intestinal barrier function, and direct pathogen-targeting mechanisms. However, the precise molecular mechanism through which probiotics reduce gastrointestinal cell apoptosis following viral infection remains unclear. Future research should explore the effects and mechanisms of different probiotic strains on various pathogens, such as *Helicobacter pylori*-induced gastrointestinal infections, and their impact on intestinal cell apoptosis.

8 Probiotic, postbiotic, and safety

Probiotics and postbiotics offer numerous health benefits; however, their potential adverse effects in specific conditions should not be overlooked. First, individuals with underlying diseases and immunocompromised conditions may be at risk of infections when consuming probiotics, particularly those with weakened immune systems, such as chemotherapy patients, pediatric patients, and individuals with HIV (135). In some cases, commercially available probiotics have been associated with infections, such as *Lactobacillus rhamnosus*, which has been reported to cause bacteremia (136). Second, excessive or long-term probiotic consumption may lead to mild gastrointestinal symptoms, such as bloating and indigestion, particularly in individuals with inflammatory bowel disease (IBD). However, these symptoms generally subside as the gut microbiota adapts (137). Furthermore, probiotics may influence drug interactions, potentially reducing the efficacy of concomitant medications. For example, in antibiotic-associated diarrhea, probiotics may delay the restoration of normal gut microbiota (138).

It is also important to consider the potential negative effects of postbiotics. Certain postbiotics may influence autoimmune diseases by either enhancing or suppressing the immune system (139). Additionally, probiotics may be involved in the production of toxic metabolites, such as histamine, which can trigger allergic reactions, headaches, and itching. Recent studies have shown that *Lactobacillus reuteri* plays a role in histamine activation and metabolism, activating the H2 receptor to exert anti-inflammatory effects (140). Moreover, other studies suggest that while the colibactin-producing *Escherichia coli strain Nissle 1917 (EcN1917)* exhibits significantly reduced genotoxic activity compared to other *EcN strains*, it may still increase the likelihood of hazardous mutations through mutagenic mechanisms (141).

In summary, for the general population, probiotics and postbiotics can serve as beneficial dietary supplements for clinical applications. However, their use should be approached with caution in immunocompromised individuals, critically ill patients, infants, and those with severe allergies due to potential risks (142).

9 Conclusions and future prospects

Growing evidence indicates a growing interest in probiotic preparations for improving gastrointestinal health. As viable candidates for treating digestive diseases, probiotics exhibit diverse mechanisms and beneficial effects, including modulating immunity, reducing inflammation, improving gut health, alleviating oxidative stress, and regulating apoptosis, with minimal safety risks (5, 143). This review systematically analyzes the ability of different probiotic types and strains to regulate apoptosis, both individually and synergistically. Different strains of the same species, probiotics themselves, and postbiotics may exhibit varying anti-apoptotic effects in disease development and progression. These effects may collectively influence specific signaling pathways and targets, particularly membrane receptors, Bcl-2 family proteins, mitochondria, and

TABLE 6 Probiotics and postbiotic regulate cell apoptosis to alleviate infectious diarrhea.

Probiotic-based preparation	Category	Probiotic type	The specific molecular mechanism of apoptosis	References
Bacillus clausii Mixed strains (*O/C, T, SIN, N/R*) and superserum	Probiotic Postbiotic	*Bacillus*	Human: Apoptosis may be associated with activation of the cell TLR3 pathway, involving down-regulation of NF-κB1, TRAF6, and MyD88 expression	Paparo et al. (132)
Heat killing and spore forms of *Bacillus subtilis* Gbi-*30*, *Bacillus indiensis ATCC 6633* and *Bacillus coagulans IBRC*-M *10981*	Probiotic	*Bacillus*	Human: By inhibiting the growth of pathogenic bacteria	Kawarizadeh et al. (134)
LGG and *MLGG*	Probiotic	*Lactobacillus*	Human: Possibly inhibiting the NF-kB signaling pathway, leading to down-regulation of caspase-3 expression	Buccigrossi et al. (129)
LGG	Probiotic	*Lactobacillus*	Animals: The up-regulation of Bcl-2 gene expression and the down-regulation of Bax gene expression	Chakravortty et al. (131)
L. paracasei ATCC334, which produces recombinant 3D8 single-chain variable region protein	Postbiotic	*Lactobacillus*	Animals: The expression of anti-apoptotic protein survivin was increased	Hoang et al. (133)

caspases. Currently, most research has been conducted in animal models, with only a limited number of studies focusing on apoptosis in cancer at the *in vitro* cellular level. Due to interspecies differences, the complexity of how probiotics regulate apoptosis in human diseases remains incompletely understood. Furthermore, some studies lack comprehensive evidence supporting the regulation of apoptosis. Therefore, careful interpretation of study results is crucial. These phenomena can be partially attributed to the intrinsic properties of probiotics, as well as the high diversity of the human gut microbiota, shaped by factors such as population, sex, diet, and other variables. It is essential to rigorously screen for effective and safe probiotics and postbiotics, either administered through fecal microbiota transplantation (FMT) (144) or directly demonstrating therapeutic effects in animal studies. A systematic transition from small-scale safety assessments to large-scale efficacy validation is necessary to confirm their effectiveness and safety in humans.

Finally, probiotics hold promise as dietary supplements for advancing the treatment of digestive diseases, marking a step toward precision and personalized medicine. Most importantly, further clinical studies are needed to validate the beneficial effects observed in animal models.

Author contributions

QX: Data curation, Writing – original draft. JL: Writing – original draft. PY: Resources, Software, Visualization, Writing – review & editing. TQ: Writing – review & editing. SJ: Writing – review & editing. RY: Conceptualization, Funding acquisition, 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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A study of correlation of the dietary index for gut microbiota with non-alcoholic fatty liver disease based on 2007–2018 National Health and Nutrition Examination Survey

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Objective: To explore the correlation of dietary index for gut microbiota (DI-GM) with non-alcoholic fatty liver disease (NAFLD).

Methods: Data of 6,711 participants were extracted from the National Health and Nutrition Examination Survey (NHANES) during 2007–2018. A weighted logistic regression analysis was employed for assessment of the correlation of DI-GM with NAFLD, and a restricted cubic spline (RCS) analysis was implemented to examine potential non-linear associations. Subgroup analyses were conducted to identify particularly susceptible groups. Additionally, the synergistic effects of different DI-GM components on NAFLD risk was assessed by weighted quantile sum (WQS) regression.

Results: The DI-GM exhibited statistically significant correlation with NAFLD [OR (95%CI):0.91 (0.85, 0.98), $p = 0.015$]. The results of the RCS analysis indicated a linear correlation of DI-GM and NAFLD ($p = 0.810$ for non-linearity). Further stratified analyses indicated that the negative correlation of DI-GM with NAFLD were significant and consistent for all subgroups. The results of WQS regression revealed that soybean (27%), refined grains (17%), coffee (16%), and red meat (9%) had the highest contribution weights to NAFLD.

Conclusion: As an important tool for assessment of the influences of diet on gut microbiota, DI-GM is negatively correlated with NAFLD risk factors. Soybean, refined grains, coffee, and red meat are key factors influencing NAFLD. The direct correlation of DI-GM with NAFLD shall be explored and the effectiveness of prevention and treatment of NAFLD shall be evaluated by improving DI-GM scores via dietary interventions.

KEYWORDS

dietary index for gut microbiota, non-alcoholic fatty liver disease, cross-sectional study, National Health and Nutrition Examination Survey, gut microbiota, dietary

Introduction

As a liver disease correlated to metabolic disorders, non-alcoholic fatty liver disease (NAFLD) is characterized by abnormal fat accumulation in liver induced by factors other than alcohol consumption (1–3). NAFLD comprises non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), and it may further progress to hepatocellular carcinoma (1, 4–6). The incidence of NAFLD is approximately 38% globally, with regional variations ranging from 25.10% in Western Europe to 44.37% in Latin America (7). The prevalence of NAFLD and NASH in key regions such as China and USA is expected to increase by up to 30–56% from 2016 to 2030 (8). NAFLD has been demonstrated to be correlated with various factors, including insulin resistance (9, 10), lipotoxicity (11), inflammatory response (12), genetic polymorphisms (13), epigenetics (14), adipokines (15), myokines (15), hepatokines (15, 16), bile acids (17, 18), and gut microbiota (19–22).

Gut microbiota (GM), also known as the gut flora, refers to the microbial community in human intestine, and it plays a significant role in digestion and metabolism (23–25). Gut microbiota coexists symbiotically with the human body as the "second genome" (22). Previous studies have shown that GM is a dominant factor influencing incidence and progression of NAFLD (19–22). Diet determines the composition and diversity of GM (26–28). Furthermore, dietary interventions that can alter gut microbiota have attracted great attention (28). Kase et al. (26) reported a review of 14 dietary components with different influences on gut microbiota. The Dietary Index for the Gut Microbiota (DI-GM) was proposed for assessment of dietary quality associated with gut microbiota (26). Contrary to other dietary indices such as healthy eating index-2015 (HEI-2015) and alternative healthy eating index-2010 (AHEI-2010), DI-GM was established on the basis of gut microbiota but not food groups. Moreover, DI-GM had a positive correlation with urinary levels of intestinal diols and lactones, both of which are markers of gut microbiota diversity, indicating that DI-GM is associated with the diversity of GM (26). Hence, DI-GM can be used to effectively evaluate the influences of dietary patterns on GM (26) and serve as a standardized tool for diet assessment. Recent studies have shown that high DI-GM indicates low risk of accelerated aging (29), and DI-GM is inversely related to the prevalence of depression (30). Furthermore, compelling evidences have revealed that gut microbiota generates a variety of bioactive substances that interact with the host liver cells through the portal vein, which may lead to inflammation and further liver damage (31). However, the specific correlation of DI-GM with NAFLD remains unclear.

In this study, the correlation of DI-GM and NAFLD was investigated on the basis of the data extracted from the National Health and Nutrition Examination Survey (NHANES), and the potential of DI-GM which has been widely used for diet assessment for GM in prevention and dietary treatment of NAFLD was explored.

Materials and methods

Target community

Information acquired from public files for NHANES data cycles in 2007–2018 was analyzed. Across these cycles, 40,959 participants were enrolled, while the final cohort comprised 6,711 subjects only (Figure 1) as participants who provided incomplete data of fatty liver index (FLI) (n = 23,670), incomplete data of DI-GM (n = 845), with viral hepatitis (n = 315), excessive alcohol consumption (n = 4,527), or incomplete data for any covariate (n = 4,891) were excluded.

Diagnosis of NAFLD

The FLI was employed for non-invasive diagnosis of NAFLD (32), and its accuracy and clinical significance in screening and diagnosis of NAFLD have been demonstrated in various studies (33). The FLI can be determined by: FLI = ($e^{0.953 \times \log e [\text{Triglycerides (TG)}] + 0.139 \times \text{Body mass index (BMI)} + 0.718 \times \log e [\gamma\text{-glutamyltransferase (GGT)}] + 0.053 \times \text{Waist circumference (WC)} - 15.745}$)/($1 + e^{0.953 \times \log e [TG] + 0.139 \times BMI + 0.718 \times \log e [GGT] + 0.053 \times WC - 15.745}$) × 100, and the FLI of 60 or higher indicates a high risk of NAFLD (32).

Assessment of DI-GM

Fourteen food or nutrients constituting key elements of DI-GM were identified (26). Beneficial elements include chickpeas, avocados, coffee, broccoli, fermented dairy products, cranberries, soybean, green tea, fiber, and whole grains. Meanwhile, detrimental elements such as refined grains, processed meat, red meat, and a high-fat diet were identified (26). The DI-GM was determined on the basis of the dietary recall data of NHANES 2007–2018, as shown in Supplementary Table S2. For food items with positive impacts on gut microbiota, 1 point was assigned when the gender-specific median was reached for intake, otherwise 0; for food items with negative impacts on gut microbiota, 0 was assigned when the gender-specific median was reached for intake or it accounts for 40% of total caloric intake (for high-fat diets), otherwise 1 point (26). A DI-GM score ranging from 0 to 13 (ranging from 0 to 9 for promoting the gut microbiota and 0 to 4 for negatively affecting the gut microbiota) was determined based on these scores, and then the participants were classified into Groups A (0–3), B (4), C (5) and D (≥6) (30), as presented in Supplementary Table S1.

Covariables

In this survey, several potential confounding factors were considered: (1) Demographic factors: age, gender (male/female), race, marital status, and education. (2) Lifestyle factors: Smoking history (never, now, former). (3) Financial status: family poverty income ratio (PIR) (low, middle, high). (4) Health comorbidities: hypertension, cardiovascular disease (CVD), and diabetes mellitus (DM). (5) BMI (kg/m^2). (6) Liver function indicators: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (both in U/L). Detailed information could be achieved in Supplementary Table S2.

Statistical analysis

Recommended sample weights were employed to enhance accuracy and undesired influences were mitigated by intricate multistage sampling. The results of categorical variables were

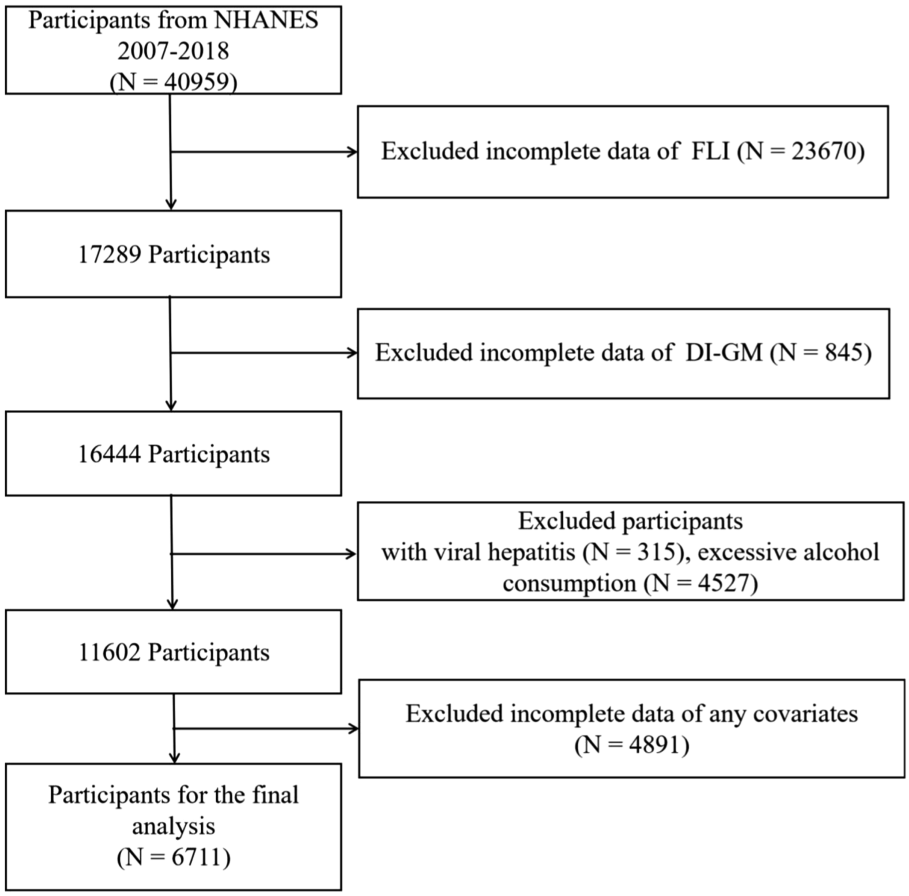

FIGURE 1
Flow diagram of study cohort selection.

denoted as weighted percentages, while the results of continuous variables were presented as weighted means ± standard deviations. To clarify the correlation of DI-GM and NAFLD, weighted multivariate logistic regression analyses were conducted. Herein, various confounding factors were considered and adjusted for. Three models were employed in this study. Model 1 was non-adjusted; Model 2 incorporated adjustments for key demographic variables; Model 3 additionally adjusted for BMI, PIR, smoking history, hypertension, DM, CVD, ALT and AST. Weighted restricted cubic spline (RCS) curves were introduced to Model 3 to investigate the correlation of DI-GM and NAFLD. Additionally, subgroup analyses were executed based on age, race, gender, education, marital status, smoking history, and common chronic diseases (hypertension and CVD). Considered the potential influences of physical activity, alcohol intake, medication use on the results. Accordingly, we further adjusted for these variables as outlined in Model 3 to assess the robustness of our findings in sensitivity analysis. Weighted quantile sum (WQS) regression models were employed for assessment of the synergistic effects of different DI-GM components on the risk of NAFLD, and the WQS index was determined on the basis of 60% training dataset, 40% validation dataset and 1,000 bootstrapping (34). To address the multicollinearity, weights were assigned to components according to their contributions to the results. The integrity of the statistical computations was validated using R software, with $p < 0.05$ denoting statistical significance.

Results

Characteristics of the participants

Table 1 shows the characteristics of the 6,711 subjects enrolled grouped by DI-GM. The average age was 51.06 ± 0.30 years. 51.28% were male and 48.72% were female. Significant differences were detected ($p < 0.05$) across DI-GM for demographic characteristics, financial status (PIR), lifestyle (smoking history), physical well-being (NAFLD, hypertension, CVD, DM), and anthropometric measures (BMI).

Association of DI-GM and NAFLD

Logistic modeling was executed to explore the association of DI-GM and NAFLD. According to Table 2, a significant negative correlation was identified [OR (95%CI): 0.88 (0.85, 0.92), $p < 0.001$], and the negative correlation remained robust after variable adjustment [OR (95%CI):0.91 (0.85, 0.98), $p = 0.015$] in Model 3, suggesting a 9% relative risk reduction per DI-GM unit which is modest at the individual level and meaningful

TABLE 1 Weighted characteristics of the study population according to the DI-GM group[a].

Characteristics	DI-GM					*p*-value
	Overall	0–3	4	5	>=6	
Number	6,711	1,246	1,436	1,526	2,503	
Age (years)	51.06 (0.30)	49.76 (0.62)	50.21 (0.67)	49.88 (0.62)	52.72 (0.44)	<0.001
Sex (%)						0.205
Male	3,354 (51.28)	667 (54.11)	707 (51.71)	760 (52.36)	1,220 (49.23)	
Female	3,357 (48.72)	579 (45.89)	729 (48.29)	766 (47.64)	1,283 (50.77)	
Race (%)						<0.001
Non-Hispanic White	3,068 (70.32)	505 (65.29)	617 (65.49)	681 (69.75)	1,265 (75.26)	
Non-Hispanic Black	1,270 (10.25)	321 (15.02)	302 (12.35)	287 (10.31)	360 (7.12)	
Mexican American	893 (6.50)	179 (7.86)	224 (8.23)	225 (7.39)	265 (4.52)	
Other Race	1,480 (12.92)	241 (11.82)	293 (13.93)	333 (12.55)	613 (13.10)	
Educational attainment (%)						<0.001
High school or less	2,956 (36.26)	673 (47.22)	732 (43.09)	701 (37.27)	850 (27.51)	
More than high school	3,755 (63.74)	573 (52.78)	704 (56.91)	825 (62.73)	1,653 (72.49)	
Marital status (%)						0.030
Married or living with partner	4,282 (68.21)	794 (66.14)	865 (65.63)	958 (67.09)	1,665 (71.05)	
Living alone	2,429 (31.79)	452 (33.86)	571 (34.37)	568 (32.91)	838 (28.95)	
PIR (%)						<0.001
Low	1,270 (12.35)	266 (15.32)	311 (13.81)	333 (14.88)	360 (8.88)	
Middle	3,542 (48.21)	731 (55.54)	803 (52.66)	775 (44.44)	1,233 (44.98)	
High	1899 (39.43)	249 (29.14)	322 (33.52)	418 (40.68)	910 (46.14)	
Smoking status (%)						0.003
Never	4,143 (62.93)	730 (62.03)	860 (61.27)	954 (62.94)	1,599 (64.15)	
Now	871 (11.70)	188 (13.59)	215 (13.87)	216 (13.19)	252 (8.93)	
Former	1,697 (25.38)	328 (24.38)	361 (24.86)	356 (23.88)	652 (26.92)	
NAFLD (%)						<0.001
No	3,769 (56.27)	624 (49.59)	771 (52.70)	847 (53.28)	1,527 (62.67)	
Yes	2,942 (43.73)	622 (50.41)	665 (47.30)	679 (46.72)	976 (37.33)	
Hypertension (%)						0.021
No	3,558 (58.35)	623 (56.28)	740 (54.42)	824 (59.22)	1,371 (60.74)	
Yes	3,153 (41.65)	623 (43.72)	696 (45.58)	702 (40.78)	1,132 (39.26)	
CVD (%)						0.251
No	5,833 (89.09)	1,056 (87.90)	1,236 (87.86)	1,332 (89.55)	2,209 (89.97)	
Yes	878 (10.91)	190 (12.10)	200 (12.14)	194 (10.45)	294 (10.03)	
DM (%)						<0.001
No	5,075 (81.40)	886 (77.58)	1,048 (79.29)	1,173 (81.31)	1968 (84.15)	
Yes	1,636 (18.60)	360 (22.42)	388 (20.71)	353 (18.69)	535 (15.85)	
BMI (kg/m^2)	29.11 (0.13)	29.74 (0.24)	29.79 (0.23)	29.43 (0.23)	28.32 (0.19)	<0.001
ALT (U/L)	24.01 (0.18)	24.11 (0.51)	24.65 (0.46)	23.80 (0.50)	23.76 (0.32)	0.436
AST (U/L)	24.19 (0.20)	23.83 (0.54)	24.37 (0.34)	23.75 (0.43)	24.50 (0.34)	0.509

DI-GM, dietary index for gut microbiota; PIR, family poverty income ratio; NAFLD, nonalcoholic fatty liver disease; CVD, cardiovascular disease; DM, diabetes mellitus; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

[a]Values are weighted means (standardized errors) or number of participants (weighted percentages) unless otherwise indicated.

TABLE 2 Weighted multivariate logistic regression analysis of DI-GM and NAFLD[a].

Characteristic	Model 1 OR (95%CI), *p*-value	Model 2 OR (95%CI), *P*-value	Model 3 OR (95%CI), *P*-value
DI-GM (continuous)	0.88 (0.85,0.92), <0.001	0.88 (0.85,0.92), <0.001	0.91 (0.85,0.98), 0.015
DI-GM (categorical)			
0–3	Reference	Reference	Reference
4	0.88 (0.73,1.07), 0.196	0.89 (0.74,1.08), 0.253	0.86 (0.62,1.20), 0.372
5	0.86 (0.70,1.05), 0.148	0.88 (0.72,1.08), 0.216	0.88 (0.64,1.22), 0.453
>=6	0.59 (0.48,0.71), <0.001	0.59 (0.48,0.72), <0.001	0.65 (0.47,0.90), 0.010
P for trend	<0.001	<0.001	0.013

DI-GM, dietary index for gut microbiota; PIR, family poverty income ratio; NAFLD, nonalcoholic fatty liver disease; CVD, cardiovascular disease; DM, diabetes mellitus; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a]Model 1: unadjusted; Model 2: adjusted for age, sex, race, educational attainment, and marital status; Model 3: adjusted for age, sex, race, education attainment, marital status, BMI, PIR, smoking status, hypertension, DM, CVD, ALT and AST.

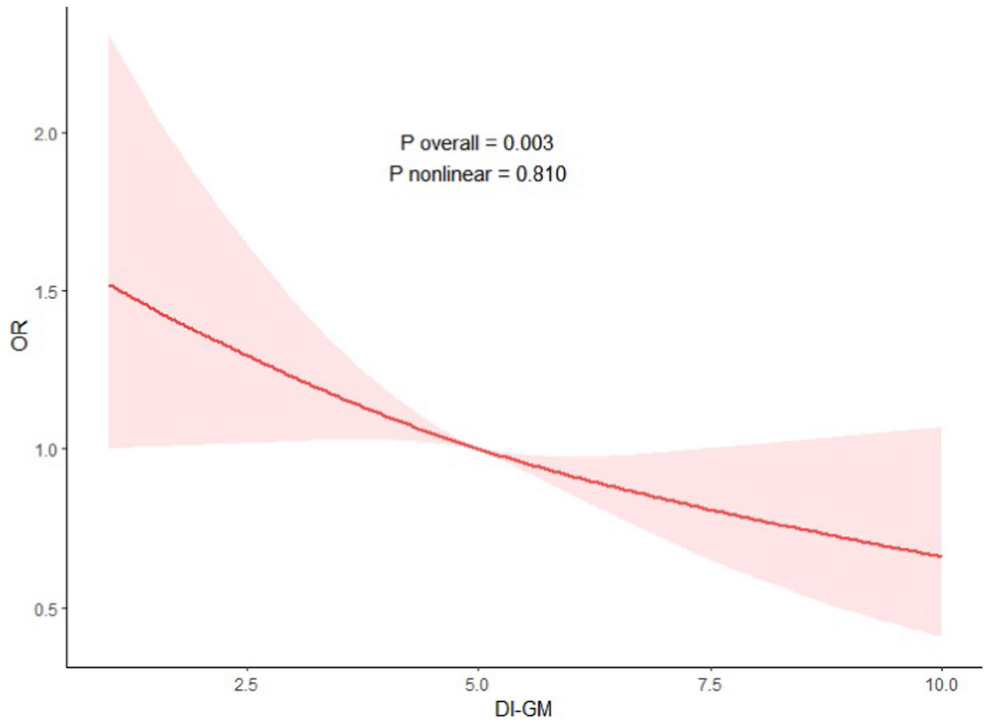

FIGURE 2
Association between DI-GM with NAFLD. Adjustment factors included age, sex, race, education attainment, marital status, BMI, PIR, smoking status, hypertension, DM, CVD, ALT and AST.

at the population level. Furthermore, slight increase in DI-GM could also offer significant benefits for NAFLD, especially for the implementation of large scale and high-risk populations strategies. Meanwhile, the percentage of participants with DI-GM ≥ 6 had a significantly negative correlation with NAFLD [OR (95%CI):0.59 (0.48, 0.71), $p < 0.001$], and the negative correlation remained robust after variable adjustment [OR (95%CI):0.65 (0.47, 0.90), $p = 0.010$] in Model 3.

Non-linear correlation

To explore the non-linearity of the associations of DI-GM and the NAFLD, a weighted multivariable–adjusted RCS analysis was executed in this study. As shown in Figure 2, no non-linear correlation of DI-GM and NAFLD was detected ($p = 0.810$ for non-linearity), and a negative dose–response correlation of DI-GM and NAFLD was identified ($p = 0.003$).

Subgroup analyses and sensitivity analysis

In this study, subgroup analysis was used to explore the specific correlations of DI-GM and NAFLD in different subgroups, indicating that the negative correlation of DI-GM and NAFLD was consistent (Figure 3). The results of the sensitivity analyses indicate that, even after further adjusting for physical activity, alcohol intake, medication

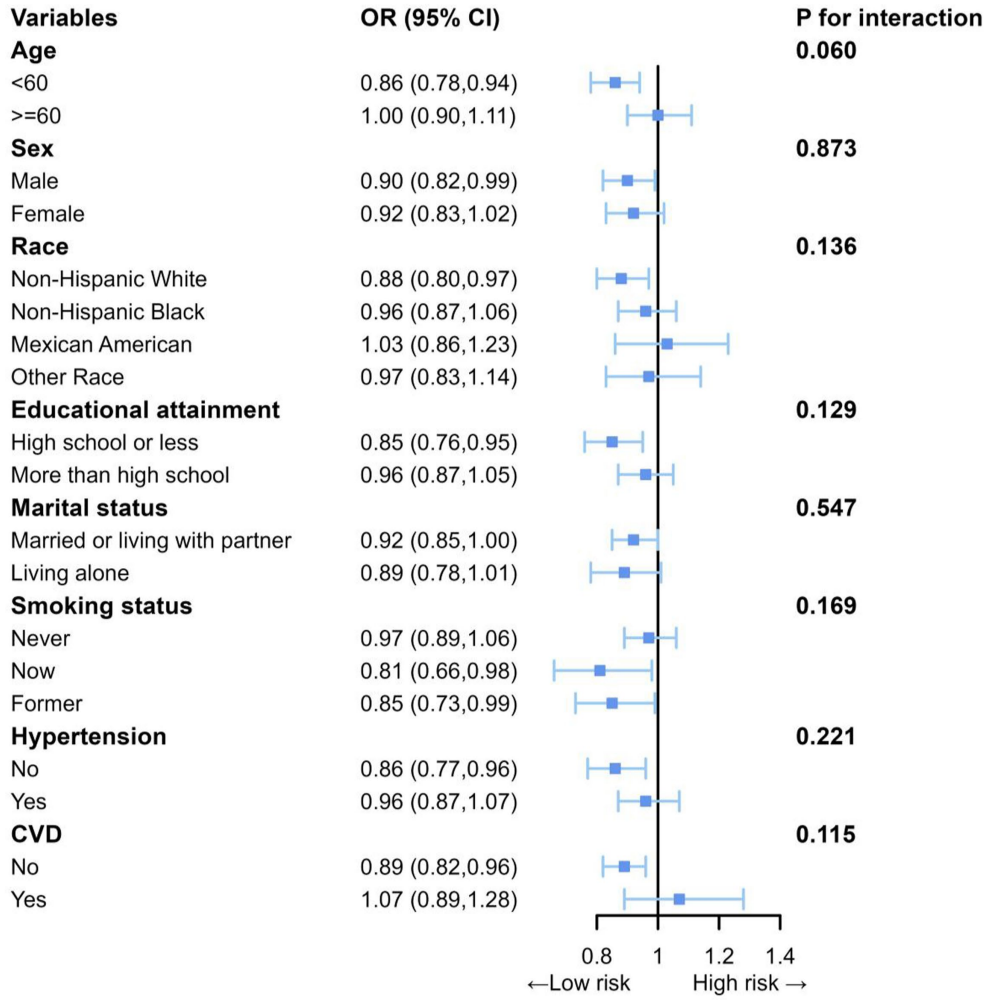

FIGURE 3
Subgroup analyses between DI-GM with NAFLD. Each subgroup analysis adjusted for age, sex, race, education attainment, marital status, BMI, PIR, smoking status, hypertension, DM, CVD, ALT and AST.

use, the positive correlation between DI-GM and NAFLD persists (Supplementary Table S3).

WQS regression

A weighted index was developed based on the WQS regression to assess the impacts and weight contributions of different DI-GM components on NAFLD (Figure 4). Herein, consistent variable adjustments were applied. The results demonstrated that soybean (27%), refined grains (17%), coffee (16%) and red meat (9%) had high weight contributions, while fiber (0%) and whole grains (0%) had low weight contributions.

Discussion

As demonstrated, DI-GM remained an independent risk factor for NAFLD after variable control. According to the RCS curves, DI-GM had negligible non-linear, dose-dependent association with NAFLD. Additionally, DI-GM was negatively related to the probability of NAFLD, suggesting that DI-GM can serve as an effective indicator for NAFLD, and adjustment of the diet structure could aid in preventing NAFLD.

In previous studies, DI-GM has been found to be associated with biological age through the mediation role of body mass index, while biological age is linked with various biomarkers possibly in relation with NAFLD (29). After consulting relevant literature, gut microbiota can interact with the pathogenesis of NAFLD by various mechanisms: (1) gut microbiota translocation: dysbiosis of gut microbiota induces translocation of bacteria or their metabolites from gut to liver, causing liver inflammation and damage (35–38); (2) production of endogenous ethanol: gut microbiota can produce endogenous ethanol, which may affect the metabolism and inflammatory response of liver (36, 39–44); (3) abnormal regulation of bile acid and choline metabolism: gut microbiota participates in the metabolism of bile acids and choline, and changes in these metabolites may affect liver health (22, 36, 41, 45–48); (4) endotoxemia: dysbiosis of gut microbiota could result in increased level of endotoxins (e.g., lipopolysaccharides), resulting in inflammation and insulin resistance (36, 49–53). Overall, GM is closely

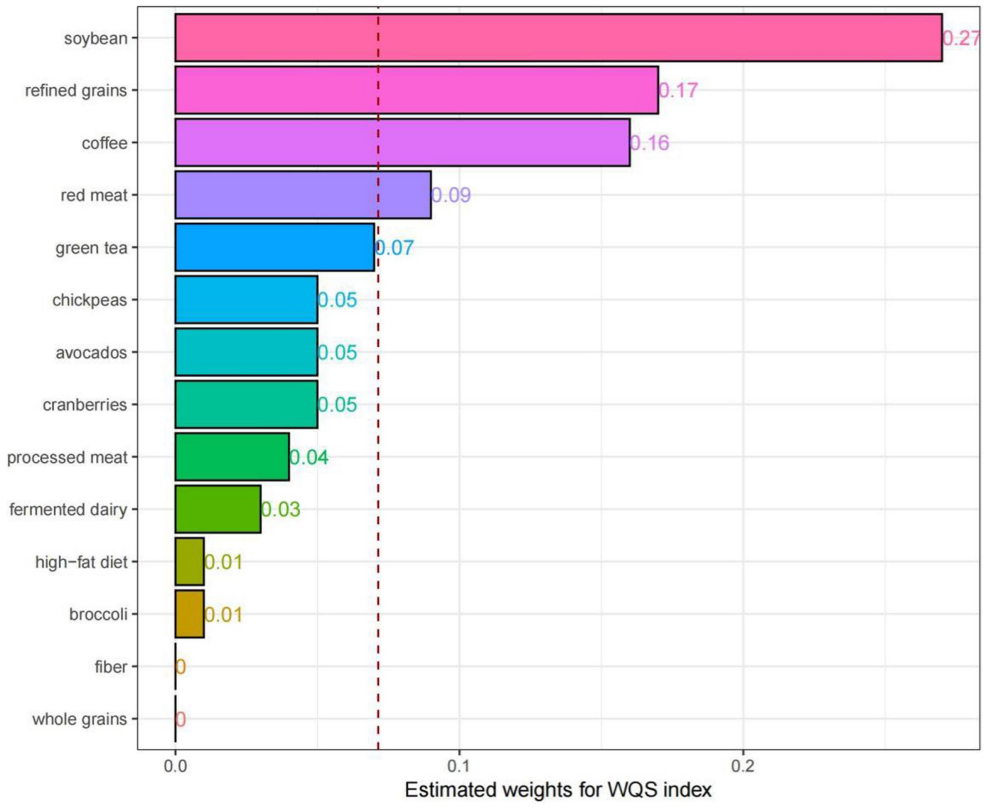

FIGURE 4
Weighted quantile sum (WQS) model regression index weights for the NAFLD, adjusted for age, sex, race, education attainment, marital status, BMI, PIR, smoking status, hypertension, DM, CVD, ALT and AST.

related to NAFLD, with a two-way influence through the gut-liver axis regulatory mechanism (22, 35, 36, 46, 49, 54–57). Therefore, a balanced gut microbiota helps maintain hepatic homeostasis, while disruption of GM may promote the incidence and progression of NAFLD.

The associations of various dietary indicators and the NAFLD have been thoroughly investigated. It has been demonstrated that specific dietary patterns could significantly reduce the risk of MASLD and relevant liver fibrosis (MASLD-LF) (58). Meanwhile, high scores of the HEI or the AHEI were significantly correlated with low incidence of MAFLD (59), and high-quality diets led to low mortality rates of MAFLD (60). To date, several dietary assessment tools, including HEI and AHEI, have been employed to evaluate dietary quality. Nevertheless, they barely considered the specific correlations of dietary components with gut microbiota, which are essentially important for liver health (61).

The DI-GM is distinctive in that it specifically examines the impact of the gut microbiota, addressing a gap in existing dietary indices. Based on the integrated effects of 14 key dietary components on gut microbiota, the DI-GM's structured approach not only offers insights into dietary impacts on the microbiota but also reflects overall dietary quality (26). The DI-GM provides an assessment framework, making it exceedingly valuable for exploration of the relationship between microbiota and metabolic health. Its correlation with the HEI exemplifies the dual applicability and scientific relevance of the DI-GM in assessing nutritional status and gut microbiota (26). Therefore, future dietary interventions could leverage the DI-GM to optimize dietary structures, thereby improving the conditions of GM and relieving NAFLD.

The results of the WQS regression model indicate that soy, refined grains, coffee, and red meat are key food components influencing NAFLD. Among them, soybean and coffee, as beneficial components of the DI-GM, may play critical roles in the prevention and management of NAFLD. Indeed, soybean can regulate lipid metabolism and oxidative stress, thus protecting the liver of NAFLD patients (62). Consequently, soybean may serve as an effective dietary intervention for NAFLD (62). Some studies claimed that coffee consumption could reduce the risk of NAFLD (63), while conflicting results regarding its impact on prevention of NAFLD have also been reported. For NAFLD patients, coffee intake can relieve liver fibrosis (64). On the contrary, refined grains and red meat, as detrimental components of the DI-GM, may elevate the risk of NAFLD. Indeed, the consumption of refined grains led to increased incidence of NAFLD, while the intake of whole grains may positively affect the clinical conditions of NAFLD patients and relieve disease progression (65). Furthermore, the consumption of red meat has been confirmed to have a positive correlation with NAFLD risk (66). Eating one serving of legumes per week instead of red or processed meats or poultry led to reduced incidence of NAFLD (67). However, the mechanism by which these foods influence the pathogenesis of NAFLD by modulating gut microbiota remains unclear. Moreover, the content of GM in dietary fiber and whole grains were relatively low, which may lead to the 0% of dietary fiber and whole grains of the DI-GM in the contributions of NAFLD.

This study provides epidemiological evidence supporting the inverse association between DI-GM scores and NAFLD prevalence.

These findings underscore the potential utility of DI-GM optimization as a preventive strategy in metabolic health management. To advance clinical translation, future longitudinal investigations should employ standardized DI-GM assessments across diverse cohorts to establish causal relationships, validate risk prediction models, and facilitate early risk stratification in susceptible populations.

Contributions and limitations

This study made contributions in several aspects. First, a broad cross-sectional approach leveraging NHANES data was used for the first time, revealing a linear negative correlation of the DI-GM related to GM diversity and NAFLD. Second, sampling weights, variable adjustment, and statistical tools were involved, resulting in significantly improved precision and robustness. Third, a WQS regression model was employed to evaluate the overall impacts and their weights of different DI-GM components on NAFLD.

Nevertheless, this study has several limitations: (1) the causality of DI-GM and NAFLD cannot be determined due to the cross-sectional approach used; (2) the findings may not be applicable for other populations; (3) recall bias arises from self-reported 24-h dietary records, which weakens the reliability of our results; (4) the confounding effects induced by measurement errors in unknown confounding factors or unmeasured variables cannot be completely ruled out.

Conclusion

DI-GM can effectively assess the influences of diet on GM, and it is negatively correlated with NAFLD risk. Soybean, refined grains, coffee, and red meat are key food components influencing NAFLD. Future studies may focus on the direct correlation of DI-GM with NAFLD, assessment of the effectiveness of prevention and treatment of NAFLD by improving DI-GM scores through dietary interventions.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found: the official website of the National Center for Health Statistics (URL: https://www.cdc.gov/nchs/nhanes).

Ethics statement

This study utilized anonymized data from the publicly accessible NHANES database, fundamentally ensuring the privacy and safety of the research subjects. The study has undergone strict ethical approval by the National Center for Health Statistics Ethics Review Committee, which conducted a comprehensive assessment and review of the research plan in accordance with internationally accepted ethical guidelines and regulatory requirements. Moreover, this study strictly adhered to the norms established by the Declaration of Helsinki, ensuring ethical compliance throughout the research process. Importantly, all participants in the NHANES project signed legally binding and ethically binding written consent forms before participating in the study. This consent procedure was completed properly and in strict accordance with standard processes before the initiation of data collection, ensuring that the research subjects were fully informed about the purpose, methods, risks, and benefits of the study through a comprehensive information disclosure and communication mechanism, and that they explicitly consented to participate in the survey on a fully voluntary and autonomous basis. 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 in accordance with the national legislation and institutional requirements.

Author contributions

YW: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Methodology. BZ: Writing – review & editing, Supervision, Validation, Funding acquisition. LF: Writing – review & editing, Supervision, Project administration, Validation. CC: Writing – review & editing, Funding acquisition, Project administration, Supervision, Validation. XF: Data curation, Formal analysis, Investigation, Writing – original draft, Methodology, Resources, Software, Visualization.

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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/fnut.2025.1573249/full#supplementary-material

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Association between the dietary index for gut microbiota and female infertility: a cross-sectional study of NHANES 2013–2018

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Background: Infertility poses a substantial societal and economic burden; however, current preventive strategies are limited. Recently, the relationship between gut microbiota and infertility has garnered increasing attention. The dietary index for gut microbiota (DI-GM) is a new index that reflects the diversity of the gut microbiota. However, its association with female infertility remains unclear.

Methods: This cross-sectional study included 3,053 women aged 18–45 years from the National Health and Nutrition Examination Survey (NHANES) database between 2013 and 2018. Infertility was defined based on responses to a questionnaire on reproductive health. The DI-GM score was calculated by averaging the intake from two 24-h dietary recall interviews. Weighted multivariable logistic regression, restricted cubic splines (RCS), and subgroup analyses were used to investigate the association between DI-GM and female infertility.

Results: Based on self-reported data, 370 participants (12.12%) were classified as infertile. A higher proportion of participants with lower DI-GM scores experienced infertility. Multivariable logistic regression analysis indicated a negative association between DI-GM and the risk of female infertility, regardless of whether the independent variable was analyzed as a continuous variable or in quartiles in the fully adjusted model (Model 3, continuous variable: OR = 0.89, 95% confidence interval (CI): 0.80–0.98, $p = 0.025$; Q4 *vs.* Q1: OR = 0.63, 95% CI = 0.42–0.94, $p = 0.032$, p for trend = 0.013). The RCS curves demonstrated a non-linear relationship between the DI-GM scores and infertility risk. Subsequent subgroup analyses corroborated the robustness of these findings.

Conclusion: These findings suggest a non-linear relationship between DI-GM and the risk of infertility in females, with lower DI-GM scores associated with a higher risk of infertility.

KEYWORDS

DI-GM, dysbiosis, gut microbiota, female infertility, NHANES

Introduction

Infertility is a widespread chronic condition defined as the inability to achieve clinical pregnancy after 12 months of regular unprotected sexual intercourse. Globally, approximately one in eight couples of childbearing age experience infertility or difficulty in maintaining pregnancy (1, 2). Female infertility is influenced by genetic, environmental, and lifestyle factors. Common etiologies include ovulatory dysfunction, endometriosis, polycystic ovary syndrome (PCOS), fallopian tube abnormalities, and immunological disorders (3). Numerous studies have identified various lifestyle factors, including dietary patterns, that are correlated with infertility in women (4–6). Factors such as smoking, alcohol consumption, obesity, chronic stress, and inadequate sleep adversely affect reproductive health. Inappropriate dietary habits are often associated with either excessive or insufficient calorie intake. Deficiency in essential nutrients can delay the onset of puberty and elevate the risk of ovulation disorders, thereby reducing fertility in women (7, 8). Given that infertility has emerged as the third most significant health issue following cancer and cardiovascular diseases, it presents medical challenges and engenders social issues with substantial economic and psychosocial implications (9). There is an urgent need to develop effective strategies to prevent and manage infertility, which poses a substantial threat to public health.

The gut microbiota is essential for human health and affects various physiological processes, such as nutrient absorption, intestinal mucosal growth, glycolipid metabolism, neurological function, and immune regulation (10–12). Recent studies have indicated that an imbalance in gut microbiome composition is closely linked to female reproductive diseases such as endometriosis, chronic pelvic pain, premature ovarian failure, ovarian aging, and PCOS (13–18). Notably, Qi et al. demonstrated that patients with PCOS have elevated levels of the gut microbe *Bacteroides vulgatus* and decreased concentrations of glycodeoxycholic acid and tauroursodeoxycholic acid. Mice that received fecal microbiota transplants from patients with PCOS or those colonized by *Bacteroides vulgatus* exhibited decreased interleukin-22 secretion and increased impairment of ovarian function, insulin resistance, altered bile acid metabolism, and infertility (17). Mikkelsen et al. reported that preconception antibiotic use, especially macrolides and sulfonamides, is associated with increased infertility risk (19). These findings suggest that dysbiosis of the gut microbiota and its metabolites can increase the risk of infertility.

Accumulating evidence indicates that dietary patterns substantially influence the composition of the gut microbiota and have been implicated in the etiology of female infertility (20–24). The Mediterranean diet (MD) is characterized by the traditional dietary patterns of populations residing in countries bordering the Mediterranean Sea. This dietary regimen emphasizes the consistent consumption of fruits, vegetables, legumes, whole grains, nuts, and olive oil as fundamental components of the daily nutritional intake. Studies have shown that the MD significantly improves human metabolic health. The main reason for these benefits is the ability of the MD to regulate the gut microbiota structure and function by increasing the alpha diversity indices of the gut microbiome (25–27). Studies have demonstrated that the MD positively impacts reproductive health, with a higher percentage of clinical pregnancies and live births (28–30). In contrast, the Western diet (WD) is characterized by an abundance of calories and a scarcity of fruits, vegetables, whole grains, fish, nuts, and seeds. This eating pattern is often linked to obesity, which can alter the gut microbiota composition. These changes in the gut microbiome can significantly affect fertility in both females and males (31–34).

Dietary patterns play a significant role in shaping gut microbiota composition, making it crucial to use dietary indices to understand the relationship between the gut microbiome and disease risk (20, 35). Commonly used indices include the Healthy Eating Index (HEI), Dietary Approaches to Stop Hypertension (DASH), and Mediterranean Diet Score (MDS) (36). Although these indices have demonstrated utility in assessing the correlation between dietary quality and health outcomes, investigations into their relationships with gut microbiota diversity and abundance have produced inconsistent results (37, 38). Kase et al. developed a new dietary index for gut microbiota (DI-GM) to address this inconsistency. This index evaluates the influence of diet on the gut microbiota through 14 components identified as beneficial or unfavorable to gut health, effectively capturing the relationship between dietary quality and gut microbiota diversity (39).

Recent findings have demonstrated that a lower DI-GM score is associated with a higher risk of diabetes, stroke, constipation, metabolic dysfunction-associated fatty liver disease, depression, and aging (40–45). Diabetes, depression, and aging are closely associated with infertility in women. However, the relationship between DI-GM and infertility remains unexplored. This cross-sectional investigation aimed to address this knowledge gap by examining the association between DI-GM and female infertility using NHANES data. This study also sought to elucidate valuable information for developing targeted dietary interventions to mitigate infertility.

Methods

Data source

This study analyzed data from the 2013–2018 NHANES, a comprehensive cross-sectional survey conducted biennially to collect data on the dietary habits, nutritional status, health conditions, and lifestyle behaviors of the non-institutionalized U.S. population using a multistage probability sampling methodology. These data are publicly accessible through the National Center for Health Statistics (NCHS), a division of the Centers for Disease Control and Prevention (CDC). The NCHS Ethics Review Board approved the NHANES protocols, and all participants provided written informed consent. Further information can be found at http://www.cdc.gov/nchs/nhanes/index.htm.

Study design and population

Our study focused on women aged 18–45 years who were not pregnant, representing the non-institutionalized civilian population in the United States. A comprehensive dataset encompassing DI-GM components and infertility status was acquired from an initial cohort of 29,400 study participants. The study excluded several groups of participants: males (n = 14,452), females outside the age range of 18–45 years (n = 10,625), individuals lacking DI-GM components (n = 614), and those without information on infertility (n = 656). The final analysis sample comprised 3,053 eligible participants (Figure 1).

Calculation of DI-GM

This study utilized the scoring system developed by Kase et al. to calculate the DI-GM using 14 foods and nutrients (39). The DI-GM includes 10 beneficial components (avocado, broccoli, chickpeas, coffee, cranberries, fermented dairy, fiber, green tea, soy, and whole grains) and four unfavorable components (red meat, processed meat, refined grains, and high-fat diets). We averaged the results from two 24-h dietary recall interviews for each participant to calculate the DI-GM scores. Participants with only one reliable dietary recall were excluded from the analysis (39). A score of 1 indicates consumption above the median for beneficial components and below the median for detrimental components, and a score of 0 indicates the opposite. The total score ranged from 0 to 13, with higher scores suggesting a healthier gut microbiota. Based on previous studies (41–43), participants were categorized into four groups: 0–3, 4, 5, and ≥ 6.

Definition of infertility

The dependent variable, infertility, was evaluated using two questions from the Reproductive Health Questionnaire. The first question (RHQ074) asked, "Have you ever tried unsuccessfully to conceive for a minimum of 1 year?" The second question (RHQ076) inquired, "Have you ever sought medical advice from a doctor or healthcare provider regarding difficulties in becoming pregnant?" Respondents who answered either question affirmatively were categorized as experiencing infertility, whereas those who answered both questions negatively were classified as not experiencing infertility.

Covariates

This study considered various factors, including demographic characteristics (age, race, marital status, education level, and poverty income ratio), lifestyle habits (alcohol consumption and smoking), health conditions (hypertension, diabetes, and dyslipidemia), and reproductive health factors (age at first menstruation, history of pelvic infection or inflammatory disease, use of birth control pills, and hormone therapy). Comprehensive information on the methods used to collect data on these variables can be found on the official NHANES website.

Statistical analysis

The surveys utilized a complex multistage clustered design, and all statistical analyses adhered to the NHANES sampling weights, as recommended by the CDC. We compared the participants' baseline

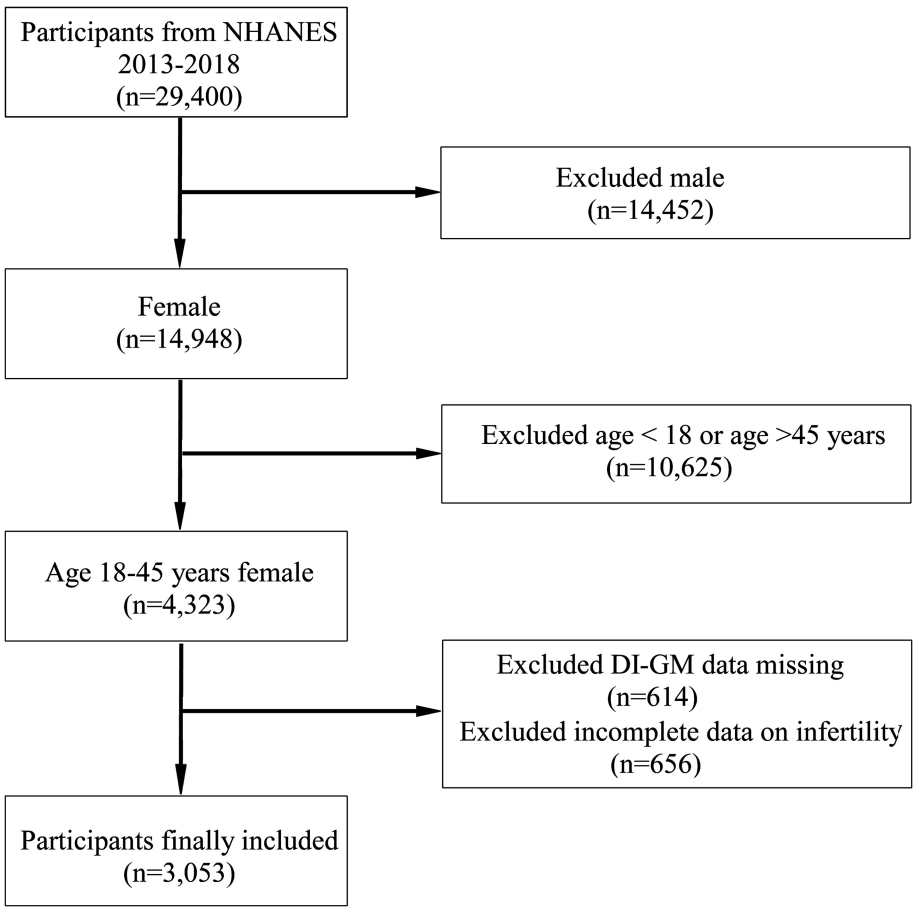

FIGURE 1
Flow chart for the inclusion and exclusion of study participants.

characteristics in the descriptive analyses based on infertility status and DI-GM quartiles. Continuous variables are reported as means with standard errors (SE), and categorical variables are expressed as numerical counts and percentage frequencies (%). A weighted linear regression model and chi-square tests were used to evaluate the baseline characteristics. For variables with missing data, continuous variables were imputed using the medians or means, depending on the distribution, and categorical variables were imputed using the modes.

We used weighted multivariate logistic regression models to examine the association between DI-GM and infertility risk while accounting for known or potential confounding variables. We also conducted subgroup analyses to explore the association between DI-GM and infertility across different demographic and clinical groups, considering factors such as age, BMI, PIR, PID, smoking, female hormones, and the presence of hypertension, dyslipidemia, and diabetes mellitus. We further employed restricted cubic spline (RCS) curves and threshold effect analysis to investigate the potential non-linear relationship between the DI-GM scores and infertility risk. Statistical analysis and data handling were conducted using R (version 4.4.0) and Zstats (version 1.0) software. Statistical significance was set at $p < 0.05$.

Results

Participant characteristics

As shown in Table 1, among the 3,053 eligible participants, 370 were infertile. Individuals in the infertile group tended to be older, have a higher body mass index (BMI), higher income, earlier age at menarche, and a higher prevalence of conditions such as being married, smoking, history of pelvic inflammatory disease (PID), use of female hormones, hypertension, dyslipidemia, and diabetes than those in the non-infertile group. Furthermore, the average DI-GM value was significantly lower in the infertility group than that in the non-infertility group (4.69 *vs.* 5.01, $p = 0.025$). Participants with lower DI-GM scores also exhibited higher triglyceride levels and fasting plasma glucose (FPG) and lower high-density lipoprotein cholesterol (HDL-C) levels ($p < 0.05$). Additionally, the prevalence of infertility among participants significantly decreased from Q1 to Q4 ($p = 0.041$, Supplementary Table S1), with notably lower rates observed in Q3 and Q4 (11.18 and 11.17%, respectively) than in Q1 and Q2 (16.32 and 16.16%, respectively). These observed variations suggest that the potential association between DI-GM and infertility requires further investigation.

Association between DI-GM and infertility

The correlation between the DI-GM score and the risk of female infertility is shown in Table 2. Logistic regression analysis revealed a significant negative association between DI-GM scores and infertility risk. When DI-GM was used as a continuous variable, the odds ratio (OR) of Model 1 (M1) was 0.89 (95% CI = 0.81–0.99, $p = 0.029$). After adjusting for demographic factors (M2) and in the fully adjusted model (M3), the OR values remained significant (M2: OR = 0.87, 95% CI = 0.79–0.96, $p = 0.010$; M3: OR = 0.89, 95% CI = 0.80–0.98, $p = 0.025$). Further analysis based on the DI-GM score groupings supported these findings. The results indicated that the highest group (Q4) was significantly associated with a reduced risk of infertility compared with the lowest group (Q1) across all three models (M1: OR = 0.65, 95% CI = 0.45–0.93, $p = 0.022$; M2: OR = 0.58, 95% CI = 0.39–0.86, $p = 0.010$; and M3: OR = 0.63, 95% CI = 0.42–0.94, $p = 0.032$). Moreover, trend analyses across all models demonstrated statistical significance ($p < 0.05$), further supporting the strong association between higher DI-GM scores and a decreased risk of infertility.

Non-linear relationship between DI-GM and infertility

RCS analysis was conducted to explore the relationship between DI-GM scores and infertility risk. The findings indicated a non-linear relationship between DI-GM and infertility risk (Figure 2). To examine this relationship in greater detail, a weighted two-segment linear regression model and a recursive algorithm were employed to conduct a threshold effect analysis. The analysis identified an inflection point at a DI-GM score of 8, with a log-likelihood ratio test showing significance at $p < 0.001$. For DI-GM scores below the threshold, each unit increase in the DI-GM was linked to a 14% reduction in the risk of infertility (OR = 0.86, 95%CI = 0.83–0.90, $p < 0.001$; Table 3). Conversely, when the DI-GM scores surpassed this threshold, each incremental unit was associated with a 197% increased probability of infertility (OR = 2.97, 95% CI = 2.15–4.12, $p < 0.001$; Table 3).

Subgroup analysis

To further investigate the association between DI-GM scores and the risk of female infertility, we analyzed various subgroups based on demographic and health factors. The results showed a significant inverse relationship between DI-GM and the risk of infertility, which remained consistent across various subgroups. These subgroups included individuals aged 35–45 years, those with obesity (BMI $\geq$ 30), individuals with lower income (PIR $\leq$ 1.3), smokers, those without a history of pelvic inflammatory disease (PID), and those not using female hormones. This association was also observed in individuals without hypertension or diabetes and those with dyslipidemia ($p < 0.05$, Figure 3). Furthermore, we found no statistically significant interactions between the DI-GM scores and covariates ($p > 0.05$, Figure 3). These extensive subgroup analyses provide strong evidence of a consistent association between DI-GM scores and the risk of female infertility across diverse population segments.

Discussion

In the present study, we demonstrated a significant association between DI-GM and female infertility. Our findings showed that the non-infertile group had significantly higher DI-GM scores than the infertile group. Higher DI-GM scores were significantly inversely associated with the risk of female infertility. We found that DI-GM was protective in subgroups aged 35–45 years, with BMI $\geq$ 30, PIR $\leq$ 1.3, smokers, without PID history, and not using female hormones. Using RCS analysis, we identified a non-linear relationship between DI-GM scores and infertility risk. Furthermore, we found a significant negative association between DI-GM and female infertility risk for scores below the inflection point of eight. However, above this threshold, a positive association with infertility risk was observed.

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TABLE 1 Basic characteristics of participants according to infertility status*.

Variable	Total (*n* = 3,053)	Non-infertility (*n* = 2,683)	Infertility (*n* = 370)	*p* value
Age, mean (SE), year	31.36 (0.26)	30.84 (0.26)	34.74 (0.56)	<0.001
BMI, mean (SE), kg/m^2	29.27 (0.28)	28.83 (0.27)	32.17 (0.81)	<0.001
PIR, mean (SE)	2.60 (0.07)	2.56 (0.07)	2.81 (0.11)	0.040
Menarche, mean (SE), year	12.58 (0.04)	12.61 (0.04)	12.36 (0.13)	0.048
Triglyceride, mean (SE), mg/dL	88.90 (1.51)	87.25 (1.71)	99.77 (5.51)	0.048
Fasting blood glucose, mean (SE), mg/dL	97.88 (0.43)	97.52 (0.42)	100.28 (1.32)	0.045
HDL-C, mean (SE), mg/dL	57.22 (0.49)	57.68 (0.50)	54.24 (1.29)	0.013
Race, *n* (%)				0.380
Mexican American	532 (13.17)	475 (13.37)	57 (11.84)	
Other Hispanic	316 (7.25)	289 (7.51)	27 (5.57)	
Non-Hispanic White	1,007 (54.88)	858 (54.01)	149 (60.61)	
Non-Hispanic Black	689 (13.91)	612 (14.17)	77 (12.24)	
Other Race - Including Multi-Racial	509 (10.78)	449 (10.94)	60 (9.73)	
Marital status, *n* (%)				<0.001
Married	1,361 (46.55)	1,137 (44.29)	224 (61.45)	
Widowed	14 (0.56)	11 (0.30)	3 (2.30)	
Divorced	207 (6.63)	185 (6.67)	23 (6.35)	
Separated	120 (3.05)	107 (3.12)	13 (2.59)	
Never married	928 (29.92)	865 (32.08)	63 (15.61)	
Living with partner	423 (13.29)	379 (13.53)	44 (11.69)	
Education level, *n* (%)				0.152
Less than high school	513 (12.19)	461 (12.33)	52 (11.33)	
High school or equivalent	684 (21.25)	613 (21.97)	71 (16.47)	
College or above	1856 (66.56)	1,609 (65.70)	247 (72.20)	
Hypertension, *n* (%)				<0.001
Yes	435 (11.69)	351 (10.37)	84 (20.41)	
No	2,618 (88.31)	2,332 (89.63)	286 (79.59)	
Dyslipidemia, *n* (%)				0.001
Yes	387 (12.10)	315 (10.99)	72 (19.48)	
No	2,666 (87.90)	2,368 (89.01)	298 (80.52)	
Diabetes, *n* (%)				<0.001
Yes	125 (3.52)	96 (2.94)	29 (7.30)	
No	2,928 (96.48)	2,587 (97.06)	341 (92.70)	
PID, *n* (%)				<0.001
Yes	145 (4.86)	108 (3.84)	37 (11.62)	
No	2,908 (95.14)	2,575 (96.16)	333 (88.38)	
Smoking status, *n* (%)				<0.001
Yes	795 (29.27)	664 (27.78)	131 (39.13)	
No	2,258 (70.73)	2019 (72.22)	239 (60.87)	
Drinking status, *n* (%)				0.109
Yes	2,113 (74.88)	1837 (74.26)	275 (78.91)	
No	940 (25.12)	846 (25.74)	95 (21.09)	

(Continued)

TABLE 1 (Continued)

Variable	Total (*n* = 3,053)	Non-infertility (*n* = 2,683)	Infertility (*n* = 370)	*p* value
Birth control pills, *n* (%)				0.071
Yes	1955 (71.42)	1,676 (70.62)	280 (76.65)	
No	1,098 (28.58)	1,007 (29.38)	90 (23.35)	
Female hormones, *n* (%)				0.033
Yes	197 (6.08)	167 (5.37)	30 (10.77)	
No	2,856 (93.92)	2,516 (94.63)	340 (89.23)	
DI-GM MEAN, mean (SE)	4.97 (0.05)	5.01 (0.05)	4.69 (0.13)	0.025

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; PIR, poverty impact ratio; PID, pelvic infection/inflammatory disease; DI-GM, dietary index for gut microbiota.
*Percentage estimates are nationally representative using survey weights.

TABLE 2 Association between DI-GM and female infertility.

Variables	Model 1		Model 2		Model 3	
	OR (95%CI)	*p* value	OR (95%CI)	*p* value	OR (95%CI)	*p* value
DI-GM	0.89 (0.81–0.99)	0.029	0.87 (0.79–0.96)	0.010	0.89 (0.80–0.98)	0.025
DI-GM group						
Q1 (0–3)	1.00 (Reference)		1.00 (Reference)		1.00 (Reference)	
Q2 (4)	0.99 (0.72–1.36)	0.944	0.99 (0.69–1.41)	0.947	1.05 (0.73–1.50)	0.812
Q3 (5)	0.65 (0.39–1.06)	0.090	0.60 (0.37–0.97)	0.044	0.61 (0.38–0.98)	0.050
Q4 (≥6)	0.65 (0.45–0.93)	0.022	0.58 (0.39–0.86)	0.010	0.63 (0.42–0.94)	0.032
p for trend	0.012		0.004		0.013	

Model 1: Crude.
Model 2: Adjusted for age, ethnicity, education level, PIR, and marital status.
Model 3: Further adjusted for smoking, drinking, dyslipidemia, diabetes, hypertension, menstrual status, PID, birth control pills, and female hormones.

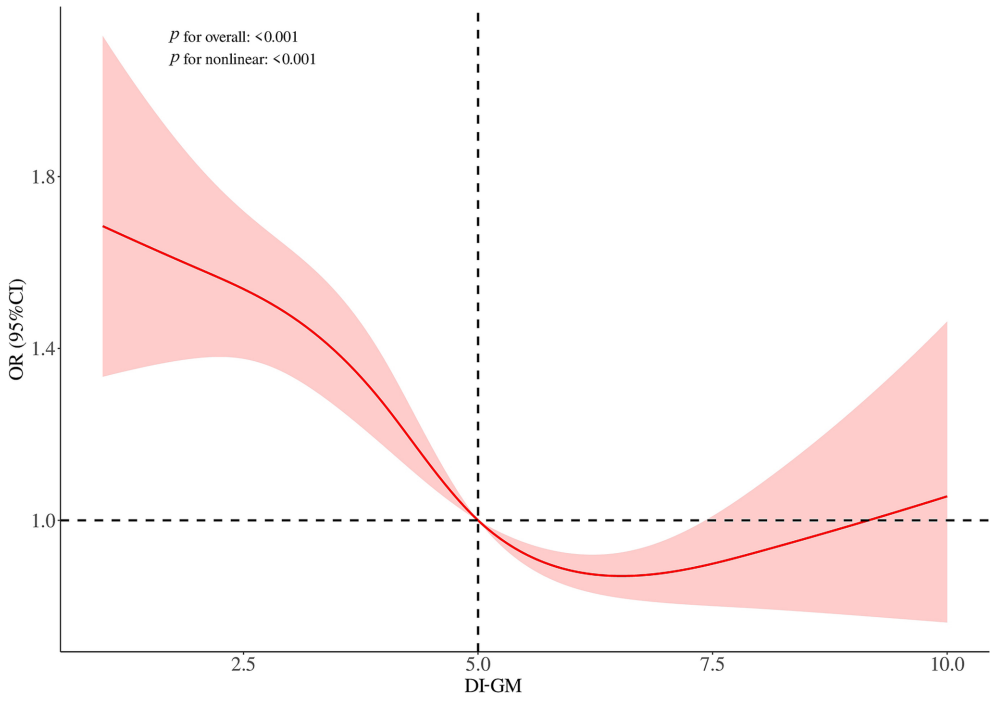

FIGURE 2
Restricted cubic spline plots for the association between DI-GM and infertility in women. Adjusted for age, ethnicity, education level, PIR, marital status, smoking, drinking, dyslipidemia, diabetes, hypertension, menstrual status, PID, birth control pill use, and female hormone use.

TABLE 3 Threshold effect analysis of DI-GM and female infertility*.

Outcome	Effect OR (95% CI)	*p* value
DI-GM		
Model 1	0.89 (0.86–0.91)	<0.001
Model 2		
Inflection point	8	
<8	0.86 (0.83–0.90)	<0.001
≥8	2.97 (2.15–4.12)	<0.001
p for likelihood test		<0.001

Model 1: Fitting model using standard linear regression.
Model 2: Fitting model using two-piecewise linear regression.
*Adjusted for age, ethnicity, education level, PIR, marital status, smoking, drinking, dyslipidemia, diabetes, hypertension, menstrual status, PID, birth control pills, and female hormones.

Recent studies suggest that dysbiosis of the gut microbiota may be a potential pathogenic factor in the development of PCOS, a prevalent endocrine disorder linked to a heightened risk of infertility (17, 46, 47). Qi et al. found a notable increase in *Bacteroides vulgatus* in the gut microbiota of individuals with PCOS, leading to a decrease in interleukin-22 (IL-22) secretion in the serum and follicular fluid, which plays a crucial role in mitigating the PCOS phenotype. Wu et al. identified enriched gut *Aspergillus tubingensis* in patients with PCOS, and this fungus induced a PCOS-like phenotype by inhibiting IL-22 secretion from intestinal group 3 innate lymphoid cells (ILC3s) via inhibition of the Aryl hydrocarbon receptor (AhR)-interleukin (IL)-22 pathway in mice. Furthermore, the gut microbiota influences reproductive health through various mechanisms, including the regulation of circulating sex hormone levels, immune system function, insulin sensitivity, and interactions with the gonadal microbiota. Additionally, healthy gut microbiota may contribute to a decreased risk of female infertility by modulating estrogen metabolism and controlling systemic inflammation (13, 14, 48, 49).

Evidence suggests that dietary practices substantially influence the composition of the gut microbiota, highlighting the critical role of dietary indices in elucidating the relationship between the gut microbiome and disease risk (20, 35). A healthy gut microbiome is characterized by high richness and diversity of microorganisms. The gut microbiota influences the host response to diet, while the host can also modify the gut microbiota through changes in dietary habits. An unhealthy diet high in fat and sugar may lead to decreased microbial diversity, reduced production of metabolites that support gut permeability, damage to the mucus layer, increased bacterial translocation, and higher lipopolysaccharide levels. These changes can trigger endotoxemia, chronic subclinical inflammation, and metabolic disorders (50, 51). The health of the intestinal microbiota is significantly associated with female infertility (13, 14, 17). Therefore, evaluating gut microbiota health by assessing dietary habits among women of reproductive age is valuable for public health (4). The DI-GM is a new dietary pattern index designed to predict gut microbiota health by identifying 14 dietary components that can have beneficial or unfavorable effects on the gut microbiome (39). This study examined the relationship between DI-GM and female infertility. We found that DI-GM has a non-linear inverse associated with the risk of female infertility. Our findings suggest that elevated DI-GM scores may protect against female infertility. Additionally, we observed that individuals with higher DI-GM scores had lower triglyceride and FPG levels and higher HDL-C levels. This may be linked to insulin resistance (IR), a common cause of infertility in PCOS, suggesting that a higher DI-GM may be associated with lower IR (52).

The association between higher DI-GM scores and a lower risk of female infertility highlights the potential of dietary interventions to improve gastrointestinal health. However, we observed a positive association between DI-GM and female infertility risk when the DI-GM scores were greater than eight in the threshold effect analysis. Based on the DI-GM calculation method, as the score increases, the consumption of dietary components with high energy densities, such as red meat and high-fat milk, declines (39). Our results suggest that inadequate energy intake may occur in women of childbearing age when the DI-GM scores exceed eight. These findings indicate that the best approach for women of reproductive age is to balance beneficial ingredients for gut health, represented by DI-GM, with less favorable ingredients. A balanced and healthy diet can boost fertility and improve the chances of conception by enhancing nutritional status. This balance is essential for reproduction as it helps regulate energy and nutrition. Additionally, the interactions between diet and microbiota, which influence human metabolism, should align with the physiological needs related to reproduction (20, 53–55).

This study had several strengths. After controlling for confounding factors, this is the first study to demonstrate a significant association between DI-GM and female infertility. These results indicate that lower DI-GM scores are associated with an increased risk of infertility in females. Additionally, this study established a non-linear relationship between DI-GM and infertility risk. Subgroup analysis further reinforced the robustness of these findings. Future longitudinal studies should explore the combined effects of dietary and gut microbiota interventions on reproductive health in diverse populations to validate these findings.

Our study had several limitations. First, the cross-sectional study design restricts the ability to establish a causal relationship between DI-GM and female infertility, underscoring the need for future longitudinal and prospective studies. Second, the DI-GM scores were calculated based on the intake data from 14 food components, leading to participant exclusion when data were missing, which may have introduced a selection bias. Moreover, the dependence on self-reported dietary information and the assessment of infertility using a reproductive health questionnaire increased the potential for recall and social desirability bias in this study. Third, despite adjustments for numerous potential confounders, the possibility of residual confounding and

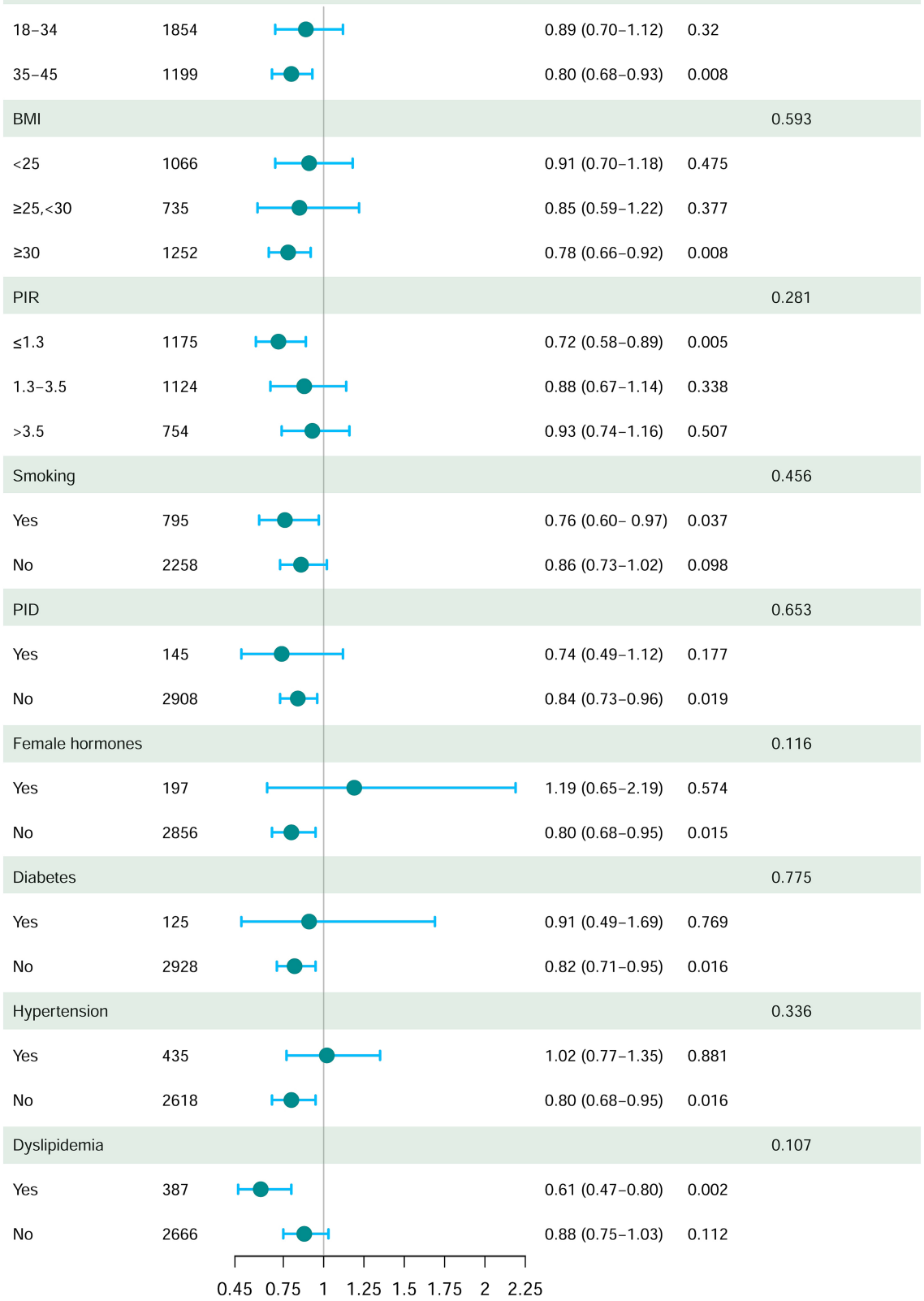

FIGURE 3
Forest plot of stratified analysis and interaction effects on the association between DI-GM and infertility in women. The model was adjusted for age, ethnicity, education level, PIR, marital status, smoking, drinking, dyslipidemia, diabetes, hypertension, menstrual status, PID, birth control pill use, and female hormone use.

unmeasured factors, such as dietary supplement use and undiagnosed reproductive disorders, cannot be entirely excluded. Fourth, DI-GM reflects dietary habits during data collection rather than long-term patterns; however, most adults maintain consistent diets unless they experience significant health issues, suggesting that the DI-GM reasonably represents typical dietary habits. Fifth, female infertility is affected by lifestyle factors such as occupational stress and physical activity. However, the DI-GM score does not comprehensively capture the influence of these factors on female infertility risk. Finally, the generalizability of the study findings is constrained because significant associations were primarily observed in the U.S. population. A more diverse representation of populations is necessary to validate these findings and enhance our understanding of the relationship between diet, gut microbiota, and the risk of female infertility. To determine the causal relationship between DI-GM and female infertility, future research should consider longitudinal study designs or microbiome sequencing data.

Conclusion

This study found a significant negative association between DI-GM and the risk of infertility in women. Interestingly, the relationship between the DI-GM scores and infertility risk demonstrated a non-linear pattern. As a new dietary quality index that reflects gut microbiota diversity, further research and interventions using DI-GM could help develop strategies to prevent and reduce the risk of female infertility.

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://wwwn.cdc.gov/nchs/nhanes/Default.aspx.

Ethics statement

The studies involving humans were approved by the National Center for Health Statistics Ethics Review Committee. 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

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Conflict of interest

The authors declare that this study was conducted without any commercial or financial relationships that could be construed as potential conflicts of interest.

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Supplementary material

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Therapeutic efficacy of fecal microbiota transplantation in severe food intolerance: a case report

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This report presents the first documented application of fecal microbiota transplantation (FMT) for the management of extensive multi-food intolerance involving 52 specific foods in a pediatric patient with autism spectrum disorder (ASD). A 7 years-old autistic child was diagnosed with food intolerance to 52 items, presenting with generalized rashes, diarrhea, and malnutrition (BMI of 12.9) upon exposure or ingestion of the implicated foods. The child received oral fecal microbiota capsule treatment, with a daily dose of nine capsules (a total of 120 capsules per course) for two consecutive treatment courses. The rashes resolved, the child regained tolerance to previously intolerable foods, nutritional status improved, and stool consistency normalized. This case suggests that FMT may hold therapeutic potential for managing food intolerance in autistic patients.

KEYWORDS

food intolerance, fecal microbiota transplantation, gut microbiota, autism spectrum disorder, gastrointestinal symptoms

1 Introduction

Food intolerance (FI) refers to non-immune-mediated adverse reactions to food or food components at normal tolerated doses, including metabolic, toxic, pharmacological, and undefined mechanisms (1–6). It's prevalence ranges from 15% to 45% (7). The main mechanisms underlying FI include these following aspects: Firstly, Certain foods contain pharmacologically active com-pounds (e.g., histamine, monosodium glutamate, caffeine) that can induce physiological responses like smooth muscle contraction and inflammation, contributing to FI (8). Secondly, Lactase deficiency is a major cause of lactose. Similarly, deficiencies in histamine-degrading enzymes, such as diamine oxidase (DAO) and histamine-N-methyltransferase (HNMT), can lead to histamine accumulation and FI symptoms (9). What's more, Food additives and preservatives (e.g., nitrates, nitrites) may also induce FI reactions (10, 11). The precise mechanisms remain unclear,

but these components can trigger adverse reactions in susceptible individuals. Diagnosis of FI requires detailed medical history, dietary and lifestyle assessments, and laboratory tests (e.g., blood and stool analyses) or imaging to exclude organic diseases and food allergies (FA) (12). Currently, the double-blind, placebo-controlled oral challenge (DBPCFC) is considered the gold standard for FI diagnosis, involving food elimination and gradual reintroduction to identify triggers (13). Food-specific IgG or IgG4 serological testing is widely used in clinical practice (14).

The gut microbiota plays a crucial role in the pathogenesis of FI. FI is often associated with gut dysbiosis, characterized by the reduction or increase of specific microbial populations, leading to impaired gut barrier function, enhanced inflammatory responses (5, 15), and abnormal metabolic functions. FMT, as a therapeutic approach to modulate the gut microbiota, may restore microbial balance and improve FI symptoms by transplanting healthy donor microbiota into the recipient. Annabel Clancy and Thomas Borodyhave (16) demonstrated that FMT can significantly alleviate FI symptoms in patients with irritable bowel syndrome (IBS), highlighting the potential therapeutic value of gut microbiota in FI. FMT can also improve both autism symptoms and gastrointestinal symptoms in patients with ASD (17).

To date, there have been no reports on improvements in food intolerances in patients with ASD treated with FMT. Therefore, this case report of improvement in food intolerance and ASD symptoms after FMT in a male patient constitutes an important insight into a possible involvement of the gut microbiome in the pathogenesis of food intolerances.

2 Case report

The patient is a male born in 2017. Family history was negative for allergies or psychiatric disorders.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University [Approval No. 2923JS (11)]. And the informed consent form for FMT was signed by the patient's legal guardian (PDF1).

In October 2019, he was diagnosed with "Autistic Spectrum Disorder" due to symptoms including poor language communication skills, irritability, social withdrawal, loose stools, multiple scattered eczema lesions and sleep disturbances. In December 2019, a 90-item food-specific IgG antibody test identified intolerance to 52 foods (Table 1), with severe reactivity to staples like rice, wheat, and milk. Elimination of these foods improved language expression, eczema, and sleep quality. Reintroduction of intolerant foods consistently provoked rashes and sleep disturbances, confirming provocation test results. Additionally, allergen-specific IgE antibody testing for inhaled and ingested allergens showed no significant abnormalities. The delayed diagnosis of FI in this pediatric patient with ASD and communication impairments was attributable to its less overt clinical manifestations compared to food allergy, compounded by age-related diagnostic challenges. The patient adheres to a structured food diary with rotation of tolerated foods. The patient presents with persistent generalized rashes, frequent diarrheal episodes, and an immunocompromised state, leading to multiple hospitalizations for recurrent pneumonia.

In July 2024, the child received oral fecal microbiota capsule treatment.

Dosage and administration: three capsules per dose, three times daily, administered with warm water 30 min prior to meals. Each treatment course consists of 120 capsules (4.2×10^{13} CFU/course) (PDF2–Daily Microbial Suspension Logbook), with the therapy to be continued consecutively for two complete courses. Capsule specification: No. 3 pediatric-sized capsules are to be utilized for encapsulation.

The donor microbiota was sourced from a rigorously screened healthy adult who had no comorbidities or disorders known to be associated with changes in gut microbiota, were chosen as donors. Donor stools were screened for enteric pathogens including parasites (Entamoeba histolytica, Giardia) and bacteria (Salmonella, Shigella, Escherichia coli, Campylobacter, Yersinia, and C. difficile). The donors were accepted only if HAV IgM, HBsAg, anti-HCV antibodies, anti-human immunodeficiency virus antibodies, IgM antibodies against cytomegalovirus and tests for syphilis were negative. The stool sample was not accepted if donors had taken antibiotics or probiotics in previous 3 months. FMT possesses repeated microfiltration, centrifugation, washing, discarding resuspension and capsules preparation based on the automatic microfiltration machine (GenFMTer, Nanjing, China) in a biosafety level-3 laboratory (18, 19) and prepared by the Microecology Center of the First Affiliated Hospital of Guangdong Pharmaceutical University. Viable bacterial counts in all capsule preparations were validated to meet international standards (18, 20) prior to lyophilization. Furthermore, proactive donor fecal sample screening was implemented in response to real-time adverse event monitoring, with no FMT-related adverse events reported to date.

September 2024: After two courses of FMT capsules, the child's symptoms significantly improved. The child tolerated previously intolerant foods (e.g., rice, wheat, soy, peanuts, and milk). No new rashes appeared, the existing rashes resolved (Figure 1), stool consistency normalized (Figure 1), and nutritional status improved with a gradual in-crease in body mass index (BMI) (Table 2).

During the therapeutic course, the patient exhibited good tolerability and maintained high adherence to the prescribed pharmacological regimen. The legal guardian reported significant improvement in the patient's rash condition post-capsule administration, with no pruritus or other adverse symptoms, demonstrating good acceptance. No adverse reactions were observed during the treatment.

December 2024: A repeat 90-item food-specific IgG antibody test showed significant improvement after two courses of fecal microbiota transplantation (Table 1). Post-FMT reassessment of food-specific IgG antibodies demonstrated a marked reduction in both the number and magnitude of food intolerances (Table 3).

The chronological summary of the patient's previous diagnostic and therapeutic interventions is systematically outlined in Figure 2.

3 Discussion

This case report demonstrates notable clinical and methodological advancements in managing severe FI through FMT. Notably, it represents the first documented application of

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TABLE 1 Positive items of 90-item food-specific IgG antibody test.

Food	Year	
	2019	2024
Peanuts	Grade 3	Grade 3
Milk	Grade 3	Grade 2
Soybeans	Grade 3	Grade 3
Eggs	Grade 3	Grade 3
Rice	Grade 3	Grade 2
White soft cheese	Grade 3	Grade 1
Wheat	Grade 3	Grade 1
Almond	Grade 2	Grade 3
Yogurt	Grade 2	Grade 0
Sunflower seeds	Grade 2	Grade 2
Mustard	Grade 2	Grade 2
Pumpkin	Grade 2	Grade 1
Barley	Grade 2	Grade 1
Millet	Grade 2	Grade 1
Broccoli	Grade 2	Grade 1
Garlic	Grade 2	Grade 1
Black walnuts	Grade 2	Grade 1
Goat's Milk	Grade 2	Grade 0
Potatoes	Grade 2	Grade 0
Buckwheat	Grade 2	Grade 0
Tomatoes	Grade 2	Grade 0
Cashew	Grade 2	Grade 3
Mixed Peas	Grade 1	Grade 1
Onions	Grade 1	Grade 1
Cheddar cheese	Grade 1	Grade 1
Cinnamon	Grade 1	Grade 1
Eggplants	Grade 1	Grade 0
Green peppers	Grade 1	Grade 0
Parsley	Grade 1	Grade 0
Cabbage	Grade 1	Grade 0
Carrots	Grade 1	Grade 0
Pineapples	Grade 1	Grade 0
Green beans	Grade 1	Grade 0
Cantaloupe	Grade 1	Grade 0
Rye	Grade 1	Grade 0
Butter	Grade 1	Grade 0
Honey	Grade1	Grade 0
Crab	Grade 1	Grade 0
Shrimp	Grade 1	Grade 0
Cod	Grade 1	Grade 0
Clams	Grade 1	Grade 0
Sardines	Grade 1	Grade 0
Lobster	Grade 1	Grade 0
Oysters	Grade 1	Grade 0
Scallions	Grade 1	Grade 0
Lettuce	Grade 1	Grade 0
Cucumbers	Grade 1	Grade 0
Watermelon	Grade1	Grade 0
Bok choy	Grade 1	Grade 0
Pomelos	Grade 1	Grade 0
Bananas	Grade 1	Grade 0
Oranges	Grade 1	Grade 0
Chili pepper	Grade 0	Grade 1
Corn	Grade 0	Grade 1
Mangetout	Grade 0	Grade 1
Mushroom	Grade 0	Grade 1

FMT for severe FI (52 items) in pediatric ASD, addressing a critical therapeutic gap in this complex patient population. Furthermore, the non-invasive oral capsule administration protocol overcomes procedural limitations typically encountered in ASD patients, enhancing clinical feasibility while maintaining therapeutic efficacy. Importantly, the intervention achieved multidimensional improvements encompassing cutaneous (rash resolution), gastrointestinal (stool normalization), immunological (restored food tolerance), and nutritional (BMI elevation) domains, suggesting systemic biological effects beyond symptomatic relief. Additionally, the standardized dosing regimen (nine capsules/day × 120 capsules/course × 2 courses) establishes a replicable framework for future trials. This correlation between microbiota modulation and regained oral tolerance aligns mechanistically with emerging evidence on gut microbiome-mediated antigen processing, thereby strengthening the biological plausibility of FMT as a disease-modifying therapy for FI.

Food intolerance can be caused by a variety of mechanisms, including enzyme deficiencies (e.g., lactase deficiency), pharmacological effects, irritant reactions, and toxicological responses. The gut microbiota plays a pivotal role in the pathogenesis of food intolerance.

The gut microbiota is involved in the pathogenesis of food intolerance caused by lactase deficiency. Lactase deficiency leads to undigested lactose interacting with the intestinal microbiota then produce short-chain fatty acids (SCFA) (acetate, propionate) and gases (hydrogen, carbon dioxide). When the amount of lactose exceeds the fermentation capacity of the colonic microbiota, or when the absorption capacity for SCFA in the colon is overwhelmed, diarrhea occurs. Brandao Gois et al. (21) found that, higher *Bifidobacterium* abundance in lactose intolerance individuals (P Wilcox = 4.56×10^{-9}).

The gut microbiota participates in the pathogenesis of FI associated with Non-Celiac Gluten Sensitivity (NCGS): M. Daulatzai et al. (22) found that NCGS patients exhibit gut microbiota dysbiosis, characterized by reduced beneficial

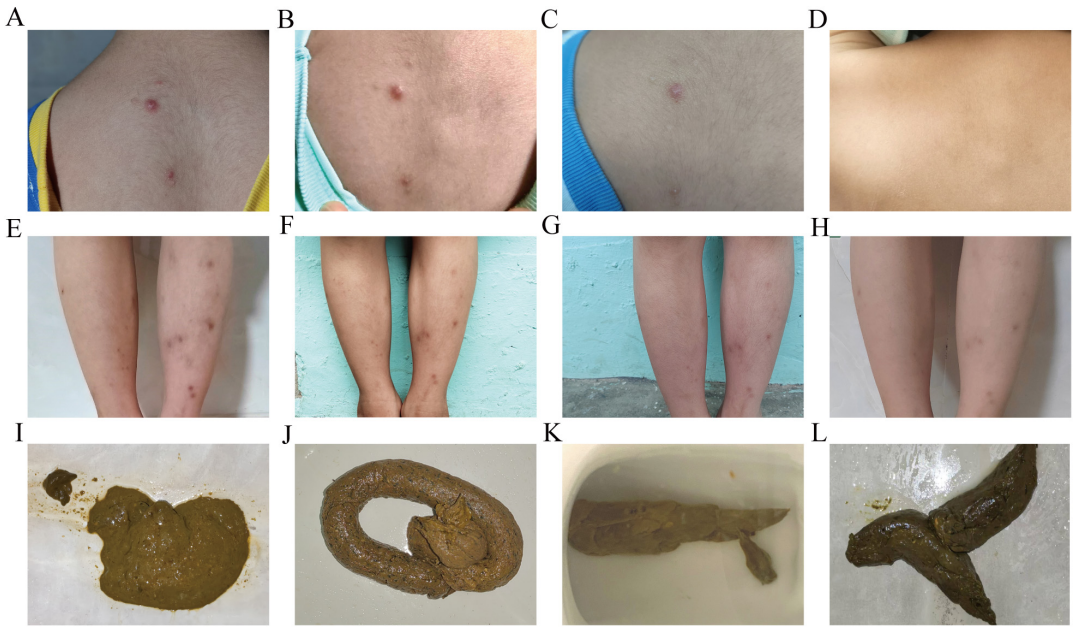

FIGURE 1
Changes in maculopapular rash and fecal characteristics after taking fecal microbiota capsules. **(A–D)** Maculopapular rash on the trapezius region: **(A)** on 2024-07-22; **(B)** on 2024-08-07; **(C)** on 2024-08-22; **(D)** on 2025-03-22. **(E–H)** Maculopapular rash on both lower legs: **(E)** on 2024-07-22; **(F)** on 2024-08-22; **(G)** on 2024-09-22; **(H)** on 2025-03-22. **(I–L)** Fecal characteristics: **(I)** on 2024-07-21; **(J)** on 2024-08-22; **(K)** on 2024-10-21; **(L)** on 2025-3-22.

bacteria (e.g., *Bifidobacterium*) and increased pro-inflammatory bacteria (e.g., *Enterobacteriaceae*, *Escherichia coli*, and *Firmicutes*). This dysbiosis may contribute to bloating through enhanced fermentation. Molecular studies highlight increased Claudin-4, a tight junction protein regulating paracellular permeability, supporting the "leaky gut" hypothesis. Hansen et al. (23) demonstrated that an 8 weeks low-gluten diet in 60 Danish adults reduced fecal *Bifidobacterium*, *Dorea*, *Blautia*, *Lachnospiraceae*, and butyrate-producing bacteria (*Anaerostipes hadrus*, *Eubacterium hallii*), alongside decreased postprandial hydrogen breath and bloating.

The gut microbiota is involved in the pathogenesis of FI induced by FODMAPs. FODMAPs, short-chain carbohydrates including lactose, fructose, sugar alcohols, fructans, and galacto-oligosaccharides (GOS), are naturally present in fruits, vegetables, grains, and dairy products. Their malabsorption results from deficiencies in brush border enzymes (e.g., lactase, sucrase) in the small intestine, leading to osmotic water retention and diarrhea. Dysregulated gut microbiota further exacerbates this condition, as pathogenic bacteria ferment undigested FODMAPs in the colon, producing excessive hydrogen, methane, and acidic by-products. This accumulation of gas, liquid, and acids stimulates the intestinal wall, causing FI symptoms such as bloating, abdominal pain, and diarrhea. The gut microbiota is implicated in the pathogenesis of histamine intolerance.

Histamine intolerance arises from impaired histamine degradation due to reduced activity or levels of histamine-metabolizing enzymes. Consumption of histamine-rich foods, or DAO-inhibiting medications elevates exogenous histamine. In these patients, gut dysbiosis is characterized by increased *Proteus* bacteria, damaging epithelial cells and DAO production. Such dysregulation exacerbates endogenous histamine levels, worsening FI symptom (24). Histamine-intolerant individuals also exhibit reduced beneficial bacteria (e.g., *Prevotellaceae*, *Ruminococcus*, *Faecalibacterium prausnitzii*) and increased histamine-secreting bacteria (e.g., *Staphylococcus*, *Proteus*, *Clostridium perfringens*, *Enterococcus faecium*) (25).

The association between gut microbiota dysbiosis and FI is increasingly recognized, highlighting the potential of microbiota-targeted therapies in managing FI. Probiotics and prebiotics have been explored as treatments since 2001 (26), with recent studies supporting probiotic supplementation for lactose intolerance (27). Animal studies further validate their efficacy. Ardizzone et al. (28) demonstrated that a novel therapeutic formulation (NTN) containing *Lactobacillus acidophilus* and *Lactobacillus reuteri* restored intestinal barrier integrity and permeability in mice with diet-induced FI, alleviating related symptoms. Ferrari et al. (29) highlighted probiotics' role in modulating gut microbiota, reducing ER stress, mitigating inflammation, and enhancing barrier function, collectively improving FI. Additionally, Besseling-van der Vaart et al. (30) showed that the multi-strain probiotic Ecologic® Tolerance (Syngut™) enhanced β-galactosidase activity, strengthened epithelial barriers, and improved resistance to digestive enzymes and bile salts *in vitro*.

These findings collectively underscore the therapeutic potential of probiotics in addressing gut microbiota dysbiosis and alleviating FI symptoms, providing a foundation for further clinical investigations.

After two consecutive treatment of oral fecal microbiota capsule, retesting of 90-food-specific IgG antibodies revealed decreased intolerance levels to most dietary antigens. Paradoxically, cashews and almonds progressed from moderate to severe

TABLE 2 Changes in body mass index (BMI) after taking in the fecal microbiota capsules.

Time	Height	Weight	BMI
22-07-2024	115	17	12.9
07-08-2024	115	17.7	13.4
22-08-2024	115	18.3	13.8
22-09-2024	116	18.75	13.9
22-10-2024	116	19	14.1
22-11-2024	116	20	14.9
22-12-2024	117	20.5	15
22-01-2025	118	21	15.1
22-02-2025	120	22	15.3
22-03-2025	120	22.2	15.4

TABLE 3 The alterations in the 90-item food-specific IgG antibody panel were analyzed before and after the administration of fecal microbiota capsules.

Grade	Year	
	2019	2024
Grade 0	38	65
Grade 1	30	16
Grade 2	15	4
Grade 3	7	5

intolerance, while corn, mushrooms, and capsaicin transitioned from tolerable to mild intolerance. We hereby propose the following discussion points.

Cashew and almond intolerance progressed from moderate to severe. These nuts are rich in lectins, which are resistant to high temperatures and enzymatic digestion in both rodents and humans (31). Emerging evidence highlights that children with ASD frequently exhibit depletion of butyrate-producing commensals, particularly *Faecalibacterium prausnitzii* (32). Post-FMT fluctuations in SCFA concentrations, notably butyrate, may activate GPR41/GPR43 receptors to modulate host energy metabolism and anti-inflammatory responses (33). However, altered intestinal transit time secondary to SCFA shifts could paradoxically prolong luminal exposure to undegraded dietary lectins. Lectins can translocate across the intestinal barrier into the bloodstream, where they deposit on blood and lymphatic vessel walls, stimulating the immune system (34), ultimately leading to elevated IgG levels. Lectins, through binding to glycans on the intestinal mucosa, may disrupt mucin polysaccharide architecture, impairing bacterial adhesion and proliferation (35), while concurrently inhibiting brush-border enzyme activity (e.g., disaccharidases), thereby exacerbating maldigestion and nutrient malabsorption (36, 37). Furthermore, lectin-mediated agglutination of beneficial symbionts may reduce their ecological fitness (38, 39), creating niches for opportunistic pathogens such as *Escherichia coli* and *Lactobacillus* spp. to proliferate. This dysbiosis disrupts microbial equilibrium, thereby contributing to fluctuations in IgG levels (40).

What's more, New-onset mild intolerance to mushroom, corn, and capsaicin was observed. Notably, chitin—a fungal polysaccharide abundant in mushrooms—alters microbial community structure (41), and incomplete engraftment of donor-derived chitinolytic taxa could manifest as transient reactivity to mushroom components. The metabolism of resistant starch (e.g., maize-derived) relies on *Clostridium butyricum*-encoded amylases (42), whereas capsaicin a TRPV1 receptor agonist, demonstrates tolerability closely linked to gut microbiota composition.(43). FMT-induced dysbiosis may disrupt these specialized metabolic pathways, potentially explaining transient post-FMT intolerances.

Current understanding of post-FMT microbiota engraftment remains incomplete. Analogous to organ transplantation, FMT faces inherent challenges of "microbiota rejection," wherein host immune and ecological factors limit donor strain persistence (44). Chen et al. demonstrated that donor strain engraftment rates rarely exceed 65%, with most clinical cohorts achieving < 30% colonization (45). While microbial network topology may attain dynamic equilibrium within weeks to 3 months post-FMT, functional stabilization (e.g., metabolic cross-feeding networks) likely requires extended timelines (46), paralleling gradual host physiological recovery. Delayed colonization of keystone taxa (e.g., *F. prausnitzii*) may thus underpin *de novo* food reactivity during this transitional phase.

Above all, these findings indicate that dysbiosis of the gut microbiota plays a significant role in the development of various types of FI. The mechanisms of FI in this case suggest a multifactorial origin of FI. FMT may be an effective intervention to restore microbial balance, reduce fermentation, repair the intestinal barrier, and reduce the levels of pro-inflammatory markers such as IFN-γ and histamine. This treatment likely contributed to the significant improvement in the child's symptoms, including the resolution of rashes, normalization of stool consistency, and improvement in BMI. The successful clinical response supports the role of microbiota modulation in the treatment of complex FIs.

Fecal microbiota transplantation is a promising therapy for chronic diseases associated with gut microbiota alterations (47). Comparative analysis of FMT across distinct disease entities is necessary. Key mechanistic insights are summarized below: The therapeutic mechanism of FMT in IBS emphasizes gut dysbiosis-driven visceral hypersensitivity, characterized by overproliferation of Gram-negative bacteria such as *Proteus mirabilis* and depletion of probiotics including *Lactobacillus rhamnosus GG*. Dysregulated microbial metabolites, such as LPS, suppress resolvin D1 synthesis in colonic tuft cells via TLR4/MyD88 signaling, perpetuating inflammatory cascades and nociception (48).

In the management of FI,FMT primarily enhances intestinal barrier function and energy metabolism through probiotic engraftment to cure FI. For instance, lactose intolerance improvement correlates with increased *Bifidobacterium* abundance, whose β-galactosidase activity facilitates lactose digestion without generating gas byproducts (e.g., hydrogen, carbon dioxide, methane) that drive bloating.

In the management of ASD,FMT reshapes gut microbiota by enriching beneficial taxa (*Bacteroides fragilis, Lactobacillus reuteri*) while suppressing pathobionts (*Clostridiales, Eubacterium coprostanoligenes*). This modulates neuroactive metabolites (4-EPS, SCFAs) and neurotransmitters (serotonin, dopamine), restoring gut barrier integrity and suppressing inflammation. These effects synergistically ameliorate ASD core symptoms and gastrointestinal

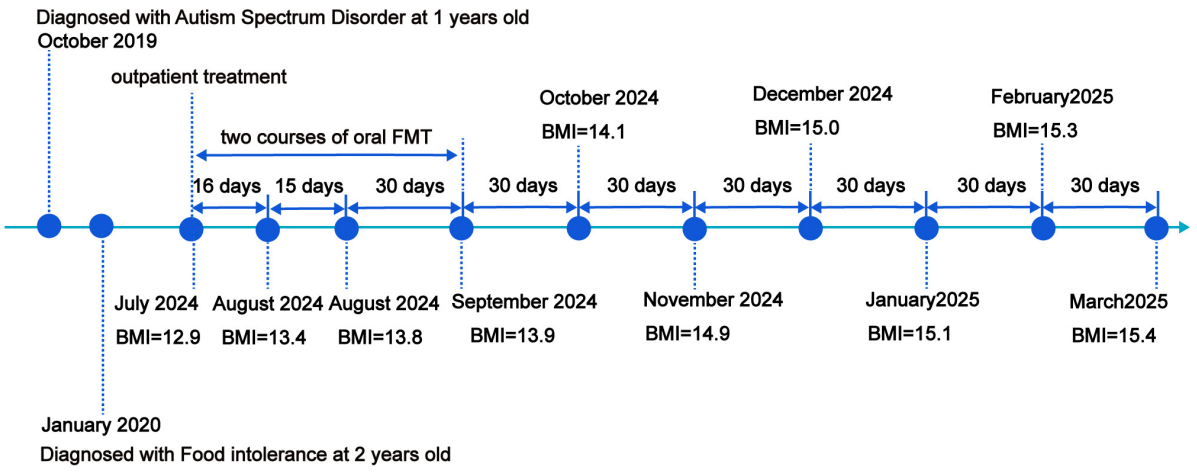

FIGURE 2
Timeline of clinical findings and nutritional intervention.

comorbidities via vagal nerve and hypothalamic-pituitary-adrenal (HPA) axis cross-talk.

Emerging evidence highlights that increased abundance of *Bifidobacterium* (49–51) in the gut microbiota is associated with clinical improvement in FI, IBS, and ASD. Notably, reduced *Faecalibacterium* (52–54) levels have been consistently reported across these three conditions, while ASD-specific dysbiosis is further characterized by overproliferation of *Sutterella* (55). Current research confirms the therapeutic efficacy of *Bacteroides fragilis* strain BF839 in ASD intervention; however, no specific microbial strains have yet been identified for targeted management of IBS or FI.

However, this study has several limitations. First, this study's sample size was small, and studies with larger sizes and control group are needed for further exploration. Second, 8 months' observations can't fully reflect the effects of FMT on developing FI symptoms. Studies with longer follow-up are needed to characterize the long-term efficacy and safety of FMT for pediatric patients. Finally, This case lacked pretreatment assessment of DAO, zonulin, endotoxin, serum histamine, SCFAs, and gut microbiota composition, The absence of zonulin and LPS measurements in this study may limit comprehensive evaluation of intestinal barrier integrity and systemic inflammation. For instance, Fasano et al. (56) demonstrated that zonulin serves as a sensitive biomarker of intestinal permeability, with dynamic changes reflecting FMT-induced mucosal repair. LPS, a key driver of gut hyperpermeability, inhibits tight junction proteins (e.g., occludin, claudin-1) via the TLR4/MyD88 pathway, directly compromising barrier function (45). The lack of LPS quantification precludes definitive conclusions regarding FMT-mediated LPS reduction and tight junction restoration.

Similarly, SCFA levels—critical mediators of microbiota-driven immune and barrier regulation (57) were not assayed. While clinical improvements (e.g., reduced diarrhea, enhanced behavioral scores) may correlate with SCFA restoration (e.g., butyrate, propionate), the absence of direct SCFA data impedes mechanistic validation. Histamine levels were also unmeasured. Histamine intolerance mechanisms include exogenous intake (high-histamine foods), dysbiosis, intestinal hyperpermeability, gastrointestinal bleeding, or mastocytosis. This omission constrains deeper mechanistic exploration (13).

Diamine oxidase levels hold significant reference value in diagnosing HIT, yet their clinical utility necessitates comprehensive multidisciplinary evaluation. Studies demonstrate that HIT patients exhibit markedly lower serum DAO levels compared to healthy controls, and strict dietary intervention correlates with DAO elevation alongside symptom remission, suggesting DAO as a biomarker for monitoring dietary compliance and therapeutic efficacy (58, 59). However, DAO's diagnostic sensitivity remains constrained: only 50%–71% of HIT patients present DAO $<$ 10 U/mL, while subnormal DAO levels are also observed in asymptomatic populations, resulting in a low positive predictive value for standalone testing (59, 60). Thus, DAO testing should serve as a complementary tool, integrated with clinical symptomatology, dietary provocation trials, and exclusion diagnostics to enhance diagnostic precision (58, 59, 61). These limitations mirror broader technical challenges in FMT research and underscore the necessity of standardized multi-omics platforms for mechanistic elucidation.

Due to the resource constraints and the patient's outpatient clinical follow-up protocol, which precluded comprehensive analysis of fecal gut microbiota composition. The absence of microbiota profiling hinders mechanistic exploration of microbial metabolites in symptom amelioration. However, the single-case design inherently limits the statistical power required for microbiota-symptom correlation analyses. Future multicenter trials with serial metagenomic and metabolomic profiling will elucidate microbial drivers of therapeutic responses.

Data availability statement

The original contributions presented in this study are included in this article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by. The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University [reference number: 2923JS(11)]. The participant provided written informed consent to participate in this study. 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. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

YH: Conceptualization, Formal Analysis, Resources, Writing – original draft, Writing – review and editing. JH: Formal Analysis, Methodology, Writing – original draft, Writing – review and editing. YL: Data curation, Writing – original draft, Writing – review and editing. TX: Data curation, Writing – original draft. GQ: Data curation Writing – original draft. PX: Data curation, Writing – original draft. XY: Writing – review and editing. ZL : Writing – review and editing. WX: Writing – review and 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/fnut.2025.1594022/full#supplementary-material

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The effects of early childhood probiotic intake on the association between prenatal micronutrient supplementation and neurobehavioral development in preschool children: a four-way decomposition analysis

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Background: Neurobehavioral developmental disorder (NDD) significantly impact children's long-term wellbeing and contribute to global disease burden. While prenatal micronutrient supplementation has shown promise in improving fetal neurodevelopment, its association with offspring's neurobehavioral outcomes remains controversial, and the potential effect of early childhood probiotic intake on this association is still underexplored. This study aimed to evaluate the association between prenatal micronutrient supplementation and neurobehavioral development in preschool children, and to explore and quantify the effect of early childhood probiotic intake on this association.

Methods: We included 15,636 mother–child dyads in Shenzhen, China, in 2022. Mothers provided information on prenatal micronutrient supplementation (calcium, folic acid, iron, and multivitamins) and early childhood probiotic intake through a structured questionnaire. Neurobehavioral development was assessed using the Ages and Stages Questionnaire (ASQ-3). Logistic regression was used to examine the association between prenatal micronutrient supplementation and NDD across crude, adjusted, and full-inclusion models. The effect of early childhood probiotic intake on the association between prenatal micronutrient supplementation and NDD was evaluated through four-way decomposition analysis and quantified using counterfactual attribution under three scenarios.

Results: Among the participants, 11.7% were identified with NDD. Prenatal multivitamin supplementation was significantly associated with a reduced risk of NDD (OR = 0.73, 95% CI = 0.66–0.81). Early childhood probiotic intake was associated with an enhanced protective effect (Total EOR = −0.33, 95% CI = −0.54 to −0.12), with 48% of the effect attributable to interactions. Early

childhood probiotic intake could prevent an additional 73 NDD cases (a 59% increase), particularly benefiting the gross motor, fine motor and personal-social domains.

Conclusion: Prenatal multivitamin supplementation has a protective effect against NDD in preschool children, and early childhood probiotic intake is associated with an enhancement of this protective effect. These findings underscore the potential effect of early-life dietary supplements for NDD prevention. Further studies are recommended to confirm these effects and explore underlying mechanisms.

KEYWORDS

neurobehavioral development, micronutrient supplementation, probiotic intake, preschool children, four-way decomposition, counterfactual attribution

1 Introduction

Neurobehavioral development refers to the development of the brain and nervous system in behavioral, cognitive, and emotional regulation, playing a crucial role in children's academic success, mental wellbeing, professional prospects, and overall quality of life (1). Despite its importance, neurobehavioral development disorders remain prevalent worldwide, characterized by key functions disorders like perception, motor skills, and language (2, 3). The 2019 World Health Organization (WHO) report estimated that approximately 58 million children (7%) globally experienced developmental disorders, with neurobehavioral disorders accounting for more than half of these cases (4). In China, the reported prevalence of such disorders ranges from 3.2 to 13.9%, with the personal-social domain being the most affected (5–8). Severe cases may manifest as conditions such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), both of which are increasing in prevalence (9–11). These conditions impose significant challenges on affected individuals, their families, and society. For example, ASD is responsible for more than 691.5 disability-adjusted life years (DALYs) per 100,000 individual globally, ranking it among the top 10 neurological disorders (12). Families of children with ASD face approximately $3,020 in additional annual healthcare costs and substantial losses in parental productivity (13). The annual social cost for all ASD patients may reach $41.8 billion, accounting for approximately 3.76% of China's total healthcare expenditure in 2020 (14). Therefore, early identification of influencing factors is essential to prevent severe neurobehavioral developmental disorders and mitigate long-term socioeconomic burdens.

Early life, including the prenatal period and early childhood, is a critical window for neurobehavioral development, during which preventive interventions can be most effective in reducing the risk and severity of these disorders (15, 16). Prenatal nutrition, particularly micronutrients, is essential in fetal neural development with long-term health implications (17). However, micronutrient deficiencies remain widespread among pregnant women globally, including in China (18–20). While some evidence suggests that prenatal iron supplementation may enhance neurobehavioral outcomes (21, 22), other studies have failed to confirm this effect (23). Similarly, randomized controlled trials (RCTs) on prenatal vitamin D supplementation have reported inconsistent results, with some showing improved motor development (24), and others finding no significant benefits (25, 26). For prenatal iodine supplementation, although certain studies suggest cognitive benefits (27, 28), a systematic review of RCTs found no impact on neurobehavioral outcomes (29). These discrepancies may be attributed to heterogeneity in study design, including confounding biases and non-standardized neurobehavioral assessments (25, 26). Therefore, further research using large sample sizes, rigorous control of confounders, and standardized assessment methods is necessary to clarify these associations.

Gut microbiota establishment and neural development share the same critical time window in early life (30). Increasing evidence suggests that gut microbiota influences neurobehavioral development via the gut-brain axis, involving mechanisms such as neurotransmitter regulation, immune modulation, production of neuroactive metabolites (e.g., short-chain fatty acids), and stress response regulation (31–34). Probiotics, which are live, nonpathogenic microorganisms that promote gastrointestinal microbial balance, have been proposed as a potential intervention to enhance neurobehavioral development by modulating gut microbiota (35, 36). Some reviews have reported therapeutic effects of childhood probiotic intake on ASD and ADHD (37–39), while an RCT has investigated its potential in preventing ADHD (40). However, some studies have failed to demonstrate significant benefits of childhood probiotic intake for ASD (41). Moreover, existing research has primarily focused on overt neurobehavioral disorders such as ASD and ADHD, whereas evidence remains limited regarding its effects during the early or subclinical stages of neurobehavioral development.

Research shows that prenatal micronutrient supplementation can benefit offspring gut microbiota, while childhood probiotic intake similarly improve gut microbiota composition in children, suggesting a potential interaction between the two in shaping neurobehavioral development via the gut–brain axis (42). Additionally, maternal self-administration of over-the-counter medications during pregnancy may influence the provision of probiotics and other nutritional supplements to their children (43). This indicates that prenatal micronutrient supplementation may also influence childhood probiotic intake, thereby potentially affecting neurobehavioral development. Figure 1 illustrates a conceptual framework of the hypothesized associations among prenatal micronutrient supplementation, early childhood probiotic intake, and neurobehavioral development.

However, existing studies have primarily focused on dietary supplements during a single developmental window—either the prenatal or early childhood. Although some studies have examined the combined effects of prenatal micronutrient supplementation and probiotic intake on maternal and infant outcomes (44–46), the relatively stable maternal microbiota and its indirect influence on the

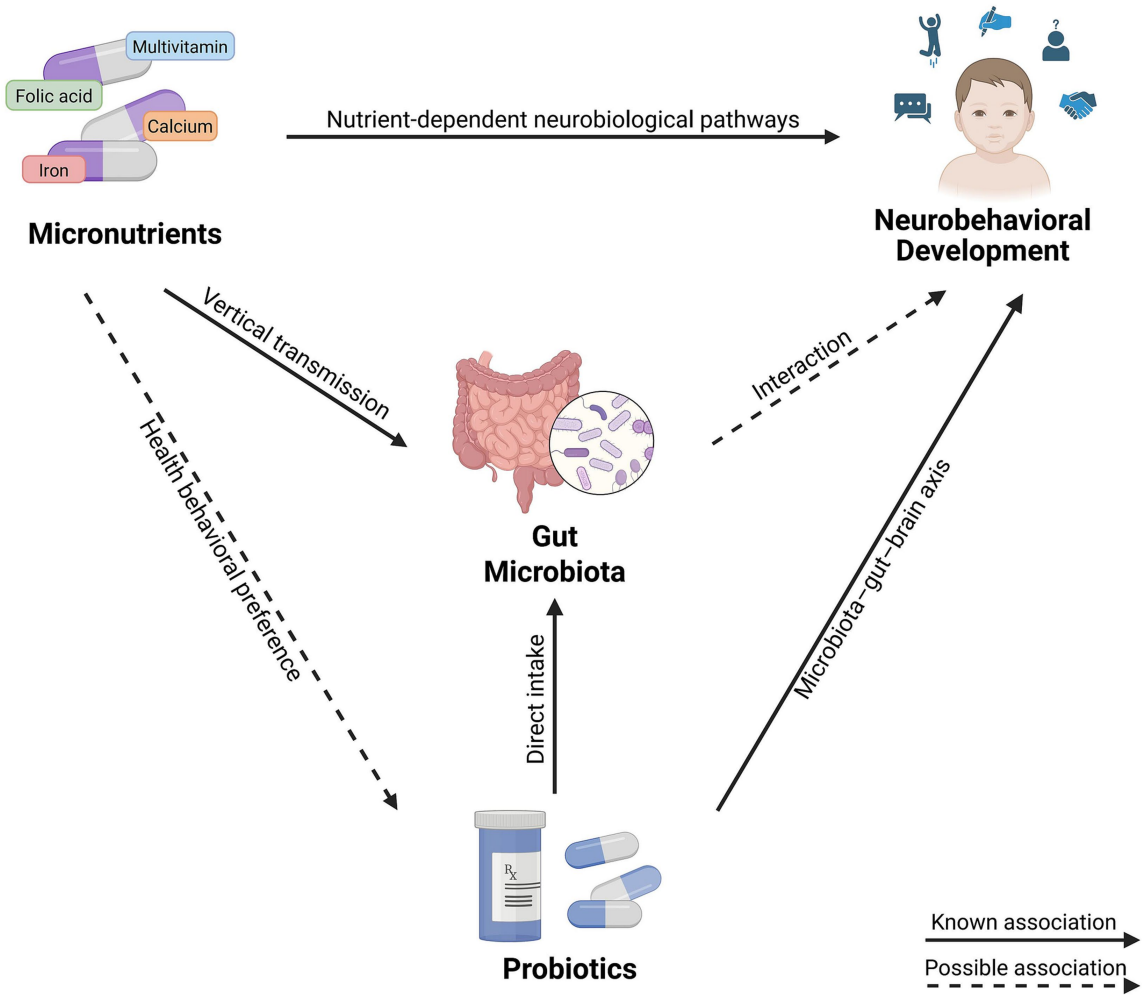

FIGURE 1
Conceptual framework of the hypothesized associations among prenatal micronutrient supplementation, early childhood probiotic intake, and neurobehavioral development. Created with BioRender.com.

fetus suggest that pregnancy may not be the optimal time for probiotic intake (47). Similarly, while other studies have explored the effects of childhood probiotic intake and micronutrient supplementation (48, 49), initiating micronutrient supplementation during childhood may have limited effects, as neurodevelopment begins in utero and largely depends on maternal nutrient stores (22, 50). Nonetheless, evidence remains limited regarding the effect of childhood probiotic intake on the association between prenatal micronutrient supplementation and neurobehavioral development in children.

Therefore, our study aimed to evaluate the association between prenatal micronutrient supplementation and neurobehavioral development in preschool children, and to explore and quantify the effect of early childhood probiotic intake on this association.

2 Methods

2.1 Participants

The study recruited participants from the 2022 survey of children aged 3–7 years, conducted in 235 kindergartens in Longhua District, Shenzhen, China, with follow-up assessment of neurobehavioral development conducted in 2023. A total of 36,220 mother–child dyads were initially enrolled, and 15,636 participants were included after excluding cases with missing follow-up data on neurobehavioral development ($n = 12,129$), no recorded prenatal micronutrient supplementation ($n = 6,624$), and incomplete childhood probiotic intake records ($n = 1,831$), as shown in Figure 2. Ethical approval was obtained from the Ethics Committee of the School of Public Health, Sun Yat-sen University, and informed consent was provided by the mothers of all participating children.

2.2 Data acquirement

Data was collected through a self-administered online structured questionnaire, which was completed by children's mothers under the supervision of childcare practitioners and kindergarten teachers. The questionnaire was developed by a multidisciplinary panel of epidemiologists, obstetricians, and pediatricians, and its clarity and readability were confirmed through a pilot test. It contained demographic characteristics, maternal condition during pregnancy

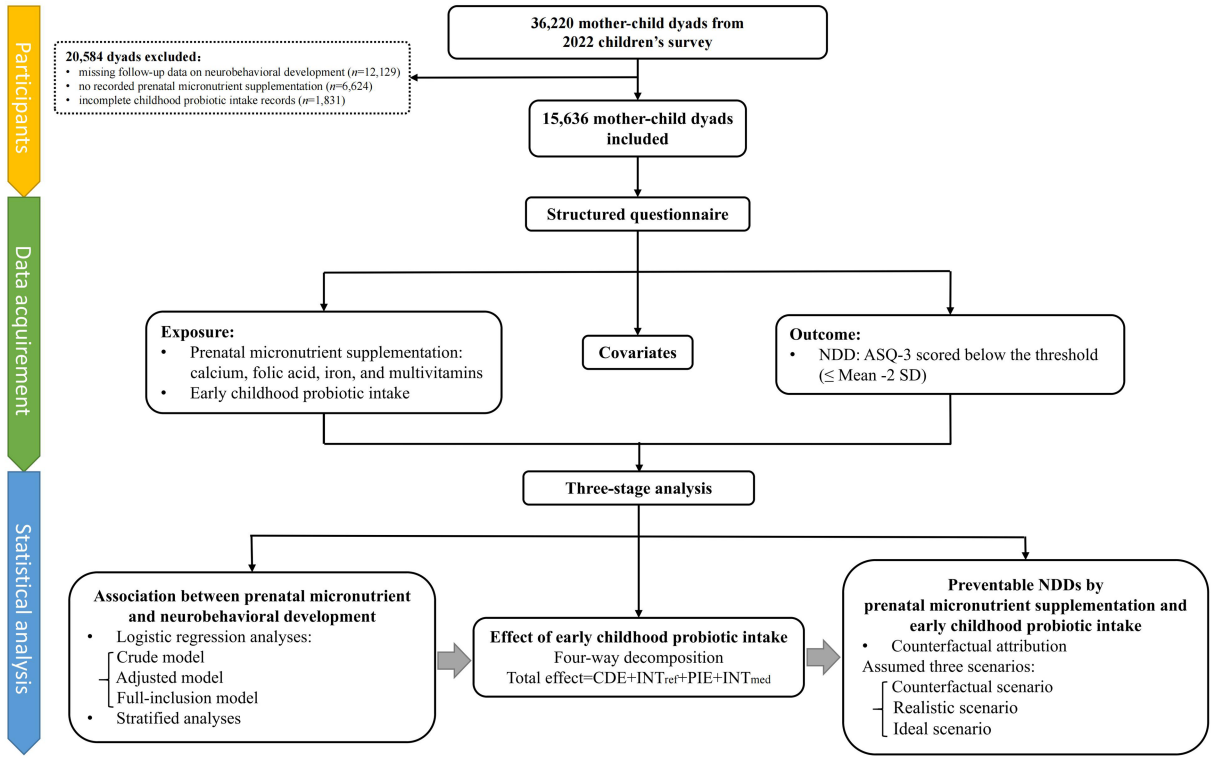

FIGURE 2
Study profile. ASQ-3, Age and Developmental Progress Questionnaire; CDE, Controlled Direct Effect; INT_{med}, mediated interaction; INT_{ref}, reference interaction; NDD, neurobehavioral developmental disorder; PIE, Pure Indirect Effect.

(e.g., micronutrient supplementation, pregnancy complications, health behaviors), parental lifestyle and health status (e.g., smoking, drinking, diseases), neonatal birth characteristics (e.g., birth weight, preterm birth, delivery mode), lifestyle and health condition at ages 0–3 years (e.g., probiotic intake, feeding pattern, nutritional condition), and children's health status at ages 3–7 years (e.g., neurobehavioral development, diseases, family function). Further details and coding are provided in Supplementary Table 1.

2.2.1 Prenatal micronutrient supplementation and early childhood probiotic intake

Prenatal micronutrient supplementation (calcium, folic acid, iron, and multivitamins) was assessed through maternal self-reported responses to four separate questions: "Did you take calcium/folic acid/iron/multivitamins during your pregnancy?" Participants answering 'YES' were assigned to the corresponding supplementation group, while all others served as the reference group.

Early childhood probiotic intake was defined based on a 'YES' response to the question: "Did your child take probiotics between the ages of 0–3 years?" Here, probiotics were specified as a single product form (capsules or sachets) not combined with other foods or supplements.

2.2.2 Outcome

The neurobehavioral development in preschool children (3–7 years old) was assessed by the Age and Developmental Progress Questionnaire-Third Edition (ASQ-3), a well-validated and widely used tool in multiple countries, including China, demonstrating good internal consistency (Cronbach's $\alpha = 0.8$) (51–53). Designed for children aged 1 to 66 months, the ASQ-3 assesses five key domains: communication, gross motor, fine motor, problem-solving, and personal-social status. Assessment results are classified into three groups: (1) scores above the threshold (>mean minus 1 standard deviation [SD]), indicating age-appropriate development; (2) scores close to the threshold (mean minus 2 SD to mean minus 1 SD), requiring further monitoring; and (3) scores below the threshold (≤mean minus 2 SD), indicating developmental disorder. In our study, the presence of neurobehavioral developmental disorder (NDD) was identified when at least one domain scored below the threshold, while others with all domains above or close to the threshold were classified as neurobehavioral developmental normality (NDN).

2.2.3 Covariates

Based on previous studies (21, 27, 54–56) and the univariate and multivariate analyses results (See Supplementary Table 2), covariates included child's demographic characteristics (age, sex, birth season, residence type), maternal demographic characteristics (education, household income, age of conception, pre-pregnancy BMI), pregnancy and perinatal characteristics (intrauterine growth restriction [IUGR], parity, preterm birth [PTB], and birth weight [BW]), and childhood family environment (parental depression, family functioning, feeding pattern). The average missing data rate for these covariates was 3.9% (range: 0–12.9%), with missing data addressed using multiple imputations through Predictive Mean Matching (PMM) (57).

2.3 Statistical analysis

We compared variables between the NDD and NDN groups using one-way ANOVA and t-tests for continuous variables and chi-square tests for categorical variables. The main analysis was conducted in three stages.

2.3.1 Association between prenatal micronutrient supplementation and neurobehavioral development in preschool children

We examined the association between maternal micronutrient supplementation during pregnancy and neurobehavioral development in preschool children using univariate and multivariate logistic regression analyses under three different models: the crude model, without adjustment for confounders; the adjusted model, adjusted for selected confounders; and the full-inclusion model, included all micronutrients in the model while adjusting for all confounders to address the confounding effects of co-supplementation. To further explore the association between prenatal micronutrient supplementation and neurobehavioral development under different probiotic intake scenarios, we conducted a stratified analysis based on probiotic intake in early childhood.

2.3.2 Effect of early childhood probiotic intake on association between prenatal micronutrient supplementation and neurobehavioral development in preschool children

Under the three models, we analyzed the effect of probiotic intake on the association between prenatal micronutrient supplementation and neurobehavioral development in preschool children through four-way decomposition, as proposed by J. VanderWeele (58). This method allows for simultaneous analysis of interaction and mediation effects, which is widely used in epidemiologic studies (59–61). The core is to decompose the total effect into four components: (1) controlled direct effect (CDE): the effect of prenatal micronutrient supplementation on neurobehavioral development independent of childhood probiotic intake, (2) reference interaction (INT_{ref}): the effect of prenatal micronutrient supplementation on neurobehavioral development only through its interaction with childhood probiotic intake, (3) pure indirect effect (PIE): the effect of prenatal micronutrient supplementation on neurobehavioral development both through an interaction with and mediation by childhood probiotic intake, and (4) mediated interaction (INT_{med}): the effect of prenatal micronutrient supplementation on neurobehavioral development only through the mediation by childhood probiotic intake. Estimates of the four components were obtained through regression analyses that included the exposure, mediator, and their interaction terms, with results presented as excess odds ratio (EOR) and proportion attributable (PA).

2.3.3 Preventable NDD by prenatal micronutrient supplementation and early childhood probiotic intake

After identifying the effect of probiotic intake, we quantified the number of preventable NDD attributable to prenatal micronutrient supplementation and early childhood probiotic intake using counterfactual attribution (62), which can be used to quantify interaction or mediation effects (63). We assumed three scenarios: (1) Counterfactual scenario (V1): no probiotic intake, (2) Realistic scenario (V2): probiotic intake in the realistic proportion of the survey population, and (3) Ideal scenario (V3): probiotic intake in all populations. Under each scenario, we estimated the effect of prenatal micronutrient supplementation and early childhood probiotic intake on NDD across three models to calculate the number of preventable NDD cases. The difference in preventable cases between the realistic and counterfactual scenarios (V2–V1) represented the additional preventable NDD due to current childhood probiotic intake proportion, while the difference between the ideal and counterfactual scenarios (V3–V1) represents the potential preventable NDD if probiotics were consumed by all populations.

Statistical analyses above were performed via R version 4.2.3. Two sided p-values <0.05 were considered significant.

3 Results

3.1 Participants' characteristics

Table 1 presents the background characteristics of the study participants, including prenatal micronutrient supplementation and childhood probiotic intake, with a comparison between NDD and NDN groups. Folic acid (88.2%) was the most widely consumed, followed by calcium (75.8%), while iron (46.0%) and multivitamin supplementation (44.4%) were relatively less common in pregnant women. A total of 80.2% of children reported taking probiotics in early childhood.

The overall average ASQ-3 score of the 15,636 children was 277 ± 26.4, with domain-specific scores ranging from 51.7 ± 10.2 in the fine motor domain to 57.6 ± 5.4 in the communication domain. A total of 11.7% of the children were identified as NDD, with the highest prevalence in the gross motor domain (8.88%) and the lowest in the problem-solving domain (0.70%), as shown in Table 2.

3.2 Association between prenatal micronutrient supplementation and neurobehavioral development in preschool children

In the crude model, prenatal calcium (OR = 0.84, 95% CI = 0.76–0.94), folic acid (OR = 0.83, 95% CI = 0.72–0.96) and multivitamin (OR = 0.73, 95% CI = 0.66–0.81) supplementation was associated with a decreased risk of NDD, whereas no significant association was observed for iron. In the adjusted and full-inclusion models, only prenatal multivitamin supplementation remained associated with a reduced risk of NDD (Adjusted Model: OR = 0.86, 95% CI = 0.78–0.96; Full-inclusion Model: OR = 0.85, 95% CI = 0.75–0.95) (Supplementary Table 3; Figure 3). In the full-inclusion model, prenatal iron supplementation was even found to be significantly associated with an increased risk of NDD (OR = 1.14, 95% CI = 1.01–1.28). Across these 5 domains, after controlling for confounders, prenatal calcium and folic acid supplementation were significantly associated with NDD in the communication and gross motor domains, folic acid and multivitamin supplementation were associated with NDD in the personal-social domain, whereas no significant associations were found in the fine motor and problem-solving domains (Supplementary Table 4).

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TABLE 1 Background characteristics of study participants in the 2022 children's survey.

Characteristic	Overall[a] (*n* = 15,636)	Outcome		*p*-value[b]
		NDD[a] (*n* = 13,804)	NDN[a] (*n* = 1,832)	
Child's age	4.6 ± 0.6	4.6 ± 0.6	4.6 ± 0.5	<0.001
Child's sex				<0.001
Male	8,346 (53.4%)	7,219 (52.3%)	1,127 (61.5%)	
Female	7,290 (46.6%)	6,585 (47.7%)	705 (38.5%)	
Birth season				<0.001
Spring	4,225 (27.0%)	3,746 (27.1%)	479 (26.1%)	
Summer	4,482 (28.7%)	3,845 (27.9%)	637 (34.8%)	
Autumn	2,945 (18.8%)	2,618 (19.0%)	327 (17.8%)	
Winter	3,984 (25.5%)	3,595 (26.0%)	389 (21.2%)	
Residence type				<0.001
Shenzhen residents	9,415 (60.2%)	8,507 (61.6%)	908 (49.6%)	
Non-Shenzhen residents	6,221 (39.8%)	5,297 (38.4%)	924 (50.4%)	
Maternal education				<0.001
Less than high school	1,614 (10.3%)	1,262 (9.14%)	352 (19.2%)	
High school and higher	14,022 (89.7%)	12,542 (90.9%)	1,480 (80.8%)	
Household income				<0.001
<RMB 20,000	7,314 (46.8%)	6,262 (45.4%)	1,052 (57.4%)	
≥RMB 20,000	8,322 (53.2%)	7,542 (54.6%)	780 (42.6%)	
Maternal conception age	34.0 ± 5.5	34.0 ± 5.5	33.7 ± 5.7	0.028
Pre-pregnancy BMI				<0.001
BMI < 18.5	2,890 (18.5%)	2,550 (18.5%)	340 (18.6%)	
18.5 ≤ BMI < 24	10,571 (67.6%)	9,392 (68.0%)	1,179 (64.4%)	
BMI ≥ 24	2,175 (13.9%)	1,862 (13.5%)	313 (17.1%)	
Intrauterine growth retardation				<0.001
No	15,497 (99.1%)	13,697 (99.2%)	1,800 (98.3%)	
Yes	139 (0.9%)	107 (0.8%)	32 (1.7%)	
Parity				0.21
Nulliparous	8,810 (56.3%)	7,752 (56.2%)	1,058 (57.8%)	
Multiparous	6,826 (43.7%)	6,052 (43.8%)	774 (42.2%)	
Preterm birth				<0.001
No	14,517 (92.8%)	12,851 (93.1%)	1,666 (90.9%)	
Yes	1,119 (7.2%)	953 (7.0%)	166 (9.1%)	
Child's birth weight	3.1 ± 0.6	3.1 ± 0.6	3.0 ± 0.7	<0.001
Parental depression				<0.001
No	13,652 (87.3%)	12,140 (87.9%)	1,512 (82.5%)	
Yes	1,984 (12.7%)	1,664 (12.1%)	320 (17.5%)	
Family function				<0.001
Normal	9,697 (62.0%)	8,772 (63.5%)	925 (50.5%)	
Dysfunction	5,939 (38.0%)	5,032 (36.5%)	907 (49.5%)	
Feeding pattern				<0.001
Breastfeeding	8,803 (56.3%)	7,815 (56.6%)	988 (53.9%)	
Formula feeding	1,665 (10.6%)	1,415 (10.3%)	250 (13.6%)	
Mixed feeding	5,168 (33.1%)	4,574 (33.1%)	594 (32.4%)	

(Continued)

TABLE 1 (Continued)

Characteristic	Overall[a] (*n* = 15,636)	Outcome		*p*-value[b]
		NDD[a] (*n* = 13,804)	NDN[a] (*n* = 1,832)	
Prenatal calcium supplementation				0.003
No	3,781 (24.2%)	3,286 (23.8%)	495 (27.0%)	
Yes	11,855 (75.8%)	10,518 (76.2%)	1,337 (73.0%)	
Prenatal folic acid supplementation				0.013
No	1,846 (11.8%)	1,597 (11.6%)	249 (13.6%)	
Yes	13,790 (88.2%)	12,207 (88.4%)	1,583 (86.4%)	
Prenatal iron supplementation				0.11
No	8,451 (54.0%)	7,428 (53.8%)	1,023 (55.8%)	
Yes	7,185 (46.0%)	6,376 (46.2%)	809 (44.2%)	
Prenatal multivitamin supplementation				<0.001
No	8,690 (55.6%)	7,549 (54.7%)	1,141 (62.3%)	
Yes	6,946 (44.4%)	6,255 (45.3%)	691 (37.7%)	
Childhood probiotic intake				0.010
No	3,098 (19.8%)	2,777 (20.1%)	321 (17.5%)	
Yes	12,538 (80.2%)	11,027 (79.9%)	1,511 (82.5%)	

[a]Data are presented as Mean ± SD or *N* (%).
[b]*p*-value was based on one-way analysis of means and Pearson's Chi-squared test where appropriate.

TABLE 2 Neurobehavioral development across five domains in the 2022 children's survey.

Domains	Description (*n* = 15,636)	
	Score, Mean ± SD	Prevalence, *n* (%)
Communication	57.6 ± 5.4	
Normal		15,457 (98.9%)
Disorder		179 (1.1%)
Gross motor	54.2 ± 8.6	
Normal		14,248 (91.1%)
Disorder		1,388 (8.9%)
Fine motor	51.7 ± 10.2	
Normal		15,214 (97.3%)
Disorder		422 (2.7%)
Problem solving	57.2 ± 5.7	
Normal		15,526 (99.3%)
Disorder		110 (0.7%)
Personal-social	56.8 ± 5.6	
Normal		15,281 (97.7%)
Disorder		355 (2.3%)
Total	277.5 ± 26.4	
Normal		13,804 (88.3%)
Disorder		1,832 (11.7%)

When the analysis was stratified by probiotic intake, we found that prenatal micronutrient supplementation was not significantly associated with NDD without childhood probiotic intake. However, with childhood probiotic intake, the association between prenatal micronutrient supplementation and NDD followed the same pattern as in the whole sample, with multivitamin supplementation associated with a decreased risk of NDD across all three models (Crude model: OR = 0.71, 95% CI = 0.63–0.79; Adjusted Model: OR = 0.84, 95% CI = 0.75–0.94; Full-inclusion Model: OR = 0.83, 95% CI = 0.74–0.94). Although no significant differences were observed between the probiotic and non-probiotic intake subgroups, most ORs were lower in the probiotic intake subgroup, with many associations showing statistical significance within this subgroup only (Figure 4). According to the stratified analysis in the five domains, compared to the non-probiotic intake subgroup, prenatal folic acid and multivitamin supplementation were significantly associated with reduced risks in the gross motor and personal-social domains in the probiotic intake group. In the communication, fine motor and problem-solving domains, though lower ORs were observed, none showed statistical significance in the probiotic intake group (Supplementary Table 5).

3.3 Effect of early childhood probiotic intake on association between prenatal micronutrient supplementation and neurobehavioral development in preschool children

In the crude model, the results showed that childhood probiotic intake was significantly associated with an enhanced protective effect of prenatal multivitamin supplementation on NDD (Total EOR = −0.33, 95% CI = −0.54 to−0.12 vs. CDE EOR = −0.22, 95% CI = −0.46 to 0.03). Most of this protective effect from childhood probiotic intake was driven by the INT_{ref} (EOR = −0.16, 95% CI = −0.49 to 0.16), accounting for 48% of the total effect. The significant mediating effect of probiotic intake was also observed (EOR = 0.10, 95% CI = 0.03–0.16). The INT_{med} followed (EOR = −0.05, 95% CI = −0.16 to 0.05), suggesting the presence of both interaction and mediation of childhood probiotic

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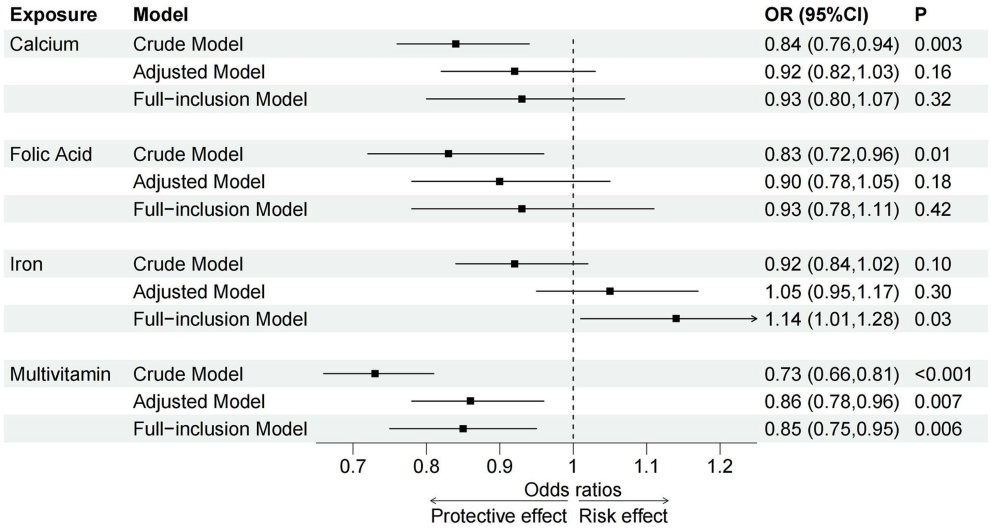

FIGURE 3
Associations between prenatal micronutrient supplementation and NDD in crude, adjusted and full-inclusion models. OR, odds ratio; CI, confidence interval; P, *p*-value.

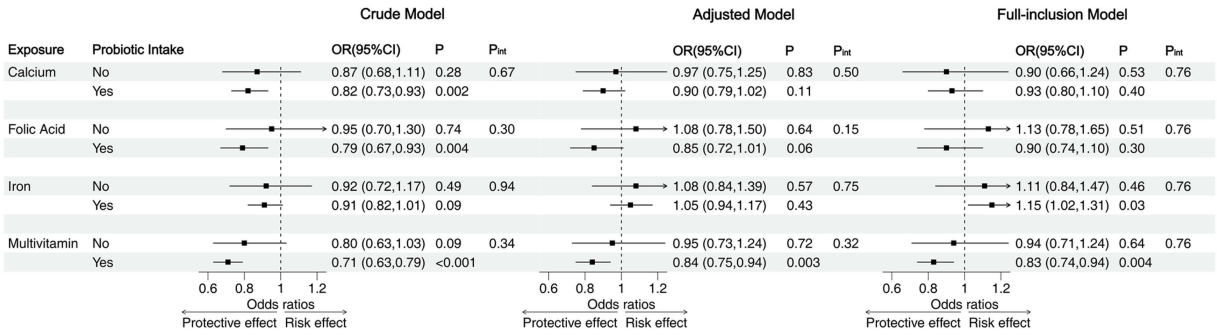

FIGURE 4
Stratified analysis of the associations between prenatal micronutrient supplementation and NDD by childhood probiotic intake. OR, odds ratio; CI, confidence interval; P, *p*-value; P_{int}, *p*-value for the interaction between prenatal micronutrient supplementation and childhood probiotic intake.

intake. No significant enhanced protective effects by childhood probiotic intake were observed in relation to prenatal supplementation of calcium, folic acid or iron (Figure 5; Table 3). Across the five domains, childhood probiotic intake was significantly associated with an enhanced protective effect of prenatal multivitamin supplementation on disorders in gross motor development (Total EOR = −0.31, 95% CI = −0.56 to −0.07) and personal-social development (Total EOR = −0.50, 95% CI = −0.97 to −0.03). The effect of prenatal folic acid supplementation on disorders of personal-social development was also significantly increased by childhood probiotic intake (Total EOR = −0.51, 95% CI = −0.88 to −0.14) (Supplementary Table 6).

3.4 Preventable NDD by prenatal micronutrient supplementation and childhood probiotic intake

In the crude model where a significant effect of childhood probiotic intake was observed on the association between prenatal multivitamin supplementation and NDD, we assumed three scenarios: In the scenario assuming no probiotic intake in early childhood (counterfactual scenario), among the 15,636 participants, prenatal multivitamin supplementation alone could prevent 123 children from developing NDD. Under current childhood probiotic intake proportion (realistic scenario), 73 additional NDD cases could be prevented compared to no probiotic intake, representing a 59% increase in preventive effect. If childhood probiotic intake were increased to a scenario where all children consume probiotics (ideal scenario), it could potentially prevent 96 more children from developing NDD compared to no probiotic intake, representing a 78% increase (Figure 6).

The highest number of preventable NDD was in the gross motor domain (47 cases in the realistic scenario and 64 in the ideal scenario), followed by the personal-social domain (19 in the realistic scenario and 24 in the ideal scenario). The highest percentage increase in NDD prevention was observed in the fine motor domain, with a 92% increase in the realistic scenario and a

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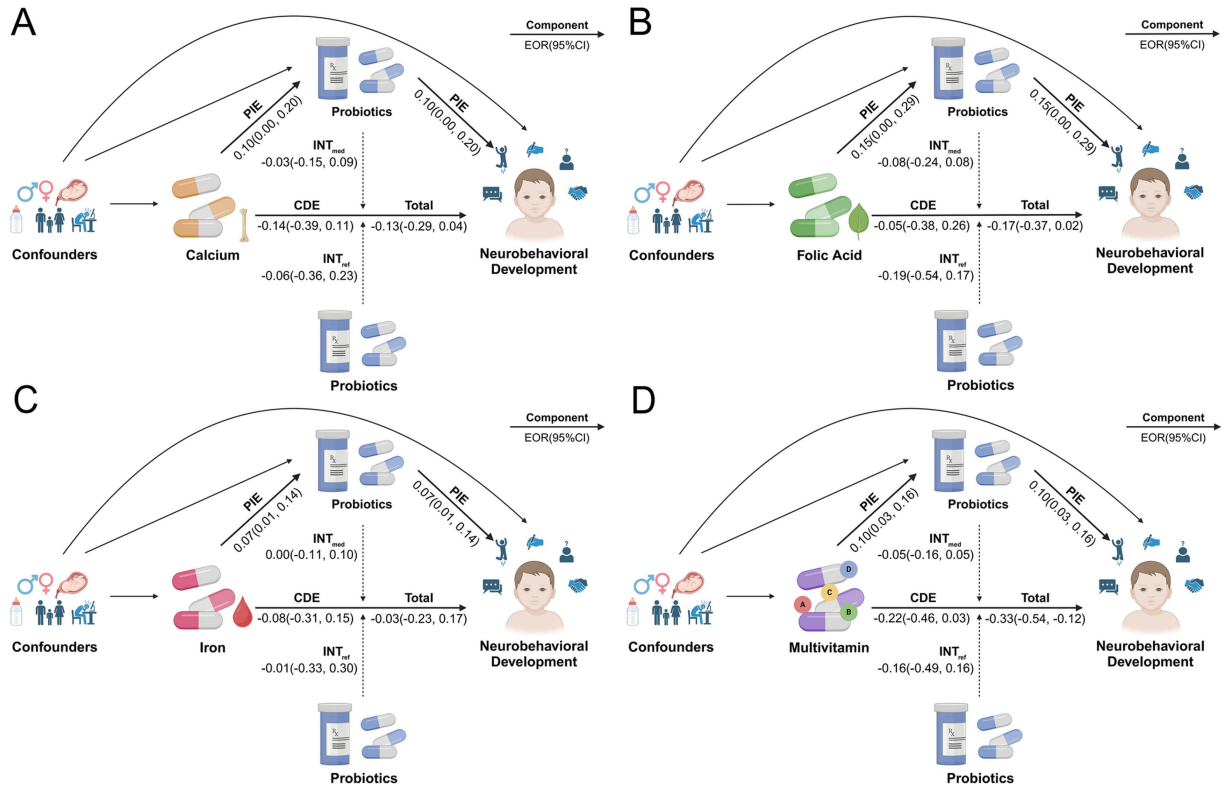

FIGURE 5
Four-way decomposition diagram of the effect of childhood probiotic intake on the association between prenatal micronutrient supplementation and NDD in the crude model. **(A)** Calcium supplementation; **(B)** Folic acid supplementation; **(C)** Iron supplementation; **(D)** Multivitamin supplementation. CDE, controlled direct effect; CI, confidence interval; EOR, excess odds ratio; INT_{med}, mediated interaction; INT_{ref}, reference interaction; OR, odds ratio; PIE, pure indirect effect. Created with BioRender.com.

117% increase in the ideal scenario, followed by the personal-social domain (80% in the realistic and 100% in the ideal scenario) (Table 4). The number of preventable NDD across the five domains under different models is detailed in Supplementary Table 7. Most prenatal micronutrient supplementation with childhood probiotic intake showed varied increases in NDD prevention in both realistic and ideal scenarios.

4 Discussion

Our study revealed that 11.7% of preschool children were identified with NDD in the 2022 children's survey. We found that prenatal multivitamin supplementation was significantly associated with a reduced risk of NDD across the crude, adjusted and full-inclusion models. When exploring the effect of probiotic intake in early childhood, our results indicated that childhood probiotic intake was associated with an enhanced protective effect of prenatal multivitamin supplementation against NDD in the crude model (Total EOR = −0.33, 95% CI = −0.54 to 0.12), with 48% of the effect attributable to interactions. Quantifying this enhanced protective effect, our study demonstrated that childhood probiotic intake contributed to the prevention of 73 (a 59% increase) additional NDD cases, with the potential to prevent 96 additional NDD cases (a 78% increase) if childhood probiotic intake were consumed by all populations.

Previous research have found that prenatal single vitamin supplementation, such as vitamins D and B12, are associated with a lower risk of NDD in children (25, 64–66), while multivitamins also significantly promote neurobehavioral development in children (67, 68), which is consistent with the results of this study. Vitamins are essential for fetal brain development, serving as cofactors in neurotransmitter synthesis and enzymatic metabolism processes (69). For instance, vitamin B12 is crucial for fatty acid metabolism necessary for myelin sheath production, while vitamin B6 functions as a coenzyme in the synthesis of various amino acid neurotransmitters, both of which can influence neurobehavioral development (70, 71). Retinoids, derived from vitamin A, contribute to neuronal differentiation and influence functions like memory and sleep (72). Some evidence suggests that multivitamin supplementation exert broader effects on neurobehavioral development because they allow multiple biological pathways for effects (71). However, other studies indicated that multivitamin supplementation not always more effective than single vitamins for cognitive function (73). Further research is warranted to clarify the comparative benefits of multivitamin supplementation versus single-vitamin supplementation in neurobehavioral development. Despite the known benefits of vitamins, our study found that fewer than half of pregnant women took multivitamins, as reported in other surveys, highlighting a need to improve multivitamin supplementation practices (66).

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TABLE 3 The effect of childhood probiotic intake on the association between prenatal micronutrient supplementation and NDD using the four-way decomposition.

Component	Crude model		Adjusted model		Full-inclusion model	
	EOR (95%CI)	PA (95%CI), %	EOR (95%CI)	PA (95%CI), %	EOR (95%CI)	PA (95%CI), %
Calcium supplementation						
CDE	−0.14 (−0.39, 0.11)	107 (304, −89)	−0.01 (−0.27, 0.23)	7 (156, −135)	0.00 (−0.27, 0.27)	−3 (163, −165)
INTref	−0.06 (−0.36, 0.23)	50 (284, −179)	−0.19 (−0.75, 0.40)	111 (437, −232)	−0.18 (−0.71, 0.37)	111 (435, −226)
INT_{med}	−0.03 (−0.15, 0.09)	20 (115, −73)	−0.04 (−0.16, 0.08)	23 (91, −48)	−0.02 (−0.07, 0.04)	10 (41, −22)
PIE	0.10 (0.00, 0.20)	−78 (−1, −157)	0.07 (−0.03, 0.16)	−40 (19, −96)	0.03 (−0.02, 0.07)	−18 (11, −45)
Total	−0.13 (−0.29, 0.04)	100	−0.17 (−0.58, 0.24)	100	−0.16 (−0.53, 0.21)	100
Folic acid supplementation						
CDE	−0.05 (−0.38, 0.26)	30 (218, −150)	0.09 (−0.23, 0.40)	−23 (58, −99)	0.14 (−0.21, 0.47)	−42 (63, −144)
INT_{ref}	−0.19 (−0.54, 0.17)	109 (309, −100)	−0.52 (−1.22, 0.22)	129 (302, −54)	−0.47 (−1.17, 0.24)	144 (359, −73)
INT_{med}	−0.08 (−0.24, 0.08)	48 (137, −45)	−0.10 (−0.25, 0.05)	26 (61, −12)	−0.02 (−0.08, 0.03)	8 (24, −9)
PIE	0.15 (0.00, 0.29)	−86 (−2, −169)	0.13 (−0.02, 0.26)	−32 (4, −64)	0.03 (−0.03, 0.09)	−9 (9, −28)
Total	−0.17 (−0.37, 0.02)	100	−0.40 (−0.89, 0.10)	100	−0.33 (−0.79, 0.13)	100
Iron supplementation						
CDE	−0.08 (−0.31, 0.15)	298 (1,110, −520)	0.08 (−0.16, 0.33)	446 (−841, 1,754)	0.17 (−0.08, 0.42)	155 (−79, 391)
INT_{ref}	−0.01 (−0.33, 0.30)	43 (1,176, −1,068)	−0.09 (−0.66, 0.48)	−478 (−3,486, 2,536)	−0.08 (−0.59, 0.44)	−72 (−551, 407)
INT_{med}	0.00 (−0.11, 0.10)	14 (380, −347)	−0.02 (−0.12, 0.09)	−88 (−650, 473)	−0.01 (−0.07, 0.05)	−9 (−66, 48)
PIE	0.07 (0.01, 0.14)	−255 (−22, −494)	0.04 (−0.03, 0.11)	220 (−134, 560)	0.03 (−0.01, 0.07)	26 (−12, 63)
Total	−0.03 (−0.23, 0.17)	100	0.02 (−0.41, 0.45)	100	0.11 (−0.24, 0.45)	100
Multivitamin supplementation						
CDE	−0.22 (−0.46, 0.03)	65 (137, −9)	−0.03 (−0.28, 0.22)	10 (85, −66)	−0.04 (−0.30, 0.23)	11 (95, −74)
INT_{ref}	−0.16 (−0.49, 0.16)	48 (148, −49)	−0.30 (−0.91, 0.30)	92 (273, −92)	−0.28 (−0.81, 0.26)	89 (261, −83)
INT_{med}	−0.05 (−0.16, 0.05)	15 (48, −16)	−0.06 (−0.18, 0.06)	18 (54, −18)	−0.04 (−0.13, 0.04)	14 (42, −14)
PIE	0.10 (0.03, 0.16)	−29 (−10, −48)	0.07 (0.00, 0.13)	−20 (1, −40)	0.05 (−0.01, 0.10)	−15 (2, −32)
Total	−0.33 (−0.54, −0.12)	100	−0.33 (−0.80, 0.15)	100	−0.31 (−0.68, 0.07)	100

Folic acid, widely recognized as an important substance in neural tube development, is supplemented at a higher prevalence, possibly because of its inclusion in the WHO's list of essential medicines for pregnant women (74–76). Similar to our findings, studies have shown that prenatal folic acid supplementation is positively associated with children's neurobehavioral development (77, 78). However, some reports suggest that excessive folic acid intake may increase the risk of ASD and food allergies, indicating a U-shaped association (79, 80). This underscores the importance of determining precise supplementation levels, especially given that nearly all prenatal foods already contain increased levels of folic acid (66). Iron and calcium, involved in neurotransmitter function, energy metabolism, and myelination, may also influence neurobehavioral development (81, 82). However, our study did not find a significant association between prenatal calcium supplementation and neurobehavioral outcomes, and even identified iron supplementation as a risk factor. Similar conclusions have been drawn in other population studies (83, 84). Variability in study populations, research design, exposure timing and neurobehavioral assessment tools may account for these results (84, 85).

Our findings further suggest that childhood probiotic intake is associated with an enhanced protective effect of prenatal multivitamin supplementation against NDD, primarily through their interaction, as suggested by previous reviews (86, 87). On the one hand, vitamins benefit maternal gut microbiota, with vitamin A and B2 increasing microbial diversity and abundance and vitamins A and D maintaining intestinal barrier integrity (88–90). Beneficial maternal microbes can be transferred to offspring through the birth canal, breast milk, and even vertically in utero (42, 91–93). Probiotic intake in early childhood may further enhance the colonization and function of these inherited beneficial microbiota, improving neurobehavioral outcomes through the gut-brain axis (55, 94). On the other hand, gut microbiota can synthesize specific vitamins such as vitamin K and the B vitamins (95), which can improve cognitive function and reduce the risk of NDD in multiple pathways (96, 97). These findings reinforce the idea that maternal nutrition during pregnancy interacts with offspring gut microbiota to influence neurobehavioral development. Additionally, our study also found that probiotics acted as a reverse mediator in the relationship between micronutrients and NDD, potentially increasing the risk of NDD. One possible explanation is that mothers who self-medicate during pregnancy are

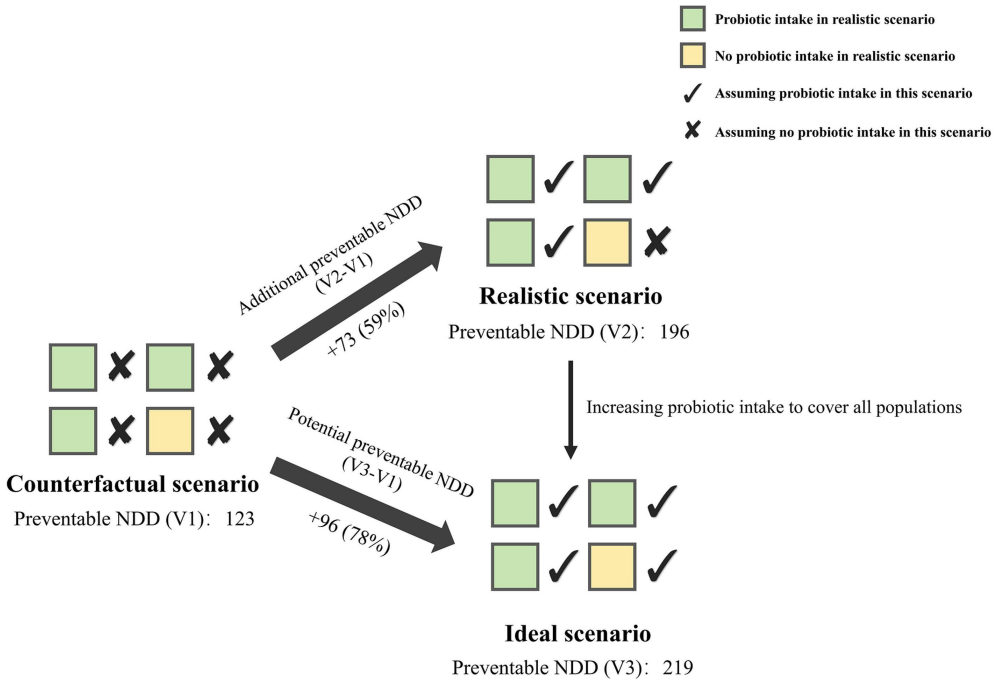

FIGURE 6
Preventable NDD by prenatal multivitamin supplementation and childhood probiotic intake in the crude model. Counterfactual scenario: without probiotic intake; Realistic scenario: with probiotic intake in the realistic proportion of the survey population; Ideal scenario: with probiotic intake in all populations. NDD, neurobehavioral developmental disorder.

TABLE 4 Number of preventable NDD by prenatal multivitamin supplementation and childhood probiotic intake in counterfactual, realistic, and ideal scenario in the crude model.

Domains	Counterfactual scenario	Realistic scenario			Ideal scenario		
	Preventable NDD (V1)	Preventable NDD (V2)	Additional preventable NDD (V2–V1)	Additional preventable percentage, %	Preventable NDD (V3)	Potential preventable NDD (V3–V1)	Potential preventable percentage, %
Communication	25	26	2	7	27	3	11
Gross motor	109	156	47	43	173	64	59
Fine motor	17	32	15	92	36	20	117
Problem solving	13	14	0	3	13	0	−2
Personal-Social	24	44	19	80	49	24	100
Total	123	196	73	59	219	96	78

more likely to administer probiotics to their children (43), often to address gut microbiota imbalances. Such pre-existing gut microbiota imbalances in children may adversely affect neurobehavioral development through oxidative stress (98).

Quantifying the enhanced protective effect of childhood probiotic intake on NDD, we found the effect was particularly present in gross motor, fine motor and personal-social developmental disorders. The reason may be that the influence of gut microbiota on the brain is mainly concentrated in the limbic system and motor cortex, which are related to emotion and motor coordination, while its effects on the prefrontal cortex and hippocampus, which are associated with problem-solving, are more indirect (99, 100). This result also echoes the protective effects of probiotic intake against Parkinson's disease, which is associated with fine motor disorders, and ASD, which is associated with emotional–social dysfunction (101, 102). In addition, we also observed that early childhood probiotic intake significantly increased the number of gross motor developmental disorders prevented by prenatal multivitamin supplementation, which happened to be the most prevalent type of NDD in our study. This suggests that early childhood probiotic intake can specifically target the prevention of neurobehavioral developmental domains that most need improvement.

This study makes several significant contributions. First, it innovatively introduces early childhood probiotic intake as a key

variable to explore its effect on the association between prenatal micronutrient supplementation and neurobehavioral development in children, addressing the limitations of previous studies focused on single time windows. Second, the study employs advanced statistical techniques, including four-way decomposition and counterfactual mediation analysis, to systematically evaluate the potential effect of probiotic intake from different perspectives. Finally, the study is based on a large-scale children's survey, enhancing the reliability and generalizability of the findings.

Nevertheless, the study has several limitations. First, as all participants were recruited from Shenzhen, China, our findings may not be generalizable to other populations. Second, data collection relied on retrospective questionnaires, which may introduce recall bias. Additionally, ASQ-3 assessments, based on maternal reports, may be subject to reporting bias compared to clinical diagnoses. Moreover, prenatal micronutrient supplementation and childhood probiotic intake were recorded as binary variables, lacking detailed dosage information and probiotic strain data. Although we adjusted for several factors that may reflect maternal health consciousness on dietary supplement use, the potential for self-selection bias remains. Lastly, although we controlled for confounders, the cross-sectional study design limits causal inference.

Therefore, future studies should focus on determining specific supplementation dosages to establish a dose–response relationship, providing clearer supplementation guidelines. Higher-evidence studies, such as RCTs, are needed to confirm the causal relationship between prenatal micronutrient supplementation, childhood probiotic intake, and NDD. In addition, molecular-level research is essential to elucidate the biological mechanisms underlying these effects and to explore the complex pathways influencing different neurobehavioral domains.

5 Conclusion

In summary, our study found that prenatal multivitamin supplementation has a protective effect against NDD in preschool children. Early childhood probiotic intake is associated with an enhancement of this protective effect, primarily driven by interaction with prenatal multivitamin supplementation. Early childhood probiotic intake could prevent up to 60% more NDD cases, with a 78% potential increase if childhood probiotic intake were consumed by all populations, particularly in the gross motor, fine motor and personal-social domains. These findings highlight the importance of early-life dietary supplements in NDD prevention, particularly the enhanced protective effect of childhood probiotic intake in combination with prenatal multivitamin supplementation. Despite the promising results, future prospective studies with detailed data are needed to confirm this enhanced effect of childhood probiotic intake and their underlying mechanisms.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Ethics statement

The studies involving humans were approved by Institutional Review Board of the School of Public Health, Sun Yat-sen University, Guangzhou, China. 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

LD: Data curation, Conceptualization, Visualization, Formal analysis, Writing – original draft, Methodology. MZ: Writing – original draft, Data curation, Visualization, Formal analysis. ES: Methodology, Supervision, Writing – review & editing. XY: Data curation, Investigation, Writing – review & editing, Resources. GW: Investigation, Resources, Writing – review & editing, Data curation. DS: Writing – review & editing, Data curation, Resources, Investigation. DX: Resources, Writing – review & editing, Data curation, Investigation. YaZ: Investigation, Writing – review & editing, Data curation, Resources. YuZ: Validation, Investigation, Writing – review & editing. FL: Investigation, Validation, Writing – review & editing. RH: Validation, Writing – review & editing, Investigation. LZ: Validation, Investigation, Writing – review & editing. WY: Writing – review & editing, Supervision, Project administration, Resources. WC: Supervision, Methodology, Funding acquisition, Conceptualization, Writing – review & editing, Resources.

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Conflict of interest

YuZ, FL, RH, and LZ were employed by Biostime (Guangzhou) Health Products 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.

Generative AI statement

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2025.1614820/full#supplementary-material

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Probiotics mitigate stress and inflammation in malnourished adults via gut microbiota modulation: a randomized controlled trial

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Objective: Malnutrition negatively affects mental health by altering neurotransmitter function and increasing stress responses. The gut-brain axis is pivotal in this process, and probiotics may mitigate stress. The current study examined the effects of multi-strain probiotic supplementation on stress levels in underweight individuals using the Perceived Stress Scale (PSS).

Methods: A double-blind, randomized, placebo-controlled trial involved 100 underweight participants were randomized to receive either a probiotic supplement (*Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus*; 3×10^9 CFU) or placebo for 8 weeks. Stress levels, anthropometric measures, and inflammatory markers (ESR, CRP) evaluated at baseline and post-intervention.

Results: Ninety participants (mean age: 26.22 ± 7.42 years) completed the study (probiotic: $n = 47$; placebo: $n = 43$). Baseline age ($p = 0.051$) and gender ($p = 0.101$) showed no significant differences. Post-intervention, the probiotic group exhibited significant weight increases ($p = 0.005$), waist circumference ($p = 0.038$), and hip circumference ($p = 0.008$), and a significant reduction in Perceived Stress Scale (PSS) scores ($p < 0.001$) in comparison to the placebo. Inflammatory markers (ESR, CRP) also decreased significantly in the probiotic group ($p < 0.001$). Within-group analysis revealed improvements in anthropometric measures and inflammatory markers in both groups ($p < 0.05$), but stress reduction was more pronounced in the probiotic group (34% vs. 9.3%, $p = 0.017$). A significant time-group interaction was observed for stress scores ($p < 0.001$).

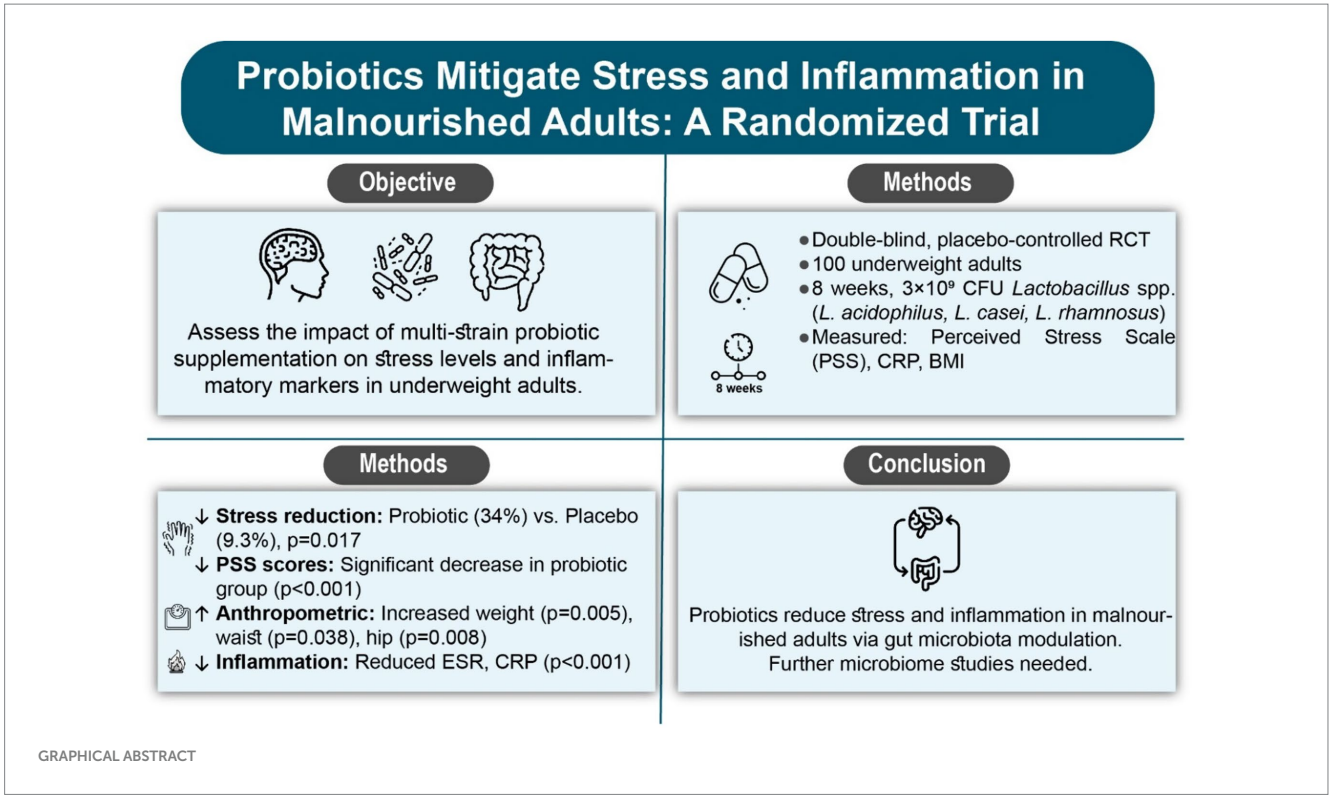
Discussion: The findings suggest that probiotic supplementation reduces stress levels in underweight individuals, possibly through gut microbiota modulation and inflammation reduction. Further research with larger samples and microbiome analysis is warranted.

Conclusion: In conclusion, administering probiotics to underweight patients positively impacts their mental health and exhibits anti-inflammatory effects.

Clinical trial registration: https://irct.behdasht.gov.ir/trial/69130, identifier IRCT20230310057667N1.

KEYWORDS

malnutrition, probiotics, perceived stress score, inflammation, stress



GRAPHICAL ABSTRACT

Introduction

Malnutrition, defined as nutrient deficiencies, excesses, or imbalances, adversely affects body composition, physiological function, and clinical outcomes (1). Undernutrition, specifically underweight conditions, is marked by body weight below healthy standards across age groups (2). In 2022, approximately 183 million women (95% CI: 169–197 million) and 164 million men (95% CI: 148–180 million) were underweight globally, down by 44.9 million women and 47.6 million men since 1990 (3).

Undernutrition disrupts neurotransmitter synthesis (e.g., serotonin, dopamine, GABA), impairing mood, sleep, and stress regulation, and increasing risks of depression and anxiety (4). Chronic malnutrition elevates cortisol, amplifies stress, and, through oxidative stress and inflammation, impairs cognitive functions like memory and attention. This creates a cycle where poor nutrition exacerbates psychological stress, further reducing appetite and worsening health (5, 6).

The gut-brain axis is critical in this interplay. Poor nutrition and gut dysbiosis, mediated by neural, metabolic, and immune pathways, contribute to stress and depression (7). Probiotics, or "psychobiotics," restore microbiome balance, modulate hormones (e.g., cortisol, serotonin), and reduce pro-inflammatory cytokines (e.g., IFN-γ, TNF-α), alleviating stress and enhancing mental well-being (7).

Probiotics also increase short-chain fatty acid production, which reduces inflammation in conditions like autoimmune disorders and inflammatory bowel disease (8).

While the gut-brain axis provides a promising framework for understanding the interplay between nutrition and mental health, the application of probiotic interventions in underweight individuals is less understood due to limited baseline data on their gut microbiota. While the gut-brain axis and probiotic interventions have been extensively studied in healthy and obese populations, data on gut microbiota alterations in underweight individuals remain limited (9). Undernutrition is associated with reduced microbial diversity, lower SCFA production, and compromised gut barrier integrity, which may uniquely influence the efficacy of probiotics in this population (10). Evidence from healthy or obese cohorts may not fully apply to underweight individuals due to these distinct microbial and physiological profiles. Consequently, this study cautiously interprets the effects of multi-strain probiotic supplementation, recognizing the need for baseline microbiota data specific to underweight individuals to enhance result validity and generalizability.

Recent studies demonstrate mental health benefits: *Lactobacillus casei Shirota* reduced anxiety by 16% and stress by 20% in athletes over 6 weeks (11), while *Lacticaseibacillus rhamnosus HN001* improved happiness and lowered stress in adults after 28 days (12). Synbiotics reduced stress and depression in adults with obesity over 8 weeks (13). Anti-inflammatory effects include lowered hs-CRP in type 2 diabetes and rheumatoid arthritis with *Bacillus coagulans* and *Lactobacillus casei* supplementation (14, 15).

However, the effects of specific multi-strain probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*) on psychological stress and inflammation in underweight individuals remain unexplored.

Abbreviations: BMI, Body Mass Index; CAD, Coronary Artery Disease; CFU, Colony Forming Units; CRP, C-reactive protein; ESR, Erythrocyte Sedimentation Rate; HPA, Hypothalamic–Pituitary–Adrenal (axis); IBD, Inflammatory Bowel Disease; IBS, Irritable Bowel Syndrome; IL, Interleukin; MDD, Major Depressive Disorder; NLR, Neutrophil-to-Lymphocyte Ratio; PLR, Platelet-to-Lymphocyte Ratio; PSS-10, 10-item Perceived Stress Scale; QOL, Quality of Life; SD, Standard Deviation; SPSS, Statistical Package for the Social Sciences; SCFAs, Short-Chain Fatty Acids; TNF-α, Tumor Necrosis Factor alpha.

This study evaluates the impact of eight-week multi-strain probiotic supplementation on psychological stress, measured via the Perceived Stress Scale, and inflammatory biomarkers in underweight adults, advancing nutritional strategies for mental and physiological health.

Methods

Study design and participants

This double-blind, randomized, placebo-controlled trial was conducted at the Specialized Nutrition Clinic of Imam Reza Hospital, Mashhad, Iran, between October 1, 2024, and February 28, 2025. The trial was registered with the Iranian Registry of Clinical Trials (IRCT Identifier: IRCT20230310057667N1). Participants were recruited through advertisements and screened for eligibility. Participants were one hundred adults (18–65 years) with undernutrition, defined by BMI <18.5 kg/m^2 and low FFMI (<17 kg/m^2 for men, <15 kg/m^2 for women), assessed to confirm reduced muscle mass. Exclusion criteria included a history of chronic diseases or gastrointestinal disorders, pregnancy, lactation, smoking, and use of antibiotics, probiotics, or foods containing probiotics within 3 months before the study. At baseline, participants completed a detailed questionnaire capturing demographic details (e.g., age, occupation, education), socio-economic status (e.g., household size, housing conditions), medical history, and current medication or supplement use. The sample size was determined based on prior research by Pan et al. (16), which investigated BMI changes following multi-species probiotic supplementation (*Bifidobacterium longum*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, 1×10^9 CFU/day) in adults undergoing peritoneal dialysis, a population with nutritional challenges. With an alpha of 0.01, power of 90% (beta = 0.10), and an effect size of 0.4, a minimum of 45 participants per group was required. Allowing for a 10% dropout, 50 participants per group were enrolled, totaling 100 participants (Figure 1).

Randomization and blinding

Participants were randomized into the probiotic or placebo group using permuted block randomization with a fixed block size

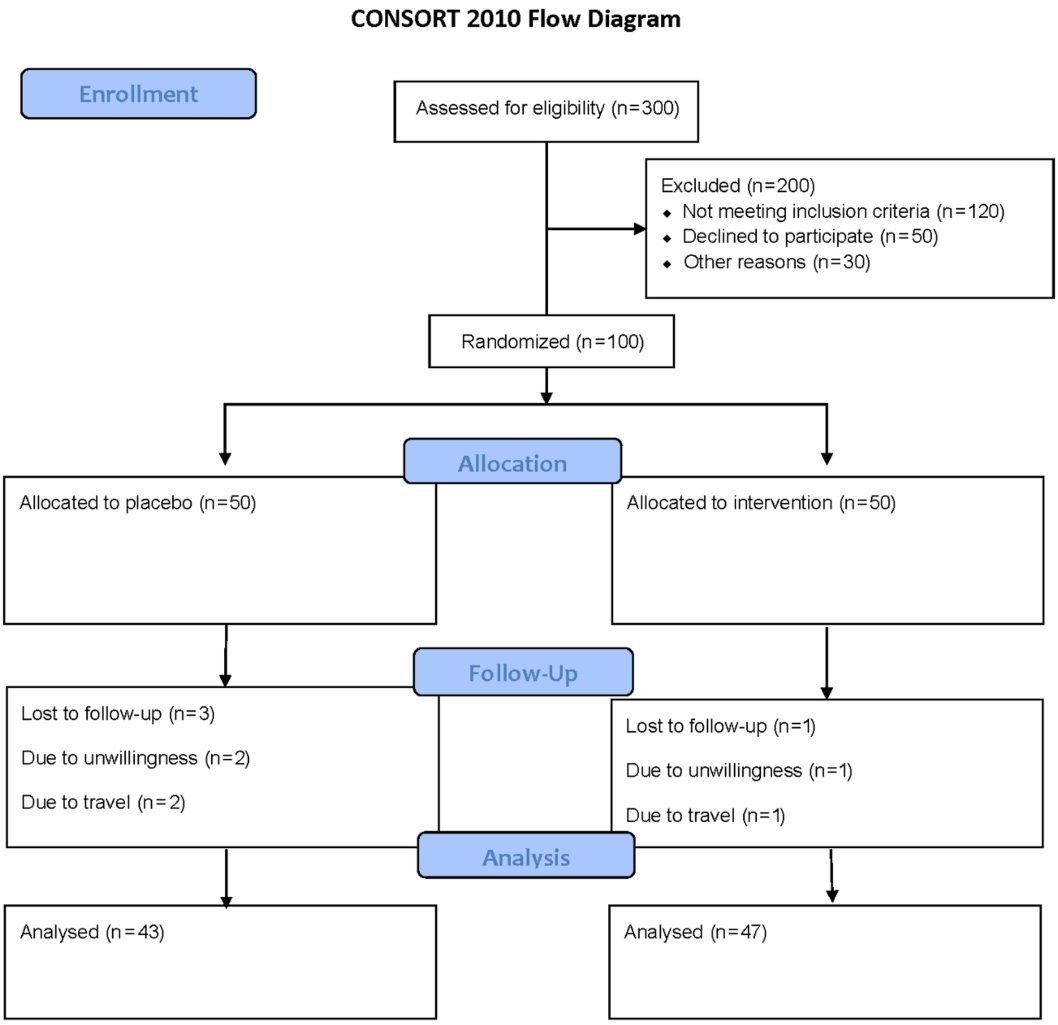


FIGURE 1
The flowchart of the study. Including patient screening, enrollment, randomization, and follow-up assessments at week 8 for outcome measurements.

of four, ensuring a 1:1 allocation ratio across 25 blocks for 100 participants (50 per group). The randomization sequence was generated by an independent statistician using a web-based platform.[1] Research assistants enrolled participants after screening, and a study coordinator assigned interventions using numbered, opaque, sealed envelopes to ensure allocation concealment. Both participants and study personnel, including those administering interventions and analyzing data, remained blind to group assignments throughout the study.

Intervention

Probiotic and placebo capsules, supplied in identical coded containers, contained 3×10^9 CFU of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and maltodextrin filler (probiotic) or maltodextrin alone (placebo), manufactured by ParsiLact Company. Participants were instructed to consume two capsules daily, one after lunch and one after dinner, for 8 weeks. Adherence was monitored via participant self-reported daily logs and capsule counts at week 8, with compliance defined as consuming ≥80% of prescribed capsules. Dietary intake was assessed at baseline and week 8 using 24-h dietary recalls to monitor potential changes. Weekly phone calls and text message reminders were used to reinforce adherence to the probiotic or placebo capsule regimen and to record any reported dietary changes.

Measurements

Perceived Stress Scale (PSS) questionnaire, blood sampling, and anthropometric indices were measured at the baseline and after 8 weeks.

Perceived stress scale

The PSS is one of the most commonly used tools for assessing the perception of stress (17, 18). The PSS-10 comprises ten items designed to evaluate the extent to which individuals perceive their life situations as stressful. Each item is scored on a scale from 0 to 4 (0 = never, 4 = very often). Scoring for the PSS-10 was conducted following the guidelines established by Cohen et al. (17) and Cohen (18). Total scores (0–40) categorize stress as none (0), low (0–13), moderate (14–26), or high (27–40).

Anthropometric indices

Height was measured to 0.5 cm using a stadiometer, weight to 0.1 kg with a digital scale (participants barefoot, lightly clothed), waist circumference at the midpoint between the lowest rib and iliac crest, and hip circumference at the widest point (19).

1 https://www.sealedenvelope.com

Blood sampling

Blood samples (8 mL) were collected at baseline and week 8 from a forearm vein by a trained technician at Navid Laboratory, Mashhad, Iran, between 8:00 and 9:30 AM. Venipuncture used 5-mL EDTA anticoagulant tubes. Samples were centrifuged at 3,000×*g* for 10 min at 4°C within 30 min of collection to separate serum and analyzed immediately. Complete blood count (CBC) was measured using Sysmex KX21, C-reactive protein (CRP) via Roche Cobas 6000 (immunoturbidimetric assay, detection limit 0.1 mg/L, intra-assay CV <5%), and erythrocyte sedimentation rate (ESR) via the Westergren method (ICSH standardized). Derived indices [neutrophil-to-lymphocyte (NLR), platelet-to-lymphocyte (PLR), monocyte-to-lymphocyte (MLR), and neutrophil-lymphocyte-platelet (NLPR) ratios] were calculated. Changes in these markers were analyzed as continuous outcomes using repeated measures ANOVA, with no predefined cut-off points applied.

Statistical analysis

Data were analyzed using SPSS v24 (IBM Corp, USA). Normality was tested with the Kolmogorov–Smirnov test. Normally distributed variables were reported as mean ± standard deviation (SD), compared within and between groups using paired and independent *t*-tests, respectively. Non-normal data were presented as median (IQR), analyzed with Wilcoxon and Mann–Whitney tests. Categorical variables were compared using chi-square tests. Repeated measures ANOVA was used to evaluate stress scores over time, with baseline variables (age, sex, socio-economic status, BMI) included as covariates to control for confounding effects. All tests were two-sided, with $p < 0.05$ considered significant.

Results

Ninety participants (mean age: 26.22 ± 7.42 years) completed the study (probiotic: $n = 47$; placebo: $n = 43$). The median age (interquartile range) was 25 (22–32) years for the probiotic group and 23 (20–27) years for the placebo group, with no significant difference ($p = 0.051$, Mann–Whitney test). Gender distribution was similar (male: female ratio was 2.92:1 in the intervention and 1.39:1 in the control group, $p = 0.101$, chi-square test).

Baseline anthropometric measures showed no significant differences ($p > 0.05$). Post-intervention, the probiotic group had significant increases in weight ($p = 0.005$), waist circumference ($p = 0.038$), and hip circumference ($p = 0.008$) compared to placebo. Within the probiotic group, significant improvements occurred in weight ($p < 0.001$), BMI ($p < 0.001$), waist circumference ($p < 0.001$), and hip circumference ($p < 0.001$). The placebo group showed improvements in weight ($p = 0.003$) and BMI ($p = 0.001$). Inflammatory markers differed significantly at baseline for ESR (1 and 2 h, $p < 0.001$ each). Post-intervention, ESR (2 h, $p < 0.001$) and CRP ($p < 0.001$) were significantly lower in the probiotic group. Within-group changes showed significant reductions in ESR (1 h: $p < 0.001$; 2 h: $p = 0.002$) and CRP ($p = 0.036$) in the probiotic group, and ESR (1 and 2 h: plinha <0.001 each) and CRP ($p = 0.017$) in the placebo group (Table 1).

A comparison of anthropometric and laboratory variables changes between the intervention and control groups is presented in Table 1.

Changes in stress scores between the intervention and control groups are presented and compared in Table 2 and Figure 2. A significant time effect ($p < 0.001$) and time-group interaction ($p < 0.001$) were observed for stress scores in the probiotic group. At the end of the study, stress scores differed significantly between groups ($p = 0.032$). Within the probiotic group, stress scores decreased significantly from baseline to week eight ($p < 0.001$), whereas no significant change was observed in the placebo group (Table 3).

Comparison of the stress levels between the intervention and control groups at baseline and the end of the study, and changes in stress category over time between groups are presented in Table 4. There was no significant difference in the distribution pattern of stress levels between groups at baseline ($p = 0.801$) or at the end of the study ($p = 0.108$). Of the participants in the control group, the stress level was reduced in four (9.3%), did not change in 37 (86%), and increased in two (4.7%) over the study duration. In contrast, in the intervention group, the stress level was reduced in 16 (34%), did not change in 30 (63.8%), and increased in one (2.2%) participant. A significant difference in stress level changes was observed between groups ($p = 0.017$, chi-square test).

Discussion

This study is the first to investigate the effects of multi-strain probiotic supplementation (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus rhamnosus*) on psychological stress and inflammatory markers in underweight adults. Our findings demonstrate significant improvements in PSS scores and reductions in CRP and ESR levels in the intervention group, highlighting probiotics' potential in addressing stress and inflammation in this population.

The gut-brain axis, a dynamic network of neural, immune, hormonal, and metabolic pathways, significantly influences mental health (20). Gut microbes regulate brain function by controlling inflammatory markers like interleukin-1, which can trigger cortisol release through the hypothalamic–pituitary–adrenal axis (21). Additionally, short-chain fatty acids (SCFAs) produced by gut microbiota contribute to mental health by modulating the immune system and neurotransmitter production. Probiotics enhance gut microbiome composition, reinforcing the intestinal barrier and producing antimicrobial substances that support mental well-being (22). To further elucidate the mechanisms underlying these effects, the potential direct and indirect actions of the probiotic strains used in this study warrant exploration. The observed improvements in PSS scores and reductions in CRP and ESR levels may result from both direct and indirect effects of the multi-strain probiotic supplementation. Directly, Lactobacillus strains may produce bioactive metabolites, such as short-chain fatty acids (SCFAs), which modulate immune responses and neurotransmitter synthesis, enhancing mental well-being (20–22). These strains may also directly interact with the hypothalamic–pituitary–adrenal (HPA) axis, potentially reducing cortisol levels by downregulating pro-inflammatory cytokines (e.g., IL-1, TNF-α) that stimulate cortisol release (21). Indirectly, probiotics may alter gut microbiota composition, strengthening the intestinal barrier and reducing systemic inflammation, although some studies suggest probiotic supplementation does not always significantly change microbiota composition (23). The absence of microbiome analysis in this study limits our ability to confirm these mechanisms. Future research should include microbial profiling to elucidate whether the observed effects are primarily driven by direct probiotic actions or microbiota-mediated changes.

SCFAs, such as acetate, propionate, and butyrate, produced by Lactobacillus strains, likely contribute to the observed reductions in PSS scores and inflammatory markers by modulating the gut-brain-immune axis. SCFAs enhance GABAergic activity by upregulating GABA receptor expression in the brain, potentially reducing stress and improving emotional regulation (24). Additionally, SCFAs inhibit pro-inflammatory cytokines (e.g., IL-6, TNF-α) by suppressing NF-κB signaling, which may explain the reductions in CRP and ESR levels (25). Although IL-6 and TNF-α were not measured in this study, their involvement in SCFA-mediated immune modulation suggests a mechanistic pathway for future investigation. These effects underscore the role of SCFAs in linking gut microbiota to brain function and systemic inflammation.

Research into natural alternatives for cognitive and mental health improvement has expanded, leading to the concept of 'psychobiotics,' probiotics that confer mental health benefits (26). Probiotics influence the gut-brain axis and provide a natural approach to managing stress and enhancing mental health outcomes (27). Several clinical trials have examined probiotics' effects on psychological health, with varying results depending on the population and probiotic strain used (12).

Studies on *Lactobacillus rhamnosus* have reported both positive and neutral results. For example, supplementation with *Lactobacillus rhamnosus* improved depressive symptoms and quality of life in post-myocardial infarction patients (28), and reduced postnatal depression and anxiety in pregnant women (29). However, no significant benefits were observed in university students or healthcare workers during the COVID-19 pandemic (30, 31). These discrepancies suggest that probiotic efficacy may depend on population characteristics and external factors. *Lactobacillus casei* supplementation has been shown to improve sleep quality and reduce stress-related symptoms in medical students during exams and athletes under competitive pressure (11, 32). Furthermore, synbiotic supplementation combining *Lactobacillus acidophilus* with other strains has demonstrated reductions in stress, anxiety, and depression in individuals with various conditions (13, 33, 34).

Regarding inflammatory markers, ESR and CRP levels decreased in the intervention group, but the between-group differences were not statistically significant, possibly due to the small sample size or low baseline inflammation levels. The use of more sensitive biomarkers, such as IL-6 or TNF-α, may better capture subtle inflammatory changes in future studies (35). This aligns with previous studies that reported reductions in hs-CRP levels following probiotic supplementation in patients with rheumatoid arthritis, coronary artery disease, and type-2 diabetes (14, 15, 36). The reason for the no significant difference observed in NLR, PLR, MLR and NLPR was hypothesized to be due to the changes in these parameters being within the normal range. Limitations include the absence of microbiome analysis to clarify mechanisms and a modest sample size that limits generalizability. Future research should involve larger samples and microbial profiling to optimize probiotics interventions. This study did not evaluate gut permeability, absorption efficacy, psychological effects and other possible factors that might affect the outcomes. Therefore, it I suggested that further studies evaluate these factors and the mechanism of the observed effects.

TABLE 1 Comparison of the demographic, anthropometric and laboratory variables between the intervention and control groups.

Variable	Time	Intervention	Control	Between-group *p*
Age (years)		25 (22–32), 95%CI: 24.39–28.55	23 (10–17), 95%CI: 23.60–28.24	0.052[a]
Gender	Male	12 (25.5%)	18 (41.9%)	0.101[c]
	Female	35 (74.5%)	25 (58.1%)	
Height (cm)	Baseline	169.77 ± 9.98, 95%CI: 166.84–172.70	166.60 ± 8.622, 95%CI: 164.68–169.93	0.115[b]
Weight (kg)	Baseline	50.37 ± 8.00, 95%CI: 47.94–52.64	47.73 ± 6.68, 95%CI: 46.34–50.40	0.094[b]
	End of study	52.78 ± 8.41, 95%CI: 50.24–55.17	48.18 ± 6.79, 95%CI: 46.77–50.40	0.005[b]*
Within group *p*		<0.001*	0.003*	
BMI (kg/m^2)	Baseline	17.6 (16.7–18.5), 95%CI: 16.93–17.81	17.4 (16.4–18.2), 95%CI: 16.88–17.63	0.487[a]
	End of study	18.18 (17.1–19.2), 95%CI: 17.74–18.66	17.5 (16.6–18.5), 95%CI: 17.04–17.94	0.052[a]
Within group *p*		<0.001*	0.001*	
FFMI (kg/m^2)	Baseline	15.60 ± 1.80, 95%CI: 15.07–16.13	15.20 ± 1.17, 95%CI: 14.86–15.54	0.205[b]
	End of study	15.46 ± 2.32, 95%CI: 14.77–16.14	15.13 ± 1.28, 95%CI: 14.76–15.50	0.406[b]
Within group *p*		0.551	0.340	
Waist circumference (cm)	Baseline	72 (68–75), 95%CI: 69.59–72.41	72 (68–75), 95%CI: 69.36–73.18	0.743[a]
	End of study	74 (70–78), 72.26–75.89	72 (67–75), 95%CI: 69.13–72.75	0.038[a]*
Within group *p*		<0.001*	0.171	
Hip circumference (cm)	Baseline	89 (86–92), 95%CI: 88.00–90.38	87 (85–89), 84.78–88.76	0.052[a]
	End of study	90 (88–94), 95%CI: 85.94–92.75	88 (85–92), 85.85–89.41	0.008[a]*
Within group *p*		<0.001*	0.110	
ESR 1 h	Baseline	5 (3–10), 95%CI: 5.36–7.91	3 (2–5), 95%CI: 3.00–4.92	<0.001[a]*
	End of study	8 (5–15), 95%CI: 3.72–5.25	5 (4–9.25), 95%CI: 4.12–6.30	0.442[a]
Within group *p*		<0.001*	<0.001*	
ESR 2 h	Baseline	3 (3–6), 95%CI: 8.26–11.40	4 (3–7), 95%CI: 5.94–9.73	0.010[a]*
	End of study	6 (4–7), 95%CI: 5.27–7.41	9 (5–13.25), 95%CI: 5.94–9.73	<0.001[a]*
Within group *p*		0.002*	<0.001*	
CRP	Baseline	1 (0.5–3), 95%CI: 1.39–2.28	1.1 (1–4.05), 95%CI: 1.88–3.06	0.198[a]
	End of study	1 (0.3–1.2), 95%CI: 0.85–1.72	4 (1.38–4.8), 95%CI: 3.01–4.76	<0.001[a]*
Within group *p*		0.036*	0.017*	–
NLR	Baseline	1.87 (1.61–2.46), 95%CI: 1.84–2.26	1.75 (1.43–2.63), 95%CI: 1.70–2.11	0.232[a]
	End of study	2.05 (1.32–2.49), 95%CI: 1.85–2.29	1.61 (1.26–2.49), 95%CI: 1.64–2.10	0.180[a]
Within group *p*		0.705	0.440	–
PLR	Baseline	7.83 ± 2.22, 95%CI: 7.17–8.48	7.48 ± 2.22, 95%CI: 6.76–8.05	0.453[b]
	End of study	7.39 (6.25–8.74), 95%CI: 7.12–8.36	7.19 (5.69–8.36), 95%CI: 6.46–8.18	0.371[a]
Within group *p*		0.832	0.340	–
MLR	Baseline	0.15 (0.13–0.20), 95%CI: 0.15–0.18	0.16 (0.11–0.22), 95%CI: 0.14–0.18	0.961[a]
	End of study	0.16 (0.13–0.20), 95%CI: 0.16–0.22	0.14 (0.11–0.19), 95%CI: 0.14–0.18	0.141[a]
Within group *p*		0.262	0.310	–
NLPR	Baseline	0.01 (0.01–0.01), 95%CI: 0.01–0.01	0.01 (0.01–0.01), 95%CI: 0.01–0.01	0.374[a]
	End of study	0.01 (0.01–0.01), 95%CI: 0.01–0.01	0.01 (0.01–0.01), 95%CI: 0.01–0.01	0.175[a]
Within group *p*		0.341	0.378	–

Between-group comparisons are presented in columns and within group comparisons are presented in rows below each variable.

[a]Median and interquartile range (IQR) were presented, and between-group comparison was performed using the Mann–Whitney test, while within-group comparison was performed using the Wilcoxon test.

[b]Mean and standard deviation (SD) were presented, and comparison was performed using an independent *t*-test, while within-group comparison was performed using the paired sample *t*-test.

[c]Frequency and percentage were presented, and comparison was performed using the chi-square test.

*Significant difference.

TABLE 2 Comparison of the changes in the anthropometric and laboratory variables between the intervention and control groups.

Variable	Intervention	Control	*p*
Weight (cm)	2.41 ± 1.59	0.45 ± 0.93	<0.001*[b]
BMI (kg/m^2)	0.8 (0.5–1.1)	0.2 (0–0.47)	<0.001*[a]
Waist circumference (cm)	3 (2–4)	0 (0–1)	<0.001*[a]
Hip circumference (cm)	2 (1–3)	0 (0–1)	<0.001*[a]
ESR 1 h	−1 (−4 to 0)	0.5 (0–2)	<0.001*[a]
ESR 2 h	−1 (−7 to 0)	2 (0–5)	0.001*[a]
CRP	−0.5 (−2 to 0.2)	0.15 (−0.53 to 3.88)	0.001*[a]
NLR	0.03 ± 0.72	−0.04 ± 0.61	0.010*[b]
PLR	0.04 (−1.84 to 1.02)	−0.29 (−1.13 to 0.94)	0.663[a]
MLR	0.01 (−0.03 to 0.51)	−0.01 (−0.05 to 0.03)	0.137[a]
NLPR	0 (0–0)	0 (0–0)	0.180[a]

[a]Median and interquartile range (IQR) were presented, and between-group comparison was performed using the Mann–Whitney test.
[b]Mean and standard deviation (SD) were presented, and comparison was performed using an independent *t*-test. *Significant difference.

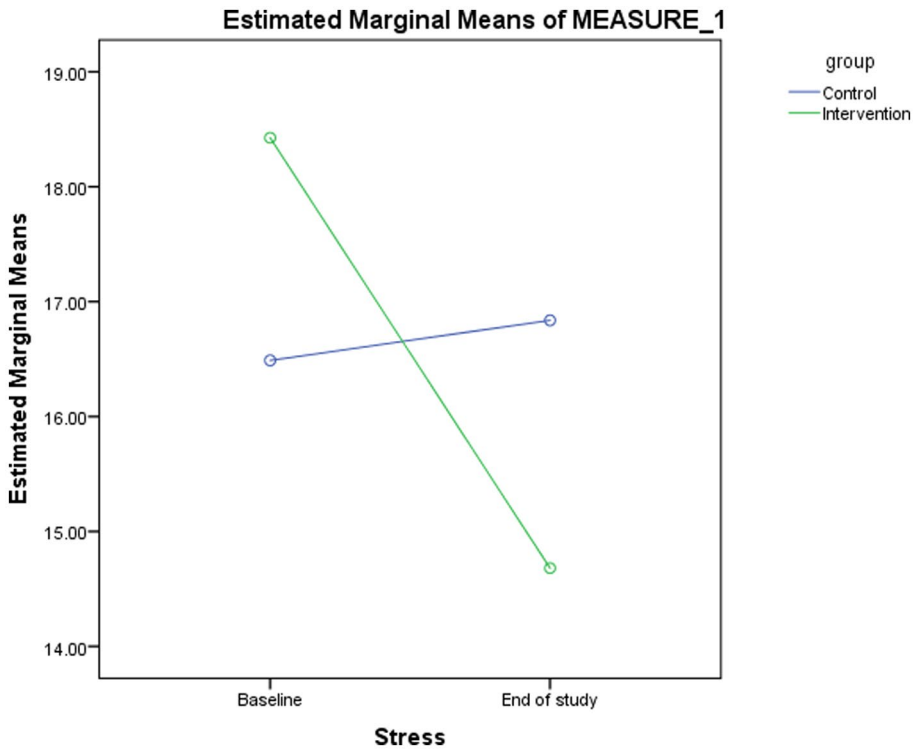


FIGURE 2
Changes in the estimated marginal means for stress score between baseline and the end of study in the intervention and control groups.

TABLE 3 Comparison of stress score between the intervention and control groups at baseline and the end of the study.

Variable	Time	Intervention	Control	Time effect *p*	Group effect *p*	Time-group interaction *p*
Stress score	Baseline	18 (16–22)[a]	15 (14–19)	<0.001*	0.905	<0.001*
	End of study	14.68 ± 4.09[ab]	16.84 ± 5.25[b]			

*Significant difference using repeated measures analysis of variance. [a]$p < 0.001$. [b]$p = 0.032$.

TABLE 4 Comparison of the stress levels between the intervention and control groups at baseline and the end of the study and changes in stress category over time between groups.

Time	Stress level	Intervention frequency (%)	Control frequency (%)	*p*
Baseline	Low	8 (17%)	9 (20.9%)	0.801
	Moderate	37 (78.7%)	33 (76.7%)	
	High	2 (4.3%)	1 (2.3%)	
End of study	Low	21 (44.7%)	12 (36.4%)	0.108
	Moderate	26 (55.3%)	29 (67.4%)	
	High	0 (0%)	2 (4.7%)	

The chi-square test was used for the comparison.

Conclusion

In conclusion, probiotic supplementation in underweight patients benefits mental health and reduces inflammation in underweight adults, offering a complementary approach to stress management. Further studies are needed to validate these findings and explore probiotics as a primary stress intervention.

Data availability statement

Data described in the manuscript, will be made available upon written request to the corresponding author and approval by the Vice Chancellor of Research and technology of the Mashhad University of Medical Sciences.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Research Vice-Chancellor Mashhad University of Medical Sciences, Mashhad, Iran. 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

MA-K: Investigation, Writing – review & editing, Data curation, Conceptualization, Writing – original draft, Project administration, Supervision. AH: Investigation, Writing – review & editing, Resources, Visualization, Writing – original draft, Data curation, Supervision, Conceptualization, Project administration. PA: Writing – review & editing, Writing – original draft. AJ: Software, Formal analysis, Methodology, Writing – review & editing. AA: Resources, Supervision, Methodology, Writing – review & editing. FJ: Writing – review & editing, Data curation. MN: Conceptualization, Resources, Funding acquisition, Project administration, Validation, Investigation, 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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Bifidobacterium longum subsp. *infantis* CCFM1426 enhances the anti-colitic effect of vitamin A via retinoic acid restoration and gut microbiota modulation in ulcerative colitis mice

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Background: Ulcerative colitis (UC) is a chronic inflammatory bowel disease with increasing global prevalence, making it a significant health concern. Although vitamin A (VA) plays a beneficial role in UC management, its therapeutic efficacy is limited by impaired absorption and disrupted retinoic acid (RA) metabolism. Gut microbiota are known to influence VA metabolic pathways, offering potential targets to enhance VA bioavailability and efficacy.

Methods: A dextran sulphate sodium (DSS)-induced mouse model of colitis was established to evaluate the therapeutic effects of co-administering *Bifidobacterium longum* subsp. *infantis* CCFM1426 with vitamin A. Body weight, disease activity index (DAI) and colon length were monitored in mice with DSS-induced colitis. Serum levels of intestinal injury markers, inflammatory cytokines, antioxidant enzymes and colonic RA levels were measured using ELISA kits. Metagenomic analysis investigated gut microbiota composition.

Results: It was indicated that the VA and CCFM1426 combination significantly improved colon length and DAI, enhanced serum levels of intestinal injury markers (lipopolysaccharide-binding protein, intestinal fatty acid-binding protein, diamine oxidase) and cytokines (IL-6, TNF-α, IL-10), and restored antioxidant capacity. The combination demonstrated superior efficacy in colonic RA levels and contributed to gut microbiota diversity restoration. Metabolomics analysis showed that colitis mice treated with the combination had higher levels of eicosapentaenoic acid, adenosine and anandamide.

Conclusion: These findings provide novel evidence that co-administration of CCFM1426 and VA synergistically alleviates colitis by enhancing RA bioavailability through microbiota-dependent pathways.

KEYWORDS

ulcerative colitis, vitamin A, retinoic acid, *Bifidobacterium longum* subsp. *infantis*, gut microbiota

1 Introduction

Inflammatory bowel disease (IBD) is a chronic condition characterised by gastrointestinal tract inflammation (1). The rising global prevalence of IBD presents significant challenges to public health and safety (2, 3). Ulcerative colitis (UC), a common type of IBD, primarily affects the colon and rectum, compromising the mucosal barrier and colonic intestinal epithelium, leading to intestinal barrier dysfunction (4). Clinical symptoms of UC include diarrhoea, bloody stools and abdominal pain, often associated with dysregulated intestinal immune systems, barrier damage and imbalanced intestinal flora homeostasis (5). At present, the clinical treatment of UC mainly relies on medications such as 5-aminosalicylic acid (5-ASA), corticosteroids, and immunomodulators (6). However, these treatment approaches are associated with issues such as drug dependence and resistance, and long-term use may even lead to serious adverse effects (7). Therefore, identifying safe and effective strategies for UC management is of critical importance.

Currently, UC treatment strategies targeting nutrition and the gut microbiota are gaining increasing attention (8, 9). Vitamin A (VA) is an essential micronutrient for growth and development with numerous physiological functions including maintenance of mucosal integrity, preservation of visual functions, enhancement of immune response and participation in cell growth and differentiation (10). Supplementation with VA has been shown to mitigate UC by restoring microbial diversity in the gut of colitis mice and alleviating intestinal damage and inflammation *in vivo* (11). In individuals with UC, VA treatment can effectively decrease the disease activity index (DAI) (12). Retinoic acid (RA), one of VA's primary active forms, is involved in crucial physiological processes (13). RA plays a vital role during embryonic and postnatal periods and is recognised as a substantial regulator of mammalian gene expression (14). Notably, RA has been shown to contribute to the maintenance of intestinal barrier function, the regulation of mucosal immune responses, and the modulation of host-microbiota interactions, thereby supporting intestinal homeostasis (15). Although VA has been shown to be beneficial in inflammatory conditions, mucosal damage in UC patients often results in decreased serum VA levels and impaired intestinal absorption (16–18). Furthermore, dysregulated RA biosynthesis in UC is further exacerbated by changes in the expression of the enzymes involved in VA metabolism, such as decreased ALDH1A1 and increased CYP26A1, which worsen inflammation and barrier dysfunction (19).

Recent studies have uncovered a critical role for gut microbiota in modulating host vitamin A metabolism. Intestinal flora exhibit host-independent metabolism of VA, producing retinaldehyde, RA and other active substances (20). These microbiota-derived retinoids can modulate the expression of serum amyloid A (SAA) and retinaldehyde dehydrogenases (ALDH) in intestinal epithelial cells, thereby regulating host RA levels, modulating intestinal immunity, and alleviating gut inflammation (21, 22). The impairment of RA-mediated signal transduction in the intestine and systemic induction in UC may be associated with disturbed VA metabolism in gut microbiota due to ecological dysregulation (16, 17). Therefore, modulating gut microbiota to regulate host VA metabolism may represent an effective strategy for alleviating colitis.

Supplementing with probiotics is an effective way to regulate the gut microbiota (23). *Bifidobacterium longum* subsp. *infantis* is a well-documented probiotic with anti-inflammatory properties and proven benefits in maintaining gut barrier integrity. The strain FJSYZ1M3 of *B. infantis* maintains the integrity of the intestinal barrier and produces beneficial metabolites such as butyrate (24). The combination of *B. infantis* and xylo-oligosaccharides has been shown to enhance colonic epithelial barrier function more effectively than either the probiotic or the prebiotic alone (25). In addition, probiotic supplementation in early infancy can enhance the protective effects of prenatal vitamin supplementation (26). The combination of lactic acid bacteria and vitamin E metabolites can exert a synergistic effect in alleviating colitis (27). Therefore, We propose that co-supplementation with VA and *B. infantis* may synergistically alleviate UC by altering the gut microbiota and promoting RA bioavailability.

Accordingly, this study explored the therapeutic effects of combined supplementation with *B. infantis* CCFM1426 and VA in a dextran sulphate sodium (DSS)-induced colitis mouse model. We hypothesised that this combination would synergistically enhance colonic RA levels, modulate gut microbiota composition, and ultimately improve colitis outcomes beyond VA supplementation alone. Furthermore, we investigated underlying mechanisms involving intestinal barrier markers, inflammatory cytokines, antioxidant enzymes, microbial shifts, and faecal metabolites to elucidate the multi-dimensional benefits of this nutrition–microbiota synergistic approach.

2 Materials and methods

2.1 Bacterial strains

The bacterial strain CCFM1426 used in this study was obtained from the Culture Collections of Food Microbiology (CCFM) at Jiangnan University, Wuxi, China. Following three successive subcultures in MRS broth, bacteria were harvested by centrifugation (8,000 rpm, 15 min, 4°C), resuspended in 30% glycerol, and stored at −80°C. The final bacterial suspension for oral gavage was standardised to approximately 109 CFU/mL.

2.2 Preparation of vitamin A and retinoic acid for oral gavage

VA was purchased from J&K Scientific Ltd. (Beijing, China), whilst RA was obtained from Titan Scientific Co., Ltd. (Shanghai, China). For each preparation, 0.5 mg of VA or RA was dissolved in 200 μL of plant oil and thoroughly vortexed until completely dissolved (28, 29). The resulting solutions were freshly prepared before administration and protected from light throughout the preparation process.

2.3 Experimental design

Six-week-old male C57BL/6 J mice were acquired from Vital River Laboratory Animal Technology Co., Ltd. (Jiaxing, China) and maintained in specific pathogen-free (SPF) conditions. The

laboratory environment was maintained at 20–26°C with 40–70% relative humidity and a 12-h light/dark cycle. All mice received ad libitum access to food and water. The experimental protocols were approved by the Ethics Committee of Experimental Animals of Jiangnan University (JN. No20220915b1281110[318]).

Following a one-week acclimatisation period, 30 mice were randomly allocated into five groups (n = 6 per group): control, model, VA, RA, and CCFM1426 + VA. The UC model was established using previously described methods (30). Briefly, mice in the model group received drinking water supplemented with 3% (w/v) dextran sodium sulphate (molecular weight, 36–50 kDa) for 7 days, followed by administration of 200 μL 0.9% saline for 6 days. The intervention doses of VA and RA were determined according to previously published methods (28, 29). The VA and RA groups received 200 μL of vegetable oil solution containing 0.5 mg of VA or RA, respectively, for six consecutive days. The CCFM1426 + VA group received 10^9 CFU/mL of CCFM1426 bacterial suspension combined with 0.5 mg VA in 200 μL of vegetable oil solution for 6 days.

2.4 Weight measurement and evaluation of colitis mice

Body weight was measured daily prior to gavage. The percentage of body weight change (%) was calculated as:

$$Percentage\ of\ body\ weight\ (\%) = (current\ weight) / (8th\ day\ weight) \times 100\%$$

DAI was assessed according to previously established methods, based on changes in body weight, presence of blood in stool, and faecal consistency. Occult blood in stool was detected using an OB (occult blood) reagent kit (Cell Science & Technology Institute, China). The DAI score was calculated based on the criteria listed in Table 1.

2.5 Histopathological changes in mouse colon tissues

Colon tissues were excised, fixed in 4% paraformaldehyde for a minimum of 24 h, and subsequently embedded in paraffin. Tissue sections were cut and mounted with neutral gum. Haematoxylin and eosin (H&E) staining was performed, and images were captured using a microscopy imaging system.

2.6 Detection of serum biomarkers for intestinal barrier damage

On day 14 of the experiment, mice were sacrificed and blood samples were collected via orbital extraction. The blood was collected in sterile centrifuge tubes and centrifuged at 3000 rpm at 4°C for 15 min. The serum was then transferred to sterile PCR tubes, with remaining aliquots stored at −80°C for subsequent analyses. Serum levels of intestinal fatty acid-binding protein (I-FABP), lipopolysaccharide-binding protein (LBP), and diamine oxidase (DAO) were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Enzyme-linked Biotechnology Co., Ltd.).

2.7 Measurement of RA, inflammatory cytokines, and antioxidant enzyme levels in the colon

Collected colon tissues were homogenised using a tissue grinder, followed by centrifugation to obtain the supernatant. Protein content in the supernatant was quantified using the BCA Protein Assay Kit (Beyotime). Enzyme-linked immunosorbent assay (ELISA) kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) were employed to assess levels of retinoic acid, glutathione peroxidase (GSH-PX), catalase (CAT), and superoxide dismutase (SOD) activity in colon tissues. Additionally, inflammatory cytokines, including tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), were measured in colon tissues using the same ELISA kits, following the manufacturer's instructions.

2.8 Untargeted metabolomics analysis of faeces

Faecal sample pre-treatment was conducted according to previously established protocols. Briefly, non-targeted metabolite analysis was performed using a Vanquish UHPLC Q-Exactive Plus MS system with a Phenomenex UHPLC Kinetex C18 column (2.6 μm, 2.1 × 100 mm). The analysis employed a binary solvent system comprising mobile phase A (0.01% acetic acid in H2O) and mobile phase B (50% ACN + 50% IPA). The gradient elution profile was programmed as follows: 0–1 min, 1% B; 1–8 min, 1 to 99% B; 8–9 min, 99% B; 9–9.1 min, 99 to 1% B; 9.1–12 min, 1% B. The column was maintained at 35°C with a flow rate of 300 μL/min and an injection volume of 2 μL. Metabolites with an mzCloud Best Match score > 85 were considered confidently

TABLE 1 Scoring criteria for calculating DAI.

Score	Weight loss (%)	Stool consistency	Blood in stools
0	1–5	Normal	No colour change upon addition of the reagent
1	5–10	Soft but well formed	Purplish-red coloration appears within 1–2 min
2	10–15	Soft but not formed	Purplish-red coloration appears within 1 min
3	15–20	Slight diarrhoea	Purplish-red coloration appears within 10 s
4	More than 20	Watery diarrhoea	Immediate purplish-red coloration upon reagent addition

identified. The processed metabolomic data were analyzed using MetaboAnalyst 6.0.[1] Prior to statistical analysis, metabolites with a relative standard deviation (RSD) >20% were excluded to ensure data quality (31). The remaining data were subjected to log10 transformation and autoscaling to normalise distributions and reduce heteroscedasticity.

2.9 Metagenomic analysis of mouse faeces

Metagenomic analysis of mouse faecal samples ($n = 5$ for CON and MOD; $n = 4$ for treatment groups) was performed by Novogene Co. Ltd. (Beijing, China). In brief, low-quality sequences from raw data generated by the DNBSEQ-T7 platform were filtered using Trimmomatic (version 0.39). Sequences with average base quality scores below 30 were trimmed, whilst those exceeding 60 bp were retained for subsequent analysis. Host genomic sequences were removed using BWA (version 0.7.17), Samtools (version 1.17), and BEDTools (version 2.30.0), and the cleaned sequences were aligned to the Genome Reference Consortium Mouse Build 39 (GRCm39). Intestinal microbiota species were annotated using MetaPhlAn4. Spearman correlation heatmaps were generated using an online tool.[2] Beta diversity and linear discriminant analysis of effect size (LEfSe) were conducted using https://www.bioincloud.tech/. The metagenomic sequencing data generated have been deposited in the NCBI under the BioProject accession number PRJNA1275596.

2.10 Statistical analysis

Data analysis and visualisation were performed using GraphPad Prism (version 9.5.0) and OriginPro 2022. Results are presented as mean ± standard error of the mean (SEM). Group differences were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Significant inter-group differences were analysed using GraphPad Prism (version 9.5.0). Unless otherwise specified, results indicate differential comparisons with the model group; "*" indicates $p < 0.05$, "**" indicates $p < 0.01$, "***" indicates $p < 0.001$, "****" indicates $p < 0.0001$.

3 Results

3.1 VA and CCFM1426 combination alleviates changes in body weight and colonic tissues in colitis mice

Body weight decreased in all treatment groups compared to the control group, although the VA and CCFM1426 combination did not significantly affect body weight relative to the model group (Figure 1A). DAI was significantly elevated in the model group compared to the control group (Figure 1B; $p < 0.0001$). Treatment with the VA and CCFM1426 combination significantly reduced DAI in colitis mice ($p < 0.0001$). As depicted in Figure 1C, colon length was significantly shorter in the model group than in the control group ($p < 0.001$). Supplementation with VA, RA, or the combination significantly increased colon length in colitis mice ($p < 0.05$). Histological examination (Figure 1D) revealed that the model group exhibited disrupted colonic crypts, upward displacement, crypt distortion, inflammatory cell infiltration, and increased basal plasma cells with haemorrhage. Both RA and the combination treatment reduced inflammation, decreased cell infiltration, restored crypt structure, and improved crypt morphology without significant bleeding.

3.2 The combination of CCFM1426 and VA reduces serum biomarkers of intestinal barrier damage in colitis mice

To investigate the effects of probiotic treatment on intestinal barrier function, serum levels of DAO, LBP, and I-FABP were measured. As shown in Figures 2A–C, the model group exhibited significantly higher serum levels of these markers compared to the control group ($p < 0.05$). In colitis mice, serum levels of DAO, LBP, and I-FABP were reduced following administration of VA, RA, or the combination. However, VA treatment alone did not significantly reduce LBP and DAO levels, whereas RA treatment significantly decreased all three markers ($p < 0.01$; Figures 2A–C). The CCFM1426 and VA combination significantly reduced all three marker levels, demonstrating superior therapeutic efficacy compared to VA alone ($p < 0.01$; Figures 2A–C).

3.3 The combination of CCFM1426 and VA modulates RA and inflammatory cytokine levels in colitis mice

The anti-inflammatory effects of VA, RA, and their combination in DSS-treated mice were assessed by measuring pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and the anti-inflammatory cytokine IL-10. The model group exhibited decreased IL-10 levels compared to the control group (Figure 3A). Furthermore, the model group demonstrated significantly elevated levels of TNF-α, IL-1β, and IL-6 in colonic tissues compared to the control group ($p < 0.01$; Figures 3B–D). Treatment with VA, RA, or the combination modulated both pro- and anti-inflammatory cytokine profiles. In colitis mice, the CCFM1426 and VA combination significantly reduced IL-6 and TNF-α levels whilst increasing IL-10 levels ($p < 0.05$; Figures 3A–C). Similarly, RA treatment significantly reduced TNF-α, IL-1β, and IL-6 levels whilst markedly increasing IL-10 levels in the model group ($p < 0.05$; Figures 3A–D). Additionally, DSS-treated mice exhibited substantially lower colonic RA levels compared to control mice ($p < 0.001$; Figure 3E). Treatment with RA or the combination significantly improved colonic RA levels ($p < 0.05$), with the combination proving more effective than VA alone, although the difference was not statistically significant.

1 https://www.metaboanalyst.ca/

2 https://www.omicstudio.cn/tool

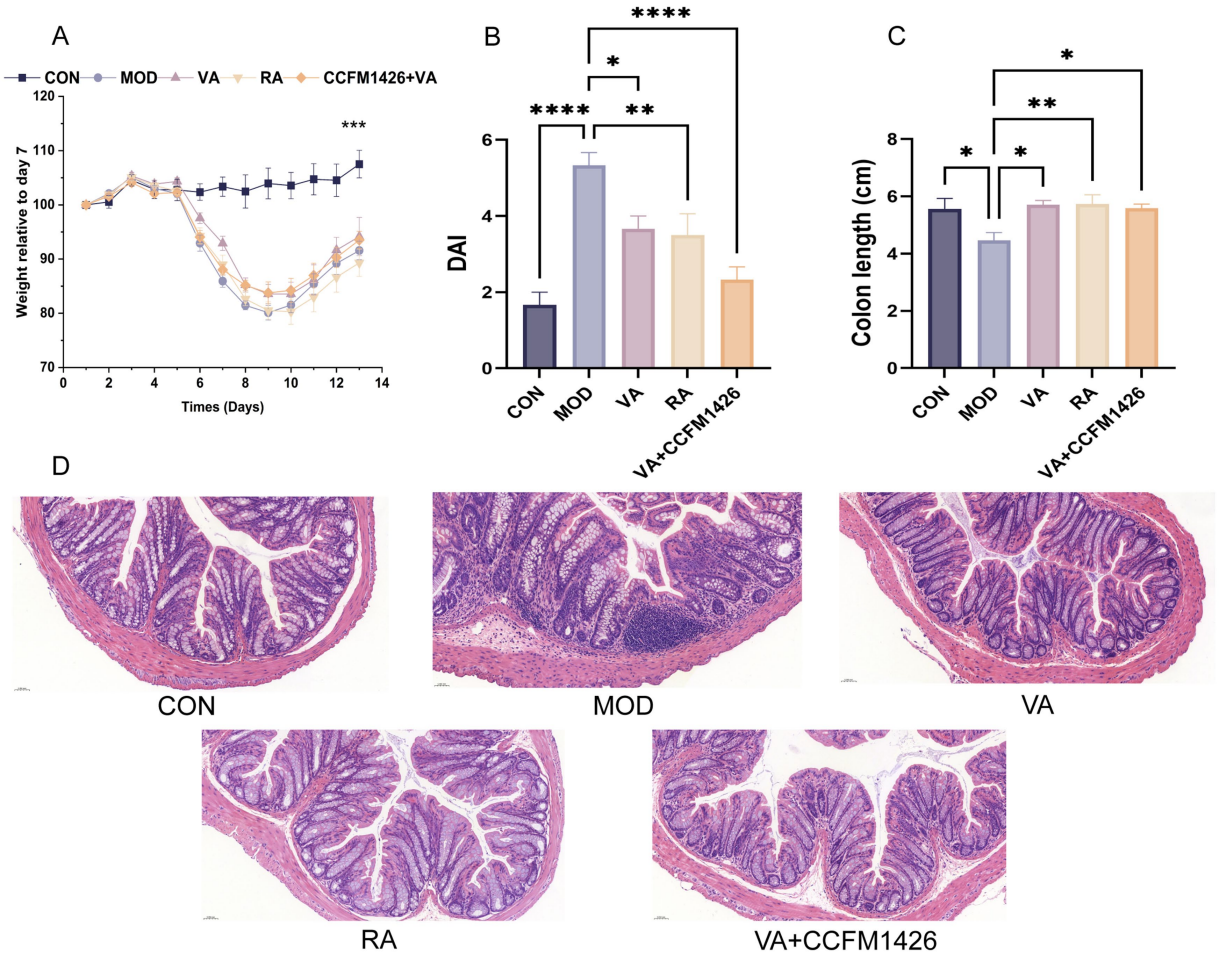

FIGURE 1
Vitamin A and CCFM1426 combination alleviates changes in body weight and colonic tissues in colitis mice. **(A)** Body weight. **(B)** DAI scores. **(C)** Colon length. **(D)** Representative images of H&E-stained colon sections, ×20.

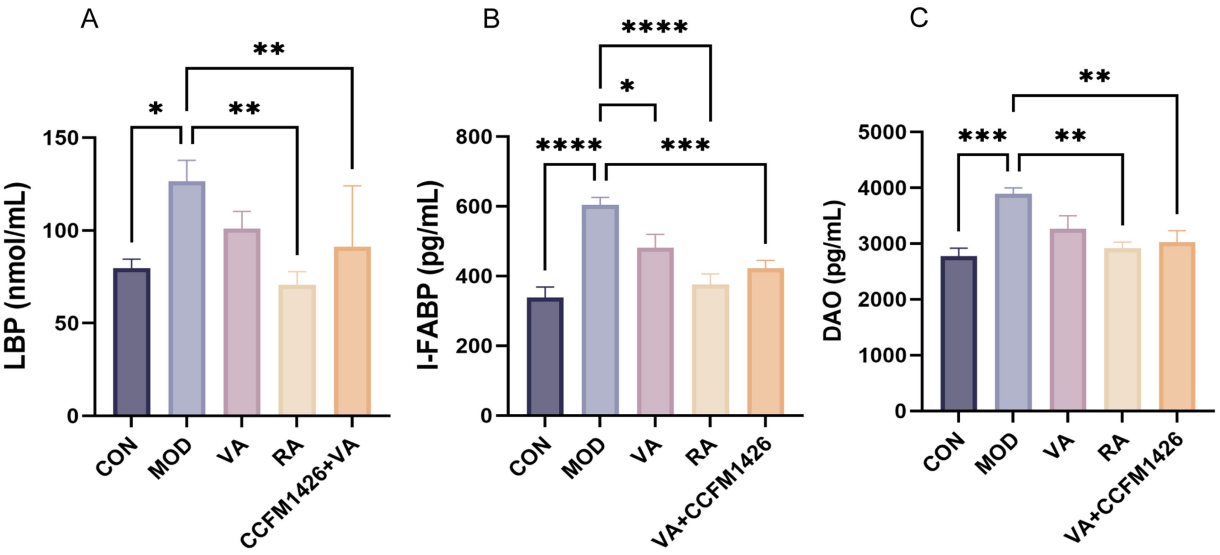

FIGURE 2
The combination of CCFM1426 and vitamin A reduces serum biomarkers of intestinal barrier damage in colitis mice. **(A)** LBP. **(B)** I-FABP. **(C)** DAO.

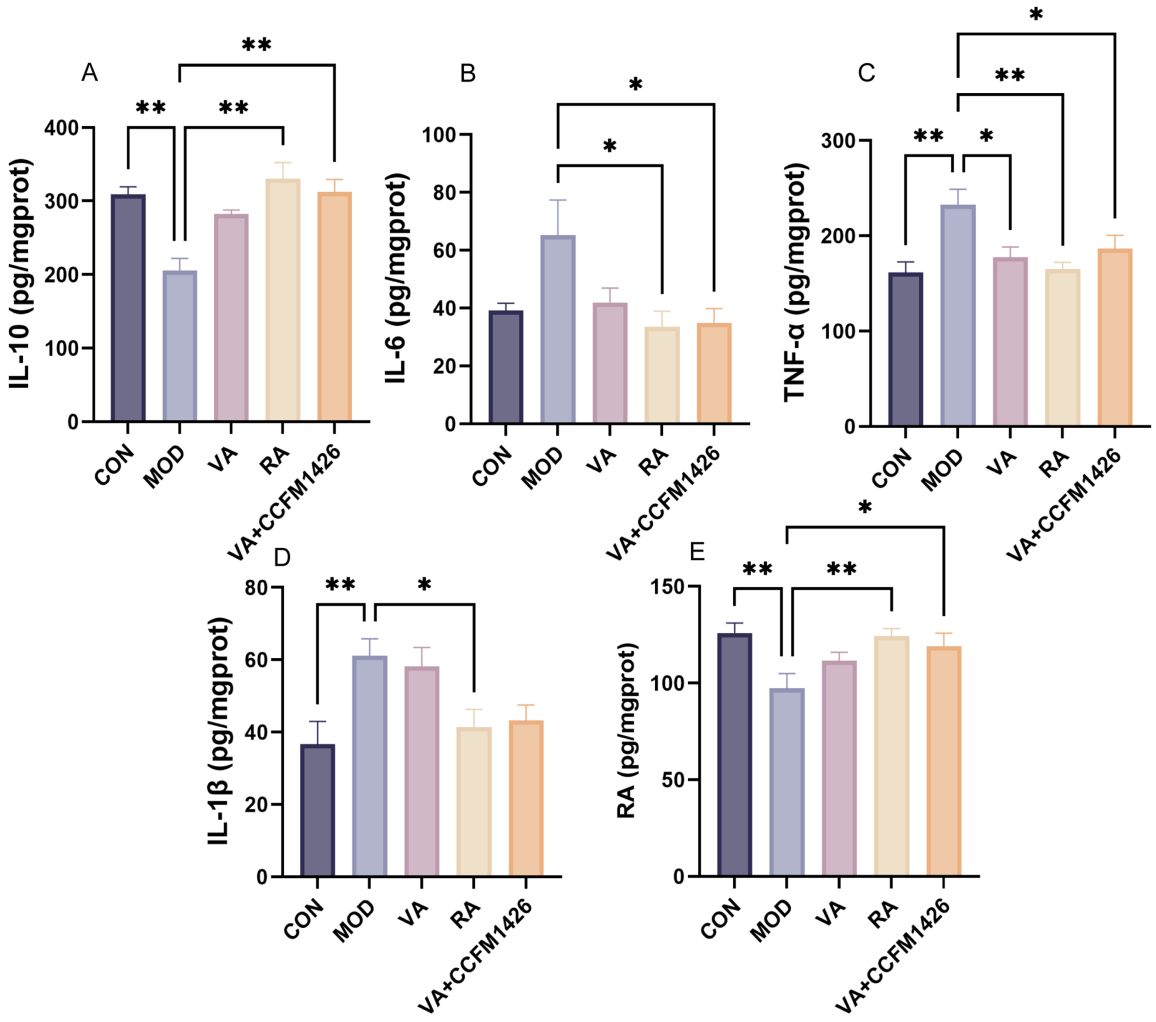

FIGURE 3
The combination of CCFM1426 and Vitamin A modulates RA and inflammatory cytokine levels in colitis mice. **(A)** IL-10. **(B)** IL-6. **(C)** TNF-α. **(D)** IL-1β. **(E)** RA.

These findings suggest that CCFM1426 enhances the VA metabolic capacity of the gut microbiota in colitis mice, thereby boosting gut-derived RA production. This RA subsequently enters intestinal tissues through the epithelium, elevating colonic RA levels, which aligns with previous research findings (21).

3.4 The combination of VA and CCFM1426 improves antioxidant enzyme levels in the colon of mice with colitis

The levels of antioxidant enzymes (CAT, GSH-PX, and SOD) were significantly lower in the colon tissues of the model group compared to the control group ($p < 0.05$; Figures 4A–C). Following treatment with RA, the activity of these enzymes was notably increased compared to the model group ($p < 0.05$; Figures 4A–C). Specifically, the concentrations of CAT and GSH-PX were significantly higher in the CCFM1426 and VA combination group compared to the model group ($p < 0.05$; Figures 4A,C). The combination exhibited superior antioxidant capabilities compared to VA alone.

3.5 The combination of vitamin A and CCFM1426 improves colitis by modulating the gut microbiota composition

Various α-diversity indices, including Shannon index, Simpson index, and richness, were calculated. As shown in Figures 5A–C, the α-diversity indices of mice in the control group were significantly higher than those in the model group ($p < 0.0001$). Treatment with the combination of CCFM1426 and VA significantly improved the Shannon and Simpson indices of mice with colitis compared to the model group, whilst the richness was not statistically significant (Figures 5A–C). The β-diversity results (Figures 5D–E) indicated that the intestinal flora of the model group exhibited significant differences compared to both the control group and the combination of CCFM1426 and VA group ($R^2 = 0.289$, $p = 0.027$). Thus, DSS-induced colitis disrupted homeostasis of the intestinal flora in mice ($p < 0.05$). At the species level, the combination induced changes in the gut microbiota composition (Figure 5F). As shown in Figure 5G, LDA score results indicated higher relative abundances of *Ligilactobacillus_murinus* and *Erysipelotrichaceae*_bacterium in

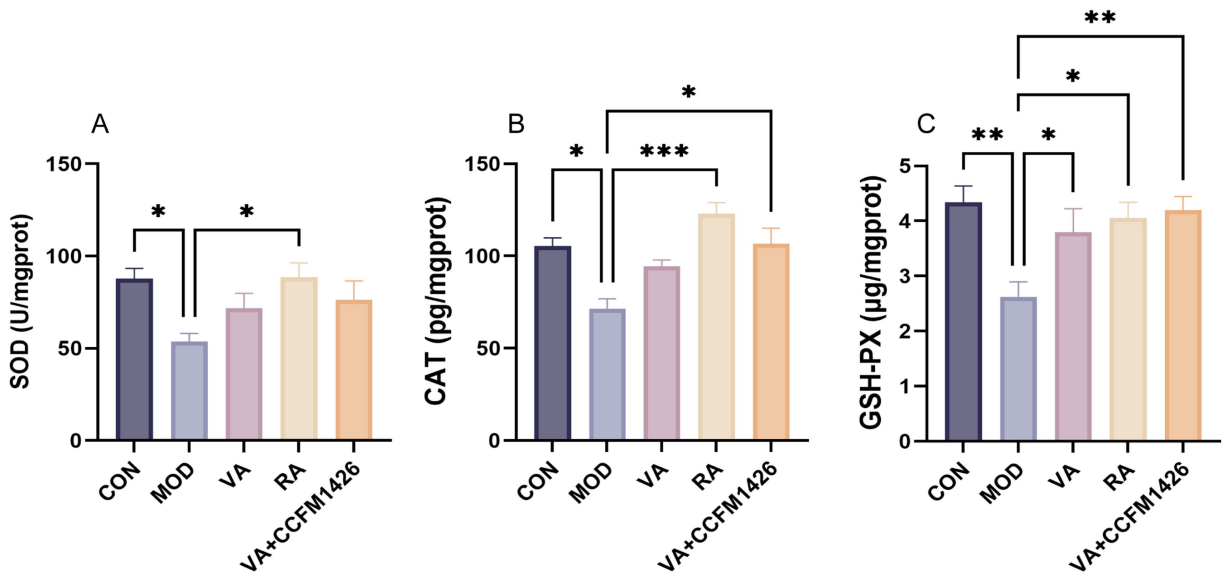

FIGURE 4
The combination of VA and CCFM1426 improves antioxidant enzyme levels in the colon of mice with colitis. **(A)** SOD. **(B)** CAT. **(C)** GSH-PX.

the model group compared to the combination groups. The administration of the combination of CCFM1426 and VA increased the abundance of *Escherichia_coli*, *Bacteroides_stercorirosoris*, *Phocaeicola_vulgatus*, *Bacteroides_congonensis*, and *Parabacteroides_merdae*.

3.6 The combination of vitamin A and CCFM1426 improves colitis by modulating the faecal metabolic phenotype

Changes in faecal metabolites following treatment with the combination of VA and CCFM1426 were further evaluated in mice using liquid chromatography-mass spectrometry (LC–MS). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) revealed that the faecal metabolite composition in the group receiving the combined intervention of vitamin A and CCFM1426 was different from that of the model group (Figures 6A,B). As described in previous studies, volcano plot analysis, applying thresholds of fold change (FC) > 1.2 or < 0.83, and a p-value <0.05, revealed 51 metabolites that were differentially abundant between the MOD group and the VA + CCFM1426 intervention group (Figure 6C) (31). KEGG pathway enrichment analysis indicated that the differentially abundant metabolites were mainly associated with several key metabolic pathways, including purine metabolism, biosynthesis of unsaturated fatty acids, as well as phenylalanine, tyrosine and tryptophan biosynthesis (Figure 6D). Specifically, guanine, adenosine, and urate were markedly enriched in the purine metabolism pathway. The significant variation in phenylalanine levels was closely associated with phenylalanine, tyrosine and tryptophan biosynthesis. In addition, the fluctuations in oleic acid, Arachidonic acid, alpha-Linolenic acid, docosahexaenoic acid and eicosapentaenoic acid (EPA) were tightly linked to the biosynthesis of unsaturated fatty acids. Notably, EPA, adenosine and anandamide (AEA) were consistently upregulated in both the blank control vs. model group and the combination vs. model group comparisons (Figure 6E).

4 Discussion

Ulcerative colitis is a chronic, relapsing inflammatory bowel disease that primarily affects the colon. It triggers the release of pro-inflammatory cytokines in the colon, leading to gut microbiota imbalances and further compromising the intestinal barrier (32, 33). Although pharmaceutical treatments are commonly used in clinical settings, many of these medications come with adverse side effects (30). Currently, nutritional strategies, particularly those targeting the gut microbiota, have emerged as promising adjunctive approaches (8, 34).

Emerging evidence suggests that patients with UC often suffer from impaired VA absorption (35). As an essential nutrient, VA not only supports normal visual function but also plays a crucial role in cell differentiation, organ development, immune defence, and the maintenance of epithelial integrity (36). Most of the non-visual physiological effects of VA are mediated by its active metabolite, RA, which contributes to the maintenance of intestinal epithelial barriers, regulation of gut immunity, and stabilisation of host-microbiota interactions (37, 38). Interestingly, gut microbiota can enhance host retinol bioavailability, thereby improving VA absorption and promoting the hepatic storage of retinyl esters (39). Previous studies have also shown that certain commensal bacteria are capable of autonomously metabolising VA, producing intermediates such as retinol, retinaldehyde, and RA, thus influencing VA metabolism within the intestinal environment (20). Leveraging probiotics with the ability to metabolise VA may provide a novel strategy for UC management by supporting vitamin metabolism, modulating immune responses, reinforcing intestinal barrier integrity, and reshaping gut microbial communities.

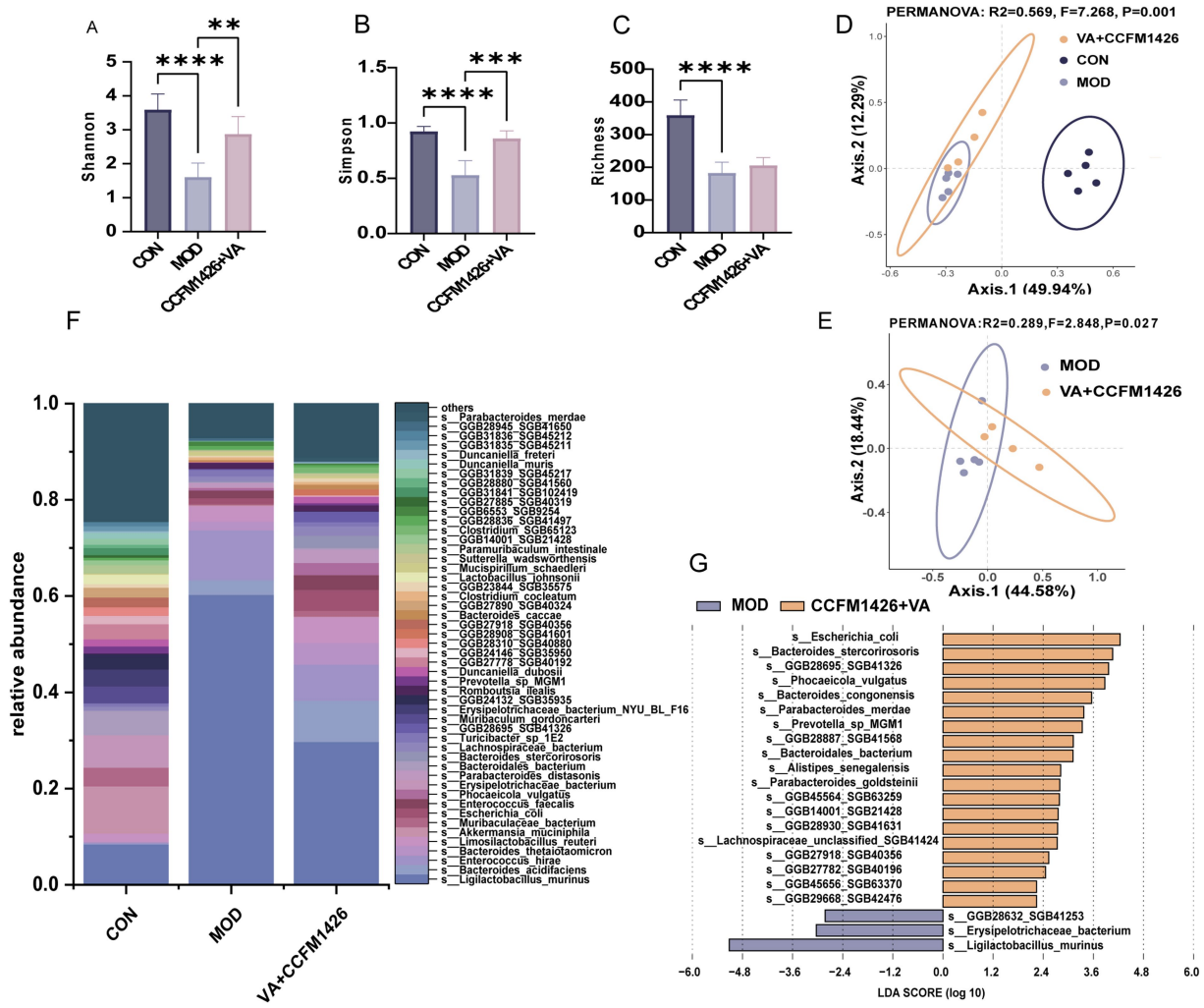

FIGURE 5
The combination of vitamin A and CCFM1426 improves colitis by modulating the gut microbiota composition. **(A)** Shannon. **(B)** Simpson. **(C)** Richness. **(D)** Bray-Curtis Principal Coordinate Analysis (PCoA) of all groups. **(E)** PCoA analysis of the CCFM1426 and Vitamin A combination group compared to the model group. **(F)** Relative abundance of the gut microbiota at the species level. **(G)** Results of linear discriminant analysis effect size (LEfSe).

In a DSS-induced colitis mouse model, RA supplementation significantly reduced inflammatory cell infiltration in colonic tissues, showing superior therapeutic effects compared to VA alone. Pathological analysis also revealed that the combination of dietary VA and probiotics effectively alleviated crypt distortion and inflammation. Furthermore, probiotic supplementation markedly increased RA levels in the colon, supporting the notion that intestinal VA metabolism can be modulated through microbial interventions.

I-FABP, LBP, and DAO were used as biomarkers to evaluate whether the combination of CCFM1426 and VA contributed to maintaining the intestinal barrier. DAO is an intracellular enzyme that exists almost exclusively in intestinal mucosal cells and is released into the bloodstream when the intestinal barrier is compromised (40). Elevated serum DAO levels indicate damage to the intestinal mucosa (40). LBP is an acute-phase protein synthesised by hepatocytes in response to bacterial infection and endotoxins; its secretion increases with intestinal barrier disruption (41). These markers, therefore, serve as indicators of intestinal permeability and mucosal injury. In this study, RA supplementation significantly reduced serum I-FABP, LBP, and DAO levels in mice with DSS-induced colitis. Notably, treatment with the combination of CCFM1426 and VA also markedly decreased serum concentrations of I-FABP, LBP, and DAO in colitis mice, suggesting a protective effect on the intestinal barrier. These findings align with previous research showing that probiotics help maintain intestinal integrity and that VA metabolites play a key role in repairing mucosal damage. Moreover, our study demonstrated that the combination exerted better efficacy than VA alone, suggesting that probiotics may enhance gut-derived RA production through modulation of the gut microbiota, thereby improving the intestinal barrier.

To further investigate the immunomodulatory and antioxidant roles of VA, RA, and their combination with CCFM1426 in colitis, the expression of key cytokines and antioxidant enzymes was evaluated. In mice with DSS-induced colitis, levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were significantly elevated, whilst the anti-inflammatory cytokine IL-10 was markedly decreased, indicating an intense inflammatory response and immune dysregulation. These findings are consistent with previous studies that

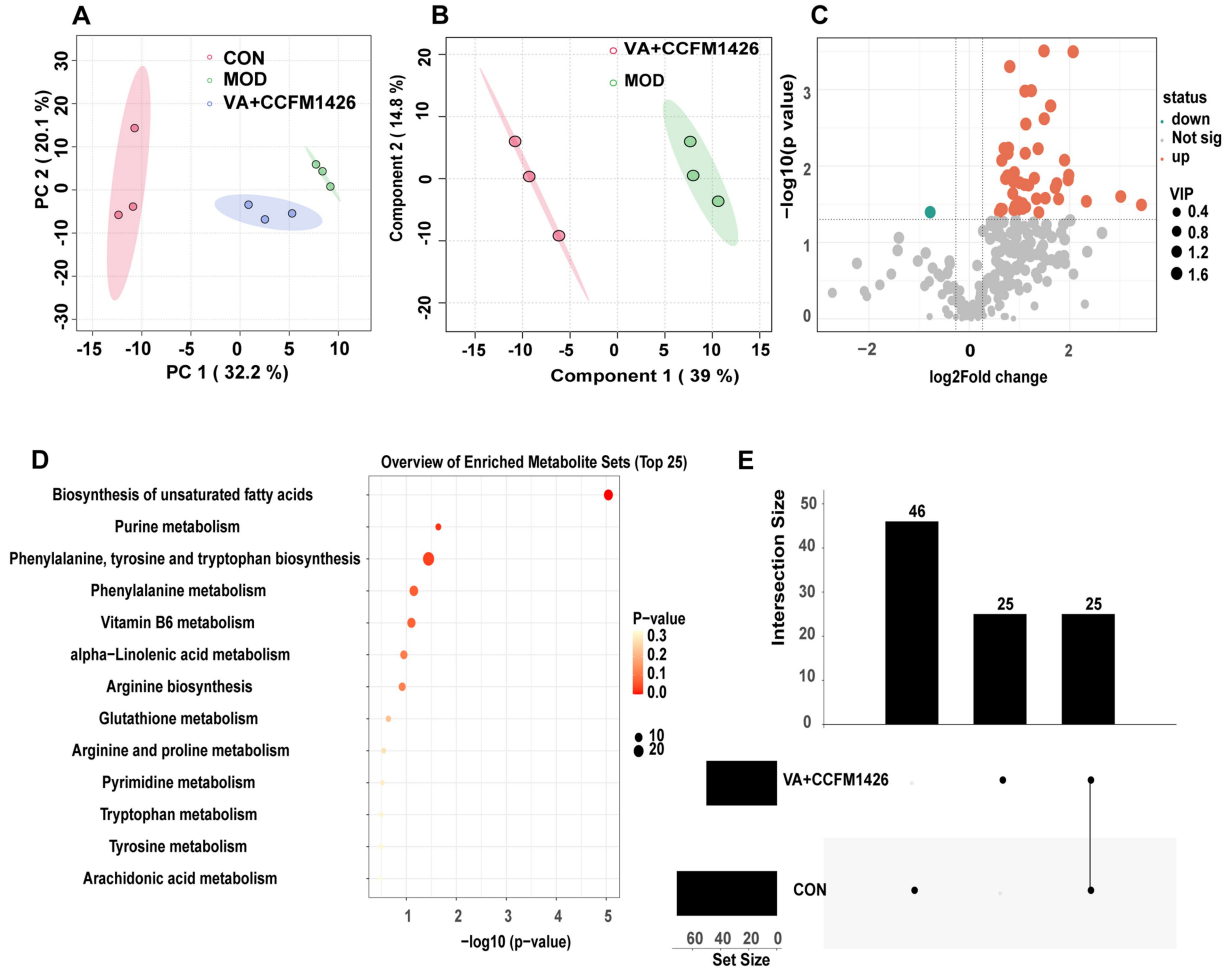

FIGURE 6
The combination of vitamin A and CCFM1426 improves colitis by modulating the faecal metabolic phenotype. **(A)** Principal component analysis (PCA) of all groups. **(B)** Partial least squares discrimination analysis of the CCFM1426 and Vitamin A combination group compared to the model group. **(C)** Volcano maps of differentially abundant metabolites of the CCFM1426 and Vitamin A combination group compared to the model group. **(D)** KEGG pathway enrichment analysis of the CCFM1426 and Vitamin A combination group compared to the model group. **(E)** UpSet plot of differential metabolites amongst groups.

reported excessive release of inflammatory cytokines leading to oxidative stress and epithelial injury in UC (42).

Treatment with VA, RA, or the combination effectively modulated cytokine levels in colitis-affected mice. Notably, the CCFM1426 and VA combination significantly reduced pro-inflammatory TNF-α and IL-6 whilst simultaneously increasing anti-inflammatory IL-10 levels ($p < 0.05$), suggesting that probiotic-enhanced VA metabolism contributed to inflammatory balance restoration. RA alone demonstrated potent efficacy by reducing TNF-α, IL-1β, and IL-6 levels whilst significantly elevating IL-10 expression ($p < 0.05$). These results align with RA's established role in inhibiting Th17-related inflammation and promoting regulatory T cell responses to maintain immune homeostasis (21, 43). Furthermore, colonic RA levels were drastically reduced in DSS-treated mice compared to controls ($p < 0.001$), whereas both RA supplementation and the combination treatment restored RA levels, with the combination outperforming VA alone. This suggests CCFM1426 enhances the gut microbiota's VA metabolic capacity, promoting endogenous RA synthesis and facilitating its transport across the intestinal epithelium for local immunomodulatory effects. Concurrently, antioxidant enzyme activities (CAT, SOD, and GSH-PX) were significantly impaired in the model group, indicating ROS accumulation and oxidative stress in inflamed colonic tissues. RA treatment markedly restored these antioxidant enzyme levels ($p < 0.05$), whilst the CCFM1426 and VA combination significantly enhanced CAT and GSH-PX activities ($p < 0.05$), suggesting superior antioxidant capacity, although no statistical significance was observed when compared to VA alone. This highlights the synergistic effect of probiotics and VA in regulating redox homeostasis, likely through increased RA production and reduced oxidative damage. These findings emphasise that probiotics targeting VA metabolism not only modulate inflammatory cytokine networks but also reinforce antioxidant defence systems to protect intestinal integrity.

Gut microbiota homeostasis plays a crucial role in regulating ulcerative colitis (UC), whilst dysbiosis contributes to colitis exacerbation. Our study revealed significantly higher Shannon, Richness and Simpson indices in the model group compared to controls ($p < 0.0001$). β-diversity

analysis using Bray–Curtis distance demonstrated that the clustering of the model group differed markedly from the CCFM1426 and VA combination groups, indicating significant differences in faecal microbiota compositional structure. The combination treatment effectively restored gut microbiota homeostasis in colitis-affected mice. Several beneficial bacteria were identified in the treatment groups: *Phocaeicola vulgatus*, which metabolises agmatine to regulate intestinal motility and maintain barrier integrity whilst providing neuromodulatory effects (44); *Parabacteroides merdae*, which produces histamine that functions as a regulatory neurotransmitter (44); *Bacteroides congonensis*, which shows increased abundance in inulin-fed mice with potential beneficial effects (45, 46). Furthermore, the combination intervention group exhibited higher relative abundance of *Limosilactobacillus reuteri* and lower abundance of *Ligilactobacillus murinus* compared to the model group. *L. reuteri* is recognised for its probiotic potential, particularly its ability to suppress inflammation by stimulating group 3 innate lymphoid cells (ILC3s) to secrete anti-inflammatory cytokines such as IL-10 and IL-22 (47, 48). Although *L. murinus* has been associated with intestinal barrier repair, excessively elevated levels may reflect incomplete recovery of gut barrier integrity and microbial balance following DSS-induced colitis. Thus, the reduced abundance of *L. murinus* in the combination group may indicate more complete restoration of intestinal homeostasis after DSS withdrawal. Notably, this combination significantly increased colonic retinoic acid levels in colitis-induced mice. Collectively, our results suggest that the VA and CCFM1426 combination alleviates gut dysbiosis by restoring microbial homeostasis through gut microbiota remodelling and enhancing gut-derived VA metabolism.

Alterations in the gut microbiota can significantly influence host metabolic phenotypes and the expression of associated metabolites (49). The study revealed that the combined use of CCFM1426 and VA markedly modulated the faecal metabolite profile in colitis-induced mice and promoted the accumulation of several beneficial metabolites. Differential metabolite analysis demonstrated that these changes were primarily enriched in biosynthesis of unsaturated fatty acids and purine metabolism. Notably, EPA, AEA and adenosine were identified as key differential metabolites, exhibiting significantly elevated levels in all intervention groups except the model group. EPA, an essential polyunsaturated fatty acid, exhibits anti-inflammatory properties that can alleviate chronic inflammatory conditions. EPA supplementation effectively mitigates inflammation in acetic acid-induced rat colitis models by modulating the TGF-β/p-EGFR and NF-κB inflammatory pathways, restoring oxidant/antioxidant balance, and enhancing colonic barrier integrity (50). Clinically, the free fatty acid form of EPA has been shown to reduce faecal calprotectin levels and prevent relapse in ulcerative colitis patients (51). Furthermore, emerging evidence indicates that EPA can potently enhance intestinal stem cell-mediated colonic epithelial regeneration through activation of the LSD1-WNT signalling pathway (52). AEA, a pivotal component of the endocannabinoid system, plays a crucial role in gut homeostasis (53) Attenuation of AEA signalling in the intestine significantly exacerbates visceral hypersensitivity (53). Adenosine is considered a pivotal immunomodulator that may control inflammation in IBD (54). Adenosine deaminase inhibition alleviates the elevation of colonic myeloperoxidase and malondialdehyde levels in rats with colitis, demonstrating that adenosine can modulate the immune system and suppress inflammation by reducing pro-inflammatory cytokine biosynthesis and regulating neutrophil function (55). Therefore, CCFM1426 and VA may improve colitis symptoms in mice by remodelling the gut microbiota and regulating metabolic phenotypes, thereby reducing intestinal permeability and exerting anti-inflammatory effects.

5 Conclusion

This study demonstrates that combined administration of VA and probiotic CCFM1426 alleviates DSS-induced colitis in mice by reducing inflammation, enhancing intestinal barrier integrity, and restoring gut microbial homeostasis. Notably, the combination elevated colonic retinoic acid levels more effectively than VA alone, suggesting enhanced VA metabolism by CCFM1426. Microbiota analysis revealed increased abundance of beneficial species such as *Bacteroides congonensis* and *Phocaeicola vulgatus*, along with improved α- and β-diversity. In addition, this combined intervention modulated gut microbiota composition and enriched faecal metabolites, including EPA, adenosine, and AEA, indicating a shift towards a health-promoting intestinal microenvironment. These findings highlight a novel microbiota-targeted nutritional strategy in which probiotics enhance the efficacy of vitamin A through synergistic regulation of retinoid metabolism and microbial homeostasis.

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, accession number PRJNA1275596.

Ethics statement

The animal study was approved by the Ethics Committee of Experimental Animals of Jiangnan University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XY: Methodology, Software, Writing – original draft. LH: Methodology, Writing – original draft. YW: Writing – review & editing. LL: Validation, Writing – review & editing, Software. WL: Validation, Writing – review & editing, Supervision, Funding acquisition. ZZ: Writing – review & editing, Project administration. HW: Supervision, Writing – review & editing, Data curation, Project administration, Funding acquisition.

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Conflict of interest

XY, YW, and LL were employed by Sinopharm Xingsha Pharmaceutical (Xiamen) 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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Bifidobacterium adolescentis CCFM1447 effectively alleviates osteoporosis by enriching intestinal flora capable of vitamin D conversion

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Introduction: Elderly individuals exhibit heightened susceptibility to osteoporosis, largely attributable to age-related declines in skin, liver, and kidney function. While vitamin D (VD) supplementation is common, its efficacy is often limited, necessitating reliance on pharmaceutical interventions. Research indicates that the intestinal flora significantly influences intestinal VD metabolism, with probiotic supplementation demonstrably impacting circulating VD levels.
Methods: We employed an fecal fermentation model to screen bacterial strains. After introducing these strains into osteoporotic mice, we tested the mice's serum and skeletal indicators. We then conducted a correlation analysis between the mice's key intestinal microbiota and serum and skeletal indicators.
Results and discussion: We identify *Bifidobacterium adolescentis* CCFM1447 for its capacity to elevate VD metabolite levels within fermented supernatants. It significantly elevated serum concentrations of 1,25-dihydroxyvitamin D. Furthermore, this intervention improved bone microarchitecture, evidenced by increased trabecular number and bone volume fraction. In addition, the intestinal flora of the osteoporotic mice was disturbed. CCFM1447 intervention increased the relative abundance of beneficial bacteria such as *Adlercreutzia equolifaciens*, *Akkermansia muciniphila* and *Pediococcus acidilactici*. And it is enriched with a part of strains that have the ability to transform VD such as Enterococcus faecalis and Pediococcus acidilactici. The above results suggest that *B. adolescentis* CCFM1447 may alleviate retinoic acid-induced osteoporosis symptoms by modulating the intestinal flora and increasing the level of active vitamin D.

KEYWORDS

osteoporosis, intestinal flora, vitamin D, active vitamins, probiotics

1 Introduction

Osteoporosis is a disorder characterized by decreased bone mass and deterioration of bone microarchitecture. It collectively contributes to a heightened risk of fractures in affected individuals (1). Common clinical manifestations include skeletal pain, loss of stature, kyphosis, fractures, and impaired pulmonary function. Although calcium supplementation is considered

a fundamental therapeutic strategy, it is insufficient as a standalone treatment, necessitating pharmacologic intervention tailored to the patient's clinical profile.

Currently, the therapeutic arsenal for osteoporosis includes bisphosphonates, selective estrogen receptor modulators (SERMs), estrogen, and calcitonin. These agents are effective in promoting osteoblast activity while suppressing osteoclast-mediated bone resorption. Nevertheless, bisphosphonates are associated with renal toxicity and may induce or worsen hypocalcemia (2). Raloxifene, a SERM, has been linked to an increased risk of stroke. Estrogen therapy elevates the risk of endometrial malignancy in women with an intact uterus, and is associated with higher rates of gallstone formation and thromboembolic events (3, 4). Similarly, the RANKL inhibitor denosumab shares adverse effect profiles with bisphosphonates. Calcitonin use can provoke allergic reactions and, in rare cases, anaphylaxis; high doses may induce hypocalcemia and have been implicated in a potential increase in cancer risk (5). Importantly, none of these anti-fracture treatments are curative, and bone degeneration tends to recur following cessation of therapy. Alarmingly, 85% of patients discharged after hip fracture repair do not receive continued anti-fracture management or pharmacologic therapy (6–8). This underscores the pressing need for new treatment modalities that are both effective and free from long-term complications.

Vitamin D (VD) is a vital micronutrient necessary for normal bone physiology, growth, and mineral ion regulation. Its significance in maintaining skeletal integrity and mineral balance is well-documented. Epidemiological studies have also linked VD deficiency to a heightened risk of various diseases, including musculoskeletal, metabolic, cardiovascular, autoimmune, malignant, and infectious conditions (9–11). The biologically active form of VD, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], functions as a ligand for the vitamin D receptor (VDR), playing a critical role in calcium homeostasis and immune modulation (12). Supplementation with VD is particularly beneficial for children and older adults, contributing to improved muscle strength, enhanced postural balance, and overall bone health.

Individuals with osteoporosis often exhibit impaired VD metabolism. As a fat-soluble vitamin, VD can be synthesized endogenously through dermal exposure to ultraviolet radiation and acquired exogenously through diet (13). To become biologically active, VD undergoes two hydroxylation steps: converting it to 25-hydroxyvitamin D [25(OH)D] in the liver (14), and then in the kidneys to form 1,25$(OH)_2$D (15). VD metabolites are excreted in bile and reabsorbed in the terminal ileum. Therefore, conditions such as ileal disease, malabsorption syndromes, or intestinal resection can reduce serum 25(OH)D levels. The decline in VD metabolism among osteoporotic patients is frequently attributed to aging and diminished renal function. Aging reduces the skin's capacity for VD synthesis, leading to lower circulating 25(OH)D, while renal impairment hampers conversion to the active 1,25$(OH)_2$D form (16). Hence, older adults and osteoporotic patients often require supplemental VD to counteract these physiological limitations. Interestingly, the American Endocrine Society recently recommended against routine screening or empirical VD supplementation in the general population (17), which further emphasizes the need to explore strategies that effectively elevate 1,25$(OH)_2$D levels to achieve its biological effects.

In this study, we identified *Bifidobacterium adolescentis* CCFM1447, a strain that significantly increases VD metabolite levels in fermentation supernatants, as demonstrated through an fecal fermentation model. We examined the effects of CCFM1447 on mice which have osteoporosis induced by retinoic acid (RA). Then we explored potential mechanisms underlying its action using Micro-CT scanning and analysis of intestinal microbiota. Findings of this study suggest that probiotics, such as *Bifidobacterium adolescentis* CCFM1447, have the potential to enhance VD activity levels, offering a promising approach for the management of osteoporosis.

2 Materials and methods

2.1 *In vitro* fermentation model screening strains

Dissolve feces in sterile PBS (1:10 w/v), mix thoroughly to homogenize. Filter the fecal slurry through two layers of gauze to remove insoluble particulate matter. This step is performed inside an anaerobic chamber. Prepare the fecal slurry. Using a 24-well plate, add 0.1 ml of bacterial solution and 0.3 ml of fecal slurry into each well. Then, add 1.6 ml of mGAM into each well. Incubate anaerobically for 36 h. Each sample is set up in triplicates. Use 0.4 ml of fecal slurry in some wells and 0.3 ml of fecal slurry and 0.1 ml of another bacterial strain in the blank controls. The entire 24-h fermentation process is conducted inside the anaerobic chamber. After fermentation, remove the 24-well plate and place it on ice for 15 min to stop fermentation. Transfer the fermented samples to 2 ml sterile centrifuge tubes. Centrifuge at 10,000 rpm for 10 min to separate the supernatant and pellet. Store the supernatant at −20°C. The levels of 25(OH)D and 1,25$(OH)_2$D in the supernatant are detected by commercial assay kits.

2.2 Preparation of gavage strains for experimentation

The strain is stored in MRS medium supplemented with 30% (v/v) glycerol at −80°C. Before experimental use, the strain undergoes three consecutive activations. The bacterial suspension is centrifuged at 8,000 × *g* for 10 min to remove the supernatant. The pellet is washed three times with sterile physiological saline solution and resuspended in a solution of defatted milk powder at a concentration of 120 g/L. The resuspended culture is stored at −80°C. Before use, dilute the bacterial suspension with sterile physiological saline solution to a concentration of 10^9 CFU/ml for further applications. All strains used in this chapter were obtained from Culture collection of food microorganisms (CCFM), Biotechnology Centre of Jiangnan University. *Bifidobacterium adolescentis* CCFM1447 has been conserved in Guangdong Microbial Strain Conservation Centre (GDMCC No: 65382) on 31st October, 2024.

2.3 Mice experimental design

The study employed SPF-grade male C57BL/6 J mice, aged 7 weeks and weighing (20 ± 5) g, procured from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. The animals were kept in a controlled environment at a temperature of (23 ± 2) °C and a relative humidity of (50 ± 10)%, with free access to standard rodent chow in accordance with national guidelines. All experiments were carried out at the

Animal Experiment Center of Jiangnan University (Wuxi, China), with approval from the Jiangnan University Experimental Animal Ethics Committee (Ethics Approval No: JN.No20231030c1360128[515] and No: JN.No20241015c1041215[533]).

The volume of the gastric lavage solution was 200 μl. The bacterial solution was removed from the refrigerator and centrifuged to remove the supernatant. It was resuspended in saline to a final concentration of 1×10^9 CFU/ml. This was used as the bacterial group gastric lavage solution.

2.3.1 Experiment 1: CCFM1447 osteoporosis relief efficacy verification

Mice were acclimatized for 1 week. Afterward, they were randomly assigned to one of four groups (n = 6): Control, Model, VD, and CCFM1447. In 1–3 weeks, all groups, except the Control group, were orally gavaged with 90 mg/kg RA daily to induce osteoporosis. Following this induction period, mice were assigned to three groups: Model, VD, and CCFM1447. In 4–6 weeks, the VD group received a daily oral dose of 0.06 μg/kg of VD. The CCFM1447 group was treated with an oral gavage of *Bifidobacterium adolescentis* CCFM1447 suspension. The Control and Model groups were orally gavaged with saline daily.

2.3.2 Experiment 2: individual effects of key differential strains

Mice were acclimatized for 1 week. They were then randomly assigned to one of eight groups (n = 6): Control, Model, Abx, Abx + Model, E.f, Abx + E.f, P.a, and Abx + P.a. In 1–3 weeks, all groups, except the Control, were given 90 mg/kg RA via oral gavage daily to induce osteoporosis. Concurrently, all antibiotic-treated groups were administered a combination of antibiotics during the last week of the modeling phase to mitigate intestinal microbiota disruption. The antibiotic cocktail included 100 mg/kg vancomycin, 200 mg/kg neomycin, 200 mg/kg ampicillin and 200 mg/kg metronidazole. In 4–6 weeks, the strain-treatment groups received an oral gavage of the corresponding bacterial strain. The remaining groups were given of saline.

At the conclusion of the study, fresh fecal samples were collected and stored at −80°C for microbiota diversity analysis. Mice were fasted for 12 h prior to being anesthetized with isoflurane, followed by euthanasia via orbital sinus puncture. Blood samples were quickly obtained from the orbital sinus, centrifuged at 5,000 *g* for 15 min at 4°C, and the serum was stored at −80°C. The femurs and tibias were carefully excised, connective tissues were removed, and the left bones were fixed in 4% paraformaldehyde in 5 ml sterile Eppendorf tubes for Micro-CT imaging. The right bones were stored at −80°C.

2.4 Determination of structural indices of mouse femur

After excising the right tibia from each mouse and fixing it in 4% paraformaldehyde, the sample was left to allow the liquid to evaporate for subsequent detection. The samples were then scanned using a Micro-CT scanner (Lateta LCT200, Hitachi-Aloka, Tokyo, Japan). The right femur from each mouse was properly positioned in the micro-CT imaging system for X-ray scanning. The scanning parameters were set as follows: 90 kV, 88 μA, a field of view of 18 mm, an acquisition time of 14 min, and a pixel size of 36 μm. Each femur underwent a 360° rotation to collect data, which were then imported into Analyze software (Version 12.0; AnalyzeDirect, Overland Park, KS, USA) for 3D reconstruction and analysis. A region of interest (ROI) 1 mm thick was selected starting 0.2 mm below the growth plate for bone parameter calculations. The following parameters were analyzed: Bone Mean (BM), Cortex Mean (CM), Trabeculae Mean (TM), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular connectivity density (Conn.D), bone surface (BS), bone volume (BV), and bone volume fraction (BV/TV).

2.5 Biochemical analysis

Serum calcium and alkaline phosphatase (ALP) levels were measured using commercial detection kits (Nanjing Jianjian Bioengineering Institute). Serum 25(OH)D and serum $1,25(OH)_2D$ were measured using commercial Mouse ELISA kits (Beyotime Biotechnology, Shanghai, China). Osteocalcin (OCN) and Procollagen type I N-propeptide (PINP) levels were measured using commercial Mouse ELISA kits (E-EL-M0864, E-EL-M0233, Elabscience Biotechnology).

2.6 Metagenomic data processing and quality control

Metagenomic sequencing was conducted on the Illumina NovaSeq 6,000 platform (Illumina Inc., San Diego, CA, USA) at Beijing Nuohe Biomedical Technology Co., Ltd. (Beijing, China). The initial sequence data underwent preprocessing, which involved several steps: Trimmomatic (version 0.39) was used to eliminate low-quality reads (18). Sequences with an average base quality score lower than 30 were trimmed, and only sequences longer than 60 bp after trimming were retained as part of the high-quality output. The filtered sequences were then aligned to the human reference genome (GRCh38/hg38) using BWA (version 0.7.17), Samtools (version 1.9), and BEDTools (version 2.30.0), successfully removing any host-derived sequences from the data [H. (19, 20)].

2.7 Detection of metabolites in supernatant from *in vitro* single-bacterium culture

After the strain frozen at −80°C was taken out, it was connected to MRS liquid medium containing 0.05% (w/v) L-cysteine at 4% inoculum in an ultra-clean bench, and placed in an anaerobic chamber at 37°C for 18~ 24 h. Subsequently, the plate was scribed and a single colony was selected to the liquid medium, and the liquid was cultured for one generation, then strain preservation and identification were performed to ensure that there was no error in the identification and then activation was repeated, and the activation was repeated twice. After activation for two times, the culture was expanded, and the culture conditions were the same as above. After the activation and identification were completed, the culture was continued and incubated in an anaerobic chamber at 37°C for 16 h. VD3 was added to each tube of bacterial liquid to a final concentration of 10 μM. After incubation in the anaerobic chamber for 2 h, the bacterial liquid was taken out and centrifuged at 10,000 r/min for 10 min at 4°C, and the supernatant was retained at −80°C in the

refrigerator for assaying, which would be reserved for the subsequent metabolite assay.

Remove the sample stored at low temperature, equilibrate to room temperature, accurately pipette 0.5 ml of the sample into a 2.0 ml Eppendorf centrifuge tube, add 1 ml of extraction solvent (hexane-ethyl acetate = 90:10), vortex vigorously for 1 min, and centrifuge at 4°C at 12,000 *g* for 15 min. Transfer 0.9 ml of the supernatant to a clean Eppendorf centrifuge tube, centrifuge at 37°C until dry, and redissolve the residue in 100 µl of methanol–water solution (methanol–water = 75:25).

Injection volume: 50 µl. Chromatography column: Phermon C18 column (Kinetex 2.6 µm, 100 × 3.0 mm). Mobile phase B: methanol solution containing 0.2 mM ammonium sulphate; mobile phase A: aqueous solution containing 0.2 mM ammonium sulphate. Flow rate: 0.5 ml/min. Detection time: 10 min. Column temperature: 40°C. The detection method used was ESI source positive ion mode MRM, with the MRM monitoring ion being m/z 423.1 → 369.0.

Prepare a series of mixed standard solutions by diluting the 1α,25(OH)$_2$D standard with methanol in a 1:1 ratio. Using Analyst 1.6.2 software, plot the standard solution concentration on the X-axis and the peak area of the standard as the Y-axis. A linear regression analysis was performed, and the regression equation was obtained using the '1/X^2' weighting. The peak area of the sample was substituted into the standard curve equation to calculate the concentration of 1α,25(OH)$_2$D in the serum sample.

2.8 Statistical analysis

Statistical and analytical evaluations were performed using GraphPad Prism 6 and SPSS software. Both control and treatment groups will be compared with the model group. Differences between groups will be assessed by one-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant. For macrogenomic data, analysis was carried out using the *psych* and *ggplot2* packages in R. * indicates significant differences from the model group: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Due to the skewed distribution of the raw microbial quantification data, data from Figures 1B,C were log-transformed (log10) to a base of 10 to meet the assumption of normality for subsequent statistical analyses. Undetected values were assigned a value of 0.001 for statistical analyses.

3 Results

3.1 Effect on VD metabolites in fermentation supernatants

We have found through recent research that some probiotics, especially *Bifidobacterium* and *Lactobacillus*, have the ability to

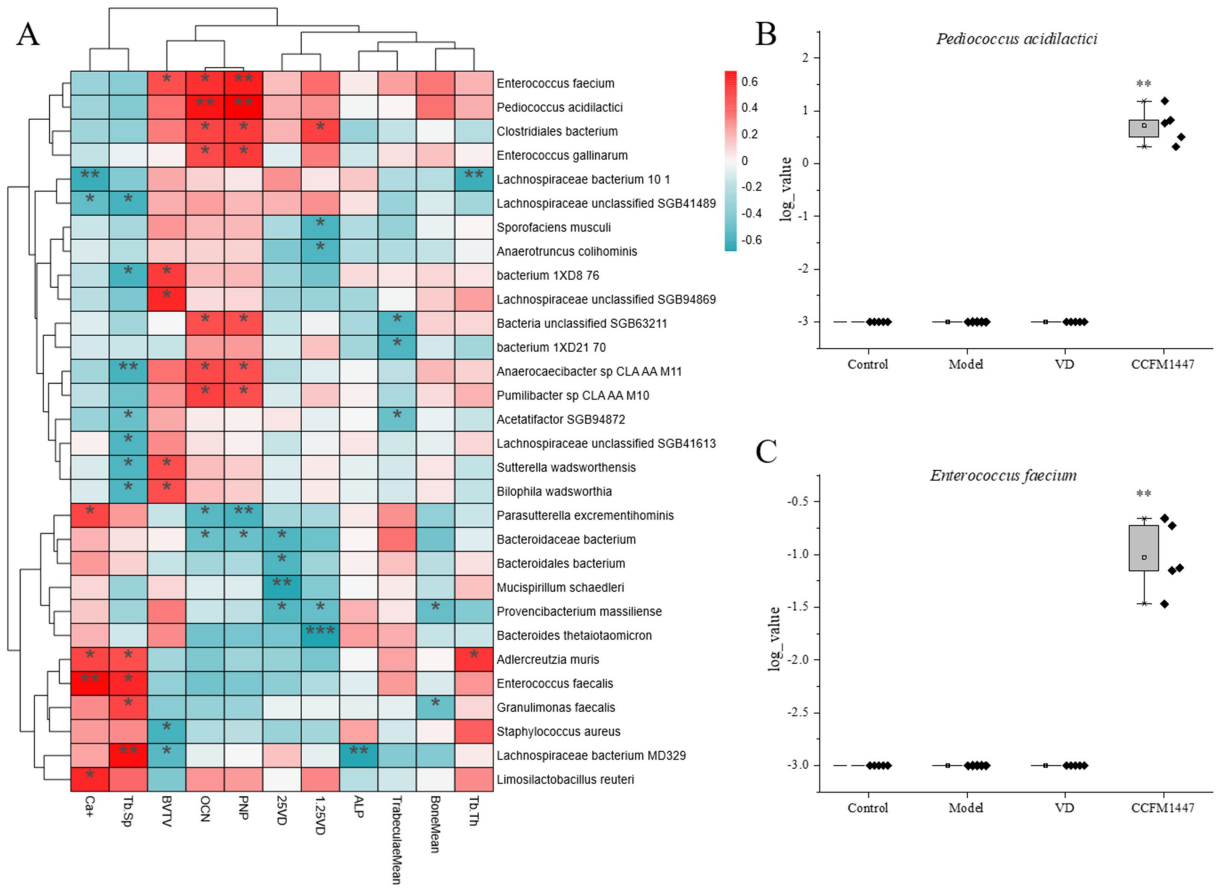

FIGURE 1
(A) Spearman's correlation analysis of the relative abundance of gut microbial species with physiological and biochemical markers; Log-transformed representation of the relative abundance of **(B)** *Pediococcus acidilactici* and **(C)** *Enterococcus faecium* in different groups.* indicates significant differences from the model group: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

improve bone health. We conjecture that this may be related to the promotion of VD metabolism by probiotics. Therefore, we selected some species of *Bifidobacterium* genus and *Lactobacillus* genus for *in vitro* fermentation experiments, including *Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Bifidobacterium. longum subsp. Infantis*, et al.

A total of 23 strains from 10 different species bacterial were selected for in-vitro fecal fermentation, and the levels of the active vitamin D form, 1,25$(OH)_2$D, in the supernatant were evaluated (Figure 2A). The addition of *Bifidobacterium adolescentis* CCFM1447 to the model resulted in a significant increase in the levels of the 1,25$(OH)_2$D in the supernatant, when compared to the Control group, which did not receive bacterial inoculation.1,25$(OH)_2$D acts as a direct agonist of the VDR. Its binding regulates gene expression crucial for maintaining skeletal health. Additionally, it is utilized as a treatment for osteoporosis. Given its superior ability to elevate 1,25$(OH)_2$D levels, *B. adolescentis* CCFM1447 was chosen to explore its potential therapeutic effects on osteoporosis in mice.

3.2 Effects on bone structures

Bone mineral density (BMD) is the main indicator for the diagnosis of osteoporosis, and its value reflects the bone metabolism of the organism. After 3 weeks of modeling and 3 weeks of strain gavage intervention, the same parts of the distal femur of the mice were taken and examined by Mirco-CT scanning. The results showed that the BM and TM of mice in the Model group decreased significantly. It indicated the successful establishment of RA-induced osteoporosis model and poor natural recovery. After gavage intervention with CCFM1447 strain, the BM, CM and TM of mice were significantly increased. The results showed superior outcomes in the BM and CM indexes compared to the VD group. Further bone microstructural analysis is presented in Figures 2B–K. In the Model group, values for Tb.Th, Tb.N, and BV/TV were lower than those in the Control group. Conn.D, BS, and BV were significantly reduced, with a notable increase in TbSp. Following treatment with CCFM1447, Tb.Th and Tb.N showed an increase. Conn.D, BS, BV, and BV/TV were significantly enhanced, while Tb.Sp decreased significantly. VD supplementation led to significant improvements only in CM, TM, Tb.N and Tb.Sp. These results suggest that RA effectively induces osteoporotic changes and decreases BMD in mice, while CCFM1447 exhibited more pronounced beneficial effects compared to VD treatment in mitigating these conditions (Figure 3).

3.3 Effects on serum bone metabolism markers

Serum 25(OH)D levels serve as an indicator of the body's VD reserves, while 1,25$(OH)_2$D is one of its most bioactive metabolites. The oral administration of 1,25$(OH)_2$D facilitates the absorption of calcium in the intestines, thereby addressing hypocalcemia and normalizing or reducing elevated serum ALP levels. Increased VD metabolites can also help lower plasma parathyroid hormone concentrations, contributing to improved bone mineralization. In the RA-induced osteoporotic mice, there was a significant reduction in serum levels of both 25(OH)D and 1,25$(OH)_2$D, indicating a decrease in both the storage and active forms of VD, impairing its physiological functions. However, after intervention with strain CCFM1447, the levels of 25(OH)D and 1,25$(OH)_2$D in the serum were significantly elevated compared to the Model group, suggesting that the strain enhanced VD metabolism.

As shown in Figures 4C–F, RA-induced osteoporosis resulted in significantly higher ALP and calcium levels, indicating bone calcium loss and damage. This indicates that the retinoic acid-induced osteoporosis model in mice has been successfully established. Following intervention, the VD group exhibited a significant reduction in ALP and calcium levels. Similarly, CCFM1447 gavage notably reduced serum calcium, though ALP levels were only marginally decreased. Osteoporosis resulted in a noticeable decrease in OCN and PINP levels. The VD-treated group exhibited significantly higher levels of OCN and PINP than the Model group, indicating improved osteoblast function. Similarly, CCFM1447 gavage led to enhanced serum OCN and PINP levels, with a more pronounced improvement han VD treatment, highlighting its superior effectiveness in stimulating osteoblast activity.

3.4 Effects on the diversity of intestinal flora

Several previous studies have demonstrated that probiotics can influence the composition of the intestinal flora, with the potential to regulate its function. The alpha diversity indices (Shannon, Simpson, and Pielou) are presented in Figures 5A–C. However, the observed differences were not statistically significant. After VD and CCFM1447 gavage, there were no significant changes in Pielou's homogeneity index, Shannon's index, and Simpson's index of the intestinal flora of mice. The reason why the diversity indices of mice in the VD and CCFM1447 groups were not significantly different from those of the model group may be related to the increase in the relative abundance of specific beneficial flora. This increase in selectivity modulates the structure of the intestinal flora as a whole and may lead to a relative decrease in other non-target microbiota, ultimately resulting in no significant change in the number of species types in the community.

This method helps to assess the differences or similarities in the intestinal flora composition between subjects. As shown in Figure 5D, the Principal coordinate analysis (PCoA) analysis revealed a marked difference in the intestinal flora structure between osteoporotic mice and healthy mice. Additionally, mice in the CCFM1447 and VD groups exhibited significant differences in microbiota composition compared to the Model group, suggesting that *Bifidobacterium adolescentis* CCFM1447 treatment effectively remodeled the intestinal flora structure in osteoporotic mice.

3.5 Effects on the structural composition of intestinal flora

The effects of VD and *Bifidobacterium adolescentis* CCFM1447 interventions on differential species of intestinal flora in osteoporotic mice were explored by LEfSe and RF analyses. As shown in Figure 6, same as the previous changes in relative abundance, there was a change in the relative abundance of *Faecalibaculum rodentium* in the

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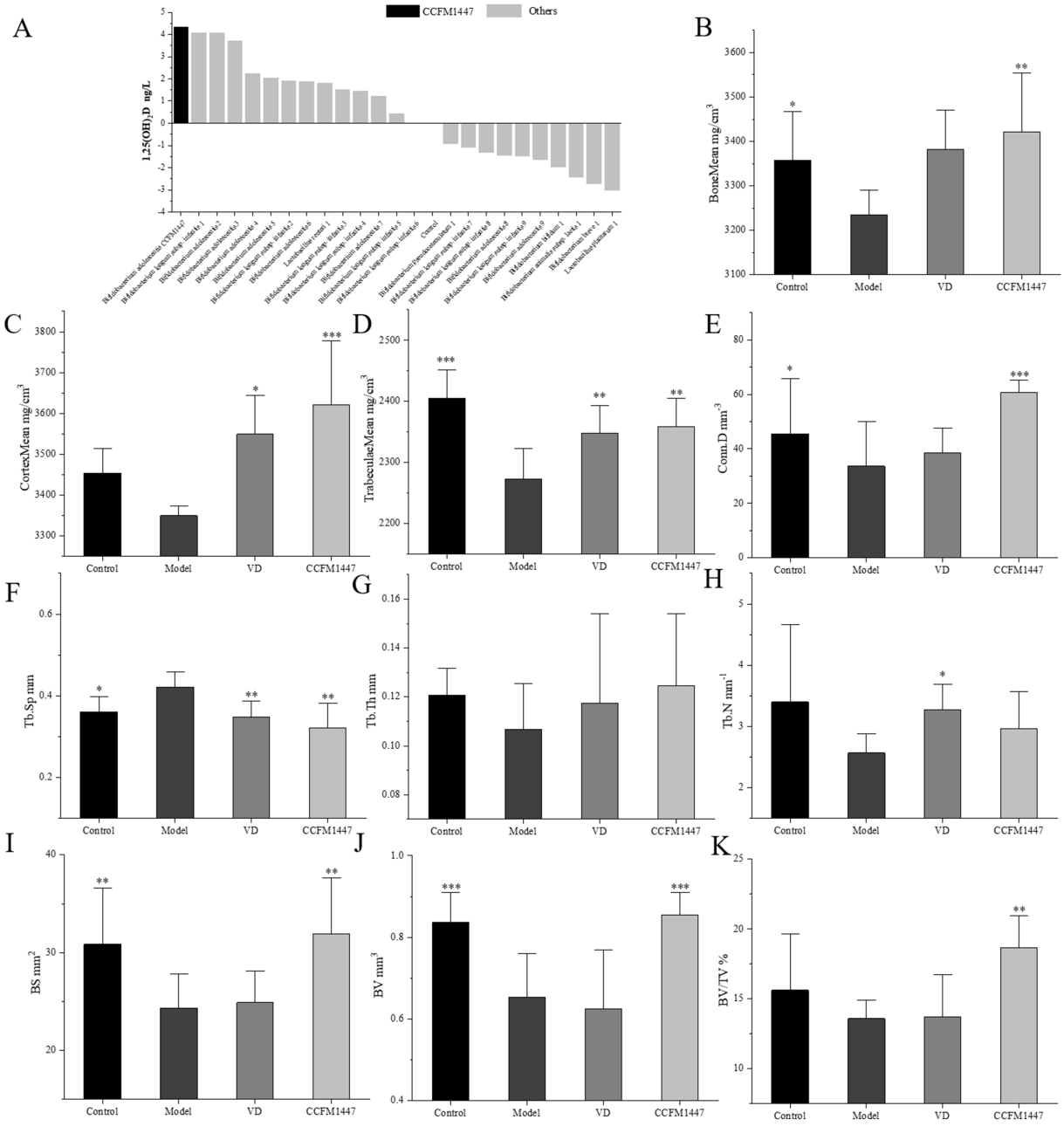

FIGURE 2
(A) Concentration of 1,25(OH)$_2$D in fermentation supernatants. BMD analysis of **(B)** total, **(C)** cortical, and **(D)** trabecular bone in the distal femur. Micro-CT-based analysis of **(E)** Conn.D, **(F)** Tb.Sp, **(G)** Tb.Th, and **(H)** Tb.N. Additional bone parameters: **(I)** BS, **(J)** BV, and **(K)** BV/TV. * indicates significant differences from the model group: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

VD group and *Pediococcus acidilactici* in the CCFM1447 group. And two-by-two comparison showed that there was a notable change in the relative abundance of *Parasutterella excrementihominis* in the intestinal flora of the model mice. These results suggest that VD and CCFM1447 decreased the relative abundance of some specific flora and increased the relative abundance of specific beneficial flora. This regulation of intestinal flora structure overall shifted the disrupted intestinal flora structure toward Control group mice. In contrast, VD and *Bifidobacterium adolescentis* CCFM1447 increased the relative abundance of specific beneficial flora in the intestinal flora and may also play a role in alleviating osteoporosis.

3.6 Correlation analysis of intestinal flora with physiological and biochemical markers

The correlation analysis of intestinal flora with physiological and biochemical markers is depicted in Figure 6. *Pediococcus acidilactici* was found to be positively correlated with beneficial indicators and negatively correlated with calcium loss and trabecular bone spacing. This aligns with the previously observed increase in *Pediococcus acidilactici* relative abundance following CCFM1447 intervention. An increase in the abundance of *Pediococcus acidilactici* may help alleviate

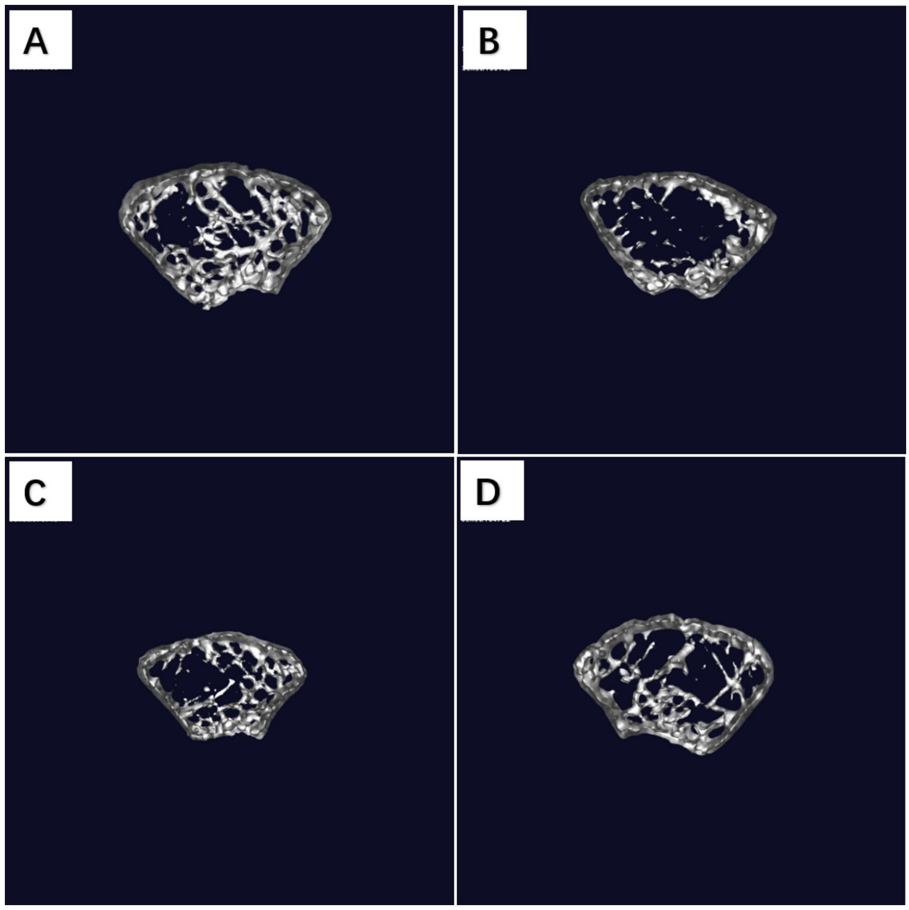

FIGURE 3
The three-dimensional reconstructions of mouse tibia. **(A)** Group Control; **(B)** group Model; **(C)** group VD; **(D)** group CCFM1447.

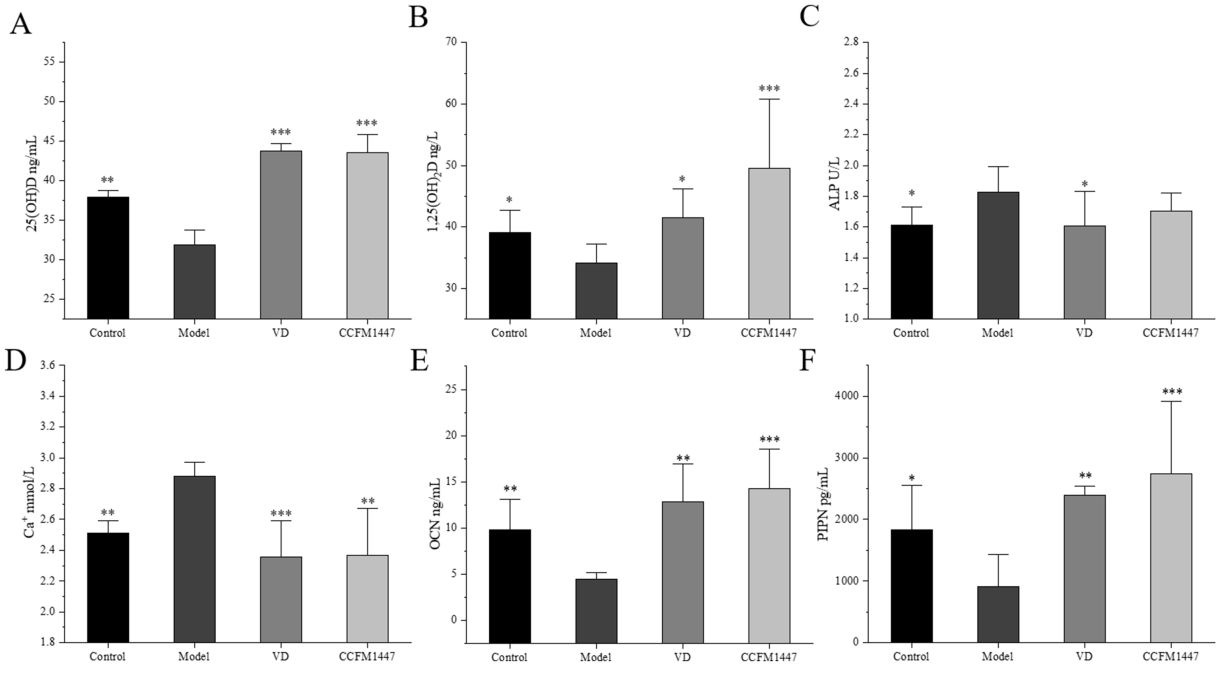

FIGURE 4
Effects on serum **(A)** 25(OH)D, **(B)** 1,25$(OH)_2$D, **(C)** ALP, **(D)** Ca^+, **(E)** OCN, **(F)** PINP concentration in mice. * indicates significant differences from the model group: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

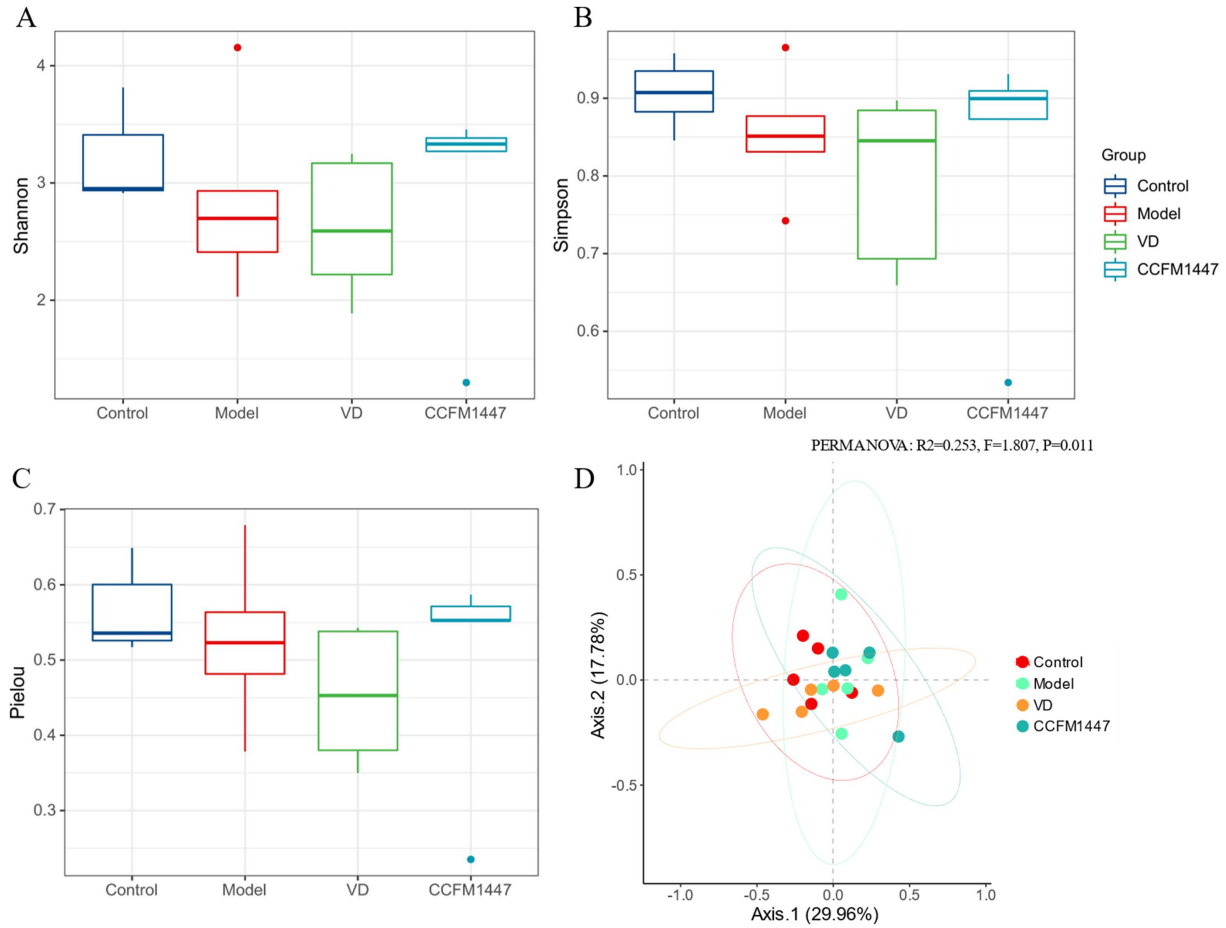

FIGURE 5
Species diversity analysis of intestinal flora across the different groups was conducted as follows: **(A)** Shannon index; **(B)** Simpson index; **(C)** Pielou index; **(D)** β-diversity based on PCoA algorithm.

osteoporosis symptoms. Conversely, the relative abundance of various Bacteroides species, including *Bacteroides thetaiotaomicron*, *Bacteroidales bacterium*, and *Bacteroidaceae bacterium*, showed a significant negative correlation with the levels of vitamin D metabolites, while being positively correlated with serum calcium levels and trabecular bone gap indices. These findings also corresponded to a notable decrease in the relative abundance of *Bacteroidales bacterium* after both vitamin D and CCFM1447 interventions. These results provide insight into the potential role of vitamin D and *Bifidobacterium adolescentis* CCFM1447 in modulating intestinal flora, offering a mechanism for their protective effects against osteoporosis through the regulation of specific microbial populations.

3.7 *Enterococcus faecalis* and *Pediococcus acidilactici* are probably the main strains in which CCFM1447 functions

Taking the intersection of LEfSe analyses, RF analyses, and association analyses of the differential groups, we found that CCFM1447 may promote VD transformation by enriching *Pediococcus acidilactici* and *Enterococcus feacium*. This was supported by the relative abundance in different groups as shown in Figures 1B,C. Gavage of CCFM1447 significantly increased *P. acidilactici* and *E. feacium* abundance compared to the model group. And this enrichment only appeared in the group with CCFM1447. This suggests that CCFM1447 may promote VD transformation and alleviate OP by specifically enriching *P. acidilactici* as well as *E. feacium*.

3.8 Functional validation of *Enterococcus faecalis* and *Pediococcus acidilactici*

Based on the key differential flora obtained in the above macrogenomic analysis, *in vitro* culture alone was performed, and the supernatant was taken for $1,25(OH)_2D$ assay at the end. The results are shown in Figure 7A, there was no significant change in $1,25(OH)_2D$ level in the supernatant of the medium of CCFM1447 after individual culture compared with the blank group. In contrast, both *Enterococcus faecalis* and *Pediococcus acidilactici* significantly increased the $1,25(OH)_2D$ level in the supernatant of the culture medium. This suggests that CCFM1447 itself does not have the ability to transform VD, and may be able to increase the ability of the intestinal flora to transform VD by increasing the abundance of *E. faecalis* and *P. acidilactici* in the intestinal flora.

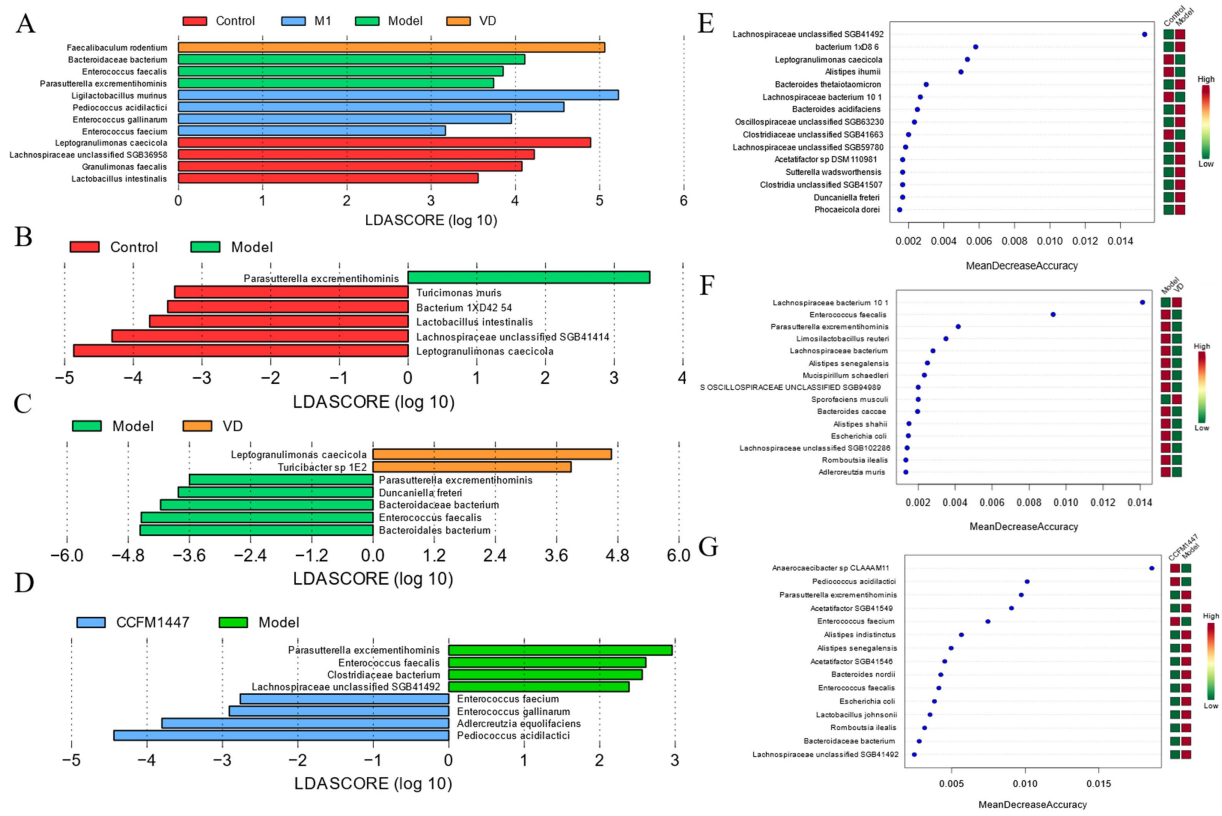

FIGURE 6
LEfSe analysis of fecal microbiota metagenomic species in mice: **(A,B,C,D)**; Importance analysis of key species characteristics based on the RF model: **(E,F,G)** (LDA > 3, $p < 0.05$).

Therefore, we induced pseudo-sterile mice by quadruple antibiotic treatment to exclude the interference of intestinal flora for further functional evaluation and validation of key differential strains. The results are shown in Figures 7B–E. Compared with the Control group, the levels of 25(OH)D and $1,25(OH)_2D$ were reduced in the Model group and Abx + Model group, but not significantly. This indicates that the intestinal flora has a certain VD conversion ability, and after the disappearance of intestinal flora, the main VD metabolism function still exists in the organism, although it will be affected to some extent. Meanwhile, the $1,25(OH)_2D$ level was more stable, probably due to the body regulation brought about by osteoporosis symptoms, which induced the depletion of the storage form 25(OH)D to be converted into $1,25(OH)_2D$, thus maintaining the stability of $1,25(OH)_2D$ level.

And compared to the model group, *E. faecalis*, Abx + *E. faecalis* and *P. acidilactici* group significantly increased the 25(OH)D and *E. faecalis*, Abx + *E. faecalis* and Abx + *P. acidilactici* group significantly increased the $1,25(OH)_2D$ levels. This suggests that *E. faecalis* and *P. acidilactici* are able to function individually in transforming VD in the presence of removal of intestinal flora, with *E. faecalis* having the most significant effect, increasing both 25(OH)D and $1,25(OH)_2D$ levels in Abx-treated mice. Similarly, the serum levels of type I procollagen amino-terminal procollagen peptide and osteocalcin were examined in the present study in all groups of mice to investigate the effect of key differential flora on the improvement of bone health in mice. Compared with the Control group, the PINP level was significantly reduced in the model group, and the OCN level was also reduced, but not significantly. And compared with the model group, the *E. faecalis*, *P. acidilactici*, Abx + *E. faecalis* and Abx + *P. acidilactici* group all showed more significant improvement in PINP and OCN levels. The above results further demonstrated that the mechanism by which CCFM1447 exerts its OP-relieving effect may be through the regulation of *E. faecalis* and *P. acidilactici* in the intestinal flora.

4 Discussion

RA has been shown to inhibit bone formation while promoting bone resorption. When administered to mice, RA induces a pathological process similar to osteoporosis, leading to altered bone structure and reduced bone density. In this model, significantly elevated serum ALP and calcium levels compared to the control group indicate extensive bone loss and confirm the successful induction of osteoporosis. Increased serum calcium levels serving as a marker for osteoporosis reflect indicating severe calcium depletion, reduced bone calcium reserves, and heightened bone turnover. Similarly, elevated ALP levels are associated with various bone disorders, including rickets, bone cancer, and bone metastases. OCN a hormone-like peptide secreted by osteoblasts, serves as a biochemical indicator of osteoblast activity. Approximately 20% of OCN produced by osteoblasts is released into the bloodstream, where its serum levels correlate with those in bone tissue, providing valuable insight into

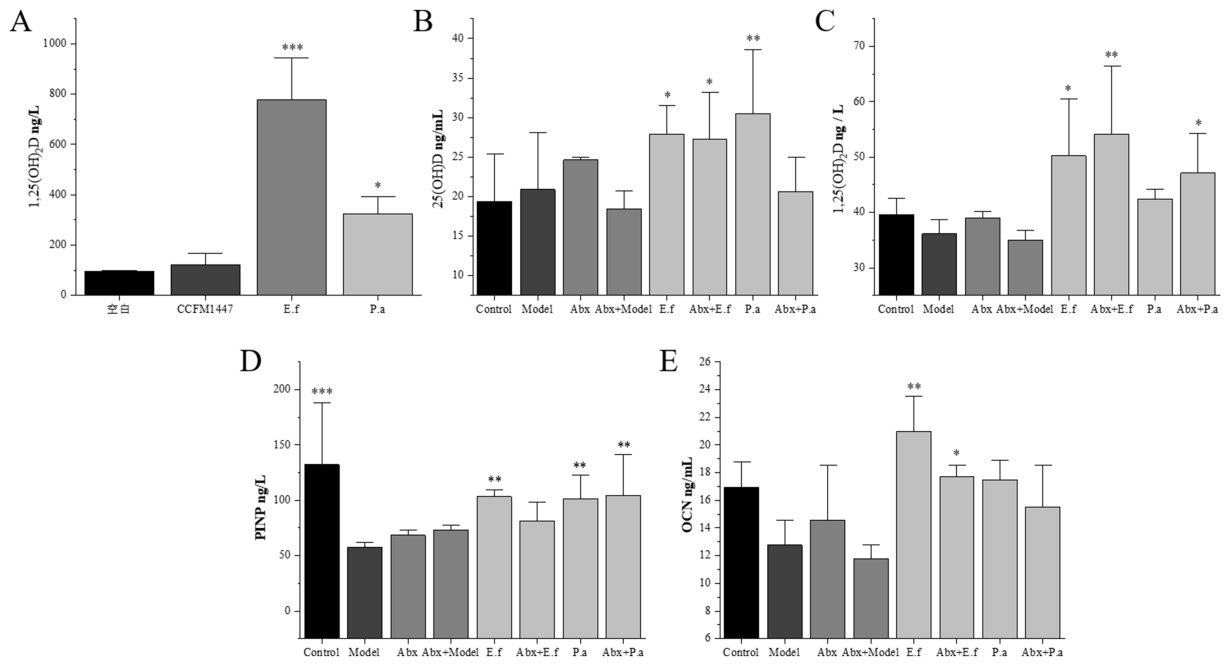

FIGURE 7
(A) Levels of 1,25(OH)$_2$D in the supernatants of key differential bacteria after separate cultures; Effects of key differential flora on serum **(B)** 25(OH)D, **(C)** 1,25(OH)$_2$D, **(D)** OCN, **(E)** PINP concentration in mice. * indicates significant differences from the model group: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

osteoblast function. Likewise, the expression of PINP, a marker of new bone formation, decreases as osteoblast activity diminishes, reflecting changes in the production of type I collagen.

Bifidobacterium adolescentis CCFM1447 was selected using an in-vitro fermentation model due to its ability to enhance vitamin D metabolite levels and alleviate osteoporosis symptoms. The effects of *Bifidobacterium adolescentis* CCFM1447 on bone structural parameters and serum biochemical markers were assessed in the RA-induced osteoporotic mice. The results demonstrated that *Bifidobacterium adolescentis* CCFM1447 significantly elevated the serum levels of 25(OH)D and 1,25(OH)$_2$D. These findings suggest that *Bifidobacterium adolescentis* CCFM1447 significantly mitigates the symptoms of RA-induced osteoporosis and plays a regulatory role in bone metabolism.

The alleviating effect of *Bifidobacterium adolescentis* CCFM1447 can be attributed to several key factors. First, VD helps with calcium uptake from the intestines into the bloodstream and directs calcium into the bones, especially benefiting calcium-deficient individuals such as children and older adults (21, 22). This function helps prevent excessive calcium loss from osteoblasts and reduces the risk of osteoporosis and bone development disorders in children. However, a large clinical trial revealed that VD supplementation did not significantly improve health outcomes like heart disease or cancer (23–26). Interestingly, some individuals are categorized as low, medium, or high responders to VD3 supplements, with 25% of people showing minimal response. This suggests that these low responders may require higher daily doses of VD than the standard recommendations (27–30). Furthermore, the body's ability to metabolize VD is as important as VD supplementation (31).

Bifidobacterium adolescentis CCFM1447 has been shown to increase the levels of VD metabolites. Clinically, 25(OH)D levels are used to assess VD deficiency, with 25-hydroxy VD3 and VD2 being key indicators (31–33). The activation rate of VD is determined by the ratio of 1,25(OH)$_2$D to 25(OH)D, which helps gauge VD metabolism. Clinically, measuring 1,25(OH)$_2$D levels provides insights into the active VD status in conditions like metabolic bone disease (34). *Blautia* and *Ruminococcus* have the ability to metabolize VD, and the levels of active VD in the blood correlate with these microorganisms (35). This suggests that intestinal flora is important in the bioavailability of VD, highlighting the potential of modulating the intestinal flora to enhance VD function and improve bone health.

VDR is present in various organs throughout the body. This makes 1,25(OH)$_2$D crucial to numerous physiological processes, as it can perform a variety of functions through the VDR, which is present throughout the body (36). 1,25(OH)$_2$D-VDR complex can activate the transcription of various genes involved in calcium absorption (37), parathyroid hormone (PTH), bone formation, and bone resorption. For example, genes regulating oxidative stress (38), calcium channels (e.g., TRPV6) (39), fibroblast growth factor 23 (FGF23), and calcium-binding proteins (e.g., calbindin-D28k) are activated (40), promoting calcium and phosphorus absorption, which benefits bone health. Additionally, the complex stimulates bone formation genes such as osteocalcin, promoting osteoblast activity and bone matrix synthesis, thereby enhancing bone density and strength (41). Moreover, the complex inhibits genes involved in bone resorption, such as RANKL and M-CSF, reducing osteoclast formation and minimizing bone destruction (42). The complex also regulates inflammation-related genes, such as NF-κB. The anti-inflammatory effects may help protect bone tissue and reduce osteoporosis symptoms (43).

In addition we detected that VD and *Bifidobacterium adolescentis* CCFM1447 intervened to significantly increase the relative

abundance of *Adlercreutzia equolifaciens*, *Akkermansia muciniphila*, *pediococcus acidilactici* and Fae*calibaculum rodentium*. *Akkermansia muciniphila*, on the other hand, has been repeatedly reported to be associated with the gut barrier. Calcium absorption or VDR expression may be affected by the pro-inflammatory effects and damage on the intestine because of RA. *Akkermansia muciniphila* may play a role in this regard (44). *Pediococcus acidilactici* is a beneficial flora in both maintenance of intestinal flora diversity and intestinal homeostasis (44). *Faecalibaculum rodentium* plays a key role in modulating RA signaling to maintain eosinophil-dependent intestinal epithelial homeostasis, which contributes to the regulation of the intestinal barrier and overall homeostasis (45).

Additionally, $1,25(OH)_2D$ is recognized not only for its essential role in maintaining bone health, but also for its involvement in cell proliferation, differentiation, and immune function (46). Recent studies have emphasized the essential role of $1,25(OH)_2D$ as a key hormone in immune system regulation, similar to the Vitamin D Receptor (VDR), which is expressed across various organs. Additionally, the enzyme 1-alpha hydroxylase (CYP27B1), which is responsible for converting vitamin D into its active form, $1,25(OH)_2D$, has been identified in several tissues beyond the kidneys. These include tumor cell supernatants, monocytes, macrophages, placenta, keratinocytes, and lymph nodes in individuals with nodal disease (36, 47). Notably, CYP27B1 expression has also been found in colon epithelial cells (48). Given the multifaceted roles of $1,25(OH)_2D$ in bone metabolism, immune modulation, and cellular differentiation, combined with the presence of CYP27B1 and VDR in the colon, it suggests a deeper and more intricate connection between gut health, intestinal barrier function, and the physiological effects of active vitamin D. This connection could provide insight into how *Bifidobacterium adolescentis* influences vitamin D metabolism in the gut by interacting with the intestinal flora. Moreover, the observed changes in intestinal flora after the intervention with *Bifidobacterium adolescentis* CCFM1447 appear to be closely linked to the regulation of gut barriers and homeostasis.

In this study, *Bifidobacterium adolescentis* CCFM1447 was experimentally found to have an excellent effect on improving OP symptoms and to enhance VD physiological activity by up-regulating the abundance of intestinal flora with the ability to transform VD. However, its mechanism of action to increase the abundance of these intestinal flora has not been clarified and will be further explored in depth in the future. In addition, the human body occupies a more dominant ability to metabolise VD, and further research is needed to improve VD physiological activity by regulating the human body and thereby increasing VD physiological activity. In this paper, we searched for key differential bacterial groups by analysing group-specific macrogenomes and non-target metabolomes and verified their functions by *in vitro* single-bacterial cultures, but the mechanism of transforming VD by differential bacterial groups is not clear. Therefore, in the future, these differential bacterial groups can be subjected to gene matching or transcriptome sequencing in order to further reveal their mechanism of transforming VD and lay a theoretical foundation for finding other strains with the ability to transform VD. These results provide a novel way of thinking about how probiotics affect VD metabolite levels by regulating intestinal flora to maintain bone health and gut homeostasis.

5 Conclusion

In conclusion, we identified a strain of *Bifidobacterium adolescentis* with the ability to enhance the activity of VD. The impact of this strain was further evaluated in a RA-induced osteoporotic mouse model. Significant bone loss and reduced serum VD metabolites were observed in the mice after 3 weeks of RA gavage. However, after 3 weeks of continuous *Bifidobacterium adolescentis* CCFM1447 intervention, we observed notable improvements in serum calcium, BMD, Tb. N, levels, decreased ALP levels, enhanced osteoblast activity, and increased VD metabolite levels, as well as modulation of intestinal flora. Compared to VD as a positive control, *Bifidobacterium adolescentis* CCFM1447 provided better improvements in osteoporosis-related symptoms. Additionally, the intervention's effect on the intestinal flora revealed that CCFM1447, combined with VD, significantly increased the abundance of *Adlercreutzia equolifaciens*, *Akkermansia muciniphila*, *Pediococcus acidilactici*, and *Faecalibaculum rodentium*. These bacteria are closely linked to intestinal barrier function, the maintenance of gut microbiota balance, and possibly estramustine production. Given the multifaceted roles of $1,25(OH)_2D$ in bone health, immune modulation, and cellular differentiation, along with the presence of CYP27B1 and VDR in the colon, there is likely a more complex relationship between gut health, intestinal barrier integrity, and the physiological actions of vitamin D. This connection suggests that the metabolism of vitamin D, particularly its active form, may not only influence bone and immune functions but also play a critical role in preserving gut homeostasis and overall gastrointestinal health. Further studies are needed to test this hypothesis and evaluate whether these microbial species could be targeted to improve VD metabolism. Therefore, we verified their role in transforming vitamin D from both *in vitro* monoculture and antibiotic-treated model mice. We found that one possibility for CCFM1447 to enhance the physiological activity of VD is through up-regulation of the abundance of *Enterococcus faecalis* and *Pediococcus acidilactici* in the intestinal flora. These bacteria possess the capability to metabolize vitamin D and enhance the levels of its active metabolites, thereby mitigating symptoms of osteoporosis in mice. This offers a theoretical foundation for boosting vitamin D activity as a strategy to combat osteoporosis. The potential applications of this approach are significant, particularly in the development of products aimed at enhancing the physiological activity of vitamin D to prevent or treat osteoporosis and bone loss. This area holds great promise and warrants further investigation.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found in here: https://www.ncbi.nlm.nih.gov/, accession number PRJNA1248171.

Ethics statement

The animal study was approved by Jiangnan University Experimental Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XY: Conceptualization, Data curation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. GT: Conceptualization, Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. YW: Conceptualization, Methodology, Software, Writing – original draft. LL: Data curation, Formal analysis, Investigation, Writing – original draft. LH: Conceptualization, Formal analysis, Investigation, Software, Writing – original draft. YurZ: Conceptualization, Data curation, Methodology, Writing – original draft. LF: Conceptualization, Formal analysis, Supervision, Writing – original draft. YuhZ: Conceptualization, Methodology, Writing – original draft. HF: Methodology, Project administration, Resources, Supervision, Writing – review & editing. WL: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. SL: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. HW: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

XY, YW, and LL were employed by Sinopharm Xingsha Pharmaceutical (Xiamen) 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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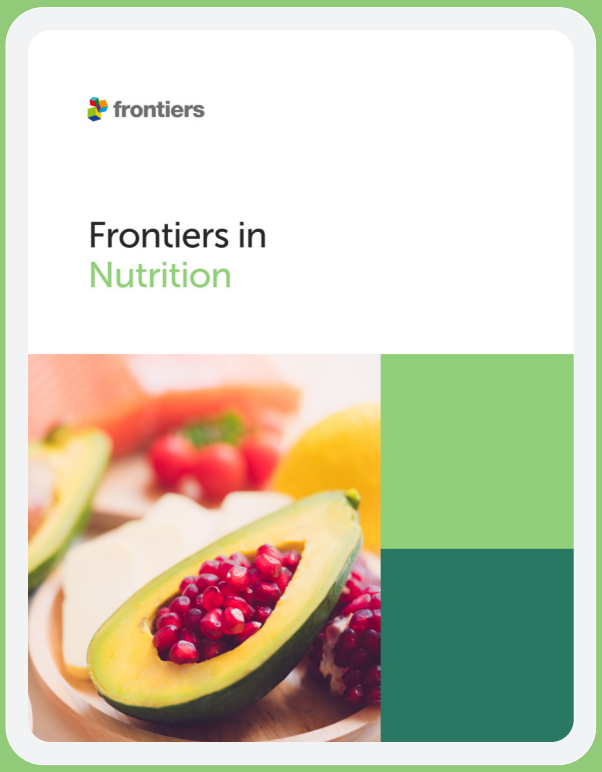

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