frontiers | Research Topics

Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications

Edited by
Nazarii Kobyliak, Ludovico Abenavoli, Tetyana Falalyeyeva and Emidio Scarpellini

Coordinated by
Ghizlane Bendriss

Published in
Frontiers in Microbiology
Frontiers in Microbiomes



FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-8325-7052-4
DOI 10.3389/978-2-8325-7052-4

Generative AI statement
Any alternative text (Alt text) provided alongside figures in the articles in this ebook has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications

Topic editors

Nazarii Kobyliak — Bogomolets National Medical University, Ukraine
Ludovico Abenavoli — Magna Græcia University, Italy
Tetyana Falalyeyeva — Taras Shevchenko National University of Kyiv, Ukraine
Emidio Scarpellini — KU Leuven, Belgium

Topic coordinator

Ghizlane Bendriss — Weill Cornell Medicine, Qatar

Citation

Kobyliak, N., Abenavoli, L., Falalyeyeva, T., Scarpellini, E., Bendriss, G., eds. (2025). *Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-7052-4

Table of contents

06 Editorial: Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications
Emidio Scarpellini, Ludovico Abenavoli, Tetyana Falalyeyeva, Nazarii Kobyliak and Ghizlane Bendriss

09 Fecal microbial transplantation limits neural injury severity and functional deficits in a pediatric piglet traumatic brain injury model
Madison M. Fagan, Christina B. Welch, Kelly M. Scheulin, Sydney E. Sneed, Julie H. Jeon, Morgane E. Golan, Savannah R. Cheek, Deborah A. Barany, Georg Oeltzschner, Todd R. Callaway, Qun Zhao, Hea Jin Park, Jeferson M. Lourenco, Kylee J. Duberstein and Franklin D. West

29 Fecal microbiota transplantation from HUC-MSC-treated mice alleviates acute lung injury in mice through anti-inflammation and gut microbiota modulation
Feng Hua, Enhai Cui, Lu Lv, Bin Wang, Liqin Li, Huadong Lu, Na Chen and Wenyan Chen

44 The causality between gut microbiome and anorexia nervosa: a Mendelian randomization analysis
Xuan Xia, Shu-yang He, Xiao-Lin Zhang, Decheng Wang, Qian He, Qing-Ao Xiao and Yong Yang

54 Fecal microbiota transplantation: a novel strategy for treating Alzheimer's disease
Wu Xiang, Han Xiang, Junyu Wang, Yiqin Jiang, Chuanhui Pan, Bingjin Ji and Anren Zhang

63 Efficacy and safety of fecal microbiota transplantation for chronic insomnia in adults: a real world study
Haiming Fang, Tingting Yao, Wanli Li, Na Pan, Hang Xu, Qian Zhao, Yuan Su, Kangwei Xiong and Jiajia Wang

78 *Bacteroides acidifaciens* and its derived extracellular vesicles improve DSS-induced colitis
Cihua Zheng, Yuchun Zhong, Jian Xie, Zhuoya Wang, Wenming Zhang, Yiming Pi, Wenjun Zhang, Li Liu, Jun Luo and Wei Xu

91 Early fecal microbiota transplantation from high abdominal fat chickens affects recipient cecal microbiome and metabolism
Jiani Song, Chaowei Luo, Zhijie Liu, Jingshou Liu, Li Xie, Xing Zhang, Zhuojun Xie, Xiangkun Li, Zheng Ma, Jinlong Ding, Hua Li and Hai Xiang

103 Effects of fecal microbiota transplantation on metabolic health of DBA mice
Wenxin Ye, Jinghui Fan, Wenzi Wu, Zhuo Chen, Qixin Huang and Lichun Qian

118 Alternative treatment of recurrent *Clostridioides difficile* infection in adults by fecal transplantation: an overview of phase I–IV studies from Clinicaltrials.gov
Najla A. Obaid

126 The interplay between the microbiota and opioid in the treatment of neuropathic pain
Zexiong Gong, Qingsheng Xue, Yan Luo, Buwei Yu, Bo Hua and Zhiheng Liu

140 Encapsulation protocol for fecal microbiota transplantation
Dávid Sipos, Adorján Varga, Ágnes Kappéter, Bernadett Halda-Kiss, Péter Kása, Szilárd Pál, Béla Kocsis and Zoltán Péterfi

149 Characterization of double humanized BLT-mice with stable engraftment of a human gut bacterial microbiome
Lance Daharsh, Saroj Chandra Lohani, Amanda E. Ramer-Tait and Qingsheng Li

165 Perspective: on the future of fecal microbiota transplantation
Olaf F. A. Larsen and Robert J. M. Brummer

169 Effect of intestinal microbiota transplantation on chronic hepatitis B virus infection associated liver disease
Lisi Deng, Xiaozhen Guo, Jiehua Chen, Baoyi Li, Na Liu, Jinyu Xia, Mengdang Ou and Zhongsi Hong

178 Suppressed oncogenic molecules involved in the treatment of colorectal cancer by fecal microbiota transplantation
Xing Han, Bo-Wen Zhang, Wei Zeng, Meng-Lin Ma, Ke-Xin Wang, Bao-Juan Yuan, Dan-Qi Xu, Jia-Xin Geng, Chao-Yuan Fan, Zhan-Kui Gao, Muhammad Arshad, Shan Gao, Liangliang Zhao, Shu-Lin Liu and Xiao-Qin Mu

188 Comprehensive effects of fecal microbiota transplantation on cynomolgus macaques across various fecal conditions
Philyong Kang, Gyu-Seo Bae, Eunsu Jeon, Jeonghwa Choi, Eun-Ha Hwang, Green Kim, Seung Ho Baek, Kyuyoung Shim, You Jung An, Kyung Seob Lim, Yujin Kim, Taehwan Oh, Jung Joo Hong, Wan-Kyu Lee, Seok-Hwan Kim and Bon-Sang Koo

201 Integrative analysis of intestinal flora and untargeted metabolomics in attention-deficit/hyperactivity disorder
Jiamin Lu, Maoying Jiang, Dingyue Chai, Yuzi Sun and Lihui Wu

215 Fecal microbiota transplantation: application scenarios, efficacy prediction, and factors impacting donor-recipient interplay
Yaxin Liu, Xinru Li, Yuchao Chen, Qinyan Yao, Jinjie Zhou, Xiaoxuan Wang, Qingguo Meng, Jiaxuan Ji, Zihan Yu and Xin Chen

238 Fecal microbiota transplantation alleviates lipopolysaccharide-induced osteoporosis by modulating gut microbiota and long non-coding RNA *TUG1* expression
Pengcheng Ma, Ruoyi Wang, Huizhi Chen, Jiachun Zheng, Weijie Yang, Bo Meng, Yifan Liu, Yao Lu, Jing Zhao and Hongwei Gao

251 Cold water swimming reshapes gut microbiome to improve high-fat diet-induced obesity
Jie Men, Chenglong Cui, Hao Li, Zhaowei Li, Yu Zhang, Zhiyu Liu, Qi Wang, Penghong Liu, Shuangling Zou, Zhengyang Yu, Yuxi Zhang, Simin Wu, Guoyu Zhu, Pengbo Wang and Xiaoli Huang

267 Prolonged effect of antibiotic therapy on the gut microbiota composition, functionality, and antibiotic resistance genes' profiles in healthy stool donors
Ramin Karimianghadim, Reetta Satokari, Sam Yeo, Perttu Arkkila, Dina Kao and Sepideh Pakpour

280 The role of gut microbiota in autoimmune disease progression and therapy: a comprehensive synthesis
Mohammad Adawi

287 Fecal microbiota transplantation for chronic constipation: a systematic review and meta-analysis of clinical efficacy, safety, and microbial dynamics
Ke Wang, Chun Gao, Li Zhu, Min Chen, Yi Xin Tong and Sheng Zhang

299 Therapeutic effect of fecal microbiota transplantation on hyperuricemia mice by improving gut microbiota
Songjian Yuan, Wenting Jia, Xiaomei Liu, Ruzhen Liu, Man Cao, Yuting Wu, Yuantao Li, Wei Xu, Chuanxing Xiao, Zhenqiang Hong and Bangzhou Zhang

311 Fecal microbiota transplantation augments 5-fluorouracil efficacy in pancreatic cancer via gut microbiota modulation
Rui Li, Yaoyuan Hu, Yixian Liu and Xiaodong Tan

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 10 October 2025
DOI 10.3389/fmicb.2025.1704947

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
Sinead Waters,
University of Galway, Ireland

*CORRESPONDENCE
Ghizlane Bendriss
ghb2002@qatar-med.cornell.edu

RECEIVED 14 September 2025
ACCEPTED 26 September 2025
PUBLISHED 10 October 2025

CITATION
Scarpellini E, Abenavoli L, Falalyeyeva T, Kobyliak N and Bendriss G (2025) Editorial: Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications. *Front. Microbiol.* 16:1704947. doi: 10.3389/fmicb.2025.1704947

COPYRIGHT
© 2025 Scarpellini, Abenavoli, Falalyeyeva, Kobyliak and Bendriss. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Editorial: Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications

Emidio Scarpellini[1], Ludovico Abenavoli[2], Tetyana Falalyeyeva[3,4], Nazarii Kobyliak[4,5] and Ghizlane Bendriss[6]*

[1]Translational Research in GastroIntestinal Disorders (TARGID), Biomedical Sciences Group, KULeuven, Leuven, Belgium, [2]Department of Health Sciences, University Magna Graecia of Catanzaro, Catanzaro, Italy, [3]Department of Biomedicine, Taras Shevchenko National University of Kyiv, Kyiv, Ukraine, [4]Department of Pathology, Medical Laboratory Care and Safe Diagnostics (CSD), Kyiv, Ukraine, [5]Endocrinology Department, Bogomolets National Medical University, Kyiv, Ukraine, [6]Premedical Department, Weill Cornell Medicine Qatar, Doha, Qatar

KEYWORDS

fecal microbiota transfer (FMT), gut microbiome, precision medicine, clinical trial, artificial intelligence

Editorial on the Research Topic
Fecal Microbiota Transplants: challenges in translating microbiome research to clinical applications

Gut microbiota encompasses a complex ecosystem including bacteria, viruses, protozoa, yeasts, fungi, and archea (Seekatz, 2025). It has a multifaceted functional profile affecting human host metabolism, nutrient absorption, immune system education and tolerance and nervous system functioning, with impact on autism, depression (Li et al., 2025). With accumulating evidence on the extra-digestive communication between the gut and other organs via short-chain fatty acids, the vagus nerve, or cytokines, the gut microbiome has become a key therapeutic target (Bendriss et al., 2023).

This Research Topic aims at gathering multidisciplinary insights into the clinical application of fecal microbiota transplantation (FMT) across a broad range of conditions. Lately, FMT has been implemented to treat *Clostridium difficile* resistant infections, with excellent results, leading to its recognition by regulatory bodies as a life-saving therapy (Herman et al., 2025). Nevertheless, its broader use brings forward important questions regarding efficacy, safety, donor screening, and regulatory frameworks. With over 500 clinical trials currently investigating its potential in gastrointestinal, neurological, metabolic, and immune-mediated disorders, FMT is increasingly recognized as a central tool in the translation of microbiome research into practice. The contributions in this Research Topic explore key methodological, mechanistic, ethical, and clinical aspects of FMT—ranging from delivery strategies and treatment variability to host–microbiome interactions. Several studies also consider the role of computational tools, including artificial intelligence and metabolomic profiling, in enhancing predictability and safety. Together, these works reflect the growing need to standardize, refine, and ethically frame FMT as part of a precision medicine approach. Its modulation via prebiotics, probiotics, and postbiotics has seen several advancements in human disease over the last decades (Scarpellini et al., 2021). In detail, an increasingly personalized approach has taken place in research and current clinical practice (Baldi et al., 2025).

FMT has been explored in treatment of Crohn's disease, ulcerative colitis, irritable bowel syndrome, and neurological conditions such as Alzheimer's and Parkinson's disease, to improving response to cancer immunotherapy treatments (Al-Ali et al., 2021). However, there is a lack of objective markers of its efficacy across such a wide spectrum of research and clinical applications. In particular, it is crucial to measure the extent of engraftment following FMT to assess patients' response to treatment. More specifically, the gray zones in FMT investigation are community coalescence, which aims to study microbiome shifts following FMT engraftment; indicator features, which aim to assess specific microbiome features as a signal of engraftment; resilience, which aims to assess post-FMT recipients' microbiomes' resistance to shifts (Cymbal et al., 2025).

The contributions gathered in this Research Topic extend well beyond the established use of FMT for recurrent *Clostridioides difficile* infections and illustrate the breadth of its translational potential. Evidence now supports meaningful clinical benefits in functional and metabolic disorders: a systematic review demonstrated that FMT provides remission and symptom improvement in chronic constipation with favorable safety and microbial remodeling (Wang et al.), while preclinical work showed that gut microbial transfer can ameliorate hyperuricemia (Yuan et al.) and high-fat diet–induced obesity (Men et al.). Neurological and psychiatric conditions have also emerged as promising targets, with studies documenting improvements in chronic insomnia (Fang et al.), disease modification in Alzheimer's models (Xiang et al.), and integrative microbiome–metabolome signatures linked to attention-deficit/hyperactivity disorder (Lu et al.), alongside genetic evidence for causal associations between the gut microbiome and anorexia nervosa (Xia et al.).

In oncology, microbial modulation is increasingly recognized as a key determinant of therapeutic efficacy. FMT was found to suppress oncogenic programs in colorectal cancer (Han et al.) and to enhance the efficacy of 5-fluorouracil in pancreatic cancer (Li et al.) highlighting opportunities to optimize immuno- and chemotherapy responsiveness. Similarly, immune and autoimmune conditions are gaining attention: a comprehensive synthesis outlined the role of gut microbiota in shaping autoimmune disease progression and treatment strategies (Adawi), while experimental work demonstrated that FMT could alleviate lipopolysaccharide-induced osteoporosis by regulating microbial communities and lncRNA-TUG1 (Ma et al.). Microbiota transfer has also been shown to benefit chronic liver disease associated with hepatitis B virus infection, with improvements in both metabolic and microbial parameters (Deng et al.).

These advances also underscore the importance of donor factors and methodology. A study on healthy donors revealed that prior antibiotic exposure can durably alter microbial composition, phage dynamics, and resistance gene profiles, raising critical safety considerations (Karimianghadim et al.). At the same time, innovative delivery methods are being developed: an encapsulation protocol has demonstrated feasibility for stable, capsule-based administration (Sipos et al.). Expanding the scope even further, animal models illustrate how FMT influences systemic immune and neural processes, with demonstrated protection against pediatric traumatic brain injury (Fagan et al.) and mitigation of acute lung injury through anti-inflammatory and microbial pathways (Hua et al.).

Across this Research Topic, mechanistic insights converge on the role of transferable metabolic signatures—including SCFA production, bile acid pathways, and amino acid metabolism—in mediating host responses (Deng et al.; Lu et al.; Men et al.). Finally, forward-looking perspectives emphasize the urgent need for standardization and predictive tools: multi-omics integration and intelligence are proposed as key strategies to monitor engraftment, anticipate therapeutic outcomes, and tailor donor–recipient matching, bringing FMT closer to a precision medicine framework (Larsen and Brummer; Liu et al.; Cantón et al., 2024). Together, these studies demonstrate both the promise and complexity of FMT, reinforcing the need for careful methodological refinement and ethical oversight as the field moves toward clinical translation.

This Research Topic allows experts in the field to update their knowledge on the concept of gut microbiota, FMT, its use in clinical and research practice to beneficially affect human health.

Author contributions

ES: Conceptualization, Writing – original draft, Writing – review & editing. LA: Writing – review & editing. TF: Writing – review & editing. NK: Writing – review & editing. GB: Conceptualization, Investigation, Supervision, Writing – review & editing.

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 author(s) declare that no Gen AI was used in the creation of this manuscript.

Any alternative text (alt text) provided alongside figures in this article has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Al-Ali, D., Ahmed, A., Shafiq, A., McVeigh, C., Chaari, A., Zakaria, D., et al. (2021). Fecal microbiota transplants: a review of emerging clinical data on applications, efficacy, and risks (2015-2020). *Qatar Med. J.* 2021:5. doi: 10.5339/QMJ.2021.5

Baldi, S., Sarikaya, D., Lotti, S., Cuffaro, F., Fink, D., Colombini, B., et al. (2025). From traditional to artificial intelligence-driven approaches: revolutionizing personalized and precision nutrition in inflammatory bowel disease. *Clin. Nutr. ESPEN* 68, 106–117. doi: 10.1016/j.clnesp.2025.05.012

Bendriss, G., MacDonald, R., and McVeigh, C. (2023). Microbial reprogramming in obsessive-compulsive disorders: a review of gut-brain communication and emerging evidence. *Int. J. Mol. Sci.* 24:11978. doi: 10.3390/IJMS241511978

Cantón, R., De Lucas Ramos, P., García-Botella A, García-Lledó A, Hernández-Sampelayo T, Gómez-Pavón J, et al. (2024). Human intestinal microbiome: Role in health and disease. *Rev Esp Quimioter*. 37, 438–453. doi: 10.37201/req/056.2024

Cymbal, M., Chatterjee, A., and Baggott, B. (2025). Fecal microbiota transplantation: current evidence and future directions. *Cleveland Clin. J. Med.* 92, 421–428. doi: 10.3949/CCJM.92A.24107

Herman, C., Barker, B. M., Bartelli, T. F., Chandra, V., Krajmalnik-Brown, R., Jewell, M., et al. (2025). A review of engraftment assessments following fecal microbiota transplant. *Gut Microbes* 17:2525478. doi: 10.1080/19490976.2025.2525478

Li, P., Li, M., and Chen, W. H. (2025). Best practices for developing microbiome-based disease diagnostic classifiers through machine learning. *Gut Microbes* 17:2489074. doi: 10.1080/19490976.2025.2489074

Scarpellini, E., Rinninella, E., Basilico, M., Colomier, E., Rasetti, C., Larussa, T., et al. (2021). From pre- and probiotics to post-biotics: a narrative review. *Int. J. Environ. Res. Public Health* 19:37. doi: 10.3390/IJERPH19010037

Seekatz, A. M. (2025). Straining to define a healthy microbiome. *MSphere* 10:e0079724. doi: 10.1128/MSPHERE.00797-24

frontiers | Frontiers in Neuroscience

TYPE Original Research
PUBLISHED 28 September 2023
DOI 10.3389/fnins.2023.1249539

Check for updates

OPEN ACCESS

EDITED BY
Hailong Song,
University of Pennsylvania, United States

REVIEWED BY
Xianghao Zhan,
Stanford University, United States
Kate Wofford,
University of Pennsylvania, United States

*CORRESPONDENCE
Franklin D. West
✉ westf@uga.edu

†These authors have contributed equally to this work

RECEIVED 28 June 2023
ACCEPTED 12 September 2023
PUBLISHED 28 September 2023

CITATION
Fagan MM, Welch CB, Scheulin KM, Sneed SE, Jeon JH, Golan ME, Cheek SR, Barany DA, Oeltzschner G, Callaway TR, Zhao Q, Park HJ, Lourenco JM, Duberstein KJ and West FD (2023) Fecal microbial transplantation limits neural injury severity and functional deficits in a pediatric piglet traumatic brain injury model.
Front. Neurosci. 17:1249539.
doi: 10.3389/fnins.2023.1249539

COPYRIGHT
© 2023 Fagan, Welch, Scheulin, Sneed, Jeon, Golan, Cheek, Barany, Oeltzschner, Callaway, Zhao, Park, Lourenco, Duberstein and West. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbial transplantation limits neural injury severity and functional deficits in a pediatric piglet traumatic brain injury model

Madison M. Fagan[1,2,3†], Christina B. Welch[3†], Kelly M. Scheulin[1,2,3], Sydney E. Sneed[1,3], Julie H. Jeon[4], Morgane E. Golan[1,2,3], Savannah R. Cheek[1,3], Deborah A. Barany[1,5], Georg Oeltzschner[6], Todd R. Callaway[3], Qun Zhao[1,7], Hea Jin Park[4], Jeferson M. Lourenco[3], Kylee J. Duberstein[1,2,3] and Franklin D. West[1,2,3*]

[1]Regenerative Bioscience Center, University of Georgia, Athens, GA, United States, [2]Biomedical and Health Sciences Institute, University of Georgia, Athens, GA, United States, [3]Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences, University of Georgia, Athens, GA, United States, [4]Department of Nutritional Sciences, College of Family and Consumer Sciences, University of Georgia, Athens, GA, United States, [5]Department of Kinesiology, College of Education, University of Georgia, Athens, GA, United States, [6]Russell H. Morgan Department of Radiology and Radiological Science, The Johns Hopkins University School of Medicine, Baltimore, MD, United States, [7]Department of Physics and Astronomy, Franklin College of Arts and Sciences, University of Georgia, Athens, GA, United States

Pediatric traumatic brain injury (TBI) is a leading cause of death and disability in children. Due to bidirectional communication between the brain and gut microbial population, introduction of key gut bacteria may mitigate critical TBI-induced secondary injury cascades, thus lessening neural damage and improving functional outcomes. The objective of this study was to determine the efficacy of a daily fecal microbial transplant (FMT) to alleviate neural injury severity, prevent gut dysbiosis, and improve functional recovery post TBI in a translational pediatric piglet model. Male piglets at 4-weeks of age were randomly assigned to Sham + saline, TBI + saline, or TBI + FMT treatment groups. A moderate/severe TBI was induced by controlled cortical impact and Sham pigs underwent craniectomy surgery only. FMT or saline were administered by oral gavage daily for 7 days. MRI was performed 1 day (1D) and 7 days (7D) post TBI. Fecal and cecal samples were collected for 16S rRNA gene sequencing. Ipsilateral brain and ileum tissue samples were collected for histological assessment. Gait and behavior testing were conducted at multiple timepoints. MRI showed that FMT treated animals demonstrated decreased lesion volume and hemorrhage volume at 7D post TBI as compared to 1D post TBI. Histological analysis revealed improved neuron and oligodendrocyte survival and restored ileum tissue morphology at 7D post TBI in FMT treated animals. Microbiome analysis indicated decreased dysbiosis in FMT treated animals with an increase in multiple probiotic *Lactobacilli* species, associated with anti-inflammatory therapeutic effects, in the cecum of the FMT treated animals, while non-treated TBI animals showed an increase in pathogenic bacteria, associated with inflammation and disease such in feces. FMT mediated enhanced cellular and tissue recovery resulted in improved motor

function including stride and step length and voluntary motor activity in FMT treated animals. Here we report for the first time in a highly translatable pediatric piglet TBI model, the potential of FMT treatment to significantly limit cellular and tissue damage leading to improved functional outcomes following a TBI.

KEYWORDS

traumatic brain injury, porcine (pig) model, fecal matter transfer, MRI, microbiome gut-brain axis, behavior analysis, gait analysis

Introduction

Pediatric traumatic brain injury (TBI) is a substantial contributor to death and disability worldwide. Each year in the United States alone there are over 2.8 million TBI hospital visits of which over 850,000 are children (CDC Injury Center, 2022). TBI results in brain lesioning, edema, hemorrhage, cerebral blood flow disruption, and inflammation that can have widespread effects on the body including gut dysbiosis (Feighery et al., 2008; Sundman et al., 2017) and loss of gastrointestinal tract (GIT) integrity (Hang et al., 2003). In children, TBI can interrupt and irrevocably alter ongoing neural development, thus leading to lifelong deficits in learning (Prasad et al., 2017), memory (Prasad et al., 2017), motor function (Beretta et al., 2009), and behavior (Li and Liu, 2013). As such, TBI is considered a chronic disease as opposed to a static injury (Masel and DeWitt, 2010). Although TBI is a worldwide epidemic, there is no FDA-approved treatment. Therefore, there is a significant need for the development of novel and innovative TBI therapies that will lead to improved patient recovery. Recent studies in rodent TBI models have demonstrated that manipulating the GIT microbiome through fecal microbiota transplantation (FMT) (Du et al., 2021; Davis et al., 2022) or introduction of specific probiotic species, *Lactobacillus acidophilus* (Sun et al., 2015; Ma et al., 2019) or *Clostridium butyricum*, (Li et al., 2018) may lead to reduced intracerebral brain damage and improved cognitive and motor function outcomes, making the GIT microbiome a novel TBI therapeutic target.

A number of recent studies have discovered significant connections between the gut microbiome and brain health (Tremlett et al., 2017). The gut microbiome consists of over a trillion microorganisms that primarily inhabit the lower portion of the GIT system. Connected through the microbiota-gut-brain-axis (MGBA), bi-directional communication exists in which the gut microbiome communicates to the brain through the immune system, enteric nervous system, and hypothalamic-pituitary-adrenal (HPA) axis using complex signaling molecules (Rice et al., 2019). Indeed, TBI can result in changes to the gut microbiome which have been detected as early as 2 h after experimental TBI (Crapser et al., 2016; Fung et al., 2017; Nicholson et al., 2019; Jeon et al., 2020). These changes often include an increase in opportunistic pathogenic bacteria commonly associated with disease and a corresponding decrease in healthy, commensal bacteria resulting in a population imbalance, termed dysbiosis. GIT dysbiosis leads to the activation of GIT inflammatory cells, breakdown of the intestinal wall, and leakage of intestinal contents and bacteria into the circulatory system which results in systemic inflammation that further exacerbates TBI induced inflammation in the brain. Additionally, the vagus nerve and HPA axis are activated in a positive feedback loop which perpetuates inflammation in both the brain and the GIT following TBI (Thayer and Sternberg, 2009). Therefore, a microbial based therapy may act on each arm of the MGBA to reduce the TBI-induced secondary injury cascade and improve outcomes.

As interest in the gut microbiome-health connection increases, evidence has grown that introduction of key gut bacteria may contribute to the treatment of numerous central nervous system (CNS) pathologies such as Alzheimer's Disease, Parkinson's Disease, and TBI (Vendrik et al., 2020). Recent studies have demonstrated that FMT may be a potent microbiome altering therapeutic option for TBI (Du et al., 2021; Davis et al., 2022). FMT is the ingestion of a healthy bacterial population isolated from donor stool with the goal of replenishing a dysbiotic gut microbiome. FMT studies in controlled cortical impact (CCI) TBI mouse models showed that TBI induced significant gut dysbiosis, including changes in microbial population alpha- and beta-diversity, with increasing levels of dysbiosis correlating with increased brain lesion volumes (Li et al., 2018). FMT treatment restored gut microbial populations back to a pre-injury state (Du et al., 2021; Davis et al., 2022). FMT-induced restoration of the microbiome was associated with decreased oxidative stress, cortical volume loss, and attenuated white matter damage in the brain (Du et al., 2021; Davis et al., 2022). FMT-treated animals also showed reduced neurological deficits as measured by improved modified neurological severity scores, decreased anxiety-like behavior as determined by open field testing, and improved spatial memory and learning as determined by Morris Water Maze testing (Du et al., 2021; Davis et al., 2022). These studies demonstrate that FMT is a promising therapeutic that can limit TBI pathogenesis and lead to improved recovery in rodent models.

Despite highly compelling FMT TBI therapeutic data in rodent systems, significant differences exist in human and rodent anatomy and physiology that may decrease the translational potential of FMT into human patients, particularly in the pediatric population. Porcine models are increasingly utilized in neural injury studies due to key similarities in human and pig anatomy and physiology (Kinder et al., 2019c; Kaiser and West, 2020). In fact, humans and pigs share remarkably similar neuroanatomical architecture, including both possessing gyrencephalic brains, similar white matter composition (>50%), and cerebral vasculature (Ahmad et al., 2015; Kaiser and West, 2020). Evaluating the effects of TBI

in a gyrencephalic brain is critically important as gyrification significantly influences the movement of the brain in the skull during TBI, including maximum mechanical stress in tissues, with considerably more brain deformation occurring and deeper transmission of forces relative to a lissenciphalic brain like those found in typical rodent models (Vink, 2018). The human brain is approximately 650 times the size of the average rodent brain, while only 7.5 times larger than the pig brain- a size comparable to non-human primate models (Lind et al., 2007). Brain size is an important factor when evaluating TBI effects on brain pathophysiology as smaller rodent brains can tolerate much greater angular acceleration forces than animals with larger brains as shearing forces and inertial loading are related to brain mass (Vink, 2018). Moreover, the piglet brain shows a more similar time course in neural development, maturation, and myelination to human adolescents as compared to the rodent model (Flynn, 1984; Conrad et al., 2012). Humans and pigs also share key GIT features including similarities in body size resulting in more relatively proportional intestinal size, length, and transit time as opposed to rodents (Heinritz et al., 2013). Pigs and humans share more similar digestion strategies as opposed to rodents. For example, fermentation occurs primarily in the colon of pigs and humans versus cecal fermentation in rodents (Miller and Ullrey, 1987). Studies have also demonstrated similarities in the gut bacterial populations in humans and pigs when compared to rodents (Lamendella et al., 2011). Both adult human and pig GIT microbial populations are dominated by Firmicutes and Bacteroidetes and show similar levels of microbial diversity (Lamendella et al., 2011). Furthermore, >95% of functional genes in the human gut metagenome are present in the pig, thus demonstrating significant overlap between the functional capacity of pig and human microbiomes and further supporting the use of porcine preclinical microbial models whereas less microbial functional gene overlap is observed between humans and rodents (Xiao et al., 2016). Finally, the pediatric piglet TBI model has also demonstrated relevant functional deficits after TBI including loss of motor coordination and behavioral changes (Baker et al., 2019; Kinder et al., 2019a,b; Wang et al., 2021). Taken together, these key similarities in human and pig brain, GIT, microbiome, and functional outcomes enable the pig to be an invaluable tool in preclinical neural injury research, specifically in modeling gut mediated therapies.

The objective of this study was to determine the efficacy of a daily FMT to alleviate neural injury severity, reverse GIT dysbiosis, and improve functional recovery post TBI in a translational pediatric porcine model. In this study, we demonstrate that FMT treatment leads to a significant reduction in lesion volume, midline shift, hemorrhage, and edema in our piglet TBI model. Furthermore, we demonstrate that FMT treatment prevents an acute increase in potentially harmful fecal microbial species and promotes an increase in cecal therapeutic probiotic species post-TBI. These FMT-mediated improvements in injury severity and gut dysbiosis led to enhanced recovery in motor function and behavioral outcomes in TBI piglets.

Materials and methods

Animals and housing

Four-week-old castrated male crossbreed piglets (N = 18) were used as experimental animals in this study due to animal availability. Previous research from our lab indicated that crossbreed animals showed similar brain pathophysiology and motor outcomes following TBI as Yucatan biomedical pigs (Sneed et al., 2020). Additional castrated male piglets (N = 9) were kept from each litter as healthy fecal matter donor pigs. All piglets were born from the same genetic lineage and maintained in an environmentally controlled room to minimize microbiome variability. This study was performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals guideline. All procedures were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol A2019 07-007-Y1-A9). Pigs were housed in Public Health Service (PHS) and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility with a 12-h light/dark cycle maintained at room temperature (27°C). Pigs were provided free access to water and fed standard pig starter I diets. Additionally, all pigs received daily enrichment through human contact and toys.

Study design

A total of 18 animals were enrolled in this randomized single blinded study. To minimize day effects, this study was conducted in a randomized block design in which animals were randomly assigned to one of three treatment groups (Sham craniectomy + saline, TBI + saline TBI + FMT). Treatments were administered in an unblinded fashion; however, all data was anonymized and underwent blinded analysis. Beginning the day after birth, piglets were socialized and acclimated to human interaction in order to reduce handling stress during future procedures. Starting at 2 weeks of age, piglets were individually habituated to the gait track and underwent gait training 5 days per week for 2 weeks. After weaning at approximately 4 weeks of age, pre-injury gait and behavior data were collected, and pigs were randomly assigned to either TBI + FMT (FMT; n = 6), TBI + saline (TBI; n = 6) or craniectomy + saline (Sham; n = 6) experimental groups. FMT or saline treatments were administered 2-h post TBI or Sham surgery, and once a day thereafter. MRI collection occurred 1 and 7 days post-surgery. Gait, behavior, and fecal matter collections took place 1, 3, and 7 days post-surgery. All experimental animals were sacrificed 7 days post-surgery for tissue analysis and cecal collection.

Controlled cortical impact

Approximately 12-h prior to surgery a transdermal fentanyl patch (12 mcg/h; Covetrus) was applied to each pig for pain management. Pre-induction analgesia and sedation for TBI or Sham surgery was achieved using xylazine (2 mg/kg IM; VetOne)

and midazolam (0.2 mg/kg IM; Heritage). Prophylactic lidocaine (0.5 mL 2% lidocaine VetOne) was administered topically to laryngeal folds, and propofol (0.5 mL to effect IV; Zoetis) to facilitate intubation. Anesthesia was maintained with isoflurane (1.0–2.0% Abbott Laboratories) in oxygen. Vitals including heart rate, respiration, and temperature were continuously monitored throughout surgery and maintained within normal parameters.

Using aseptic sterile technique, 4 cm left-sided incision was made at the top of the cranium to expose underlying skull. Bupivacaine (0.5% bupivacaine, 2 mg/kg; Pfizer) was applied as a periosteal block for desensitization of the area. A 20 mm craniectomy was performed at the left anterior junction of the coronal and sagittal sutures, exposing the underlying dura of the motor cortex. Each pig was then secured in in a controlled cortical impact device (University of Georgia Instrument Design and Fabrication Shop, Athens, GA, USA). A 15 mm blunt impactor tip was centered over the exposed dura to induce a motor cortex TBI with the following parameters: 4 m/s velocity, 9 mm depth of depression, 400 ms dwell time. Studies conducted previously by our lab determined these parameters generate a moderate-severe TBI (Baker et al., 2019; Kinder et al., 2019a). Sham animals received craniectomy only with no TBI. After TBI or Sham induction, the injury site was flushed with sterile saline. A second dose of Bupivacaine (0.5% bupivacaine, 2 mg/kg; Pfizer) was administered as an incisional block prior to closing the tissue with surgical sutures.

Post-operatively, pigs were monitored continuously until extubated. Once returned to their pen, pigs were monitored every 15 min until vitals returned to normal, then every 4 h for 24-h, and twice daily thereafter.

Gut microbial transplant

Collection

Healthy male piglets were kept from each litter as sex- and age-matched fecal matter donor pigs. Fecal matter was collected once per day beginning on the day of TBI induction, through day 6 of the study for the administration of fresh solution each day. For collection, a trained handler restrained each donor pig as a second trained handler cleaned the rectum with a 70% ethanol wipe. A sterile swap was used to stimulate the rectum. Fecal matter was collected into a sterile 50 mL conical tube and immediately placed on ice until all collections were complete and transported for processing.

Processing

All processing occurred in an anaerobic chamber. Fecal matter from each donor pig was combined and pooled into one sample to obtain a homogenous mixture to ensure all FMT treated piglets received the same treatments. Pooled fecal matter was then weighed and placed in a commercial blender (Oster Classic Series). Sterile saline was added to the blender based on weight to result in a 50 g fecal matter per 250 mL sterile saline ratio (Ponte et al., 2015), and blended on low for 30 s until a smooth texture was achieved. The mixture was then filtered through sterile gauze three times to ensure removal of particulate matter. Approximately 30 mL of microbial transplant material was placed into sterile syringes and immediately administered to FMT piglets.

Administration

Piglets were placed in individual pens for treatment administration in which 25 mL of the prepared 30 mL of microbial transplant material or saline were administered to FMT or saline piglets, respectively. Treatments were slowly dispensed orally via syringe feeding. Treatments were administered at 2 h post TBI or craniectomy, and once a day thereafter. All piglets were monitored for approximately 15 min after treatment to ensure no adverse effects (vomiting, diarrhea) occurred. However, no adverse events were noted for the duration of the study.

Magnetic resonance imaging (MRI) acquisition and analysis

Magnetic resonance imaging was preformed 1 and 7 days post TBI using a General Electric 32-channel fixed-site Discovery MR750 3.0 Tesla MRI magnet and an 8-channel knee coil placed over the head. All pigs were sedated as previously described for craniectomy surgery, with the addition of ketamine (4 mg/kg IM; Henry Schein) at the 7 day scan as fentanyl was no longer in use. Pigs were placed in supine recumbency, and mild anesthesia was maintained via inhalation isoflurane (1.5%; Abbott Laboratories) in oxygen. Vitals were monitored for the duration of the scan, and until pigs recovered and were ambulatory. Multiplanar MRI sequences were acquired including Fast Spin Echo T2-Weighted (FSE T2W), susceptibility-weighted angiography (SWAN), diffusion weighted imaging (DWI), diffusion Tensor Imaging (DTI), and magnetic resonance spectroscopy (MRS). Sequence analysis was conducted using Osirix software (Version 12.5.2) default thresholds and Fiji ImageJ software (Version 2.0.0).

Lesion volume and midline shift were calculated from coronal FSE T2W sequences using Osirix software. To determine lesion volume, hypointense and hyperintense areas in the ipsilateral hemisphere were manually traced. Computer-generated lesion volumes were automatically calculated and reported. Similarly, each slice of the ipsilateral hemisphere was manually traced to calculate the percentage of which the lesion occupied (lesion volume divided by ipsilateral hemisphere volume). The midline shift (MLS) was measured at the falx cerebri and septum pellucidum (Yang et al., 2018). Specifically, the distance from each structure to the lateral border of the ipsilateral and contralateral hemisphere was measured. The MLS was then calculated using the formula: (total diameter/2)–contralateral diameter (Wang et al., 2021). Intracranial hemorrhage volume was measured with Osirix from coronal SWAN sequences. Similar to lesion volume, hypointense areas were manually outlined on each ipsilateral slice and automated computer-generated volumes produced. Apparent diffusion coefficient (ADC) maps were generated from DWI sequences and utilized within ImageJ software to measure changes in water diffusivity, corresponding to cytotoxic (hypointense regions) and vasogenic (hyperintense regions) edema. Specifically, each hemisphere was manually defined. Using the contralateral hemisphere as an internal control, the percent of abnormal (low or high, respectively) signal intensity was automatically calculated for each slice and averaged for a final value.

Proton magnetic resonance spectroscopy (1H-MRS) and unsuppressed water reference data were acquired in two voxels ($16 \times 20 \times 25$ mm^3) using a standard point-resolved single voxel spectroscopy (PRESS) sequence (TR/TE = 2,000/35 ms; 8 averages; 5 kHz bandwidth with 4,096 data points; scan time 2:56 min per voxel). The first voxel was placed in the ipsilateral hemisphere to cover the lesioned area, and the second voxel was placed symmetrically in the contralateral hemisphere, with adjustments to avoid the skull. The voxel size was selected to best fit within the piglet brain and to maximize signal-to-noise and spectrum quality. Pre-scan shimming was performed to achieve a suitable line width (FWHM $<$ 13 Hz). All MRS data were analyzed offline in Osprey software (version 1.1.0) (Oeltzschner et al., 2020) using the standard processing pipeline. Briefly, preprocessing steps include spectral frequency- and phase correction, spectral alignment and averaging, Fourier transformation, residual water removal, and linear baseline correction. The processed averaged spectra were then fitted using LCModel's default linear-combination algorithms (Provencher, 1993) and baseline knot spacing of 0.40 ppm, as implemented in Osprey. Levels of total N-acetyl aspartate (NAA) and N-acetyl aspartyl glutamate (NAAG) (NAA + NAAG), and creatine and phosphocreatine (Cr + PCr) were estimated using water as a reference. For these peaks, model fit quality was high, as indicated by low Cramer-Rao Lower Bounds (<15% for all metabolites on all spectra). We also estimated levels of Cr and PCr separately, Lactate (Lac), and glutamate (Glu) as exploratory analyses; however, the quality of the model fits for these metabolites were often lower (Cramer-Rao Lower Bound exceeding $>$ 15%). Though metabolite levels are commonly expressed as a ratio relative to total creatine (Cr + PCr), it is not used here as creatine levels can be variable, especially after brain injury (Ross et al., 1998; Rae, 2014).

Histology

All pigs were euthanized following the 7 day post TBI MRI via euthanasia solution (1 mL/10 lbs IV). Immediately after euthanasia, pigs' brains were removed and coronally sectioned at the level of the optic chiasm. The rostral portion of the brain was placed in 10% buffered formalin for immunohistochemical analysis. After fixation, the brain was further sectioned in which two serial ipsilateral slices at the level of the lesion which were routinely processed, embedded in paraffin, and stained with antibodies specific to NeuN (1:500), glial fibrillary acidic protein (GFAP; 1:4,000), Olig2 (1:400), Iba1 (1:8,000), and doublecortin (DCX; 1:2,000). Additionally, 5 cm of the distal ileum were dissected, cleaned, and placed in 10% buffered formalin. After fixation, three serial slices of the distal end of the ileal segment were routinely processed, embedded in paraffin wax, and stained with hematoxylin/eosin (H&E) for morphological analysis.

All slices of both gut and brain tissue were imaged using a Cytation 5 Imaging Multi-mode reader (BioTek, Vermont) in montage mode at 4 × magnification. Using Gen5 Software (Biotek, Vermont), images were digitally stitched together to create a single image. Quantification of brain cells was performed using ImageJ software by counting cell numbers (NeuN + neurons) or calculating the number of stained pixels per brain slice as indicated by percent positive staining (Olig2 + oligodendrocytes, Iba1 + microglia, GFAP + astrocytes, and DCX + neuroblasts). Villus height (measured from the top of the villus to the villus-crypt junction), and crypt depth (measured from the villus crypt junction to the base of the crypt) were measured using Gen5 Software. Measurements were conducted on 15 well-oriented, intact villi and their associated crypt for each pig.

Microbiome analysis

Fecal collection and storage

Fecal samples were collected pre, 1, 3, and 7 days post TBI. Fecal samples were collected using aseptic equipment directly from the rectum of piglets. Piglets were restrained by a trained handler during collection. Prior to collection, the piglet's rectum was cleaned using 70% ethanol wipes to avoid contamination. Sterile swabs were utilized to stimulate defecation, and feces was collected and stored in sterile 50 mL conical tubes. Samples were immediately frozen and stored at −80°C until further analysis.

Cecal content collection and storage

On day 7 after euthanized, the abdominal cavity of all piglets was dissected using sterilized tools. The cecum of all piglets was located by identifying the ileocecal junction. Using sterilized scissors, the cecum was opened and cecal contents were collected directly into sterile 50 mL conical tubes. Samples were immediately frozen and stored at −80°C until further analysis.

DNA extraction, sequencing, and analysis

Deoxyribonucleic acid extraction was performed on fecal and cecal samples following a previously described protocol with slight modifications (Welch et al., 2020). Briefly, 350 mg of sample was placed in a 2 mL Lysing Matrix E tube (MP Biomedicals LLC, Irvine, CA, USA), and homogenized using a QIAGEN vortex adapter (QIAGEN, Venlo, Netherlands) to disrupt the cells. Enzymatic inhibition was achieved by using InhibitEX Buffer (QIAGEN, Venlo, Netherlands), and DNA elution and purification was carried out using a spin column and a series of specialized buffers according to manufacturer's specifications (QIAamp Fast DNA Stool Mini Kit; QIAGEN, Venlo, Netherlands). Determination of DNA concentration and purity in the resulting eluate was performed spectrophotometrically using the Synergy LX Multi-Mode Microplate Reader in conjunction with the Take3 Micro-Volume Plate (BioTek Instruments Inc., Winooski, VT, USA). Samples with a minimum volume of 100 μL and 10 ng/μL of DNA were stored at −20°C.

Following DNA extraction, samples underwent library preparation and 16S ribosomal ribonucleic acid (rRNA) gene sequencing. The library preparation step included polymerase chain reaction (PCR) replications using the forward: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and reverse: S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primer pairs (Klindworth et al., 2013), followed by a PCR clean-up using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). A second PCR step was then carried out to attach Illumina's indices and sequencing adapters (Nextera XT Index Kit; Illumina Inc., San Diego, CA, USA), followed by

another PCR clean-up step using AMPure XP beads. Following the final clean up, the library was quantified using quantitative PCR (qPCR), and the nucleotides were sequenced using an Illumina MiSeq instrument and a MiSeq v3 reagent kit (Illumina Inc., San Diego, CA, USA). A well-characterized bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA, USA) was used as a control for the sequencing runs.

Sequencing data was demultiplexed and converted to FASTQ files. Pair-end reads were converted into QIIME 2 artifacts (Bolyen et al., 2019) for further processing as described previously (Akerele et al., 2022). Sequences were denoised, dereplicated, and chimera-filtered using the DADA2 plugin (Callahan et al., 2016). A pre-trained Naïve Bayes classifier (Pedregosa et al., 2011) was utilized to assign taxonomies using the SILVA 138 SSU database (Quast et al., 2013). Even sequencing depth was set to 28,145 sequences per sample for computation of alpha and beta diversity indexes.

Gait analysis

Pigs underwent gait analysis collection on three separate days pre-TBI, as well as 1, 3, and 7 days post-injury. Data were recorded using a GAITFour electronic pressure sensitive mat (CIR Systems, Inc.) that is 7.01 × 0.85 meters in size. The mat contains a 6.10 × 0.61 meter active area will a total of 23,040 sensors. Before weaning, pigs were habituated to the gait mat in small groups. Approximately 2 weeks before TBI, pigs were individually trained to travel across the mat at a consistent two beat trotting gait. After training, pigs were asked to travel across the mat until 5 consistent runs were collected, or for a maximum of 15 min. Individual runs with less than 10% variability in stride cycle velocity were kept for potential analysis. Runs were then selected for analysis based on the individual stride cycle velocity falling within 20% of the mean stride cycle velocity on each trial day. Trials were normalized to pre-TBI values to account for high levels of inter-pig variability.

Gait was semi-automatically analyzed using GAITFour Software to provide quantitative measurements for all limbs. Parameters analyzed include stride length (the distance between successive ground contact of the same hoof) and step length (the distance between corresponding successive points of contact of opposing hooves) for all four limbs of each animal.

Open field testing

Pigs underwent open field behavior testing as an additional measure of functional outcomes pre-TBI as well as 1, 3, and 7 days post. All tests occurred in a 2.7 × 2.7 meter arena. Trials lasted 10 min and were recorded using EthoVision video tracking software (Noldus Systems) to obtain objective and quantifiable measures of behavioral characteristics such as velocity, distance traveled, and movement. Exploratory behaviors (sniffing) were manually tracked and coded by trained personnel.

Statistical analysis

All quantitative data were analyzed using Minitab® Statistical Software (version 21.1.1.0, Pennsylvania). For MRI, fecal microbiome, and behavior data, a mixed effects model was used in which treatment nested in pig was a random factor and time, treatment, and treatment by time were fixed effects. Differences were measured with *post-hoc* Tukey–Kramer pairwise comparisons. For cecal microbiome and histological measurements, statistical significance between groups was determined with one-way ANOVA and *post-hoc* Tukey–Kramer pairwise comparisons. Due to high levels of individual gait variability, significance between groups was measured at each timepoint using one-way ANOVA with *post-hoc* Fisher pairwise comparison. All results are reported as mean ± standard error of the mean (M ± SEM) using model adjusted values; however, figures are presented using the raw unadjusted values. P-values < 0.05 were considered significantly different, and p-values between 0.05 and 0.10 were considered trending.

TABLE 1 Frequently used abbreviations.

Abbreviation	Meaning
TBI	Traumatic brain injury
GIT	Gastrointestinal tract
MGBA	Microbiota-gut-brain-axis
HPA	Hypothalamic-pituitary-adrenal
FMT	Fecal microbial transplant
CCI	Controlled cortical impact
FSE T2W	Fast Spin Echo T2-Weighted
SWAN	Susceptibility-weighted angiography
DWI	Diffusion weighted imaging
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MLS	Midline shift
ADC	Apparent diffusion coefficient
NAA	N-acetyl aspartate
NAAG	N-acetyl aspartyl glutamate
Cr	Creatine
PCr	Phosphocreatine
Lac	Lactate
Glu	Glutamate
GFAP	Glial fibrillary acidic protein
DCX	Doublecortin

Results

FMT decreases lesion volume and midline shift

To evaluate the effect of FMT (fecal microbiota transplant abbreviation; Table 1) treatment on structural TBI pathology in the brain, FSE T2W MRI sequences were evaluated at 1 and 7 days post TBI (Figures 1A–F). Lesion volume was significantly decreased in FMT treated animals at 7 days post injury as compared to

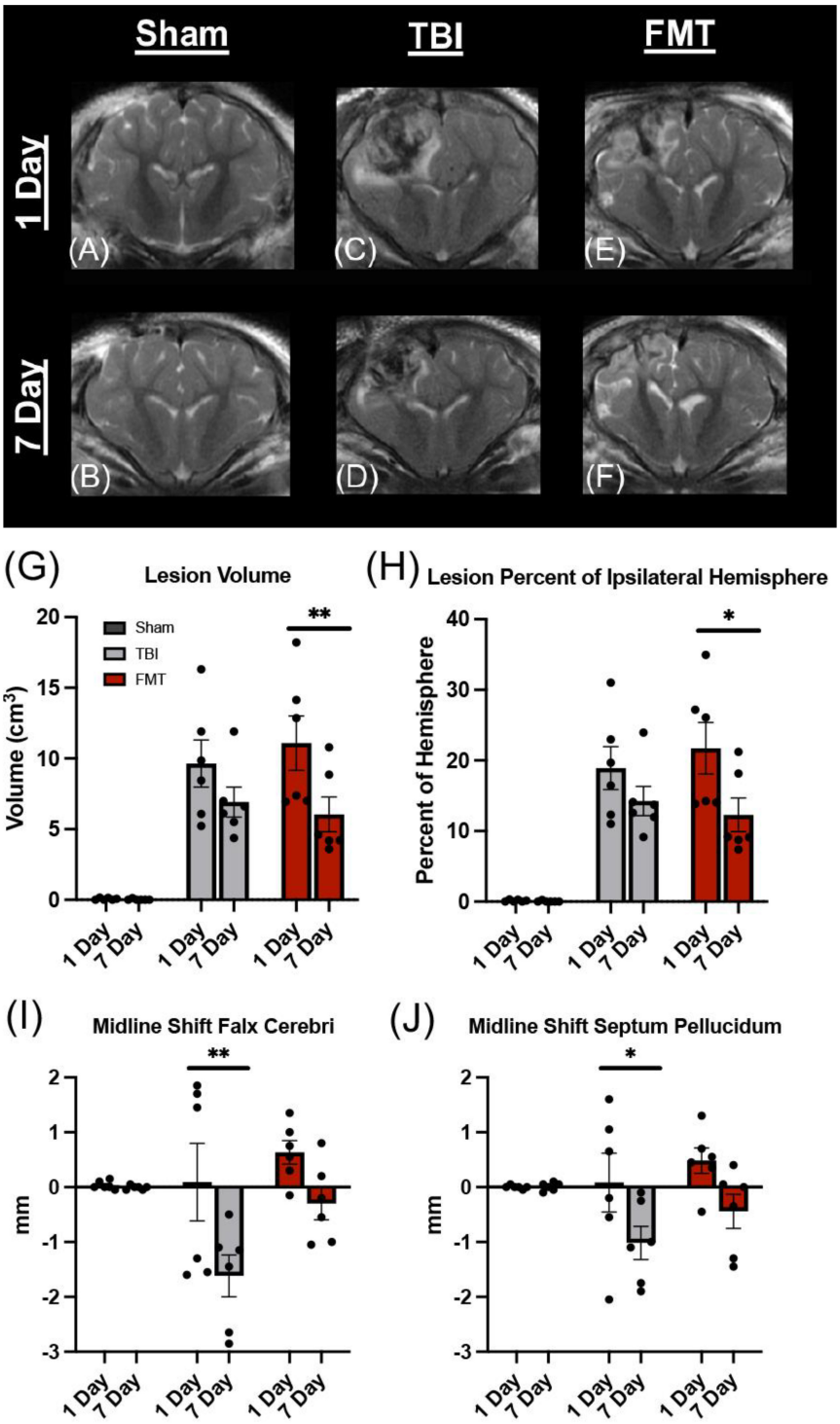


FIGURE 1
FMT significantly reduced lesion volume and MLS following TBI. Coronal T2W MRI scans revealed minimal to no lesion present in Sham animals at 1 and 7 days **(A,B)**. Hyperintense and hypointense ipsilateral lesioning characteristic of heterogenous TBI were present at 1 and 7 days in TBI non-treated **(C,D)** and to a lesser degree in FMT treated **(E,F)** animals. FMT treatment led to a significantly reduced lesion volume **(G)** and lesioned percent of the ipsilateral hemisphere **(H)** at 7 days as compared to 1 day post TBI. TBI non-treated animals experienced a significant change in MLS as measured at the falx cerebri **(I)** and at the septum pellucidum **(J)**, while FMT treated animals showed no changes in MLS. All data is expressed as mean ± SEM. Bar denotes a time effect within treatment. $^{**}p < 0.01$ and $^{*}p < 0.05$.

1 day post (6.041 ± 1.233 vs. 11.089 ± 1.233 cm^3, respectively, $p = 0.001$; Figure 1G), while non-treated TBI control animal lesion volumes did not differ between day 7 and day 1 (6.918 ± 1.233 vs. 9.636 ± 1.233 cm^3, respectively, $p = 0.079$; Figure 1G). Nominal lesion injuries were detected in Sham animals due to craniectomy surgery and lesions did not change at 7 days as compared to 1 day

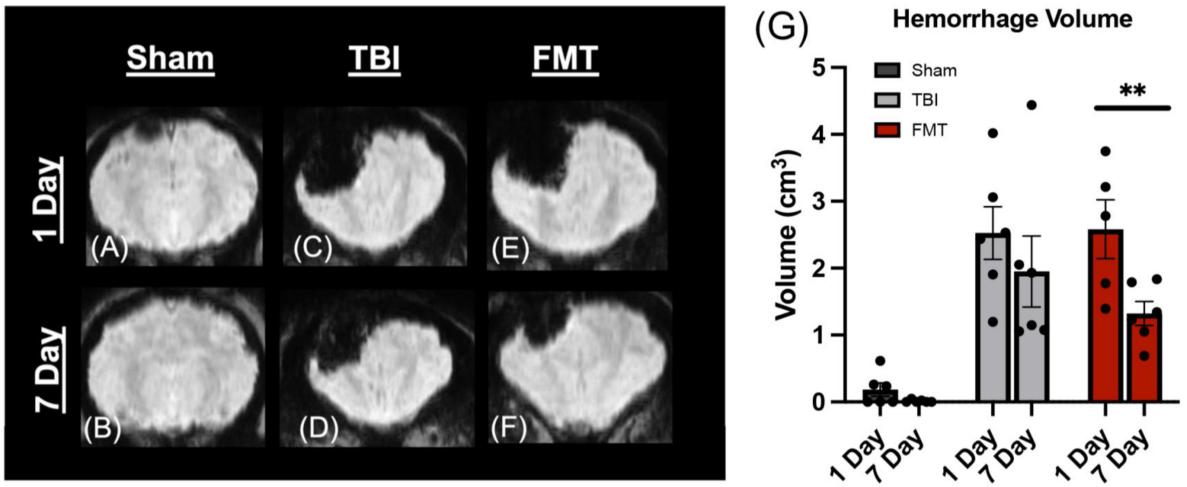


FIGURE 2
FMT significantly reduced hemorrhage volume in TBI piglets. Coronal SWAN images showed hypointense regions indicative of intracerebral blood and blood products **(A–F)**. Sham animals show minimal hemorrhage at 1 day **(A)** and 7 days **(B)**. TBI non-treated animals showed a slight decrease in hypointense hemorrhagic lesion volume from 1 day **(C)** to 7 days **(D)** post TBI whereas FMT treated animals showed a significant reduction in hypointense hemorrhagic lesion volume from 1 day **(E)** to 7 days **(F)** post TBI **(G)**. Data is presented as mean ± SEM. $^{**}p < 0.01$.

post-surgery (0.021 ± 1.233 vs. 0.062 ± 1.233 cm^3, respectively, $p = 1.000$; Figure 1G).

In order to account for possible differences in brain size, the percent of ipsilateral tissue occupied by the lesion was calculated for all groups. The percent of ipsilateral brain containing lesion tissue was significantly less in FMT pigs at 7 days as compared to 1 day post TBI (12.299% ± 2.327% vs. 21.727% ± 2.327%, respectively, $p = 0.020$; Figure 1H). No significant changes were observed from 7 to 1 day in TBI non-treated control (14.255% ± 2.327% vs. 18.908% ± 2.327%, respectively, $p = 0.118$; Figure 1H). Additionally, Sham animals did not exhibit a change in lesion tissue from 7 days as compared to 1 day (0.041% ± 2.327% vs. 0.124% ± 2.327%, respectively, $p = 1.000$; Figure 1H) post TBI. This data suggests FMT contributed to a reduction in lesion volume following TBI.

Midline shift severity is directly correlated with poor clinical outcomes making it an important injury/recovery biomarker (Palekar et al., 2021). MLS was calculated at 1 and 7 days post injury utilizing the falx cerebri, as the injury was more dorsal, as well as the more commonly utilized septum pellucidum. The midline significantly shifted between 1 and 7 days post injury in the non-treated TBI group (0.233 ± 0.443 vs. −1.858 ± 0.44 mm, respectively, $p < 0.001$; Figure 1I) measured at the falx cerebri and at the level of the septum pellucidum (−0.082 ± 0.388 vs. −1.417 ± 0.388 mm, respectively, $p = 0.044$; Figure 1J). There was no significant difference in MLS from 1 to 7 days post injury in the FMT treated group at the falx cerebri (0.483 ± 0.443 vs. −0.525 ± 0.443 mm, respectively, $p = 0.111$; Figure 1I) or the septum pellucidum (0.475 ± 0.388 vs. −0.650 ± 0.388 mm, respectively, $p = 0.112$; Figure 1J). The Sham group demonstrated no change between the 1 and 7 day timepoints at the falx cerebri (0.008 ± 0.443 vs. −0.033 ± 0.443 mm, respectively, $p = 1.00$; Figure 1I) nor the septum pellucidum (−0.008 ± 0.388 vs. 0.008 ± 0.388 mm, respectively, $p = 1.00$; Figure 1J). Negative values demonstrate a shift toward the ipsilateral hemisphere, indicative of tissue atrophy. Therefore, our MLS data suggest FMT-treated pigs may have experienced less tissue atrophy 7 days post injury.

FMT reduces intracranial hemorrhage volume

The SWAN MRI sequence is a sensitive measure of cerebral hemorrhage (Docampo et al., 2013). Others have reported a reduction in hemorrhagic brain injury as a result of microbiota transplant (Yu et al., 2021), therefore we investigated the effects of FMT on hemorrhage volume 1 and 7 days post injury (Figures 2A–F). A decrease in hemorrhagic volume was noted in FMT treated pigs at 7 days as compared to 1 day post injury (1.323 ± 0.321 vs. 2.543 ± 0.337 cm^3, respectively; $p = 0.003$; Figure 2G). A significant decrease was not observed in non-treated TBI pigs at 7 days as compared to 1 day post injury (1.950 ± 0.321 vs. 2.523 ± 0.321 cm^3, respectively; $p = 0.212$ Figure 2G). As expected, Sham animals showed minimal cerebral hemorrhage at 1 day and 7 day (0.186 ± 0.321 cm^3 vs. 0.012 ± 0.321 cm^3, respectively; $p = 0.975$; Figure 2G). These results indicate FMT therapy may alleviate intracranial hemorrhage associated with moderate-severe TBI.

FMT reduces cytotoxic and vasogenic edema

Apparent diffusion coefficient maps generated from DWI MRI sequences were used to evaluate changes in water diffusivity in the ipsilateral hemisphere (Figures 3A–F). TBI causes a heterogeneous injury that includes hypointense and hyperintense regions of cytotoxic and vasogenic edema, respectively. Therefore, ipsilateral hemisphere analysis was used to identify and quantitate

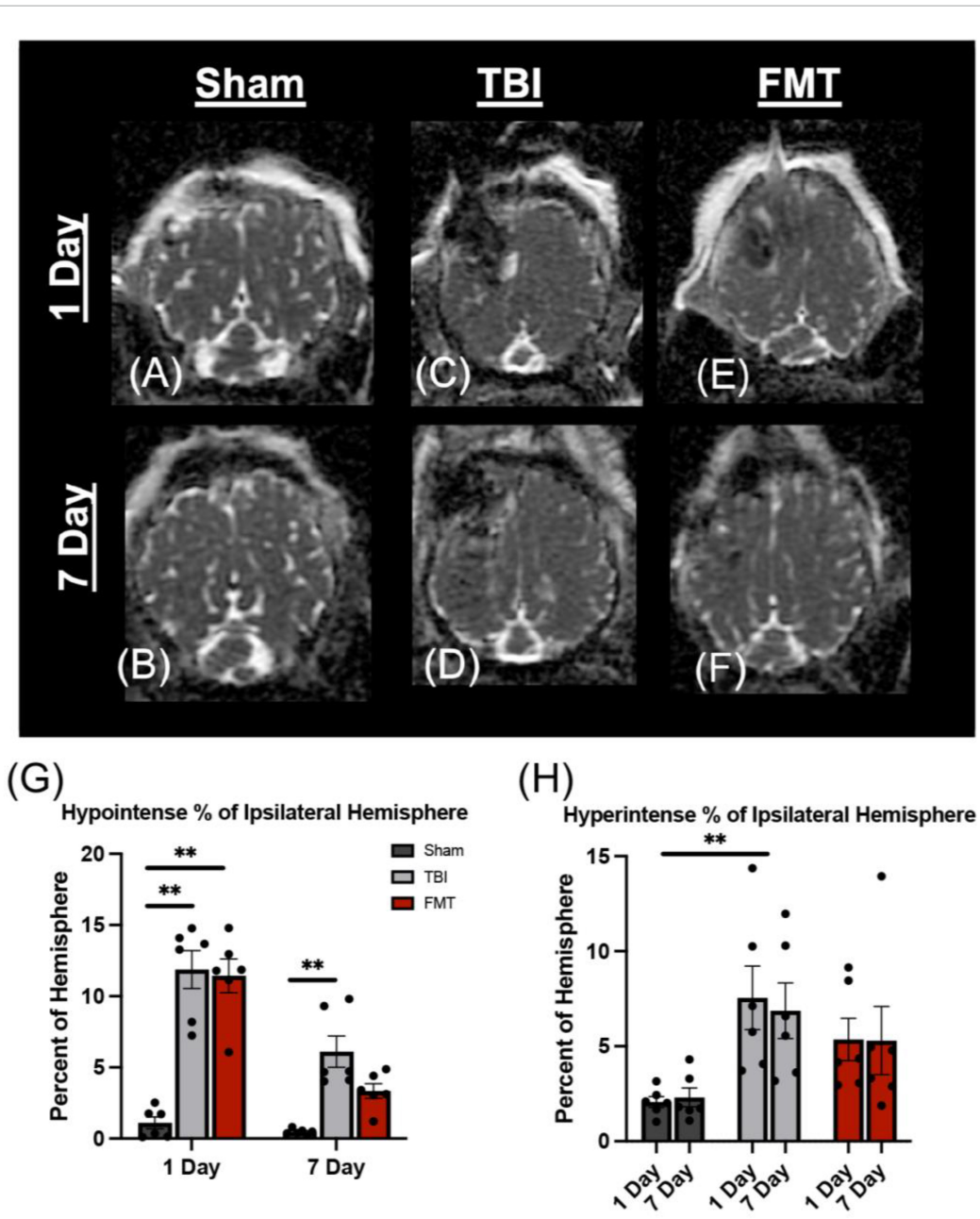


FIGURE 3
FMT ameliorates cytotoxic and vasogenic edema. Axial ADC maps showed minimal edema at 1 day **(A)** and 7 days **(B)** in Sham animals. However, the percentage of the ipsilateral hemisphere occupied by cytotoxic edema (hypointense regions) was significantly elevated in TBI non-treated **(C)** and FMT treated **(E)** animals at 1 day post TBI as compared to Sham **(A,G)**. However, the percentage of the ipsilateral hemisphere occupied by cytotoxic edema was not significantly different between Sham **(B)** and FMT **(F)** treated animals at 7 days post TBI, while cytotoxic edema remained significantly elevated in TBI non-treated animals **(D,G)**. An overall treatment effect was reported in the percent of the ipsilateral hemisphere occupied by vasogenic edema (hyperintense regions) in which TBI non-treated animals **(C,D)** displayed a significantly greater area of hyperintensity whereas there was no difference in vasogenic edema in FMT treated animals **(E,F)** compared to Sham animals **(A,B,H)**. Data is presented as mean ± SEM. Treatment effect within day is compared to Sham. Bracket indicates treatment difference compared to Sham. $^{**}p < 0.01$.

changes in hypointense and hyperintense regions. At 1 day post TBI, as compared to Sham (1.100 ± 0.912%), both FMT treated (11.436 ± 0.912%, $p < 0.001$) and TBI non-treated (11.998 ± 0.912%, $p < 0.001$) animals had significantly increased levels of hypointensity (Figure 3G). At 7 days post TBI, a decrease was observed in which FMT treated pigs did not differ from Sham (3.343 ± 0.912 vs. 0.474 ± 0.912%, respectively, $p = 0.283$), but non-treated TBI pigs hypointense regions remained increased as compared to Sham (6.104 ± 0.912% vs. 0.474 ± 0.912%, respectively, $p = 0.006$; Figure 3G). An overall treatment difference was observed for hyperintensity ($p < 0.001$), indicative of vasogenic edema in the ipsilateral hemisphere. TBI non-treated animals (7.549 ± 1.077%) showed a significant ($p = 0.004$) increase in hyperintensity relative to Sham (2.069 ± 1.077%; Figure 3H). FMT treated animals (5.358 ± 1.077%) showed no difference as compared to Sham ($p = 0.070$) or TBI non-treated

animals (p = 0.341; Figure 3H). Together these results indicate administration of FMT may lead to the reduction of cytotoxic and vasogenic edema at 7 days post TBI.

FMT alters key brain metabolite levels

Magnetic resonance spectroscopy enables non-invasive, quantitative evaluation of brain metabolites, which have been reported to be altered after TBI (Croall et al., 2015; Kinder et al., 2019b). Commonly measured metabolites include N-acetylaspartate (NAA; neuron death and dysfunction), N-acetyl aspartyl glutamic acid (NAAG; membrane turnover), NAA and NAAG (NAA + NAAG), lactate (Lac; glycolysis), glutamate (Glu; neuronal death), phosphocreatine (PCr), creatine (Cr), and the sum of creatine and phosphocreatine (Cr + PCr; cellular energy). Although no treatment by time interactions were observed, a significant treatment effect was noted for multiple metabolites. The NAAG concentration was significantly affected by treatment (p = 0.021) as TBI non-treated animals exhibited lower NAAG concentration as compared to Sham (1.080 ± 0.209 vs. 1.974 ± 0.195 IU, respectively, p = 0.019) but FMT treated pigs did not statistically differ from Sham (1.739 ± 0.199 vs. 1.974 ± 0.195 IU p = 0.682; Supplementary Figure 1A), which may indicate a neuroprotective effect (Neale et al., 2011). A significant decrease was observed in PCr (p = 0.022) in TBI non-treated pigs compared to Sham (4.503 ± 0.490 vs. 6.411 ± 0.457 IU, p = 0.017), but FMT treated pigs did not statistically differ from Sham (5.376 ± 0.468 vs. 6.411 ± 0.457 IU, respectively, p = 0.262; Supplementary Figure 1B), suggesting FMT promoted proper energy metabolism in the TBI brain. A significant treatment difference was also detected in NAA + NAAG concentration (p = 0.013) in which TBI non-treated (14.279 ± 0.476 IU, p = 0.017) and FMT treated (14.629 ± 0.454 IU, p = 0.042) animals were lower relative to Sham (16.339 ± 0.446 IU; Supplementary Figure 1C). No difference was reported for Lac between TBI non-treated, Sham, and FMT treated animals (0.489 ± 0.258 vs. 1.271 ± 0.241 vs. 0.787 ± 0.246 IU, respectively, p = 0.097; Supplementary Figure 1D). A significant treatment effect was noted for NAA (p = 0.043) in which the concentration of FMT treated animals was significantly decreased as compared to Sham (14.369 ± 0.442 vs. 16.009 ± 0.434, respectively, p = 0.046), but TBI non-treated animals did not significantly differ from Sham (14.707 ± 0.464 vs. 16.009 ± 0.434, respectively, p = 0.135; Supplementary Figure 1E). The concentration of Cr + PCr approached a trending (p = 0.097) treatment difference, but *post-hoc* analysis revealed no statistical differences between the three groups (p > 0.100; Supplementary Figure 1F). No statistically significant differences between groups were noted for Glu (p = 0.672; Supplementary Figure 1G) or Cr (p = 0.534; Supplementary Figure 1H). These data suggest FMT treatment may contribute to moderate recovery of brain metabolism following TBI.

FMT improved neuron and oligodendrocyte survival

Ipsilateral tissue sections underwent histological assessment to determine cellular level changes in neuron survival, gliosis, microglia and astrocyte activation, and neuroblast stimulation in response to FMT treatment (Supplementary Figure 2). There was a difference between treatments in number of surviving NeuN+ neurons (p = 0.007). A decrease was observed in the number of surviving NeuN+ neurons in TBI non-treated animals (p = 0.005) but not in FMT treated animals (p = 0.181), as compared to Sham (45133.000 ± 4121.000 and 53990.000 ± 4514.000 vs. 65090.000 ± 4121.000 cells, respectively; Figures 4A–D), indicating less neuronal death in FMT treated animals compared to TBI non-treated. Similarly, the percent area positive for Olig2+ oligodendrocytes was different (p = 0.018) between treatments. There was a decrease in the percentage of area positive for Olig2+ oligodendrocytes in TBI non-treated animals (p = 0.013) but not in FMT treated animals (p = 0.214) as compared to Sham (1.126 ± 0.134 and 1.371 ± 0.134 vs. 1.697 ± 0.134%, respectively; Figures 4E–H), demonstrating improved oligodendrocyte survival in FMT treated animals compared to TBI-non-treated. TBI induced an increase in the percent of Iba1+ microglia activated (p < 0.001) in TBI non-treated animals and FMT treated animals as compared to Sham (9.779 ± 0.864 and 8.526 ± 0.864 vs. 4.339 ± 0.864%, respectively, p < 0.005; Figures 4I–L). This data demonstrates TBI led to an increase in the percent of activated microglia thus increasing harmful neural inflammation. FMT did not decrease astrocyte activation as TBI non-treated and FMT-treated animals both demonstrated significantly increased GFAP + percent area as compared to Sham (11.859 ± 0.655 and 11.397 ± 0.655 vs. 6.441 ± 0.655%, respectively; (p < 0.001); Figures 4M–P). However, there was not a difference in GFAP + percent area between TBI non-treated and FMT treated animals (11.859 ± 0.655 vs. 11.397 ± 0.655%, respectively; (p = 0.622); Figures 4M–P) demonstrating FMT treatment did not influence subacute astrocyte activation. Finally, the number of DCX + neuroblasts was evaluated at the subventricular zone. TBI non-treated and FMT treated animals showed a significant increase in the percent area of DCX + neuroblasts as compared Sham animals (15.555 ± 0.859 vs. 13.377 ± 0.859 and 8.003 ± 0.859%, $p \leq 0.004$; Figures 4Q–T). It should be noted that changes Olig2 + oligodendrocytes, Iba1 + microglia, GFAP + astrocytes, and DCX + neuroblast markers may be due to an increase in cell number or a change in morphology as staining was quantified on a per pixel basis. Collectively, these data indicate that FMT treatment improves neuron survival and has limited effects on other cellular responses in TBI animals.

FMT did not alter alpha diversity or phylum level in feces

No changes in fecal sample alpha diversity indices were detected as measured by number of features, Faith's Phylogenetic Diversity (PD) index, Shannon index, and Evenness index in 1, 3, and 7 day post TBI fecal samples (Supplementary Table 1) or 7 day post TBI cecal samples (Supplementary Table 2). The microbial composition of donor FMT treatment (Supplementary Figure 3A) contained 20 different phyla at relative abundances of 63.2% Bacillota (formerly Firmicutes), 30.5% Bacteroidota, 2.5% Spirochaetota, 2.4% Actinobacteriota, and 0.7% Pseudomonadota

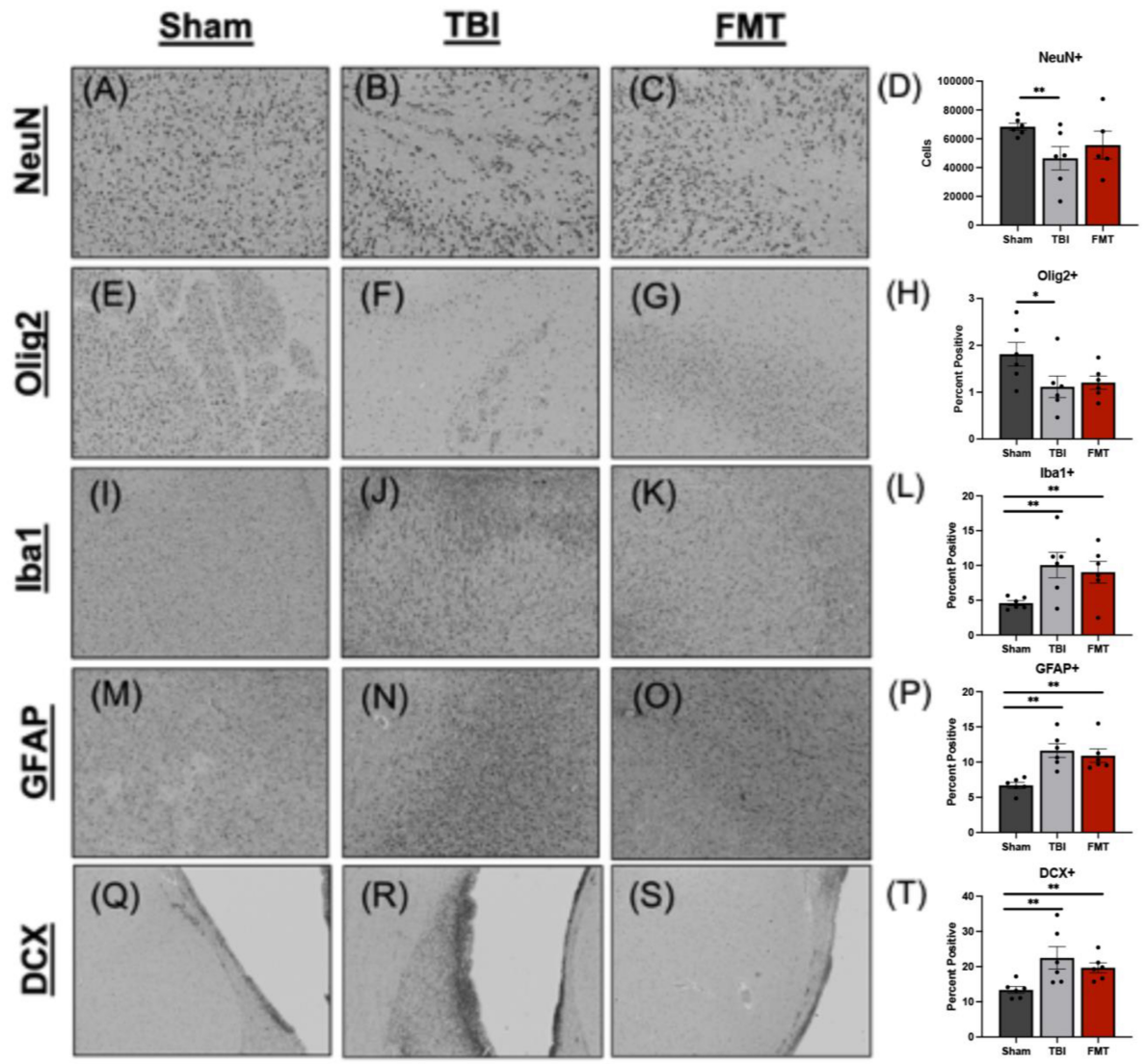


FIGURE 4
FMT increases neuron survival in TBI animals at 7 days post TBI. Histological evaluation of NeuN+ neurons in Sham **(A)**, TBI non-treated **(B)**, and FMT treated animals **(C)** showed TBI non-treated animals demonstrated a significant decrease in NeuN+ neurons relative to Sham animals, while FMT treated animals **(C)** showed no difference from Sham **(D)**. Histological assessment of Olig2+ oligodendrocytes in Sham **(E)**, TBI non-treated **(F)**, and FMT treated animals **(G)** showed TBI non-treated animals demonstrated a significant decrease in Olig2+ relative to Sham animals, while FMT treated animals did not **(H)**. Iba1+ activated microglia in Sham **(I)**, TBI non-treated **(J)**, and FMT treated **(K)** animals showed TBI non-treated and FMT treated animals displayed a significant increase in Iba1+ microglia cells as compared to Sham animals **(L)**. GFAP+ astrocytes in Sham **(M)**, TBI non-treated **(N)**, and FMT treated **(O)** animals demonstrated TBI non-treated and FMT treated animals showed a significant increase in GFAP+ astrocyte activation as compared to Sham animals **(P)**. DCX+ neuroblasts in Sham **(Q)**, TBI non-treated **(R)**, and FMT treated **(S)** animals demonstrated TBI non-treated animals showed a significant increase in DCX+ cells as compared to FMT treated and Sham animals **(T)**. Additionally, FMT treated animals had a significant increase in DCX+ cells as compared to Sham **(T)**. All images were captured at 10 × magnification. Data is presented as mean ± SEM. Treatment effects are as compared to Sham. $^{**}p < 0.01$ and $^{*}p < 0.05$.

(formerly Proteobacteria) with all other phyla comprising less than 0.5% of the total relative abundance. The overall composition of day 7 cecal bacterial phyla can be found in Supplementary Figure 3B for Sham, FMT, and TBI piglets and Supplementary Figure 3C for individual piglets. The overall composition of fecal bacterial phyla can be found in Supplementary Figure 3D for Sham, FMT, and TBI piglets pre-TBI and 1, 3, and 7 days post TBI and Supplementary Figure 3E for individual piglets pre-TBI and 1, 3, and 7 days post TBI. Visual differences were observed between phyla; however, to draw more meaningful conclusions, further analyses of the gut microbiome were focused on bacterial species.

FMT prevented pathogenic bacterial increase in feces

Fecal bacterial species relative abundances (Figure 5) were measured via 16S rRNA sequencing to detect acute changes in GIT microbiota post TBI and measure effects of FMT treatment

on these populations. *Actinobacillus indolicus* (Figure 5A), a known pathogenic bacterium, made up a relatively small portion of the microbial population of Sham, TBI and FMT piglets prior to surgery (0.000 ± 0.002 0.001 ± 0.002 and 0.000 ± 0.002, respectively). However, there was a significant increase (0.015 ± 0.002; p = 0.007) in the relative abundance of *A. indolicus* 1 day post TBI in TBI non-treated piglets compared to FMT treated and Sham piglets (0.001 ± 0.002 and 0.002 ± 0.002, respectively). Fecal relative abundances of *Actinomyces howellii* (Figure 5B) was increased (0.002 ± 0.004) 1 day post TBI in TBI non-treated piglets compared to Sham and FMT treated piglets (0.000 ± 0.004 and 0.000 ± 0.004, respectively; p = 0.043). Similarly, *Bifidobacterium animalis* (Figure 5C) had a trending increase in relative abundance 1 day post TBI in TBI non-treated piglets compared to FMT treated and Sham piglets (00.048 ± 0.0085 vs. 0.000 ± 0.0085 and 0.000 ± 0.0085, respectively; p = 0.067). Fecal relative abundances of *Streptococcus hyointestinalis* (Figure 5D) increased 3 days post TBI in TBI-non treated piglets compared to FMT treated and Sham piglets (0.044 ± 0.008 vs. 0 ± 0.008 and 0.003 ± 0.008, respectively; p = 0.020). Collectively, these data indicated TBI resulted in an acute increase in harmful bacteria which can be prevented by FMT treatment.

FMT increased probiotic cecal bacteria

Cecal relative abundances of bacterial species (Figure 6) were measured 7 days post TBI to determine the effects of FMT on intestinal microbial populations. A number of differences were measured in cecal relative abundances of beneficial bacteria. The relative abundance of *L. amylovorus* (Figure 6A) and *L. mucosae* (Figure 6B) was increased in FMT treated piglets compared to TBI non-treated piglets (2.637 ± 0.784 vs. 0.971 ± 0.784; p = 0.024 and 2.38 ± 0.607 vs. 0.000 ± 0.607; p = 0.023, respectively). *Lactobacilli coleohominis* (Figure 6C) and *L. pontis* (Figure 6D) was increased in cecal contents of FMT treated piglets compared to TBI non-treated and Sham piglets (0.049 ± 0.014 vs. 0.006 ± 0.014 and 0.002 ± 0.014; p = 0.027 and 0.698 ± 0.159 vs. 0.173 ± 0.159 and 0.000 ± 0.159; p = 0.006; respectively). Collectively, these results indicated FMT treatment may be capable of shifting specific species in gut populations to an increased proportion of potentially beneficial bacteria, thus preventing gut dysbiosis.

FMT restores gut villi morphology

Ileal tissues were collected and analyzed for changes in villi morphology 7 days post TBI (Figure 7A). A significant difference was observed in TBI non-treated animals in villi length to crypt depth ratio (p = 0.018). No differences were noted between Sham and FMT treated animals (3.083 ± 0.351 vs. 2.493 ± 0.351 μm, respectively, p = 0.412), but TBI non-treated animals (1.480 ± 0.351 μm) demonstrated a significantly reduced ratio as compared to Sham (p = 0.015; Figure 7B). However, there was no treatment differences detected for villi length (p = 0.180; Figure 7C) between Sham (314.400 ± 37.500 μm), TBI non-treated (236.300 ± 37.500 μm), FMT (335.000 ± 37.500 μm) animals. Additionally, no treatment differences were noted (p = 0.351) for crypt depth of TBI non-treated (113.800 ± 13.300 μm, p = 0.330) or FMT treated (109.500 ± 13.300 μm, p = 0.869) animals as compared to Sham (124.500 ± 713.300 μm) (Figure 7D). Together, these findings suggest FMT administration contributes to an improvement of gut villi morphology ratios.

FMT promotes motor function recovery

Step length and stride length were normalized to pre-TBI values and analyzed 1, 3, and 7 days post TBI as markers of gait deficits and FMT-mediated recovery. Step length (Figure 8A) is defined as the distance between corresponding contact of opposing hooves, with decreased step length indicating instability. There was a difference in left front step length detected between treatment groups over time (p = 0.038; Figure 8B). However, there was no difference in right front (p = 0.131; Figure 8C), right hind (p = 0.052; Figure 8E), or left hind (p = 0.103; Figure 8D) step length between treatments. At 1 day post TBI, TBI non-treated and FMT treated pigs demonstrated a reduction in step length in the left front (0.901 ± 0.042 and 0.927 ± 0.042 vs. 1.045 ± 0.042, $p \leq 0.048$; Figure 8B) as compared to Sham animals. At 3 days post TBI, TBI non-treated pigs demonstrated a reduction in step length in the left front (0.931 ± 0.042 vs. 1.106 ± 0.042, p = 0.005; Figure 8B) as compared to Sham animals, while FMT (1.009 ± 0.042 vs. 1.106 ± 0.042, p = 0.005; Figure 8B) did not. At 7 days post TBI, TBI non-treated pigs demonstrated a reduction in step length in the left front (0.916 ± 0.042 vs. 1.060 ± 0.042 and 1.054 ± 0.042, $p \leq 0.024$; Figure 8B) as compared to Sham and FMT treated animals.

Stride length (Figure 8F) measures the distance between consecutive contacts of the same hoof with a decrease often reported after TBI indicating reduced stability (Neumann et al., 2009; Peri et al., 2019). There was a trending difference in right front (p = 0.068; Figure 8H), left front (p = 0.067; Figure 8G), and left hind (p = 0.085; Figure 8I) stride length between treatment groups. However, there was no difference in right hind stride length between treatments (p = 0.102; Figure 8J). At 1 day post TBI, TBI non-treated animals exhibited a reduction in stride length as compared to Sham animals in the right front (0.902 ± 0.041 vs. 1.034 ± 0.041, p = 0.029; Figure 8G) limb, but FMT treated animals did not statistically differ from Sham in any limb. At 3 days post TBI, TBI non-treated animals exhibited a reduction in stride length as compared to Sham animals in the left front (0.930 ± 0.041 vs. 1.097 ± 0.041, p = 0.029; Figure 8G) and right front (0.933 ± 0.042 vs. 1.100 ± 0.042, p = 0.038; Figure 8H), but FMT treated animals did not statistically differ from Sham in any limb. At 7 days post TBI, TBI non-treated animals exhibited a significant reduction in stride length as compared to Sham animals in the left front (0.917 ± 0.041 vs. 1.043 ± 0.041, p = 0.035; Figure 8G) and right front (0.918 ± 0.042 vs. 1.045 ± 0.042, p = 0.040; Figure 8H). Additionally, the stride length of TBI non-treated animals was decreased as compared to FMT treated animals in the left front (0.917 ± 0.041 vs. 1.045 ± 0.041, p = 0.033 Figure 8G), right front (0.918 ± 0.042 vs. 1.048 ± 0.042, p = 0.036; Figure 8H) and left hind (0.899 ± 0.037 vs. 1.056 ± 0.045, p = 0.014; Figure 8I) limbs. Overall, FMT treated pigs displayed less gait impairment as measured by step length and stride length 3 and 7 days post injury as compared to TBI treated animals, which is indicative of improvement in motor function following TBI.

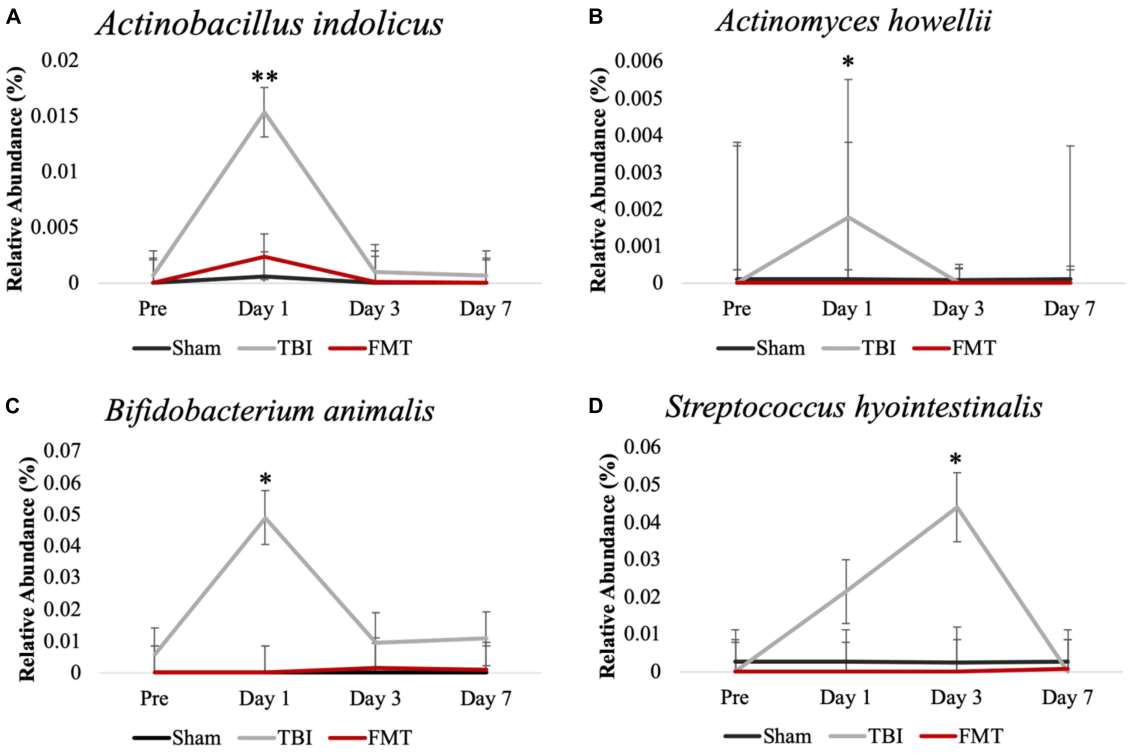


FIGURE 5
FMT prevented pathogenic bacterial increase in feces of FMT treated piglets at acute timepoints. Fecal microbiome analysis of *Actinobacillus indolicus* **(A)**, *Actinomyces howellii* **(B)**, *Bifidobacterium animalis* **(C)**, and *Streptococcus hyointestinalis* **(D)** showed that TBI non-treated animals had an acute increase in potentially pathogenic bacteria compared to FMT treated and Sham animals. Data is presented as mean ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$.

FMT improves behavioral deficits caused by TBI

Traumatic brain injury related behavioral changes were assessed by open field testing pre TBI and 1, 3, and 7 days post TBI. Furthermore, as pigs are inherently exploratory animals, the amount of time an animal spent sniffing the wall of the behavior arena was quantified. There were treatment differences detected overtime for distance traveled ($p = 0.004$; Figures 9A–C), velocity ($p = 0.004$), movement duration ($p = 0.033$), sniff duration ($p = 0.007$; Figures 9D–F), percent of trial moving ($p = 0.033$), and time not moving ($p = 0.033$). In comparing treatments at each timepoint, as expected no treatment differences were observed pre-injury in any measured parameter. At 1 day post TBI, no significant difference was noted between Sham and FMT treated pigs in velocity (0.182 ± 0.019 vs. 0.097 ± 0.019 m/s, respectively, $p = 0.105$; Figure 9H), movement duration (295.980 ± 32.412 s vs. 168.200 ± 32.412, respectively, $p = 0.220$; Figure 9I), sniffing duration (194.193 ± 22.333 vs. 142.127 ± 22.333 s, respectively; $p = 0.881$; Figure 9J), percent of trial moving (49.330 ± 5.402 vs. 28.033 ± 5.402 percent, respectively; $p = 0.220$; Figure 9K), or time not moving (304.020 ± 32.403 vs. 431.800 ± 32.403 s, respectively; $p = 0.219$; Figure 9L). However, at 1 day post TBI, TBI non-treated animals showed a decrease as compared to Sham in velocity (0.065 ± 0.019 vs. 0.182 ± 0.019 m/s, respectively, $p = 0.004$; Figure 9H), movement duration (136.540 ± 32.412 vs. 295.980 ± 32.412 s, respectively, $p = 0.046$; Figure 9I), sniffing duration (52.340 ± 22.333 vs. 194.193 ± 22.333, respectively; $p = 0.003$; Figure 9J), percent of trial moving (22.757 ± 5.402 vs. 49.330 ± 5.402 percent, respectively; $p = 0.046$; Figure 9K). Meanwhile, at 1 day post TBI, TBI non-treated animals had an increase in time not moving compared to Sham (463.460 ± 32.403 vs. 304.020 ± 32.403 s, respectively; $p = 0.046$; Figure 9L). Similarly, 3 days post injury Sham and FMT treated animals only exhibited a trending difference in distance traveled (99.364 ± 11.470 vs. 44.902 ± 11.470, respectively, $p = 0.062$; Figure 9G) and velocity (0.167 ± 0.019 vs. 0.075 ± 0.019, respectively, $p = 0.059$; Figure 9H). Additionally, 3 days post injury Sham and FMT treated animals had no difference in movement duration (277.360 ± 32.412 vs. 135.080 ± 32.412 s, respectively, $p = 0.113$; Figure 9I), percent of trial moving (46.227 ± 5.402 vs. 22.513 ± 5.402 percent, respectively; $p = 0.113$; Figure 9K), or time not moving (322.640 ± 32.403 vs. 464.920 ± 32.403 s, respectively; $p = 0.113$; Figure 9L). However, at 3 days post TBI, TBI non-treated animals showed a decrease relative to Sham in distance traveled (33.322 ± 11.470 vs. 99.364 ± 11.470, respectively, $p = 0.009$; Figure 9G), velocity (0.056 ± 0.019 vs. 0.167 ± 0.019 m/s, respectively, $p = 0.008$; Figure 9H), movement duration (102.053 ± 32.412 vs. 277.360 ± 32.412 s, respectively, $p = 0.018$; Figure 9I), and percent of trial moving (17.009 ± 5.402 vs. 46.227 ± 5.402 percent, respectively; $p = 0.018$; Figure 9K). At 3 days post TBI, TBI non-treated animals had an increase compared to Sham in time spent not moving (497.947 ± 32.403 vs. 464.920 ± 32.403 s, respectively; $p = 0.018$; Figure 9L). There

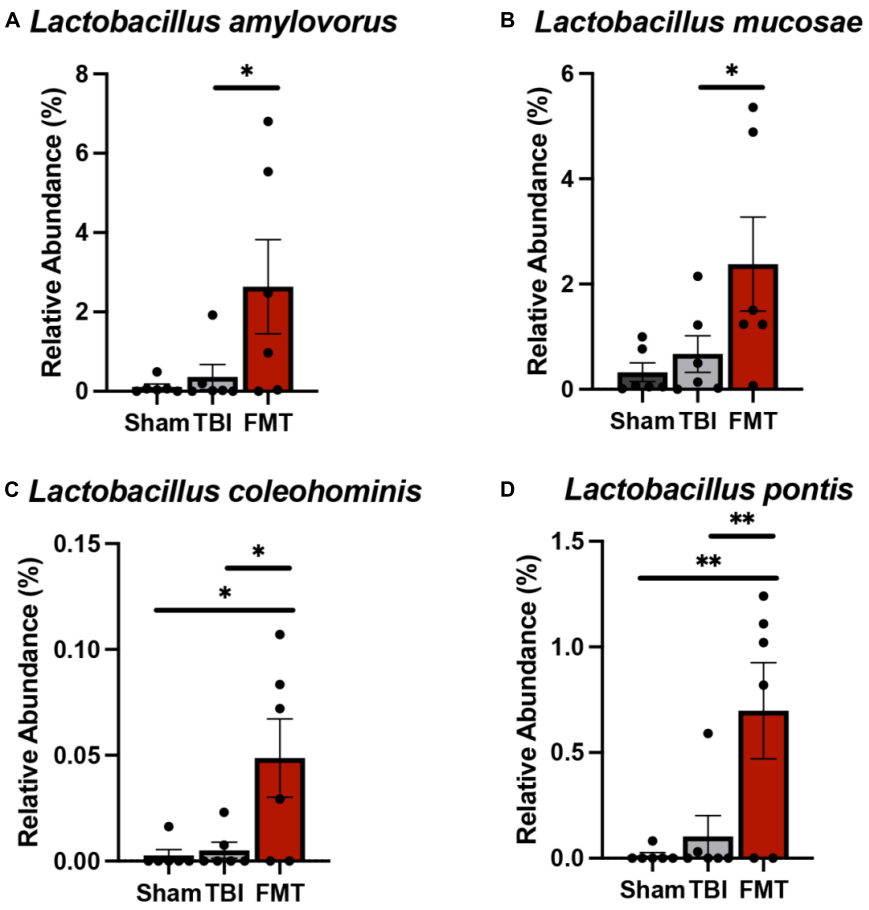


FIGURE 6
FMT increased probiotic cecal bacteria at 7 days post TBI. Cecal microbiome analysis revealed significant increases in probiotic species *L. amylovorus* **(A)** and *L. mucosae* **(B)** in the cecum of FMT treated animals versus TBI non-treated animals. *L. coleohominis* **(C)** and *L. pontis* **(D)** were increased in FMT treated animals compared to TBI non-treated and Sham animals. Data is presented as mean ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$.

was no significant difference between any of the three treatment groups in behavioral parameters measured 7-days post injury, most likely due to habituation to the behavior arena as has been noted previously (Xiao et al., 2016). Together, this data indicates FMT leads to improved recovery of spontaneous movement and characteristic porcine exploratory behavior.

Discussion

In this study, we provide, for the first-time, pivotal evidence that FMT can improve cellular and tissue damage, gut microbiota dysbiosis, and functional recovery in a translational pediatric piglet TBI model. Advanced MRI analysis revealed FMT treatment was associated with reduced lesion volume, MLS, hemorrhage volume, and cytotoxic and vasogenic edema. Histological analysis demonstrated that FMT had a neuroprotective leading to increased neuron and oligodendrocyte survival. At the level of the gut, FMT treatment prevented an increase in harmful bacteria, increased beneficial bacterial populations, and restored villi morphology ratios to a pre-TBI like state in treated pigs. Ultimately, these FMT mediated cellular and tissue level improvements were associated with enhanced functional recovery. Gait analysis showed preserved step and stride length and open field testing showed increased voluntary movement including distance traveled, velocity, percent of trial moving, movement duration while decreasing time not moving. Together these findings suggest that daily FMT may be a highly effective therapy for pediatric TBI patients, highlighting the potential of this treatment as a promising avenue for future clinical research.

Magnetic resonance imaging analysis demonstrated significant improvements in TBI-related tissue damage as a result of FMT administration. Lesion volume and MLS, reliable clinical predictors of TBI outcomes in patients (Chastain et al., 2009; Wang et al., 2021), showed FMT administration led to a 45% reduction in lesion volume and a 32 and 54% decrease in MLS at the falx cerebri and septum pellucidum, respectively, at the 7 day timepoint. Similarly, others have reported a reduced lesion volume via *ex vivo* brain tissue analysis at 3 and 7 days post severe CCI as a result of *Lactobacillus acidophils* probiotic administration in a rodent model (Ma et al., 2019). To the best of the author's knowledge, this is the first study that has reported MLS as a metric of FMT-mediated recovery. However, as a related metric, FMT administration reportedly attenuated cortical volume loss, which is known to be a contributing factor to MLS, at 3 days post severe CCI injury as compared to TBI-injured mice (Davis et al., 2022).

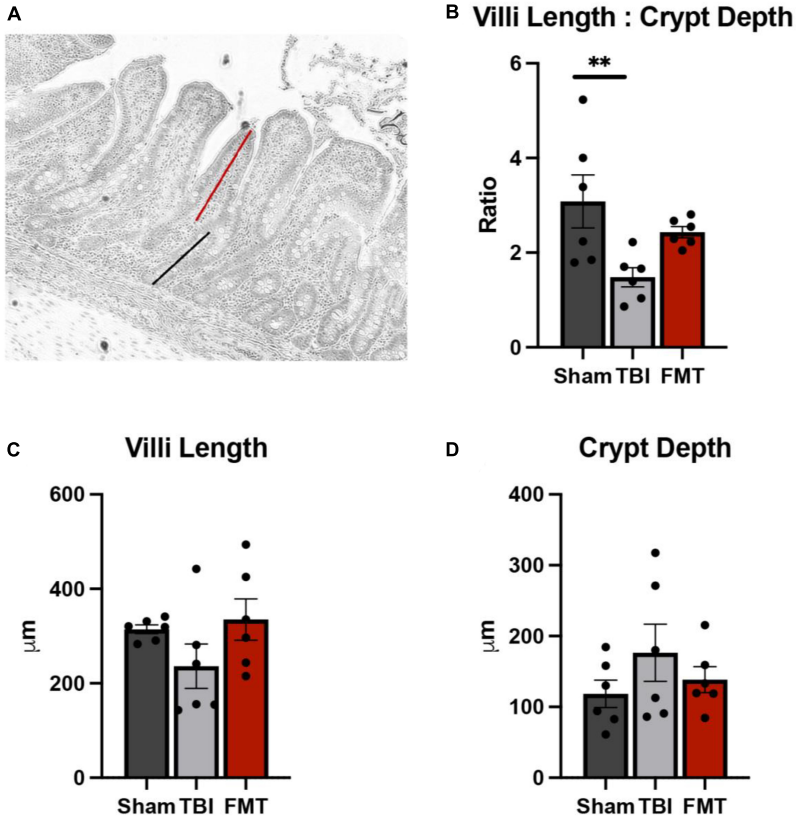


FIGURE 7
FMT treatment restores gut villi morphology. At 7 days post TBI, ileum tissue was acquired, and morphological measurements including villi height (red line), **(A)** and crypt depth (black line), **(A)** were obtained on 15 intact villi. TBI non-treated animals showed a significant reduction in villi length to crypt depth ratio, while FMT treated animals showed no significant difference **(B)**. No differences were noted between any treatment group in villi length **(C)** and crypt depth **(D)**. Data is presented as mean ± SEM. Treatment effects are as compared to Sham. $^{**}p < 0.01$.

Intracerebral hemorrhage occurs in approximately 40% of children with TBI and are directly related to increased morbidity and mortality in pediatric TBI patients (Tong et al., 2004; Beauchamp et al., 2013). Hemorrhagic transformation has been shown to be closely associated with gut microbiome perturbations (Huang et al., 2022). The present study showed, for the first time, FMT of healthy bacterial populations induced a 48% decrease in TBI hemorrhage volume along with functional recovery.

Apparent diffusion coefficient maps allow for quantification of cytotoxic (restricted diffusion) and vasogenic (increased diffusion) edema (Ashwal et al., 2006) which constitutes excessive intra- and extra-cellular water in the brain (Michinaga and Koyama, 2015). Such fluid accumulation has been reported to begin earlier, to be detected more frequently, and is associated with worst outcomes and higher risk for mortality following pediatric TBI as opposed to adult (Tong et al., 2004; Ichkova et al., 2017). This study found FMT treated pigs exhibited a greater reduction in cytotoxic and vasogenic edema (70% vs. 50% reduction, respectively) as compared to TBI non-treated animals, potentially due to restoration of the blood brain barrier (BBB) (Galea, 2021). This data is in agreement with other preclinical studies that demonstrated probiotic administration decreased edema and improved BBB functionality as compared to non-treated TBI rodents at 24 h and 3 days after TBI (Li et al., 2018; Ma et al., 2019). Together, our clinically relevant, multisequence MRI evaluation approach revealed that FMT led to significant improvements in brain tissue following TBI.

Neural injury has been well documented by our group and others to induce gastrointestinal distress including a shift in microbial population, with dysbiosis correlated to increased lesion volume in porcine and rodent neural injury models (Nicholson et al., 2019; Jeon et al., 2020). Our study demonstrated the ability of FMT to restore specific populations of gut bacterial species after dysbiosis occurred post TBI including reducing acute increases of bacteria that can have harmful effects on the host while increasing probiotic *Lactobacilli* species. Currently, research focusing on specific bacterial species impact on the gut-brain axis is limited. Most research has focused on bacterial phylum level differences. However, with the diverse array of bacterial species within a phylum, including those that are pathogenic and therapeutic, drawing conclusions at higher taxonomic levels can be misleading. Many of the bacterial species that acutely increased in TBI non-treated piglets post TBI have previously been reported to have potentially harmful effects on the host (Jeon et al., 2022). Previously, *A. indolicus* populations were decreased in the gut of piglets receiving colistin, an anti-inflammatory feed additive used to promote animal growth and health (Ali et al., 2022), suggesting this bacterium is associated with an inflamed GIT tract. Similarly, *A. howellii* has been associated with the disease actinomycosis (Ali et al., 2022) in humans which is indicative of this

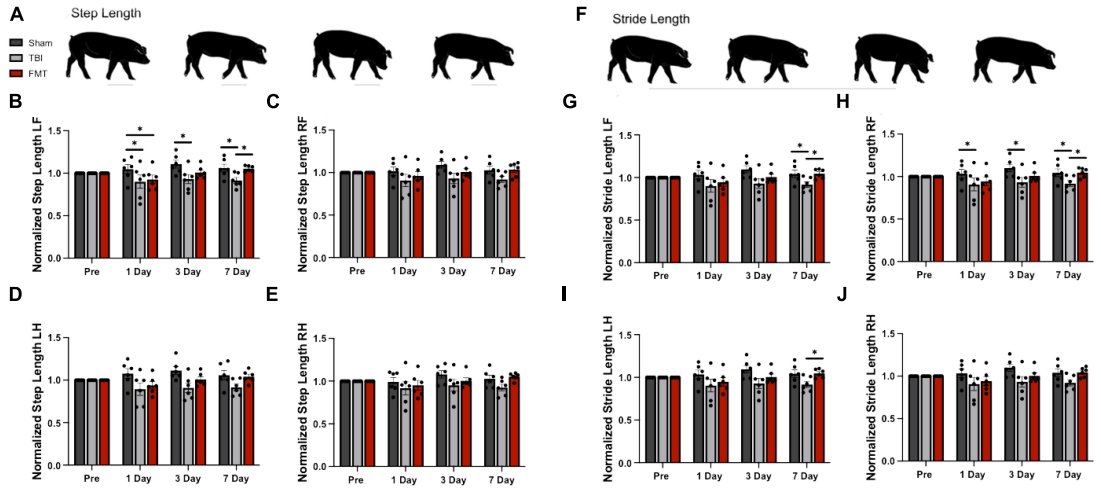


FIGURE 8
FMT improves step and stride length following TBI. Step length is the measured distance between heel strikes of opposite hooves **(A)**. At 1 day post TBI a decrease in left front step length was noted in TBI non-treated and FMT treated animals as compared to Sham **(B)**. At 3 days post TBI, TBI non-treated animals displayed a decreased step length in the left front **(B)** limbs as compared to Sham. At 7 days post TBI, TBI non-treated animals displayed a decreased step length in the left front **(B)** limbs as compared to Sham and FMT treated animals. However, there was no difference in right front **(C)**, right hind **(E)** or left hind **(D)** step length between treatments. Stride length measures the distance between consecutive heel strikes of the same limb **(F)**. At 1 day post TBI, TBI non-treated pigs exhibited a reduced stride length in the right front **(H)** limb as compared to Sham. At 3 days post TBI, TBI non-treated pigs exhibited a reduced stride length in the left front **(G)** and right front **(H)** limb as compared to Sham. At 7 days post TBI, as compared to Sham and FMT treated animals, TBI non-treated animals showed a decreased stride length in the left front **(G)** and right front **(H)** limbs. At 7 days post TBI, TBI non-treated animals showed a decreased stride length in the left hind **(I)** limbs as compared to FMT treated animals. However, there was no difference in right hind stride length between treatments **(J)**. All data is presented as mean ± SEM. Treatment effects are as compared to Sham. $*p < 0.05$.

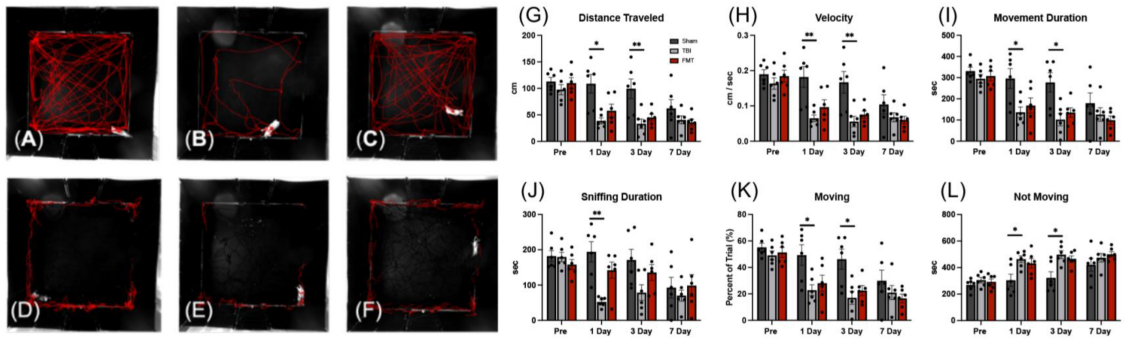


FIGURE 9
FMT improved voluntary motor activity and exploratory sniffing behavior following TBI. Representative tracking images of distance traveled **(A–C)**; (red lines), and time sniffing **(D–F)**; (red lines) are shown at 1 day post TBI in Sham **(A,D)**, respectively, TBI non treated **(B,E)**, respectively, and FMT treated **(C,F)**, respectively animals. At 1 and 3 days post TBI, TBI non-treated animals traveled significantly less distance **(G)**, moved at a slower velocity **(H)**, spent less time moving during their trial **(I)**, spent a smaller percent of the trial moving **(K)**, and spent more time not moving **(L)** as compared to Sham animals, while FMT treated animals were more comparable to sham. Additionally, at 1 day post TBI, TBI non-treated animals spent less time sniffing **(J)** compared to Sham. Data is presented as mean ± SEM. Treatment effects are as compared to Sham. $**p < 0.01$ and $*p < 0.05$.

bacteria's pathogenic nature suggesting the gut of TBI non-treated piglets in the current study were in a dysbiotic diseased state. Similarly, administration of *B. animalis* subsp. *Animalis* previously caused duodenal inflammation and mild colitis in gnotobiotic mice (Moran et al., 2009) and increased in abundance in gnotobiotic mice that have colitis compared to mice without colitis (Bibiloni et al., 2005). Based on previous research on *B. animalis*' effects on inflammation in mice, TBI non-treated piglets in the current study could have had an increase in the relative abundance of *B. animalis* subsp. *Animalis* due to its associated with inflammation and GIT distress. However, further research is needed to confirm the bacterial subspecies. Finally, increases in *S. hyointestinalis* observed in TBI non-treated piglets at 3 days post TBI may be indicative of gut dysbiosis that has systemic impacts on host health because this bacterium has previously been correlated to peripheral (Duarte and Kim, 2022) and neuroinflammation (Zhou et al., 2020) as well as depressive-like behaviors in mice (Tian et al., 2019). Collectively, our results indicate that FMT treatment may inhibit the growth of potentially harmful bacteria that are associated with disease, inflammation, and GIT distress.

Lactobacillus, a bacterial genus from the most abundant phylum Bacillota, has been identified as an important probiotic for gut health due to its anti-inflammatory effects. Specific species such as *L. acidophilus* show promising neuroprotective effects and the ability to restore gut dysbiosis and intestinal smooth muscle contractility post TBI (Masel and DeWitt, 2010; Davis et al., 2022). The present study identified four *Lactobacilli, L. amylovorus, L. coleohominis, L. mucosae,* and *L. pontis*, that were increased in cecal relative abundances in FMT piglets, suggesting their gut populations were less dysbiotic and may have a neuroprotective effect. *Lactobacilli amylovorus* has previously been shown to decrease inflammation by preventing activation of toll-like receptor 4 (TLR4) signaling pathways (Finamore et al., 2014) while also inhibiting growth of many intestinal porcine pathogens *in vitro* (Hynönen et al., 2014). *Lactobacilli coleohominis,* which increased in abundance in the gut microbiota of piglets overtime, produces antimicrobial metabolites (Hu et al., 2016) that can prevent dysbiosis and improve intestinal health. *Lactobacilli mucosae* has been identified to reduce acute inflammation in piglets following a lipopolysaccharide (LPS) challenge by increasing mucosal production of immunoglobulin A (Li et al., 2021). Additionally, it has been shown to alleviate neuropsychiatric disorders in mice by modulating their gut microbiota (Kim et al., 2020) by preventing GIT pathogen colonization and subsequent LPS production that causes GIT and hippocampal inflammation. *Lactobacilli pontis,* which was increased in the cecum of FMT-treated piglets in the current study, was recently isolated from the gut of pigs after probiotic administration. However, this bacterium's role in the gut microbiota and its effects on immunity has not been fully elucidated (Satora et al., 2020). Our results are indicative of the ability of FMT to increase the relative abundance of many *Lactobacilli* species, thus improving the health of the gut and ultimately the host.

Recovery of gross and fine motor skills and motor coordination is often stated as a patient's primary goal of TBI rehabilitation, yet severe TBI frequently results in long term gait impairment in children despite significant rehabilitation efforts (Katz-Leurer et al., 2008; Prasad et al., 2017). Our study found TBI induced a significant decrease in step and stride length, consistent with gait changes in piglet (Baker et al., 2019; Kinder et al., 2019b; Sneed et al., 2020) and human (Kuhtz-Buschbeck et al., 2003; Katz-Leurer et al., 2008) pediatric TBI populations. Additionally, we observed decreases in open field voluntary motor parameters including distance traveled time spent moving and exploratory behaviors, such as sniffing, following TBI. In the present study, FMT treatment led to significant gait improvements as well as in voluntary movement and exploratory behavior measures. To date, no rodent studies have conducted gait analysis following FMT or probiotic therapy for TBI. However, rodent studies of gut microbial therapeutics following TBI have reported beneficial functional outcomes including reduced anxiety and improved neurologic function as measured by neurological severity score (Li et al., 2018; Ma et al., 2019; Du et al., 2021), rotarod performance (Ma et al., 2019), Morris Water Maze, (Du et al., 2021), and Elevated Zero Maze (Davis et al., 2022). Maintained exploratory behavior assessed by open field testing was noted here and by Davis et al. (2022) following FMT administration. Therefore, based on the improvement in gait and voluntary movement tests observed herein, FMT treatment shows promise for promoting clinically relevant functional recovery in TBI patients.

Fecal microbial transplant treatment led to significant recovery in TBI animals, which is likely mediated through the MGBA. However, questions remain as to which MGBA component mediates the FMT therapeutic effect- stimulation of the enteric nervous system, activation of the hypothalamic pituitary adrenal axis, dampening systemic inflammation, or potentially all 3 arms of the MGBA axis. The authors hypothesize that FMT administration may contribute to improvement following TBI via anti-inflammatory mediators. Inflammation is a key consideration in the progression of TBI, as early inflammation is important to clear dead cells and debris, however, extended inflammation can exasperate brain damage (Simon et al., 2017). As demonstrated by the current study and others, brain damage can result in changes to the gut (Crapser et al., 2016; Fung et al., 2017; Nicholson et al., 2019). This can lead to an increased systemic inflammatory response, which may further exacerbate neural damage. Increased levels of systemic cytokines have been detected in serum over a year following TBI (Kumar et al., 2015). At the level of the gut, probiotic bacteria have been shown to interact with toll-like receptors to stimulate the immune system, thus altering levels of circulating cytokines (Kashiwagi et al., 2015; Maranduba et al., 2015). In rodent TBI models, administration of *Lactobacillus acidophilus (L. acidophilus)* or *Clostridium butyricum* (*C. butyricum*) inhibited the inflammatory response leading to alterations in the gut microbiome and correlating brain and functional recovery (Li et al., 2018; Ma et al., 2019). In a mouse TBI weight drop model, animals treated with *L. acidophilus* showed higher levels of GIT tight junction protein occludin and prevention of inflammatory leaky gut syndrome as indicated by reduced circulating endotoxin levels (Ma et al., 2019). This resulted in decreased circulating TNF-α levels and brain inflammatory activity including microglia proliferation and *Tlr4* and *Myd88* gene expression. Changes in neuroinflammation were associated with a reduction in BBB damage and edema and ∼50% reduction in lesion volume. These cellular and tissue level changes led to significant improvements in neurological severity scores (NSS) and motor function. Similarly, treatment with *C. butyricum* in a mouse TBI weight drop model decreased IL-6 levels in the colon and increased occludin levels, thus indicating decreased GI inflammation and improved integrity GIT integrity (Li et al., 2018). *Clostridium butyricum* treatment also correlated with a significant reduction in BBB damage, edema, cortical apoptosis, and neurodegeneration relative to controls. These results again correlated with a significant improvement in NSS scores. However, further studies are needed to confirm this effect in our porcine model. While positive results were noted in our all-male cohort, sex differences in response to pediatric TBI have been reported including increased neuroinflammation in males relative to females, likely due to differences in circulating hormones (Arambula et al., 2019). Furthermore, age and sex have been shown to alter the components of the gut microbiome (Yoon and Kim, 2021). Therefore, possible sex differences in response to FMT following TBI should be investigated in future studies.

The therapeutic effect of FMT in TBI animals are expected to persist even after discontinuation of the FMT treatment as the microbial bacteria population can restore the dysbioitic GIT microbiota. The increase in probiotic bacteria in the cecum of FMT treated animals demonstrates the ability of the FMT to reestablish beneficial bacteria in the microbiota. Further studies are needed to confirm the longevity of the FMT TBI therapeutic effect. Beyond FMT treatment, modulation of the gut microbiome

to limit TBI injury and promote recovery can potentially be accomplished through other approaches such as consumption of bioactive foods or probiotic supplementation. Fermented foods such as yogurt, apple cider vinegar, cheese, kimchi, kombucha, miso, and sauerkraut (NIH, 2023) contain live microorganisms that may provide a similar probiotic effect to the GIT microbiota as a FMT. However, further research needs to be conducted to determine if adding fermented foods to the diet could have a therapeutic effect following a TBI.

Conclusion

For the first time in a highly translatable pediatric piglet TBI model, our study has demonstrated the potential of FMT treatment to significantly limit tissue damage, prevent gut dysbiosis, and ultimately improve functional outcomes following TBI. Treatment with FMT resulted in significant therapeutic efficacy as assessed by neuro- and gastrointestinal-histology, advanced neuroimaging, microbiome analysis, and gait and behavior functional testing. Our study highlights the strong therapeutic potential of FMT treatment for TBI and underscores the need for further investigations into its long-term effects and mechanistic pathways. Such studies will be critical in establishing the efficacy and safety of FMT to be evaluated for potential use in human clinical trials.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/, 10.6084/m9.figshare.23681328.

Ethics statement

The animal study was approved by the University of Georgia Institutional Animal Care and Use Committee (Animal Use Protocol A2019 07-007-Y1-A9). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MF, CW, KD, and FW contributed to the conception and design of the study. MF, CW, KS, SS, JJ, MG, and SC performed the pig work. MF, CW, KS, SS, JJ, DB, GO, QZ, and JL performed the data analysis. MF and CW wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Acknowledgments

We would like to thank our team of undergraduate researchers: Abigail Sartin, Jonathan McDonald, Luke Frost, Chris Littlejohn, Mikayla Mashburn, Nik Ghayal, and Brendan Mahoney who were involved in various aspects of surgeries, post-operative care, treatment administration, pig gait/behavioral testing, and data analysis. We would also like to thank the UGA Animal Resources team for veterinary care and guidance as well as Rick Utley and Kelly Parham for their pig expertise and management skills.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1249539/full#supplementary-material

SUPPLEMENTARY FIGURE 1
FMT improves brain metabolites post TBI. As compared to Sham, TBI non-treated animals exhibited a significant decrease in NAAG **(A)** and PCr **(B)**, while FMT treated pigs did not. TBI non-treated and FMT treated pigs displayed a significant reduction in NAA + NAAG as compared to Sham **(C)**. FMT treated animals showed a significant decrease in NAA as compared to Sham animals **(E)**. No treatment differences were reported in Lac, **(D)**, Cr + PCr **(F)**, Glu **(G)**, or Cr **(H)**. Data is presented as mean ± SEM. Treatment effects are as compared to Sham. $^{**}p < 0.01$ and $^{*}p < 0.05$.

SUPPLEMENTARY FIGURE 2
A consistent location was used for histological imaging of neural cells. Following euthanasia, brains were immediately removed and placed in a custom piglet brain slicer to ensure consistency in brain sectioning. The rostral portion of the brain (black box) was placed in 10% buffered formalin for immunohistochemical analysis. After fixation, the brain was further subdivided (red box) and tissues were routinely processed, paraffin embedded, sectioned, and serial sections were stained.

SUPPLEMENTARY FIGURE 3
FMT caused visual changes at the phylum level post TBI. Microbiome analysis of FMT treatment administered to FMT treated piglets **(A)**. Microbiome analysis of the cecal [average per treatment: **(B)**; individual piglets (n = 6/treatment)]: **(C)** and fecal [average per treatment: **(D)**; individual piglets (n = 6/treatment pre-TBI and 1 and 7 days post TBI; n = 5/treatment 3 days post TBI)]: **(E)** environments at the phylum level indicated there were visual differences. There were increased cecal relative abundances of Bacteroidota and Pseudomonadota and decreased cecal relative abundances of Actinobacteriota in TBI non-treated animals 7 days post TBI (B). There were increased fecal relative abundances of Pseudomonadota and Actinobacteriota on 1 day post TBI in TBI non-treated animals and Spirochaetota on day 1 post TBI in FMT treated and TBI non-treated animals **(D)**.

References

Ahmad, A., Satriotomo, I., Fazal, J., Nadeau, S., and Doré, S. (2015). Considerations for the optimization of induced white matter injury preclinical models. *Front. Neurol.* 6:172. doi: 10.3389/fneur.2015.00172

Akerele, G., Al Hakeem, W., Lourenco, J., and Selvaraj, R. (2022). The effect of necrotic enteritis challenge on production performance, cecal microbiome, and cecal tonsil transcriptome in broilers. *Pathogens* 11:839. doi: 10.3390/PATHOGENS11080839

Ali, M., Razok, A., and Ziglam, H. (2022). A 5-year retrospective study of *Actinomyces odontolyticus* bacteremia in the state of Qatar, case series. *Ann. Med. Surg.* 76:103583. doi: 10.1016/J.AMSU.2022.103583

Arambula, S., Reinl, E., El Demerdash, N., McCarthy, M., and Robertson, C. (2019). Sex differences in pediatric traumatic brain injury. *Exp. Neurol.* 317, 168–179. doi: 10.1016/j.expneurol.2019.02.016

Ashwal, S., Holshouser, B., and Tong, K. (2006). Use of advanced neuroimaging techniques in the evaluation of pediatric traumatic brain injury. *Dev. Neurosci.* 28, 309–326. doi: 10.1159/000094157

Baker, E., Kinder, H., Hutcheson, J., Duberstein, K., Platt, S., Howerth, E., et al. (2019). Controlled cortical impact severity results in graded cellular, tissue, and functional responses in a piglet traumatic brain injury model. *J. Neurotrauma* 36, 61–73. doi: 10.1089/neu.2017.5551

Beauchamp, M., Beare, R., Ditchfield, M., Coleman, L., Babl, F., Kean, M., et al. (2013). Susceptibility weighted imaging and its relationship to outcome after pediatric traumatic brain injury. *Cortex* 49, 591–598. doi: 10.1016/J.CORTEX.2012.08.015

Beretta, E., Cimolin, V., Piccinini, L., Carla Turconi, A., Galbiati, S., Crivellini, M., et al. (2009). Assessment of gait recovery in children after traumatic brain injury. *Brain Inj.* 23, 751–759. doi: 10.1080/02699050903133988

Bibiloni, R., Simon, M., Albright, C., Sartor, B., and Tannock, G. (2005). Analysis of the large bowel microbiota of colitic mice using PCR/DGGE. *Lett. Appl. Microbiol.* 41, 45–51. doi: 10.1111/j.1472-765X.2005.01720.x

Bolyen, E., Rideout, J., Dillon, M., Bokulich, N., Abnet, C., Al-Ghalith, G., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Callahan, B., Mcmurdie, P., Rosen, M., Han, A., Johnson, A., and Holmes, S. (2016). Dada2: High-resolution sample inference from *Illumina amplicon* data. *Nat. Methods* 13, 581–583. doi: 10.1038/nMeth.3869

CDC Injury Center (2022). *Traumatic brain injury & concussion*. Available online at: https://www.cdc.gov/traumaticbraininjury/data/index.html (accessed on 21 Jun, 2022).

Chastain, C., Oyoyo, U., Zipperman, M., Joo, E., Ashwal, S., Shutter, L., et al. (2009). Predicting outcomes of traumatic brain injury by imaging modality and injury distribution. *J. Neurotrauma* 26, 1183–1196. doi: 10.1089/NEU.2008.0650

Conrad, M., Dilger, R., and Johnson, R. (2012). Brain growth of the domestic pig (*Sus scrofa*) from 2 to 24 weeks of age: A longitudinal MRI study. *Dev. Neurosci.* 34, 291–298. doi: 10.1159/000339311

Crapser, J., Ritzel, R., Verma, R., Venna, V., Liu, F., Chauhan, A., et al. (2016). Ischemic stroke induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice. *Aging* 8, 1049–1063. doi: 10.18632/AGING.100952

Croall, I., Smith, F., and Blamire, A. (2015). Magnetic resonance spectroscopy for traumatic brain injury. *Top. Magn. Reson. Imaging* 24, 267–274. doi: 10.1097/RMR.0000000000000063

Davis, B., Chen, Z., Islam, M., Timken, M., Procissi, D., and Schwulst, S. (2022). Fecal microbiota transfer attenuates gut dysbiosis and functional deficits after traumatic brain injury. *Shock* 57, 251–259. doi: 10.1097/SHK.0000000000001934

Docampo, J., Gonzalez, N., Bravo, F., Sarroca, D., Morales, C., and Bruno, C. (2013). Susceptibility-weighted angiography of intracranial blood products and calcifications compared to gradient echo sequence. *Neuroradiol. J.* 26, 493–500. doi: 10.1177/197140091302600501

Du, D., Tang, W., Zhou, C., Sun, X., Wei, Z., Zhong, J., et al. (2021). Fecal microbiota transplantation is a promising method to restore gut microbiota dysbiosis and relieve neurological deficits after traumatic brain injury. *Oxid. Med. Cell Longev.* 2021:5816837. doi: 10.1155/2021/5816837

Duarte, M., and Kim, S. (2022). Significance of mucosa-associated microbiota and its impacts on intestinal health of pigs challenged with F18+ E. coli. *Pathogens* 11:589. doi: 10.3390/PATHOGENS11050589

Feighery, L., Smyth, A., Keely, S., Baird, A., O'Connor, W., Callanan, J., et al. (2008). Increased intestinal permeability in rats subjected to traumatic frontal lobe percussion brain injury. *J. Trauma* 64, 131–137. doi: 10.1097/TA.0b013e3181568d9f

Finamore, A., Roselli, M., Imbinto, A., Seeboth, J., Oswald, I., and Mengheri, E. (2014). *Lactobacillus amylovorus* inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic *Escherichia coli* via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. *PLoS One* 9:e94891. doi: 10.1371/JOURNAL.PONE.0094891

Flynn, T. (1984). Developmental changes of myelin-related lipids in brain of miniature swine. *Neurochem. Res.* 9, 935–945. doi: 10.1007/BF00964525

Fung, T., Olson, C., and Hsiao, E. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nat. Neurosci.* 20, 145–155. doi: 10.1038/nn.4476

Galea, I. (2021). The blood–brain barrier in systemic infection and inflammation. *Cell. Mol. Immunol.* 18, 2489–2501. doi: 10.1038/s41423-021-00757-x

Hang, C., Shi, J., Li, J., Wu, W., and Yin, H. (2003). Alterations of intestinal mucosa structure and barrier function following traumatic brain injury in rats. *World J. Gastroenterol.* 9, 2776–2781. doi: 10.3748/wjg.v9.i12.2776

Heinritz, S., Mosenthin, R., and Weiss, E. (2013). Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. *Nutr. Res. Rev.* 26, 191–209. doi: 10.1017/S0954422413000152

Hu, J., Nie, Y., Chen, J., Zhang, Y., Wang, Z., Fan, Q., et al. (2016). Gradual changes of gut microbiota in weaned miniature piglets. *Front. Microbiol.* 7:1727. doi: 10.3389/fmicb.2016.01727

Huang, Q., Di, L., Yu, F., Feng, X., Liu, Z., Wei, M., et al. (2022). Alterations in the gut microbiome with hemorrhagic transformation in experimental stroke. *CNS Neurosci. Ther.* 28, 77–91. doi: 10.1111/CNS.13736

Hynönen, U., Kant, R., Lähteinen, T., Pietil, T., Beganovi, J., Smidt, H., et al. (2014). Functional characterization of probiotic surface layer protein-carrying *Lactobacillus amylovorus* strains. *BMC Microbiol.* 14:199. doi: 10.1186/1471-2180-14-199

Ichkova, A., Rodriguez-Grande, B., Bar, C., Villega, F., Konsman, J., and Badaut, J. (2017). Vascular impairment as a pathological mechanism underlying long-lasting cognitive dysfunction after pediatric traumatic brain injury. *Neurochem. Int.* 111, 93–102. doi: 10.1016/J.NEUINT.2017.03.022

Jeon, J., Lourenco, J., Fagan, M., Welch, C., Sneed, S., Dubrof, S., et al. (2022). Changes in oral microbial diversity in a piglet model of traumatic brain injury. *FASEB J.* 12:1111.

Jeon, J., Lourenco, J., Kaiser, E. E., Waters, E. S., Scheulin, K. M., Fang, X., et al. (2020). Dynamic changes in the gut microbiome at the acute stage of ischemic stroke in a pig model. *Front. Neurosci.* 14:587986. doi: 10.3389/FNINS.2020.587986

Kaiser, E., and West, F. (2020). Large animal ischemic stroke models: Replicating human stroke pathophysiology. *Neural Regen. Res.* 15, 1377–1387. doi: 10.4103/1673-5374.274324

Kashiwagi, I., Morita, R., Schichita, T., Komai, K., Saeki, K., Matsumoto, M., et al. (2015). Smad2 and Smad3 inversely regulate TGF-b autoinduction in *Clostridium butyricum*-activated dendritic cells. *Immunity* 43, 65–79. doi: 10.1016/j.immuni.2015.06.010

Katz-Leurer, M., Rotem, H., Lewitus, H., Keren, O., and Meyer, S. (2008). Relationship between balance abilities and gait characteristics in children with post-traumatic brain injury. *Brain Inj.* 22, 153–159. doi: 10.1080/02699050801895399

Kim, J., Lee, K., Lee, S., Jang, H., and Kim, D. (2020). Interplay between human gut bacteria *Escherichia coli* and *Lactobacillus mucosae* in the occurrence of neuropsychiatric disorders in mice. *Front. Immunol.* 11:273. doi: 10.3389/fimmu.2020.00273

Kinder, H., Baker, E., Howerth, E., Duberstein, K., and West, F. (2019a). Controlled cortical impact leads to cognitive and motor function deficits that correspond to cellular pathology in a piglet traumatic brain injury model. *J. Neurotrauma* 36, 2810–2826. doi: 10.1089/neu.2019.6405

Kinder, H., Baker, E., Wang, S., Fleischer, C., Howerth, E., Duberstein, K., et al. (2019b). Traumatic brain injury results in dynamic brain structure changes leading to acute and chronic motor function deficits in a pediatric piglet model. *J. Neurotrauma* 36, 2930–2942. doi: 10.1089/NEU.2018.6303

Kinder, H., Baker, E., and West, F. (2019c). The pig as a preclinical traumatic brain injury model: Current models, functional outcome measures, and translational detection strategies. *Neural Regen. Res.* 14, 413–424. doi: 10.4103/1673-5374.245334

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/NAR/GKS808

Kuhtz-Buschbeck, J., Stolze, H., Gölge, M., and Ritz, A. (2003). Analyses of gait, reaching, and grasping in children after traumatic brain injury. *Arch. Phys. Med. Rehabil.* 84, 424–430. doi: 10.1053/apmr.2003.50017

Kumar, R., Boles, J., and Wagner, A. (2015). Chronic inflammation after severe traumatic brain injury: Characterization and associations with outcome at 6- and 12-months post injury. *J. Head Trauma Rehabil.* 30, 369–381. doi: 10.1097/HTR.0000000000000067

Lamendella, R., Santo Domingo, J., Ghosh, S., Martinson, J., and Oerther, D. (2011). Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiol.* 11:103. doi: 10.1186/1471-2180-11-103

Li, H., Sun, J., Du, J., Wang, F., Fang, R., Yu, C., et al. (2018). *Clostridium butyricum* exerts a neuroprotective effect in a mouse model of traumatic brain injury via the gut-brain axis. *Neurogastroenterol. Motil.* 30:e13260. doi: 10.1111/NMO.13260

Li, L., and Liu, J. (2013). The effect of pediatric traumatic brain injury on behavioral outcomes: A systematic review. *Dev. Med. Child Neurol.* 55, 37–45. doi: 10.1111/J.1469-8749.2012.04414.X

Li, Y., San Andres, J., Trenhaile-Grannemann, M., van Sambeek, D., Moore, K., Winkel, S., et al. (2021). Effects of mannan oligosaccharides and *Lactobacillus mucosae* on growth performance, immune response, and gut health of weanling pigs challenged with *Escherichia coli* lipopolysaccharides. *J. Anim. Sci.* 99:skab286. doi: 10.1093/JAS/SKAB286

Lind, N., Moustgaard, A., Jelsing, J., Vajta, G., Cumming, P., and Hansen, A. (2007). The use of pigs in neuroscience: Modeling brain disorders. *Neurosci. Biobehav. Rev.* 31, 728–751. doi: 10.1016/j.neubiorev.2007.02.003

Ma, Y., Liu, T., Fu, J., Fu, S., Hu, C., Sun, B., et al. (2019). *Lactobacillus acidophilus* exerts neuroprotective effects in mice with traumatic brain injury. *J. Nutr.* 149, 1543–1552. doi: 10.1093/JN/NXZ105

Maranduba, C., De Castro, S., de Souza, G., Rossato, C., da Guia, F., Valente, M., et al. (2015). Intestinal microbiota as regulators of the immune system and neuroimmune system: Impact on host health and homeostasis. *J. Immunol. Res.* 2015:931574. doi: 10.1155/2015/931574

Masel, B., and DeWitt, D. (2010). Traumatic brain injury: A disease process, not an event. *J. Neurotrauma* 27, 1529–1540. doi: 10.1089/NEU.2010.1358

Michinaga, S., and Koyama, Y. (2015). Pathogenesis of brain edema and investigation into anti-edema drugs. *Int. J. Mol. Sci.* 16, 9949–9975. doi: 10.3390/IJMS16059949

Miller, E., and Ullrey, D. (1987). The pig as a model for human nutrition. *Annu. Rev. Nutr.* 7, 361–382. doi: 10.1146/ANNUREV.NU.07.070187.002045

Moran, J., Walter, J., Tannock, G., Tonkonogy, S., and Sartor, R. (2009). Bifidobacterium animalis causes extensive duodenitis and mild colonic inflammation in monoassociated interleukin-10-deficient mice. *Inflamm. Bowel Dis.* 15, 1022–1031. doi: 10.1002/IBD.20900

Neale, J., Olszewski, R., Zuo, D., Janczura, K., Profaci, C., Lavin, K., et al. (2011). Advances in understanding the peptide neurotransmitter NAAG and appearance of a new member of the NAAG neuropeptide family. *J. Neurochem.* 118, 490–498. doi: 10.1111/J.1471-4159.2011.07338.X

Neumann, M., Wang, Y., Kim, S., Hong, S., Jeng, L., Bilgen, M., et al. (2009). Assessing gait impairment following experimental traumatic brain injury in mice. *J. Neurosci. Methods* 176, 34–44. doi: 10.1016/J.JNEUMETH.2008.08.026

Nicholson, S., Watts, L., Burmeister, D., Merrill, D., Scroggins, S., Zou, Y., et al. (2019). Moderate traumatic brain injury alters the gastrointestinal microbiome in a time-dependent manner. *Shock* 52, 240–248. doi: 10.1097/SHK.0000000000001211

NIH (2023). *Probiotics dietary supplement fact sheets*. Available online at: https://ods.od.nih.gov/factsheets/Probiotics-Consumer/ (accessed on 30 Aug, 2023).

Oeltzschner, G., Zöllner, H., Hui, S., Mikkelsen, M., Saleh, M., Tapper, S., et al. (2020). Osprey: Open-source processing, reconstruction & estimation of magnetic resonance spectroscopy data. *J. Neurosci. Methods* 343:108827. doi: 10.1016/J.JNEUMETH.2020.108827

Palekar, S., Jaiswal, M., Patil, M., and Malpathak, V. (2021). Outcome prediction in patients of traumatic brain injury based on midline shift on CT scan of brain. *Indian J. Neurosurg.* 10, 210–215. doi: 10.1055/S-0040-1716990

Pedregosa, F., Michel, V., and Grisel, O. (2011). Scikit-learn: Machine learning in python. *J. Mach. Learn. Res.* 12, 2825–2830.

Peri, E., Panzeri, D., Beretta, E., Reni, G., Strazzer, S., and Biffi, E. (2019). Motor improvement in adolescents affected by ataxia secondary to acquired brain injury: A pilot study. *Biomed. Res. Int.* 2019:8967138. doi: 10.1155/2019/8967138

Ponte, A., Pinho, R., Mota, M., Silva, J., Vieira, N., Oliveira, R., et al. (2015). Initial experience with fecal microbiota transplantation in clostridium difficile infection-transplant protocol and preliminary results. *Rev. Esp. Enferm. Dig.* 107, 402–407. doi: 10.17235/reed.2015.3767/2015

Prasad, M., Swank, P., and Ewing-Cobbs, L. (2017). Long-term school outcomes of children and adolescents with traumatic brain injury. *J. Head Trauma Rehabil.* 32, E24–E32. doi: 10.1097/HTR.0000000000000218

Provencher, S. (1993). Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn. Reson. Med.* 30, 672–679. doi: 10.1002/MRM.1910300604

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/NAR/GKS1219

Rae, C. (2014). A guide to the metabolic pathways and function of metabolites observed in human brain 1H magnetic resonance spectra. *Neurochem. Res.* 39, 1–36. doi: 10.1007/s11064-013-1199-5

Rice, M., Pandya, J., and Shear, D. (2019). Gut microbiota as a therapeutic target to ameliorate the biochemical, neuroanatomical, and behavioral effects of traumatic brain injuries. *Front. Neurol.* 10:875. doi: 10.3389/FNEUR.2019.00875

Ross, B., Ernst, T., Kreis, R., Haseler, L., Bayer, S., Danielsen, E., et al. (1998). 1H MRS in acute traumatic brain injury. *J. Magn. Reson. Imaging* 8, 829–840. doi: 10.1002/JMRI.1880080412

Satora, M., Magdziarz, M., Rząsa, A., Rypuła, K., and Płoneczka-Janeczko, K. (2020). Insight into the intestinal microbiome of farrowing sows following the administration of garlic (*Allium sativum*) extract and probiotic bacteria cultures under farming conditions. *BMC Vet. Res.* 16:442. doi: 10.1186/s12917-020-02659-y

Simon, D., McGeachy, M., Bayır, H., Clark, R., Loane, D., and Kochanek, P. (2017). The far reaching scope of neuroinflammation after traumatic brain injury. *Nat. Rev. Neurol.* 13, 171–191. doi: 10.1038/nrneurol.2017.13

Sneed, S., Scheulin, K., Kaiser, E., Fagan, M., Jurgielewicz, B., Waters, E., et al. (2020). Magnetic resonance imaging and gait analysis indicates similar outcomes between Yucatan and landrace porcine ischemic stroke models. *Front. Neurol.* 11:594954. doi: 10.3389/fneur.2020.594954

Sun, B., Hu, C., Fang, H., Zhu, L., Gao, N., and Zhu, J. (2015). The Effects of lactobacillus acidophilus on the intestinal smooth muscle contraction through PKC/MLCK/MLC signaling pathway in TBI mouse model. *PLoS One* 10:e0128214. doi: 10.1371/journal.pone.0128214

Sundman, M., Chen, N., Subbian, V., and Chou, Y. (2017). The bidirectional gut-brain-microbiota axis as a potential nexus between traumatic brain injury, inflammation, and disease. *Brain Behav. Immun.* 66, 31–44. doi: 10.1016/J.BBI.2017.05.009

Thayer, J., and Sternberg, E. (2009). Neural concomitants of immunity—focus on the vagus nerve. *Neuroimage* 47, 908–910. doi: 10.1016/J.NEUROIMAGE.2009.05.058

Tian, T., Xu, B., Qin, Y., Fan, L., Chen, J., Zheng, P., et al. (2019). *Clostridium butyricum* miyairi 588 has preventive effects on chronic social defeat stress-induced depressive-like behaviour and modulates microglial activation in mice. *Biochem. Biophys. Res. Commun.* 516, 430–436. doi: 10.1016/J.BBRC.2019.06.053

Tong, K., Ashwal, S., Holshouser, B., Nickerson, J., Wall, C., Shutter, L., et al. (2004). Diffuse axonal injury in children: Clinical correlation with hemorrhagic lesions. *Ann. Neurol.* 56, 36–50. doi: 10.1002/ANA.20123

Tremlett, H., Bauer, K., Appel-Cresswell, S., Finlay, B., and Waubant, E. (2017). The gut microbiome in human neurological disease: A review. *Ann. Neurol.* 81, 369–382. doi: 10.1002/ANA.24901

Vendrik, K., Ooijevaar, R., de Jong, P., Laman, J., van Oosten, B., van Hilten, J., et al. (2020). Fecal microbiota transplantation in neurological disorders. *Front. Cell Infect. Microbiol.* 10:98. doi: 10.3389/fcimb.2020.00098

Vink, R. (2018). Large animal models of traumatic brain injury. *J. Neurosci. Res.* 96, 527–535. doi: 10.1002/jnr.24079

Wang, H., Baker, E., Mandal, A., Pidaparti, R., West, F., and Kinder, H. (2021). Identification of predictive MRI and functional biomarkers in a pediatric piglet traumatic brain injury model. *Neural Regen. Res.* 16, 338–344. doi: 10.4103/1673-5374.290915

Welch, C., Lourenco, J., Davis, D., Krause, T., Carmichael, M., Rothrock, M., et al. (2020). The impact of feed efficiency selection on the ruminal, cecal, and fecal microbiomes of Angus steers from a commercial feedlot. *J. Anim. Sci.* 98:skaa230. doi: 10.1093/JAS/SKAA230

Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., et al. (2016). A reference gene catalogue of the pig gut microbiome. *Nat. Microbiol.* 1:16161. doi: 10.1038/nmicrobiol.2016.161

Yang, W., Li, Q., Li, R., Liu, Q., Wang, X., Zhao, L., et al. (2018). Defining the optimal midline shift threshold to predict poor outcome in patients with supratentorial spontaneous intracerebral hemorrhage. *Neurocrit. Care* 28, 314–321. doi: 10.1007/s12028-017-0483-7

Yoon, K., and Kim, N. (2021). Roles of sex hormones and gender in the gut microbiota. *J. Neurogastroenterol. Motil.* 27, 314–325. doi: 10.5056/jnm20208

Yu, X., Zhou, G., Shao, B., Zhou, H., Xu, C., Yan, F., et al. (2021). Gut microbiota dysbiosis induced by intracerebral hemorrhage aggravates neuroinflammation in mice. *Front. Microbiol.* 12:647304. doi: 10.3389/FMICB.2021.647304

Zhou, J., Yang, J., Dai, M., Lin, D., Zhang, R., Liu, H., et al. (2020). A combination of inhibiting microglia activity and remodeling gut microenvironment suppresses the development and progression of experimental autoimmune uveitis. *Biochem. Pharmacol.* 180:114108. doi: 10.1016/J.BCP.2020.114108

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 28 September 2023
DOI 10.3389/fmicb.2023.1243102

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Qiao Shi,
Renmin Hospital of Wuhan University, China
Shenhai Gong,
Southern Medical University, China

*CORRESPONDENCE
Enhai Cui
✉ kjkceh@126.com

RECEIVED 20 June 2023
ACCEPTED 29 August 2023
PUBLISHED 28 September 2023

CITATION
Hua F, Cui E, Lv L, Wang B, Li L, Lu H, Chen N and Chen W (2023) Fecal microbiota transplantation from HUC-MSC-treated mice alleviates acute lung injury in mice through anti-inflammation and gut microbiota modulation. *Front. Microbiol.* 14:1243102. doi: 10.3389/fmicb.2023.1243102

COPYRIGHT
© 2023 Hua, Cui, Lv, Wang, Li, Lu, Chen and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbiota transplantation from HUC-MSC-treated mice alleviates acute lung injury in mice through anti-inflammation and gut microbiota modulation

Feng Hua[1], Enhai Cui[1]*, Lu Lv[1], Bin Wang[1], Liqin Li[2], Huadong Lu[1], Na Chen[1] and Wenyan Chen[1]

[1]Department of Respiratory and Critical Care Medicine, Huzhou Central Hospital, Affiliated Huzhou Hospital, Zhejiang University School of Medicine, Huzhou, China, [2]Traditional Chinese Medicine Key Laboratory Cultivation Base of Zhejiang Province for the Development and Clinical Transformation of Immunomodulatory Drugs, Huzhou, China

Introduction: Acute lung injury (ALI) is a severe respiratory tract disorder facilitated by dysregulated inflammation, oxidative stress and intestinal ecosystem. Fecal microbiota transplantation (FMT) is a rapid method for gut microbiota (GM) reconstruction. Furthermore, our previous studies have confirmed that human umbilical cord mesenchymal stromal cells (HUC-MSCs) can alleviate ALI by improving GM composition. Therefore, we aimed to explore the efficacy and mechanism of FMT from HUC-MSCs-treated mice on ALI.

Methods: In brief, fresh feces from HUC-MSCs-treated mice were collected for FMT, and the mice were randomly assigned into NC, FMT, LPS, ABX-LPS, and ABX-LPS-FMT groups ($n = 12$/group). Subsequently, the mice were administrated with antibiotic mixtures to deplete GM, and given lipopolysaccharide and FMT to induce ALI and rebuild GM. Next, the therapeutic effect was evaluated by bronchoalveolar lavage fluid (BALF) and histopathology. Immune cells in peripheral blood and apoptosis in lung tissues were measured. Furthermore, oxidative stress- and inflammation-related parameter levels were tested in BALF, serum, lung and ileal tissues. The expressions of apoptosis-associated, TLR4/NF-κB pathway-associated, Nrf2/HO-1 pathway related and tightly linked proteins in the lung and ileal tissues were assessed. Moreover, 16S rRNA was conducted to assess GM composition and distribution.

Results: Our results revealed that FMT obviously improved the pathological damage of lung and ileum, recovered the immune system of peripheral blood, decreased the cell apoptosis of lung, and inhibited inflammation and oxidative stress in BALF, serum, lung and ileum tissues. Moreover, FMT also elevated ZO-1, claudin-1, and occludin protein expressions, activating the Nrf2/HO-1 pathway but hindering the TLR4/NF-κB pathway. Of note, the relative abundances of Bacteroides, *Christensenella*, *Coprococcus*, and *Roseburia* were decreased, while the relative abundances of *Xenorhabdus*, *Sutterella*, and *Acinetobacter* were increased in the ABX-LPS-FMT group.

Conclusion: FMT from HUC-MSCs-treated mice may alleviate ALI by inhibiting inflammation and reconstructing GM, additionally, we also found that the TLR4/NF-κB and Nrf2/HO-1 pathways may involve in the improvement of FMT on ALI, which offers novel insights for the functions and mechanisms of FMT from HUC-MSCs-treated mice on ALI.

KEYWORDS

fecal microbiota transplantation, human umbilical cord mesenchymal stromal cells, acute lung injury, inflammation, gut microbiota

1. Introduction

Acute lung injury (ALI) is a serious disorder characterized by acute, inflammatory, and diffuse damage that occurs in the lungs (Jiang et al., 2021). In the United States, 200,000 patients are diagnosed with ALI, and the mortality rate is as high as 40% (Costa et al., 2017). Despite great efforts, the mortality of ALI remains considerable, and definitive therapies are still lacking (Wang et al., 2021). So far, lung protective ventilation represents the only beneficial strategy for ALI patients, but the extensive adoption of the strategy contributes to the occurrence of ventilator-induced lung injury (Li et al., 2017). Thus, new and efficacious treatment strategies for ALI are desperately required.

Following ALI, numerous inflammatory factors were generated and released, which exacerbate lung injury (Yang et al., 2020). The triggered inflammation not only causes oxidative stress but also results in apoptosis in the lungs, both of which play key roles in ALI initiation and progression (Jia et al., 2021). As is well-known, the TLR4/NF-κB pathway is a classic signal pathway responsible for regulating inflammation (Zhang et al., 2019a). Currently, research has found that inflammation in lipopolysaccharide (LPS)-induced ALI can be alleviated *via* the inhibition of the TLR4/NF-κB pathway (Liu et al., 2020b). In addition, the Nrf2/HO-1 pathway is also a crucial pathway for antioxidant defense (Su et al., 2020). Following oxidative stress, Nrf2 separates from Keap-1, combines with the antioxidant response element (ARE), and then elevates the expression of some antioxidant-encoding proteins, which protect against oxidative stress in ALI (Li Y. et al., 2023). Bi et al. have revealed that activating the Nrf2/HO-1 pathway can reduce oxidative stress in ALI mice. Hence, looking for new drugs that can suppress inflammation and oxidative stress by modulating the TLR4/NF-κB and Nrf2/HO-1 pathways is an efficient approach to treating ALI.

Gut microbiota (GM) interacts with the host and is pivotal for controlling host health (Bi et al., 2020). Recent research has demonstrated that GM can impact the lung function of ALI *via* the "gut–lung axis" (Kapur et al., 2018). Specifically, disordered GM will break down intestinal barrier integrity, so as to activate the immune system, facilitate bacterial displacement, induce migration of bacteria to the lungs, and exacerbate lung inflammation and oxidative stress (Tang et al., 2021). Fecal microbiota transplantation (FMT) is a simple and fast method for gut bacterial transplantation. Following FMT, the composition of GM in the recipient is similar to that in the donor (Le Bastard et al., 2018). Published studies have found that FMT from normal mice can relieve ALI by restoring GM composition, inhibiting inflammation (Li et al., 2020), and reducing oxidative stress (Tang et al., 2021). In addition, our previous study has also revealed that human umbilical cord mesenchymal stromal cells (HUC-MSCs) can attenuate LPS-stimulated ALI by modulating lung–gut microbiota. In addition, we also observed that compared with the sham mice without HUC-MSC treatment, there were three microflora with upregulated abundance and nine microflora with downregulated abundance in the feces of mice treated with HUC-MSCs (Supplementary Figure 1). However, the effect and mechanism of FMT from HUC-MSC-treated mice on ALI remain unknown.

Hence, in this study, we performed animal experiments to explore whether FMT from HUC-MSC-treated mice can alleviate LPS-induced ALI by modulating inflammation and GM composition. These findings may offer a novel effective therapeutic option for ALI.

2. Materials and methods

2.1. Animals

C57BL/6J mice (6–8 weeks old, 20.0 ± 2 g) were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd (China, license No. SCXK (Beijing) 2016-0006). The mice were all housed in a specific pathogen-free environment with controlled temperature (22–26°C), humidity (40%−60%), and lighting (12/12-h light–dark cycle). All mice received water and standard food freely. All animal-related experiments were carried out with the evaluation and supervision of the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center [certificate No. SYXK (Zhe) 2021-0033] and in compliance with the guidelines of the Institutional Animal Care and Use Committee.

2.2. Animal grouping and FMT treatment

All mice were allowed to acclimate to the conditions of the animal house for 7 days before the experiments. A mouse died during the adaptation phase, and subsequently, the surviving mice were randomized into five groups (n = 12/group): Group A: normal control group (NC); Group B: normal mice received FMT from HUC-MSC-treated mice group (FMT, the protocol of FMT is presented below); Group C: LPS-stimulated group (LPS); Group D: mice received antibiotics (ABX) and LPS stimulation group (ABX-LPS); Group E: mice treated with ABX, stimulated by LPS, and then received FMT from the HUC-MSC-treated mice group (ABX-LPS-FMT).

The flow of the experiment is shown in Figure 1. In brief, mice from the ABX-LPS and ABX-LPS-FMT groups received broad-spectrum antibiotics (0.1 mg/g neomycin, 0.1 mg/g ampicillin, 0.1 mg/g metronidazole, and 0.05 mg/g vancomycin) by gavage twice per day for 8 days; the step was utilized to remove the GM of the mice. The other mice were treated with an equal volume of sterile water intragastrically. Subsequently, the LPS, ABX-LPS, and ABX-LPS-FMT groups were administered with 100 μl LPS (10 mg/kg) intraperitoneally to construct the ALI model, and the remaining mice were perfused with the same amount of sterile saline (Yang et al., 2021). After 6 h, mice in the FMT and ABX-LPS-FMT groups received FMT treatment; fresh fecal samples were collected from normal mice that had received HUC-MSC treatment (in short, 6–8-week-old male C57BL/6 mice were treated with 100 μl 0.9% NaCl). After 6 h, the mice were injected intraperitoneally with 0.5 ml of phosphate-buffered saline (PBS) containing HUC-MSCs (2×10^6 cells/ml, iCell Bioscience). After 3 days of HUC-MSC intervention, the fecal samples of the mice were collected (Wu et al., 2022). Then, the fecal samples were homogenized and diluted to a final concentration of 50 mg/ml using sterile saline.

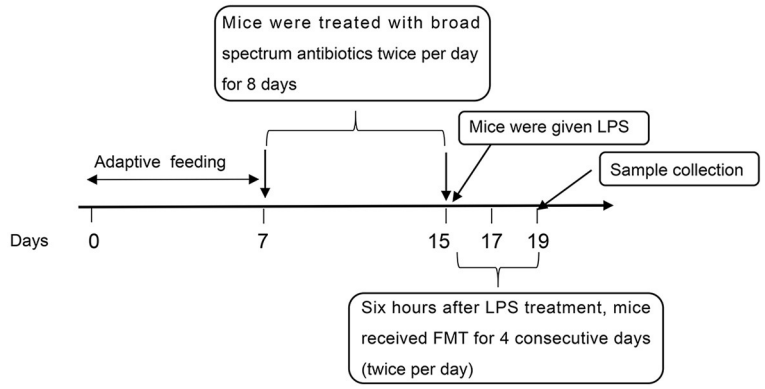


FIGURE 1
The flow of the experiment. LPS, lipopolysaccharide; FMT, fecal microbiota transplantation.

TABLE 1 qRT-PCR primers.

Gene	Forward primer	Reverse primer
Mouse IL-1β	TTGAAGTTGACGGACCCCAA	TGTCCTGACCACTGTTGTTTC
Mouse IL-6	GGCGGATCGGATGTTGTGAT	GGACCCCAGACAATCGGTTG
Mouse TNF-α	TCACTGGAGCCTCGAATGTC	TCTGTGAGAAGGCTGTGCA
Mouse iNOS	TTCACGACACCCTTCACCACAA	CCATCCTCCTGCCCACTTCCTC
Mouse Cox-2	CACCCTGACATAGACAGTGAAAG	CTGGGTCACGTTGGATGAGG
Mouse GAPDH	CGAGACACGATGGTGAAGGT	TGCCGTGGGTGGAATCATAC

Afterward, the fecal samples were centrifuged, and the supernatant filtered by filters (70 μm) was applied for FMT. FMT was achieved by giving 200 μl fecal sample supernatant to the recipient mice intragastrically for four consecutive days (twice per day) (Wu et al., 2021). Correspondingly, mice in other groups were treated with an equal volume of sterile saline. The ALI model induced by LPS is deemed to be reliable and stable (Yang et al., 2021).

2.3. Specimen collection

On the day of the last injection, fecal samples from the NC, FMT, LPS, and ABX-LPS-FMT groups were collected. Then, the mice were euthanized using 5% isoflurane, and the blood from the abdominal aorta was collected and centrifuged to obtain serum. After bronchoalveolar lavage fluid (BALF) was collected from the left lung, the right lung was harvested to evaluate the lung wet to dry (W/D) ratio and other indicators. Additionally, ileal tissues were removed from the mice and kept for further tests.

2.4. Collection and analysis of BALF

The trachea cannula of the left lung was washed with 0.7 ml of PBS repeatedly. The collected BALF was centrifuged, and the supernatant was utilized to determine BALF protein concentration and inflammatory cytokines. The cell pellets were resuspended by PBS, and then, the total cell number in BALF was counted by cell counting plate. Furthermore, the number of neutrophils in BALF was calculated by Wright–Giemsa staining.

2.5. Lung W/D ratio

The middle lobe of the right lung was excised and weighed immediately to obtain the wet mass of the lungs. Next, these lung tissues were dehydrated for 48 h at 80°C, to obtain the dry mass of the lungs. Finally, the lung W/D ratio was calculated to evaluate pulmonary edema.

2.6. Detection of malondialdehyde, glutathione, superoxide dismutase, and myeloperoxidase levels in the lung tissues

Lung tissue samples were rinsed with PBS and then minced into pieces. The tissues were fully homogenized on ice with PBS. Then, the homogenates were centrifuged; the supernatant was obtained to measure the levels of detection of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and myeloperoxidase (MPO) by commercial kits (A003-1; A006-2-1; A001-1; A044-1-1; Nanjing Jiancheng Bioengineering Institute, China).

TABLE 2 Antibody information.

Antibody	Source	Cat No.	Dilutions
Bax	Affinity	AF0120	1:1,000
Bcl-2	Affinity	AF6139	1:1,000
Caspase-3	CST	9662s	1:1,000
Cleaved-caspase-3	Abcam	ab2302	1:1,000
ZO-1	Proteintech	21773-1-AP	1:1,000
Claudin-1	Affinity	AF0127	1:1,000
Occludin	Affinity	DF7504	1:1,000
TLR4	Affinity	AF7017	1:1,000
Myd88	Affinity	AF5195	1:1,000
p-NF-κB p65	Affinity	AF2006	1:1,000
NF-κB p65	Affinity	AF5006	1:1,000
p-IκBα	Affinity	AF2002	1:1,000
IκBα	Affinity	AF5002	1:1,000
Nrf2	Proteintech	16396-1-AP	1:1,000
HO-1	Proteintech	27282-1-AP	1:1,000
COX-2	Affinity	AF7003	1:1,000
iNOS	Affinity	AF0199	1:1,000
β-actin	Affinity	AF7018	1:10,000
GAPDH	Affinity	AF7021	1:10,000

2.7. Enzyme-linked immunosorbent assay (ELISA) analysis

The homogenates of ileal tissues were prepared as lung tissue. Then, commercial enzyme-linked immunosorbent assay (ELISA) kits were applied to test the contents of interleukin-1β (IL-1β, ml063132-1), interleukin-6 (IL-6, ml002293-1), and tumor necrosis factor-a (TNF-a, ml002095-1) in the BALF, serum, lung, and ileal tissues, following the manufacturer's instructions. All ELISA kits were bought from Shanghai Enzyme-linked Biotechnology Co., Ltd (China).

2.8. Peripheral blood immune cell analysis

The whole blood of the mice was collected and anticoagulated with heparin sodium. Then, the blood was treated with corresponding antibodies for 0.5 h at room temperature. Specifically, Tc cells were evaluated by anti-CD3e (F2100301), anti-CD8a (F2100810), and anti-CD4 (F2100403) antibodies. DCs were measured using anti-CD11c (F21011C03) and anti-CD86 (F2100810) antibodies. NK cells were examined with anti-CD49b (F21049B03) and anti-CD3e antibodies. Tregs were tested using anti-CD4, anti-CD25 (F2102504), and anti-Foxp3 (F21FP302) antibodies. Finally, the mixture was centrifuged. After removing the supernatant, the pellet was resuspended in PBS and analyzed using flow cytometry. All antibodies used in the experiment were supplied by Liankebio (China).

2.9. Histological analysis

The collected lung and ileal tissues were rinsed with PBS, followed by fixation and dehydration using 4% paraformaldehyde and a series of ethanol, respectively. Thereafter, the tissues were embedded in paraffin and cut into sections (5 μm thick) for staining with hematoxylin (H3136, Sigma, USA) and eosin (HE, E4009, Sigma, USA). The histopathological changes in the lung and ileal tissues were observed using a microscope (Eclipse Ci-L, Nikon, Japan).

2.10. Terminal deoxynucleotidyl transferase-mediated nick end labeling staining

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was employed to assess the apoptosis in the lung tissues. In short, TUNEL staining was performed on the processed lung tissue sections by an *in situ* cell death detection kit (11684795910, Roche, France), as per the supplier's instructions. Finally, the slices were counterstained with 4′,6-diamidino-2-phenylindole for 10 min in the darkness to identify the nucleus. After completing staining, the non-apoptotic cell appeared blue, while the apoptotic cell was green. The slices were viewed using the microscope, and the apoptosis of the lung tissues was exhibited as the amount of TUNEL-positive cells.

2.11. Quantitative PCR (qPCR)

The total RNA was isolated from lung tissue homogenate by TRIzol reagents. Then, the total RNA was used to synthesize cDNA using reverse transcription kits (CW2569, CWBIO). Thereafter, quantitative PCR (qPCR) was conducted by SYBR Green qPCR kits (11201ES08, YE SEM, China) and carried out on real-time PCR instruments (LightCycler 96, Roche, Germany). The relative mRNA expressions were calculated as the ratio of GAPDH and analyzed by the $2^{-\Delta\Delta Ct}$ method. The qPCR primer sequences designed in the experiment are presented in Table 1.

2.12. Immunohistochemical staining

After fixation with paraformaldehyde, the lung and ileal tissues were embedded and cut as usual. Immunohistochemical staining was, then, performed to detect the expressions of TLR4, ZO-1, claudin-1, and occludin. In brief, the sections were deparaffinized and rehydrated prior to quenching in H_2O_2 (3%). Upon placing in 5% bovine serum albumin to block non-specific binding, the lung samples were probed with anti-TLR4 antibody (1:100, AF7017, Affinity, China), and the ileal samples were probed with anti-ZO-1 (1:500, ab221547, Abcam, UK), anti-claudin-1 (1:250,

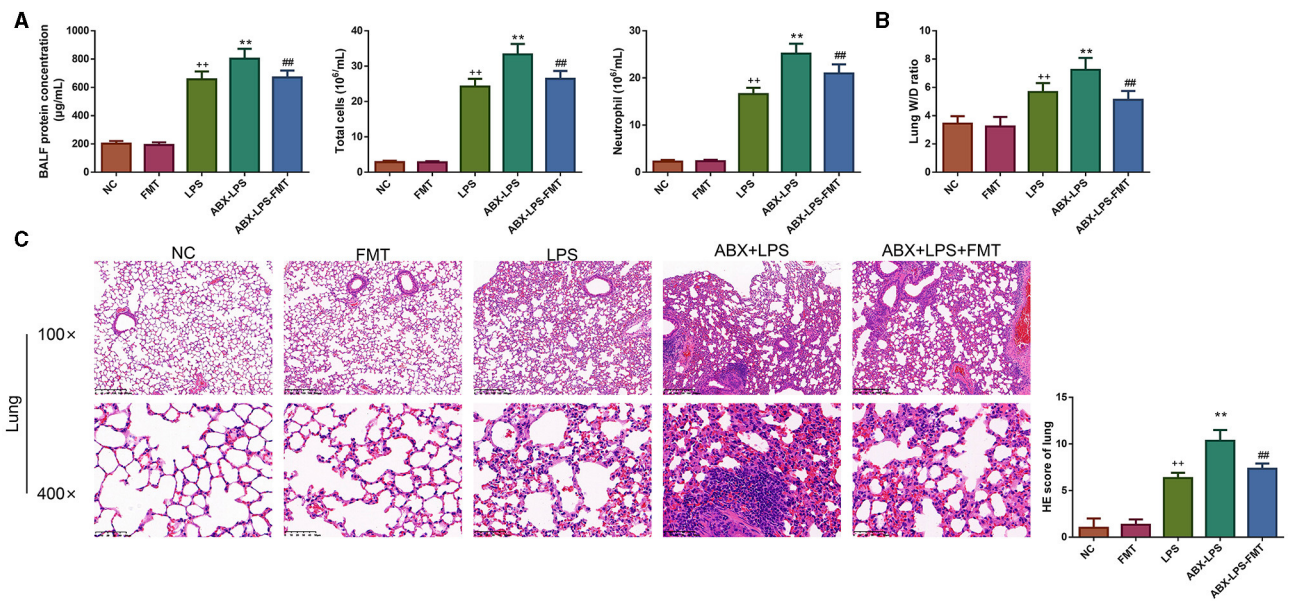


FIGURE 2
FMT from HUC-MSC-treated mice decreased BALF inflammatory cell counts and attenuated lung injury in ALI mice. Following FMT treatment, the protein concentration, total cells, and neutrophils were detected in the BALF **(A)**, and the W/D ratio in the lung was measured **(B)**, moreover, the pathological changes of the lung tissues were observed by HE staining **(C)**. The magnification for HE staining was 100× and 400×. BALF analysis and HE staining for the lung tissues were repeated three times, and the W/D ratio in the lung was detected once for every mouse. $^{++}p < 0.01$ vs. NC, $^{**}p < 0.01$ vs. LPS, $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD. HUC-MSCs, human umbilical cord mesenchymal stromal cells; BALF, bronchoalveolar lavage fluid; ALI, acute lung injury; W/D, wet to dry; HE, hematoxylin and eosin.

ab211737, Abcam, China), and anti-occludin (1:200, ab216327, Abcam, UK). The next day, the slices were washed and then reacted with secondary antibodies, stained with diaminobenzidine and hematoxylin, and finally examined under a microscope.

2.13. Western blot analysis

The total protein was isolated from lung and ileal tissue homogenate by Radioimmunoprecipitation assay buffer. Then, bicinchoninic acid kits were applied to detect protein concentration. Afterward, protein extracts were separated by SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After shaking softly in blocking solution, the membranes were incubated with primary antibodies overnight at 4°C. The following day, the membranes were washed and incubated again with secondary antibodies. After the blots were visualized using enhanced chemiluminescence, data were analyzed by ImageJ software. All information on the primary antibodies is presented in Table 2.

2.14. 16S rRNA gene amplicon sequencing

DNA extraction and 16S rRNA gene sequencing of fecal samples were conducted with the help of BioNovoGene Co., Ltd. (China). In short, the total microbial DNA was extracted from the feces and then quantified by Nanodrop. Agarose gel electrophoresis (1.2%) was utilized to evaluate the quality of extracted DNA. After completing 16S rRNA amplification by PCR, purification, and recovery by magnetic beads, the products were subjected to fluorescence quantitative analysis. Subsequently, the TruSeq Nano DNA LT Library Prep Kit provided by Illumina was employed to construct sequencing libraries.

Raw sequence reads were trimmed and filtered into effective reads, which were further clustered into operational taxonomic units (OTUs) with a similarity >97% by UPARSE. The taxonomy of OTU was carried out by the SILVA database. In addition, the α-diversity of microbiota was analyzed by Shannon and Simpson indices, and the β-diversity of microbiota was assessed by weighted UniFrac distance and visualized with non-metric multidimensional scaling (NMDS) plot and analysis of similarities (ANOSIM) test. Additionally, linear discriminant analysis effect size was applied to taxonomic discovery analysis for microbial biomarker discovery.

2.15. Statistical analysis

The data were analyzed by SPSS 19.0 and presented as mean ± SD. Differences between multiple groups were compared by analysis of variance (ANOVA) and Tukey's tests. The Kruskal–Wallis *H*-test was applied when variances were not homogeneous. $p < 0.05$ was considered statistically significant. R software was used to perform amplicon sequencing statistical analysis. Furthermore, the Wilcoxon test was utilized to calculate the microbial α-diversity index, while the ANOSIM non-parametric test was conducted to compare microbial β-diversity between the two groups.

10.3389/fmicb.2023.1243102

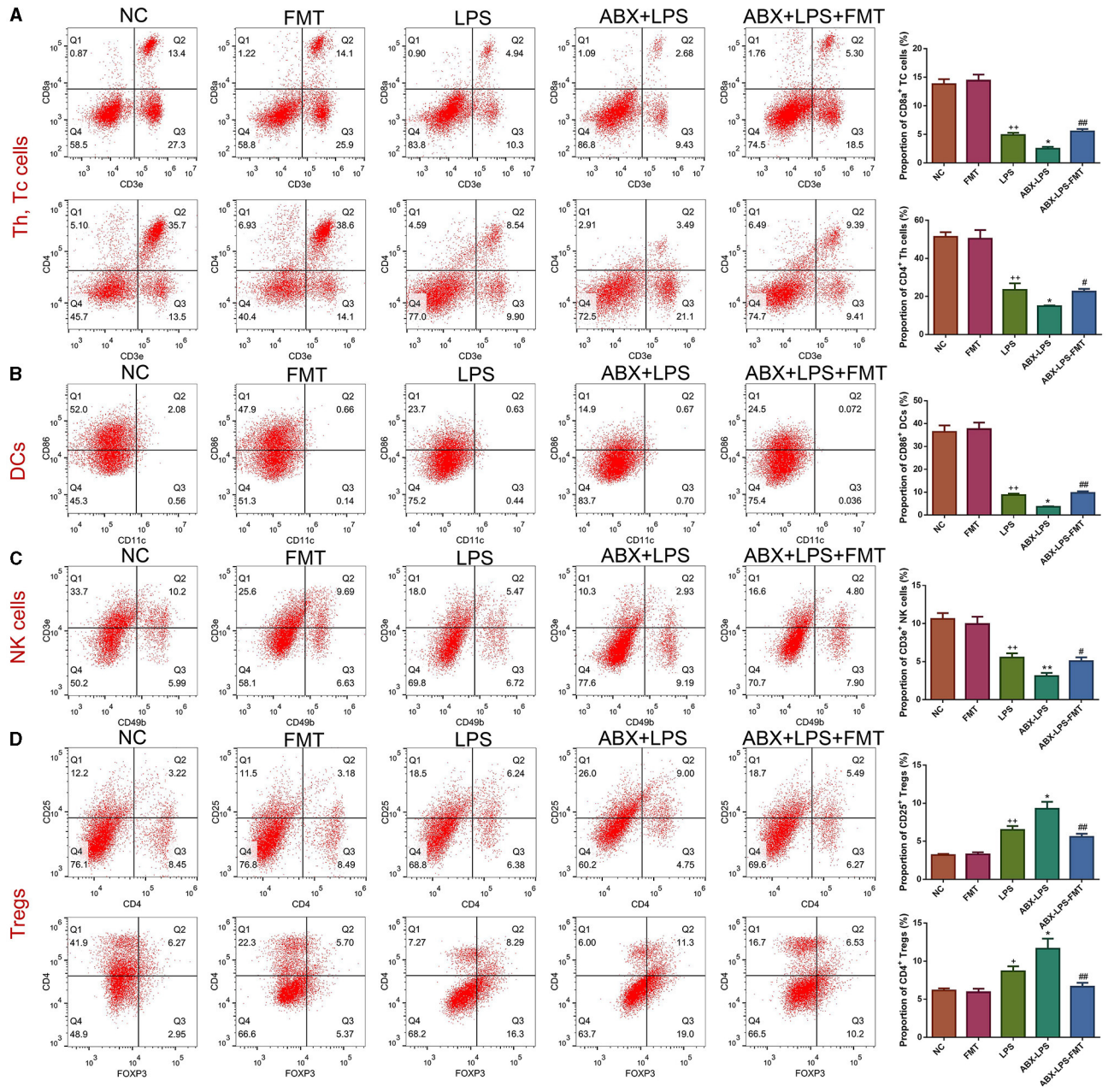


FIGURE 3
FMT from HUC-MSC-treated mice restored the immune system of the peripheral blood in ALI mice. After treatment with FMT, Th and Tc cells **(A)**, DCs **(B)**, NK cells **(C)**, and Tregs **(D)** in the peripheral blood were evaluated by flow cytometry. Peripheral blood immune cell analysis was repeated three times. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. NC, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD.

3. Results

3.1. FMT from HUC-MSC-treated mice counteracted the upregulation of BALF inflammatory cell counts and alleviated lung injury in ALI mice

As shown in Figure 2A, LPS treatment leads to an obvious upregulation of the concentrations of total protein, the counts of total cells, and neutrophils in BALF of ALI rats ($p < 0.01$). Consistent with this, after mice were exposed to LPS, the lung W/D ratio increased clearly ($p < 0.01$), and the lung exhibited serious injury (Figures 2B, C); specifically, the remarkable thickening of the alveolar septum and wall was observed. In addition, from the results of HE staining, we also observed pulmonary capillary edema and congestion in LPS mice. When LPS mice were pre-treated with ABX, all the phenomena described were more pronounced ($p < 0.01$). However, FMT treatment effectively reversed the increase of total protein, total cells, and neutrophils in BALF, reduced the W/D ratio in the lung, and improved lung injury induced by LPS and ABX; the alveolar septa were thinning, and inflammatory cell infiltration was alleviated ($p < 0.01$).

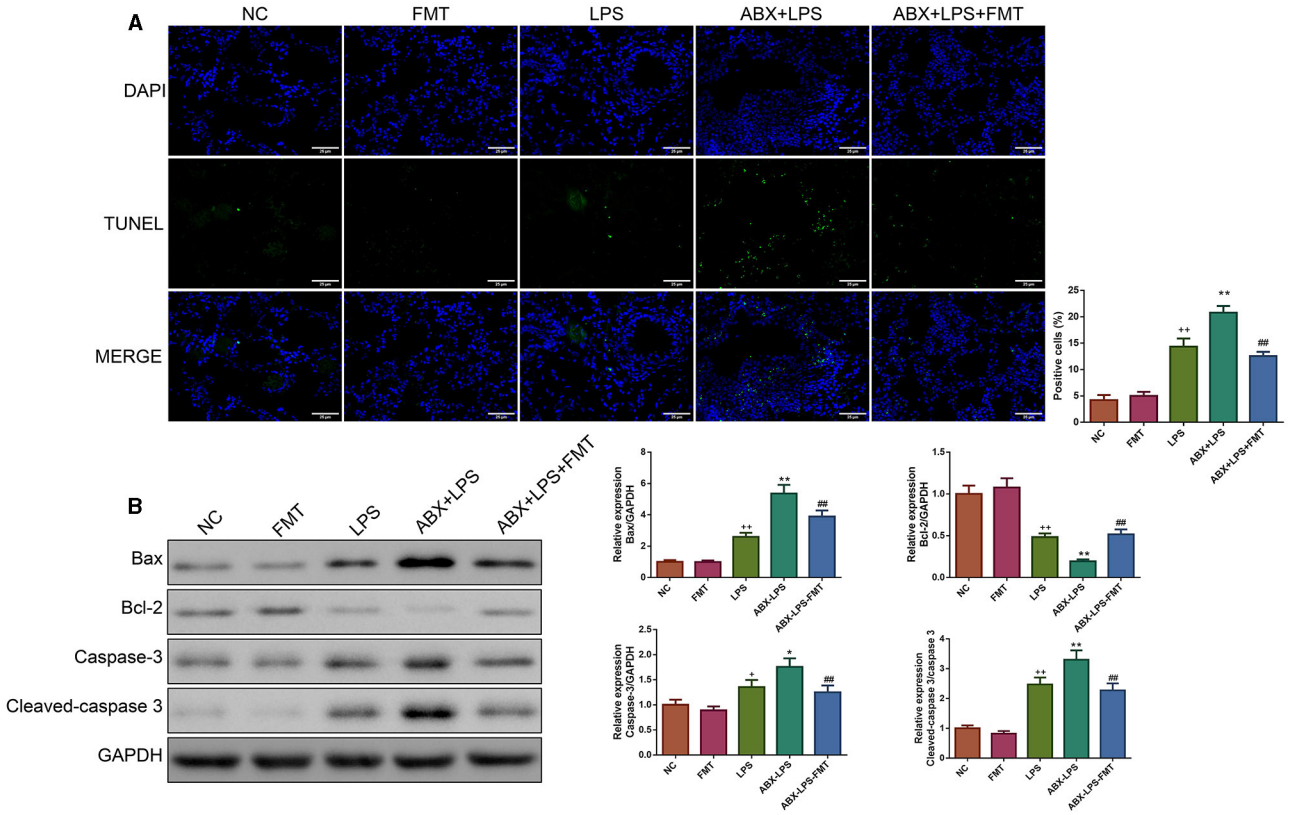


FIGURE 4
FMT from HUC-MSC-treated mice reduced the apoptosis of the lung tissues in ALI mice. After FMT, the apoptosis of the lung tissues was evaluated by TUNEL staining with the magnification of ×400 **(A)**, and the expressions of apoptosis-associated protein (including Bax, Bcl-2, caspase-3, and cleaved-caspase-3) in the lung tissues were tested by Western blot **(B)**. TUNEL and Western blot analyses for the lung tissues were repeated three times. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. NC, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS, $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD. TUNEL, Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling.

3.2. FMT from HUC-MSC-treated mice restored immune cell number in the peripheral blood of ALI mice

Given that innate immune cells are crucial in ALI development, the percentage of immune cells was detected in the study. Flow cytometry results revealed that LPS exposure caused a significant increase in the percentages of $CD4^+$ Th, $CD8a^+$ Tc, $CD86^+$ DC, and $CD3e^+$ NK cells but blocked the percentages of $CD25^+$ Tregs and $CD4^+$ Tregs cells ($p < 0.05$, Figure 3). Interestingly, ABX pre-treatment worsened this situation ($p < 0.05$). As expected, it can be observed from Figure 3 that FMT treatment moderately attenuated ABX- and LPS-stimulated immune cell changes ($p < 0.05$).

3.3. FMT from HUC-MSC-treated mice blunted the apoptosis of the lung tissue in ALI mice

The percentage of apoptotic cells was raised remarkably in the lung tissues of LPS-exposed mice ($p < 0.01$, Figure 4A). Accordingly, ABX pre-treatment deteriorated the apoptosis of the lung tissues in LPS mice ($p < 0.01$). Nevertheless, FMT treatment effectively counteracted the upregulation of apoptosis in the lung tissues after ABX and LPS stimulation ($p < 0.01$). The apoptosis-related proteins in the lung tissues were utilized to verify the extent of apoptosis. Unanimously, we found that LPS and ABX increase the Bax, caspase-3, and cleaved-caspase-3 protein expressions and decrease the Bcl-2 protein expression ($p < 0.05$, Figure 4B); however, those changes were offset by the FMT treatment ($p < 0.01$).

3.4. FMT from HUC-MSC-treated mice prevented oxidative stress and inflammation response in ALI mice

Next, the effect of FMT from HUC-MSC-treated mice on the inhibition of oxidative stress and inflammation response was determined. As shown in Figure 5A, the lung tissues of LPS-stimulated mice had higher MDA and MPO levels and lower GSH and SOD levels than normal control mice ($p < 0.05$). In addition, ELISA results also revealed that the LPS challenge led to serious inflammation in the BALF, serum, lung, and ileal tissues (Figure 5B). ABX pre-treatment further worsened those changes. However, ABX- and LPS-induced increases of MDA, MPO, IL-1β,

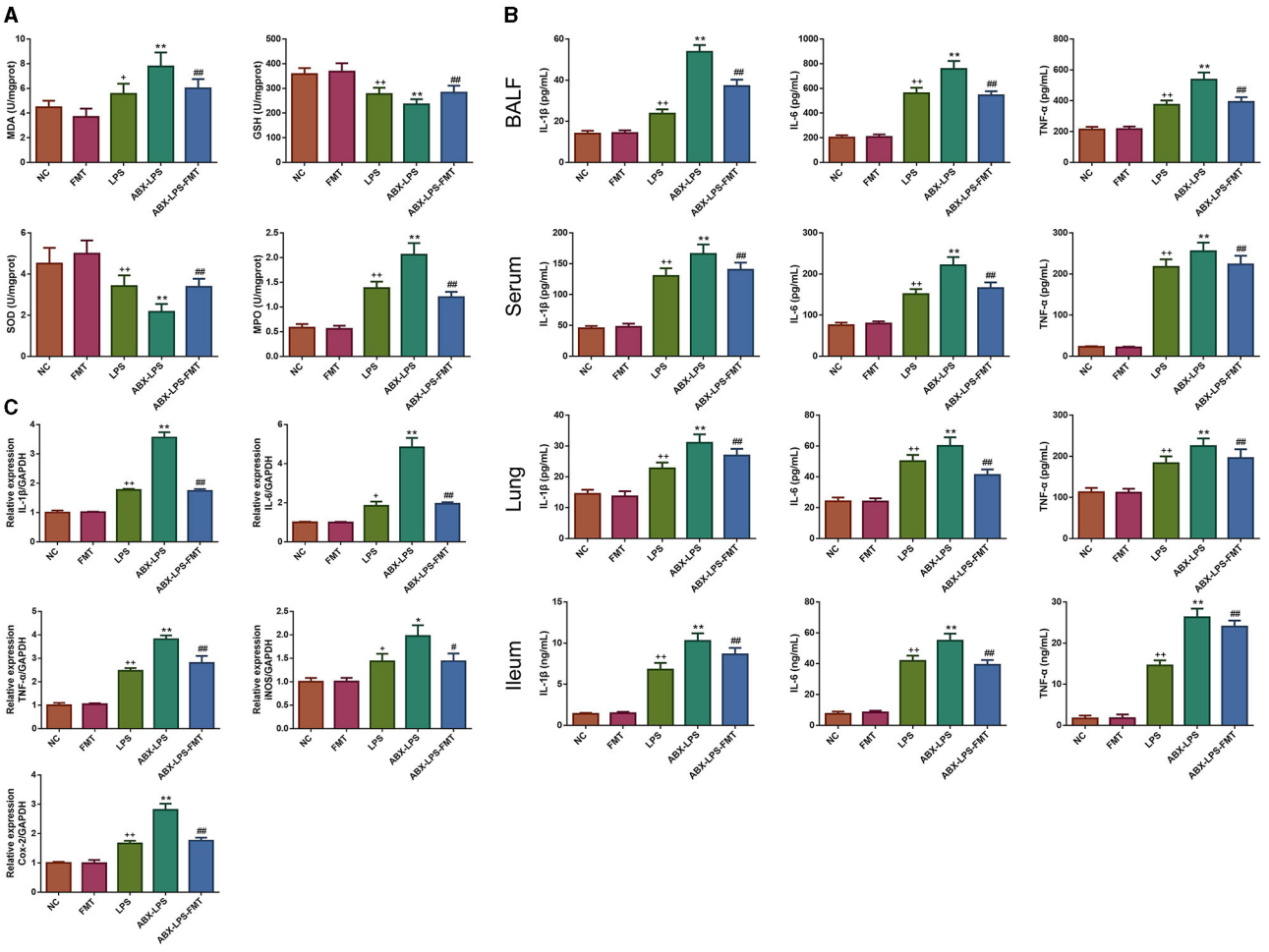


FIGURE 5
FMT from HUC-MSC-treated mice prevented oxidative stress and inflammation in ALI mice. Upon FMT treatment, the levels of MDA, GSH, SOD, and MPO were measured in the lung tissues **(A)**. In addition, the contents of IL-1β, IL-6, and TNF-a in the BALF, serum, lung, and ileal tissues were assessed by ELISA **(B)**, the expressions of IL-1β, IL-6, TNF-a, iNOS, and Cox-2 mRNAs in the lung tissues were detected using qPCR **(C)**. MDA, GSH, SOD, and MPO levels in the lung tissues and ELISA assay for the BALF, serum, and lung were measured once for every mouse; in addition, qPCR analysis for the lung tissues was repeated three times. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. NC, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD. ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative PCR.

IL-6, and TNF-α and reduction of GSH and SOD were effectively restored by FMT treatment ($p < 0.01$). Then, qPCR was conducted to further assess the antioxidant and anti-inflammatory effects of FMT treatment. It can be observed from Figure 5C that IL-1β, IL-6, TNF-α, iNOS, and Cox-2 mRNA expressions were significantly raised in the LPS and ABX-LPS groups ($p < 0.05$), but upon FMT treatment, the upregulation of these mRNA was effectively relieved ($p < 0.05$).

3.5. FMT from HUC-MSC-treated mice improved the barrier function and inhibited the apoptosis of ileal tissues in ALI mice

It is well-known that injured ileal tissues and impaired barrier function contribute to the development of ALI (Xu Y. et al., 2021). Thus, we checked whether FMT from HUC-MSC-treated mice could alleviate the injured degree, inhibit apoptosis, and improve the impaired barrier function for the ileal tissues. It can be observed that after stimulation by LPS, the ileal tissues appeared with obvious injury (Figure 6A). Accordingly, the situation was more serious in the ABX-LPS group. As expected, FMT treatment attenuated ABX- and LPS-induced ileal tissue injury. In addition, following LPS stimulation, Bax protein expression was increased, while Bcl-2 protein expression was decreased ($p < 0.01$), the situation further deteriorated in the ABX + LPS group ($p < 0.01$, Figure 6B). However, FMT from HUC-MSC-treated mice decreased Bax protein expression but increased Bcl-2 protein expression ($p < 0.01$). The expressions of ZO-1, claudin-1, and occludin are crucial for maintaining barrier function (Shi et al., 2020). The results of immunohistochemical staining revealed that ZO-1, claudin-1, and occludin protein expressions were obviously decreased upon ABX and LPS stimulation ($p < 0.05$, Figure 6C). Of note, FMT treatment elevated the decrease in ZO-1, claudin-1, and occludin protein expressions ($p < 0.05$). As expected, the Western blot results exhibited a similar trend as the results of the immunohistochemical staining (Figure 6D).

10.3389/fmicb.2023.1243102

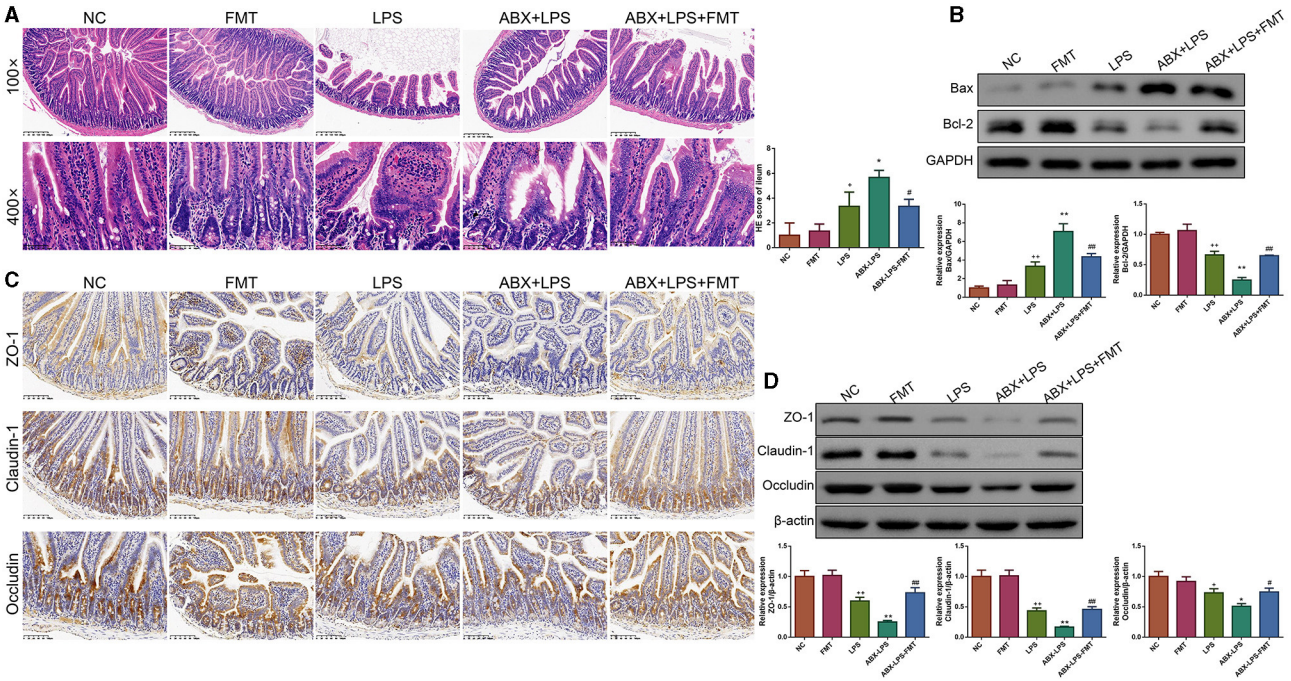


FIGURE 6
FMT from HUC-MSC-treated mice restored barrier integrity of the ileum tissues in ALI mice. After treatment with FMT, the pathological changes in the ileum tissues were analyzed by HE **(A)**, and the protein expressions of Bax and Bcl-2 in the ileum tissues were measured by Western blot **(B)**, moreover, immunohistochemical staining **(C)** and Western blot **(D)** were employed to measure ZO-1, claudin-1, and occludin protein expressions. The magnification for immunohistochemical staining was 200×, and for HE staining was 100× and 400×. HE staining, Western blot, and immunohistochemical staining for the ileum tissues were repeated three times. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. NC, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD.

3.6. FMT from HUC-MSC-treated mice restored the TLR4/NF-κb and Nrf2/HO-1 pathways in ALI mice

LPS has been found to play pro-oxidative and pro-inflammatory roles by the TLR4/NF-κB and Nrf2/HO-1 pathways (Xin et al., 2020). Therefore, the impact of FMT from HUC-MSC-treated mice on the TLR4/NF-κB and Nrf2/HO-1 pathways was tested. Immunohistochemical staining and Western blot analysis demonstrated that ABX and LPS treatments raised the protein expressions of TLR4, Myd88, COX-2, and iNOS, as well as the phosphorylation of NF-κB and IκBα in the lung tissues of mice, but decreased the protein expressions of Nrf2 and HO-1 ($p < 0.05$, Figure 7). Notably, FMT treatment restored the expressions and phosphorylation of TLR4/NF-κB and Nrf2/HO-1 pathway-related proteins in the lung tissues from ABX- and LPS-treated mice ($p < 0.05$). The results obtained from the ileal tissues were similar to those from the lung tissues (Figure 8). The mechanistic diagram is presented in Figure 9.

3.7. FMT from HUC-MSC-treated mice alleviated the dysbiosis of GM in ALI mice

Further sequencing of the 16S rRNA gene was applied to detect the microbial profiles of the fecal samples from mice in the NC, FMT, LPS, and ABX-LPS + FMT groups. The top 20 bacterial genera are presented in Figure 10A in terms of the relative abundance. In addition, microbial α-diversity analyzed by Shannon and Simpson indices revealed that the community diversity of microbe in the LPS group was higher than those of the NC and FMT groups, despite the difference was not significant (Figure 10B). However, the community diversity of microbe from ABX-LPS + FMT mice was effectively decreased ($p < 0.01$). Additionally, microbial β-diversity was also analyzed by NMDS and ANOSIM, and the results revealed that the microbial community structure of ABX-LPS + FMT mice changed obviously (Figures 10C, D). Then, we further screen the potential GM change by FMT treatment in LPS mice. LEfSe analysis was conducted to detect the bacterial taxa that differed significantly among the groups (Figures 10E, F). Specifically, at the genus level, we found that relative to the NC group, the relative abundances of *Bacteroides*, *Christensenella*, *Coprococcus,* and *Roseburia* were significantly elevated in the LPS group. However, the relative abundances of *Bacteroides*, *Christensenella*, *Coprococcus,* and *Roseburia* were decreased obviously in the ABX-LPS + FMT group. Furthermore, the relative abundances of *Xenorhabdus*, *Sutterella,* and *Acinetobacter* obviously increased in the ABX-LPS + FMT group (Figure 10G).

4. Discussion

Despite immense efforts have been made and substantial achievements have been achieved to develop new therapies for ALI, effective treatments for ALI continue to be limited (Wang et al., 2021). Recently, scholars have demonstrated that MSCs

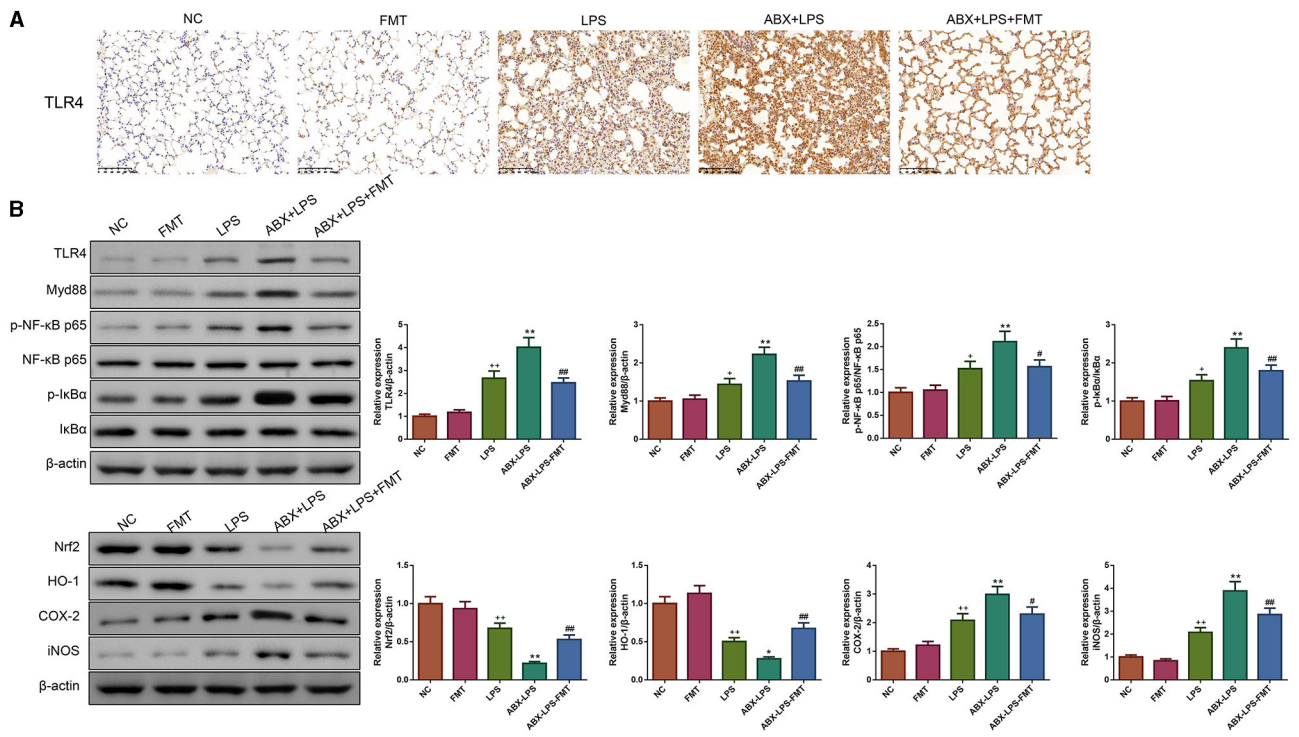


FIGURE 7
FMT from HUC-MSC-treated mice recover the TLR4/NF-κB and Nrf2/HO-1 pathways for the lung tissues of ALI mice. Immunohistochemical staining was conducted to detect the expression of TLR4 in the lung tissues with the magnification of ×200 **(A)**, and Western blot was performed to detect the protein expressions of TLR4, Myd88, Nrf2, HO-1, COX-2, and iNOS and the phosphorylation of NF-κB and IκBα in the lung tissues **(B)**. Immunohistochemical staining and Western blot for the lung tissues were repeated three times. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. NC, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD.

have excellent immunomodulatory functions in numerous lung disorders, including ALI (Peng W. et al., 2021). A published study has revealed that MSCs derived from the bone marrow, chorion, and lung can manage LPS-induced ALI by suppressing inflammatory responses and increasing the Tregs/Th17 cell ratio (Wang L. et al., 2022). In addition, our previous study has also proven that HUC-MSCs can be used to treat ALI effectively by redefining gut microbiota. At the same time, researchers have demonstrated the composition, structure, and distribution of the intestinal flora were remarkably different between the ALI and normal mice (Li et al., 2014). However, elevating short-chain fatty acid generation and remodeling intestinal flora disorder can alleviate LPS effectively (Peng L.-Y. et al., 2021). Given that FMT is an effective method to improve the altered GM diversity and exhibited more cost-effectiveness than antimicrobial treatment in some cases (Hu et al., 2019), we hypothesized that FMT from HUC-MSC-treated mice may have the potential to treat ALI.

Using appropriate animal models is crucial for studying the effects and mechanisms of drugs for diseases. Currently, LPS-induced ALI has become the most common and reliable animal model to test the potential therapies for ALI (de Souza Xavier Costa et al., 2017). It is well-known that intratracheal injection of LPS will induce the generation of inflammatory factors and stimulate the neutrophils to migrate to the lung, thereby inducing systemic inflammation and lung damage (Li et al., 2021). In this study, the BALF protein concentration, total cell number, neutrophil number, and lung W/D ratio increased obviously after LPS administration, which implied that lung damage happened in mice. The pathological injuries of the lung further demonstrated it. Nevertheless, all the changes induced by LPS were restored by FMT treatment, which indicated that FMT from HUC-MSC-treated mice can ameliorate LPS-stimulated ALI.

A body of evidence has revealed that inflammation is the key mechanism for ALI pathogenesis (Chen et al., 2022). Specifically, inflammatory responses can increase alveolar capillary permeability, which facilitates the leak of fluid, thereby promoting the formation of lung edema (Zhao and Bie, 2020). Additionally, inflammation-induced oxidative stress also plays a key function in the development of ALI (Du et al., 2021). Under a healthy condition, the scavenging and generation of reactive oxygen species (ROS) are balanced in the body; however, inflammation will disrupt this balance and stimulate the production of ROS (Lv et al., 2016). Excessive ROS will attack polyunsaturated fatty acids, generating lipid peroxides (such as MDA) and leading to the injury of tissues (Huang et al., 2016). On the other hand, excessive ROS also contributes to the release of inflammatory factors in turn, thus entering a vicious cycle (Wang et al., 2019). This study found that inflammation and oxidative stress were aggravated in response to LPS treatment, which aligned with the symptoms observed in ALI patients (Gong et al., 2023). However, FMT treatment could effectively inhibit the inflammatory response and oxidative stress stimulated by LPS, suggesting that FMT

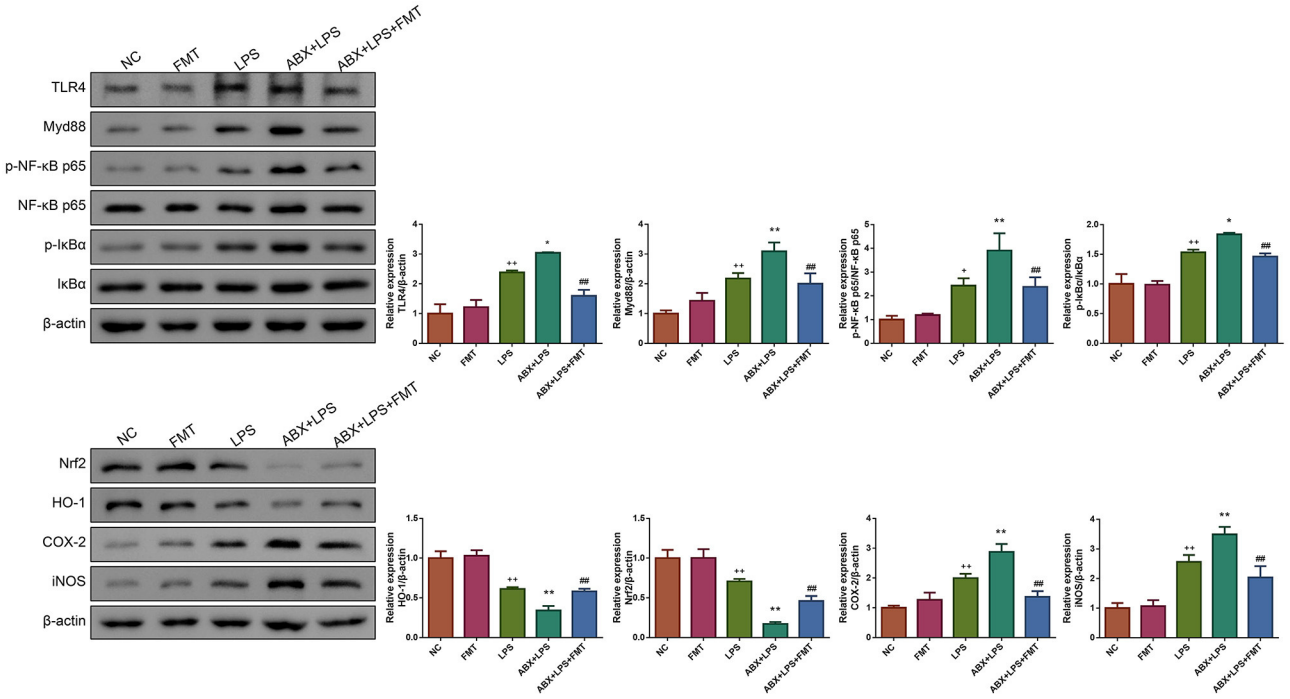


FIGURE 8
FMT from HUC-MSC-treated mice recovers the TLR4/NF-κB and Nrf2/HO-1 pathways for the ileum tissues of ALI mice. Western blot was performed to detect the protein expressions of TLR4, Myd88, Nrf2, HO-1, COX-2, and iNOS and the phosphorylation of NF-κB and IκBα in the ileum tissues. Western blot for the ileum tissues was repeated three times. $^{+}p < 0.05$ and $^{++}p < 0.01$ vs. NC, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. LPS, $^{\#\#}p < 0.01$ vs. ABX + LPS. The results were presented as mean ± SD.

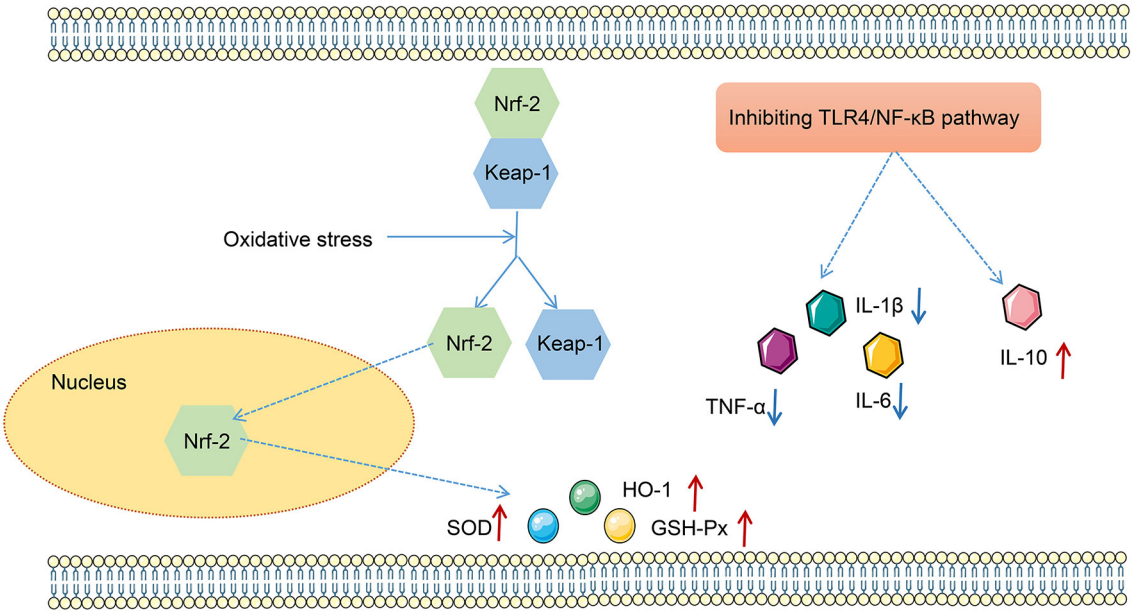


FIGURE 9
The mechanism map of effects of the TLR4/NF-κB and Nrf2/HO-1 pathways on oxidative stress and inflammation.

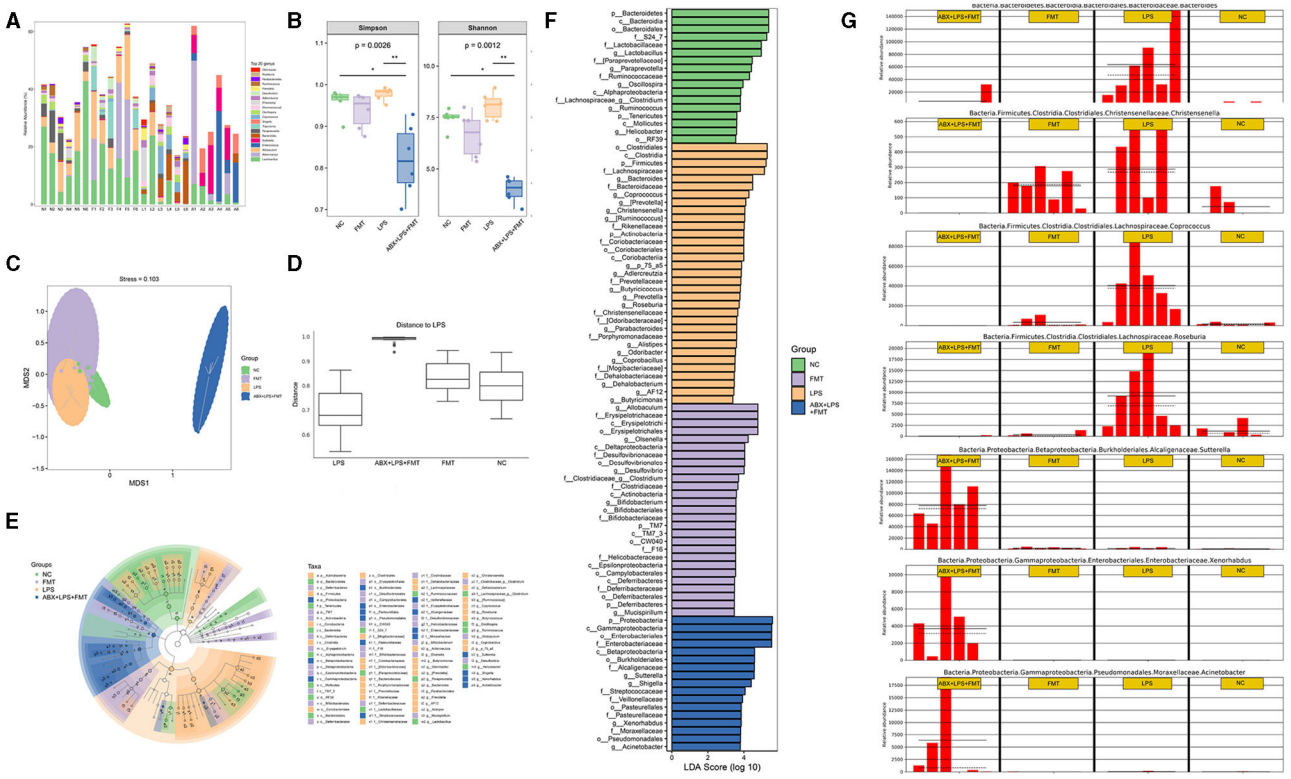


FIGURE 10
FMT from HUC-MSC-treated mice alleviated the disorder of GM in ALI mice. After FMT treatment, the top 20 bacterial genera with the highest relative abundance among the groups were tested **(A)**. The microbial α-diversity was analyzed by Shannon and Simpson indices **(B)**. The microbial β-diversity was analyzed by NMDS **(C)** and ANOSIM **(D)**. LEfSe was carried out to discriminate the different taxa from the fecal samples of the mice **(E, F)**. **(G)** The relative abundance of *Bacteroides*, *Christensenella*, *Coprococcus*, *Roseburia*, *Xenorhabdus*, *Sutterella*, and *Acinetobacter* was compared between the groups. In total, 24 fecal samples from 4 groups were applied for 16S rRNA gene amplicon sequencing. *$p < 0.05$ and **$p < 0.01$. GM, gut microbiota; NMDS, non-metric multidimensional scaling; ANOSIM, analysis of similarities; LEfSe, linear discriminant analysis effect size.

from HUC-MSC-treated mice may alleviate ALI by repressing inflammatory response.

TLR4 is thought to be the specific receptor for LPS in mammals and is essential in the development of inflammatory response, while the NF-κB pathway plays a central role in the modulation of inflammatory response (Yang W. et al., 2022). Activation of the NF-κB pathway promotes the excessive release of pro-inflammatory factors (TNF-α, IL-6, IL-1β, and so on) but inhibits the secretion of anti-inflammatory factors (interleukin 10); the imbalance between pro-inflammatory and anti-inflammatory factors will result in ALI (Han et al., 2019). Previous research by Li et al. (2016) has reported that MSCs obtained from the bone marrow have the potential to treat ALI by suppressing inflammation by downregulating the TLR2 and 4/NF-κB pathways. In addition, when the body suffers from inflammation and oxidative stress, Nrf2, a basic leucine zipper protein, will modulate the expression of proteins that participate in protection from oxidative injuries (Lei et al., 2020). In normal physiological conditions, Nrf2 exists in the cytoplasm by binding with Keap-1 (Zhang Z. et al., 2022). However, when subjected to oxidative stress, Nrf2 will dissociate from Keap-1, combined with ARE, and subsequently transported to the nucleus to induce the expressions of target genes (such as HO-1, GSH-Px, and SOD), thereby protecting cells from oxidative stress (Liberti et al., 2020). Drugs that activate the Nrf2/HO-1 pathway have been studied for treating diseases caused by oxidative stress (Xu C. et al., 2021). It has already been demonstrated that MSCs can activate HO-1 and Nrf2 and suppress inflammation, cell apoptosis, and oxidative stress in the lung tissues, thereby improving ALI (Zhang et al., 2019b). In addition, another study has revealed that pre-treating HUC-MSCs with pyrogallol improves LPS-stimulated lung injury and inflammation by activating the Nrf2/HO-1 pathway (Zhang Y. et al., 2022). Similarly, this research found that FMT from HUC-MSC-treated mice inhibited the TLR4/NF-κB pathway and activated the Nrf2/HO-1 pathway, which might explain the improving mechanism of FMT on ALI.

The intestinal barrier is pivotal in sustaining intestinal balance, and impairment of its function may result in the displacement of harmful intestinal substances to the circulatory system (Wang R. et al., 2022). The expressions of ZO-1, claudins, and occludin in the ileum are essential in modulating intestinal permeability (Li L. et al., 2023). In addition, it is widely accepted by researchers that injured ileal tissues and impaired barrier function are involved in ALI development (Xu Y. et al., 2021). In the study, we found that FMT from HUC-MSC-treated mice could improve the impaired degree of the ileum and upregulated the expressions of ZO-1, claudins, and occludin proteins in the ileum, suggesting that FMT from

HUC-MSC-treated mice can improve intestinal barrier function for ALI mice.

The composition of GM can also influence the development of ALI (Kosyreva et al., 2020). By alerting the composition and metabolisms of GM and modulating the balance between beneficial and harmful bacteria, the injured lung can be alleviated and lung immunity can be enhanced (Liu et al., 2020a). *Bacteroides* is a microbe recognized as a potential mucus degrader; research has reported that pulmonary *Bacteroides* are negatively associated with lung function (Yang Y.-S. H. et al., 2022). Additionally, *Christensenella* dominates the lungs of patients with idiopathic pulmonary fibrosis or lung cancer, while the *Roseburia* genus was elevated in the fecal sample of non-small cell lung cancer patients (D'Alessandro-Gabazza et al., 2018). Moreover, inhibiting the relative abundance of *Coprococcus* in the gut can improve immune function and lung function (Jia et al., 2022). It is well-known that *Xenorhabdus* can produce various antimicrobial compounds (Dreyer et al., 2019), and the abundance of *Sutterella* is negatively correlated with ileal-pouch inflammation (Olaisen et al., 2021). In the present study, *Bacteroides*, *Christensenella*, *Roseburia,* and *Coprococcus* were reduced, while *Xenorhabdus*, *Sutterella,* and *Acinetobacter* were increased after FMT treatment, which confirmed that FMT from HUC-MSC-treated mice alleviated LPS-induced ALI by regulating the relative abundance of specific GM. In future, we will gavage *Xenorhabdus*, *Sutterella,* and *Acinetobacter* to ALI mice, so as to further investigate whether these GM can ameliorate ALI and explore the specific mechanism.

However, the main limitation of the study is the absence of a control group, i.e., letting the mice in the ABX + LPS group receive FMT from healthy mice. In future, we will treat ABX + LPS mice with FMT from healthy mice to make the key funding of the study more convincing.

In summary, this study demonstrated that FMT from HUC-MSC-treated mice may improve ALI by inhibiting inflammation and modulating GM abundance (Figure 9). Additionally, the TLR4/NF-KB and Nrf2/HO-1 pathways may also take part in the improvement of FMT on ALI, which needed further exploration by using corresponding agonist and inhibitor. These discoveries provided powerful evidence for the protective function of FMT from HUC-MSC-treated mice on ALI.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information (NCBI) BioProject database, accession number PRJNA1016527.

Ethics statement

All animal related experiments were carried out with the evaluation and supervision of the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center [certificate No. SYXK (Zhe) 2021-0033] and in compliance with the guidelines of the Institutional Animal Care and Use Committee.

Author contributions

FH, EC, and BW: conceptualization and writing—reviewing and editing. EC, LLv, BW, LLi, HL, NC, and WC: data curation. EC and LLv: writing—original draft preparation. EC and BW: funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the Key Research and Development Project of the Science and Technology Department of Zhejiang Province [grant number 2019C03041], and the key project of Huzhou city [grant number 2023GZ84].

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1243102/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The microflora homeostasis in feces of mice treated with or without HUC-MSCs. After HUC-MSC treatment, the top 20 bacterial families with the highest relative abundance among the groups were tested **(A)**. The microbial α-diversity **(B)** and β-diversity **(C)** were statistically analyzed in the feces of different groups. The heatmap was applied to analyze the differences in the genus abundance of different microflora in feces **(D)**. $n = 6$. Note: HUC-MSCs, human umbilical cord mesenchymal stromal cells.

References

Bi, X.-G., Li, M.-L., Xu, W., You, J.-Y., Xie, D., Yuan, X.-F., et al. (2020). Helix B surface peptide protects against acute lung injury through reducing oxidative stress and endoplasmic reticulum stress via activation of Nrf2/HO-1 signaling pathway. *Eur. Rev. Med. Pharmacol. Sci.* 24, 6919–6930. doi: 10.26355/eurrev_202006_21683

Chen, Y.-B., Zhang, Y.-B., Wang, Y.-L., Kaur, P., Yang, B.-G., Zhu, Y., et al. (2022). A novel inhalable quercetin-alginate nanogel as a promising therapy for acute lung injury. *J. Nanobiotechnol.* 20, 272. doi: 10.1186/s12951-022-01452-3

Costa, S. G., Barioni, É. D., Ignácio, A., Albuquerque, J., Câmara, N. O. S., Pavani, C., et al. (2017). Beneficial effects of red light-emitting diode treatment in experimental model of acute lung injury induced by sepsis. *Sci. Rep.* 7, 12670. doi: 10.1038/s41598-017-13117-5

D'Alessandro-Gabazza, C. N., Méndez-García, C., Hataji, O., Westergaard, S., Watanabe, F., Yasuma, T., et al. (2018). Identification of halophilic microbes in lung fibrotic tissue by oligotyping. *Front. Microbiol.* 9, 1892. doi: 10.3389/fmicb.2018.01892

de Souza Xavier Costa, N., Júnior, G. R., Alemany, A. A. D., Belotti, L., Zati, D. H., Frota Cavalcante, M., et al. (2017). Early and late pulmonary effects of nebulized LPS in mice: an acute lung injury model. *PLoS ONE* 12, e0185474. doi: 10.1371/journal.pone.0185474

Dreyer, J., Rautenbach, M., Booysen, E., van Staden, A. D., Deane, S. M., and Dicks, L. M. T. (2019). *Xenorhabdus khoisanae* SB10 produces Lys-rich PAX lipopeptides and a xenocoumacin in its antimicrobial complex. *BMC Microbiol.* 19, 132. doi: 10.1186/s12866-019-1503-x

Du, J., Wang, G., Luo, H., Liu, N., and Xie, J. (2021). JNK-IN-8 treatment alleviates lipopolysaccharide-induced acute lung injury via suppression of inflammation and oxidative stress regulated by JNK/NF-κB signaling. *Mol. Med. Rep.* 23, 150. doi: 10.3892/mmr.2020.11789

Gong, L., Shen, Y., Wang, S., Wang, X., Ji, H., Wu, X., et al. (2023). Nuclear SPHK2/S1P induces oxidative stress and NLRP3 inflammasome activation via promoting p53 acetylation in lipopolysaccharide-induced acute lung injury. *Cell Death Discov.* 9, 12. doi: 10.1038/s41420-023-01320-5

Han, C.-J., Zheng, J.-Y., Sun, L., Yang, H.-C., Cao, Z.-Q., Zhang, X.-H., et al. (2019). The oncometabolite 2-hydroxyglutarate inhibits microglial activation via the AMPK/mTOR/NF-κB pathway. *Acta Pharmacol. Sin.* 40, 1292–1302. doi: 10.1038/s41401-019-0225-9

Hu, Y., Xiao, H.-Y., He, C., Lv, N.-H., and Zhu, L. (2019). Clostridium difficileFecal microbiota transplantation as an effective initial therapy for pancreatitis complicated with severe infection: a case report. *World J. Clin. Cases* 7, 2597–2604. doi: 10.12998/wjcc.v7.i17.2597

Huang, J., Li, L., Yuan, W., Zheng, L., Guo, Z., and Huang, W. (2016). κNEMO-binding domain peptide attenuates lipopolysaccharide-induced acute lung injury by inhibiting the NF-B signaling pathway. *Mediators Inflamm.* 2016, 7349603. doi: 10.1155/2016/7349603

Jia, L., Hao, H., Wang, C., and Wei, J. (2021). Etomidate attenuates hyperoxia-induced acute lung injury in mice by modulating the Nrf2/HO-1 signaling pathway. *Exp. Ther. Med.* 22, 785. doi: 10.3892/etm.2021.10217

Jia, Y., He, T., Wu, D., Tong, J., Zhu, J., Li, Z., et al. (2022). The treatment of Qibai Pingfei Capsule on chronic obstructive pulmonary disease may be mediated by Th17/Treg balance and gut-lung axis microbiota. *J. Transl. Med.* 20, 281. doi: 10.1186/s12967-022-03481-w

Jiang, Y., Xia, M., Xu, J., Huang, Q., Dai, Z., and Zhang, X. (2021). Dexmedetomidine alleviates pulmonary edema through the epithelial sodium channel (ENaC) via the PI3K/Akt/Nedd4-2 pathway in LPS-induced acute lung injury. *Immunol. Res.* 69, 162–175. doi: 10.1007/s12026-021-09176-6

Kapur, R., Kim, M., Rebetz, J., Hallström, B., Björkman, J. T., Takabe-French, A., et al. (2018). Gastrointestinal microbiota contributes to the development of murine transfusion-related acute lung injury. *Blood Adv.* 2, 1651–1663. doi: 10.1182/bloodadvances.2018018903

Kosyreva, A. M., Dzhalilova, D. S., Makarova, O. V., Tsvetkov, I. S., Zolotova, N. A., Diatroptova, M. A., et al. (2020). Sex differences of inflammatory and immune response in pups of Wistar rats with SIRS. *Sci. Rep.* 10, 15884. doi: 10.1038/s41598-020-72537-y

Le Bastard, Q., Ward, T., Sidiropoulos, D., Hillmann, B. M., Chun, C. L., Sadowsky, M. J., et al. (2018). Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice. *Sci. Rep.* 8, 6219. doi: 10.1038/s41598-018-24342-x

Lei, L., Chai, Y., Lin, H., Chen, C., Zhao, M., Xiong, W., et al. (2020). Dihydroquercetin activates AMPK/Nrf2/HO-1 signaling in macrophages and attenuates inflammation in LPS-induced endotoxemic mice. *Front. Pharmacol.* 11, 662. doi: 10.3389/fphar.2020.00662

Li, B., Yin, G.-F., Wang, Y.-L., Tan, Y.-M., Huang, C.-L., and Fan, X.-M. (2020). Impact of fecal microbiota transplantation on TGF-β1/Smads/ERK signaling pathway of endotoxic acute lung injury in rats. *3 Biotech.* 10, 52. doi: 10.1007/s13205-020-2062-4

Li, D., Pan, X., Zhao, J., Chi, C., Wu, G., Wang, Y., et al. (2016). Bone marrow mesenchymal stem cells suppress acute lung injury induced by lipopolysaccharide through inhibiting the TLR2, 4/NF-κB pathway in rats with multiple trauma. *Shock* 45, 641–646. doi: 10.1097/SHK.0000000000000548

Li, L., Wang, X. M., Feng, X. J., Liu, K., Li, B., Zhu, L. J., et al. (2023). Effects of a macroporous resin extract of *Dendrobium officinale* leaves in rats with hyperuricemia induced by anthropomorphic unhealthy lifestyle. *Evid. Based Complement. Alternat. Med.* 2023, 9990843. doi: 10.1155/2023/9990843

Li, L.-F., Kao, K.-C., Liu, Y.-Y., Lin, C.-W., Chen, N.-H., Lee, C.-S., et al. (2017). Nintedanib reduces ventilation-augmented bleomycin-induced epithelial-mesenchymal transition and lung fibrosis through suppression of the Src pathway. *J. Cell. Mol. Med.* 21, 2937–2949. doi: 10.1111/jcmm.13206

Li, Q., Fu, X., Yuan, J., and Han, S. (2021). Contribution of thrombospondin-1 and−2 to lipopolysaccharide-induced acute respiratory distress syndrome. *Mediators Inflamm.* 2021, 8876484. doi: 10.1155/2021/8876484

Li, Y., Chen, L., Zheng, D., Liu, J.-X., Liu, C., Qi, S.-H., et al. (2023). Echinocystic acid alleviated hypoxic-ischemic brain damage in neonatal mice by activating the PI3K/Akt/Nrf2 signaling pathway. *Front. Pharmacol.* 14, 1103265. doi: 10.3389/fphar.2023.1103265

Li, Y., Liu, X.-Y., Ma, M.-M., Qi, Z.-J., Zhang, X.-Q., Li, Z., et al. (2014). Changes in intestinal microflora in rats with acute respiratory distress syndrome. *World J. Gastroenterol.* 20, 5849–5858. doi: 10.3748/wjg.v20.i19.5849

Liberti, D., Alfieri, M. L., Monti, D. M., Panzella, L., and Napolitano, A. (2020). A melanin-related phenolic polymer with potent photoprotective and antioxidant activities for dermo-cosmetic applications. *Antioxidants* 9, 270. doi: 10.3390/antiox9040270

Liu, T.-H., Zhang, C.-Y., Din, A. U., Li, N., Wang, Q., Yu, J.-Z., et al. (2020a). Bacterial association and comparison between lung and intestine in rats. *Biosci Rep.* 40, BSR20191570. doi: 10.1042/BSR20191570

Liu, T.-Y., Zhao, L.-L., Chen, S.-B., Hou, B.-C., Huang, J., Hong, X., et al. (2020b). Polygonatum sibiricum polysaccharides prevent LPS-induced acute lung injury by inhibiting inflammation via the TLR4/Myd88/NF-κB pathway. *Exp. Ther. Med.* 20, 3733–3739. doi: 10.3892/etm.2020.9097

Lv, H., Yu, Z., Zheng, Y., Wang, L., Qin, X., Cheng, G., et al. (2016). Isovitexin exerts anti-inflammatory and anti-oxidant activities on lipopolysaccharide-induced acute lung injury by inhibiting MAPK and NF-κB and activating HO-1/Nrf2 pathways. *Int. J. Biol. Sci.* 12, 72–86. doi: 10.7150/ijbs.13188

Olaisen, M., Flatberg, A., Granlund, A. V., Røyset, E. S., Martinsen, T. C., Sandvik, A. K., et al. (2021). Bacterial mucosa-associated microbiome in inflamed and proximal noninflamed ileum of patients with Crohn's disease. *Inflamm. Bowel Dis.* 27, 12–24. doi: 10.1093/ibd/izaa107

Peng, L.-Y., Shi, H.-T., Tan, Y.-R., Shen, S.-Y., Yi, P.-F., Shen, H.-Q., et al. (2021). Baicalin inhibits APEC-induced lung injury by regulating gut microbiota and SCFA production. *Food Funct.* 12, 12621–12633. doi: 10.1039/D1FO02407H

Peng, W., Chang, M., Wu, Y., Zhu, W., Tong, L., Zhang, G., et al. (2021). Lyophilized powder of mesenchymal stem cell supernatant attenuates acute lung injury through the IL-6-p-STAT3-p63-JAG2 pathway. *Stem Cell Res. Ther.* 12, 216. doi: 10.1186/s13287-021-02276-y

Shi, L., Xun, W., Peng, W., Hu, H., Cao, T., and Hou, G. (2020). Effect of the single and combined use of curcumin and piperine on growth performance, intestinal barrier function, and antioxidant capacity of weaned wuzhishan piglets. *Front. Vet. Sci.* 7, 418. doi: 10.3389/fvets.2020.00418

Su, L., Cao, P., and Wang, H. (2020). Tetrandrine mediates renal function and redox homeostasis in a streptozotocin-induced diabetic nephropathy rat model through Nrf2/HO-1 reactivation. *Ann. Transl. Med.* 8, 990. doi: 10.21037/atm-20-5548

Tang, J., Xu, L., Zeng, Y., and Gong, F. (2021). Effect of gut microbiota on LPS-induced acute lung injury by regulating the TLR4/NF-kB signaling pathway. *Int. Immunopharmacol.* 91, 107272. doi: 10.1016/j.intimp.2020.107272

Wang, L., Astone, M., Alam, S. K., Zhu, Z., Pei, W., Frank, D. A., et al. (2021). Suppressing STAT3 activity protects the endothelial barrier from VEGF-mediated vascular permeability. *Dis. Model Mech.* 14, dmm049029. doi: 10.1242/dmm.049029

Wang, L., Feng, Y., Dou, M., Wang, J., Bi, J., Zhang, D., et al. (2022). Study of mesenchymal stem cells derived from lung-resident, bone marrow and chorion for treatment of LPS-induced acute lung injury. *Respir. Physiol. Neurobiol.* 302, 103914. doi: 10.1016/j.resp.2022.103914

Wang, Q.-H., Li, W., Jiang, Y.-X., Lu, X.-H., and Wang, G.-G. (2019). The extract from *Agkistrodon halys* venom protects against lipopolysaccharide (LPS)-induced myocardial injury. *BMC Complement. Altern. Med.* 19, 176. doi: 10.1186/s12906-019-2595-4

Wang, R., Yang, X., Liu, J., Zhong, F., Zhang, C., Chen, Y., et al. (2022). Gut microbiota regulates acute myeloid leukaemia via alteration of

intestinal barrier function mediated by butyrate. *Nat. Commun*. 13, 2522. doi: 10.1038/s41467-022-30240-8

Wu, K.-H., Li, J.-P., Chao, W.-R., Lee, Y.-J., Yang, S.-F., Cheng, C.-C., et al. (2022). Immunomodulation via MyD88-NFκB signaling pathway from human umbilical cord-derived mesenchymal stem cells in acute lung injury. *Int. J. Mol. Sci*. 23, 5295. doi: 10.3390/ijms23105295

Wu, Z., Huang, S., Li, T., Li, N., Han, D., Zhang, B., et al. (2021). Gut microbiota from green tea polyphenol-dosed mice improves intestinal epithelial homeostasis and ameliorates experimental colitis. *Microbiome* 9, 184. doi: 10.1186/s40168-021-01115-9

Xin, Y., Yuan, Q., Liu, C., Zhang, C., and Yuan, D. (2020). MiR-155/GSK-3β mediates anti-inflammatory effect of chikusetsusaponin IVa by inhibiting NF-κB signaling pathway in LPS-induced RAW264.7 cell. *Sci. Rep*. 10, 18303. doi: 10.1038/s41598-020-75358-1

Xu, C., Song, Y., Wang, Z., Jiang, J., Piao, Y., Li, L., et al. (2021). Pterostilbene suppresses oxidative stress and allergic airway inflammation through AMPK/Sirt1 and Nrf2/HO-1 pathways. *Immun. Inflamm. Dis*. 9, 1406–1417. doi: 10.1002/iid3.490

Xu, Y., Zhu, J., Feng, B., Lin, F., Zhou, J., Liu, J., et al. (2021). Immunosuppressive effect of mesenchymal stem cells on lung and gut CD8 T cells in lipopolysaccharide-induced acute lung injury in mice. *Cell Prolif*. 54, e13028. doi: 10.1111/cpr.13028

Yang, C., Song, C., Liu, Y., Qu, J., Li, H., Xiao, W., et al. (2021). Re-Du-Ning injection ameliorates LPS-induced lung injury through inhibiting neutrophil extracellular traps formation. *Phytomedicine* 90, 153635. doi: 10.1016/j.phymed.2021.153635

Yang, W., Shao, F., Wang, J., Shen, T., Zhao, Y., Fu, X., et al. (2022). *Artemisia argyi* ethyl acetate extract from prevents liver damage in ConA-induced immunological liver injury mice via Bax/Bcl-2 and TLR4/MyD88/NF-B signaling pathways. *Molecules* 27, 7883. doi: 10.3390/molecules27227883

Yang, Y., Ding, Z., Wang, Y., Zhong, R., Feng, Y., Xia, T., et al. (2020). Systems pharmacology reveals the mechanism of activity of *Physalis alkekengi* L. var. *franchetii* against lipopolysaccharide-induced acute lung injury. *J. Cell. Mol. Med*. 24, 5039–5056. doi: 10.1111/jcmm.15126

Yang, Y.-S. H., Chou, H.-C., Liu, Y.-R., and Chen, C.-M. (2022). Uteroplacental Insufficiency causes microbiota disruption and lung development impairment in growth-restricted newborn rats. *Nutrients* 14, 4388. doi: 10.3390/nu14204388

Zhang, L., Li, Q., Liu, W., Liu, Z., Shen, H., and Zhao, M. (2019b). Mesenchymal stem cells alleviate acute lung injury and inflammatory responses induced by paraquat poisoning. *Med. Sci. Monit*. 25, 2623–2632. doi: 10.12659/MSM.915804

Zhang, Y., Yang, J., Zhou, X., Wang, N., Li, Z., Zhou, Y., et al. (2019a). Knockdown of miR-222 inhibits inflammation and the apoptosis of LPS-stimulated human intervertebral disc nucleus pulposus cells. *Int. J. Mol. Med*. 44, 1357–1365. doi: 10.3892/ijmm.2019.4314

Zhang, Y., Yang, S., Qiu, Z., Huang, L., Huang, L., Liang, Y., et al. (2022). Pyrogallol enhances therapeutic effect of human umbilical cord mesenchymal stem cells against LPS-mediated inflammation and lung injury via activation of Nrf2/HO-1 signaling. *Free Radic. Biol. Med*. 191, 66–81. doi: 10.1016/j.freeradbiomed.2022.08.030

Zhang, Z., Xu, J., Zhang, X., Wang, J., Xie, H., Sun, Y., et al. (2022). Protective effect of SeMet on liver injury induced by ochratoxin A in rabbits. *Toxins* 14, 628. doi: 10.3390/toxins14090628

Zhao, X., and Bie, M. (2020). Predictors for the development of preoperative oxygenation impairment in acute aortic dissection in hypertensive patients. *BMC Cardiovasc. Disord*. 20, 365. doi: 10.1186/s12872-020-01652-5

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 19 October 2023
DOI 10.3389/fmicb.2023.1290246

Check for updates

OPEN ACCESS

EDITED BY
Ludovico Abenavoli,
Magna Græcia University, Italy

REVIEWED BY
Bruno Tilocca,
Magna Græcia University, Italy
Giuseppe Guido Maria Scarlata,
University Magna Graecia of Catanzaro, Italy

*CORRESPONDENCE
Qing-Ao Xiao
✉ xiaoqingao0912@163.com
Yong Yang
✉ yangyongVIP1971@163.com

†These authors have contributed equally to this work

Received 07 September 2023
ACCEPTED 03 October 2023
PUBLISHED 19 October 2023

CITATION
Xia X, He S-y, Zhang X-L, Wang D, He Q, Xiao Q-A and Yang Y (2023) The causality between gut microbiome and anorexia nervosa: a Mendelian randomization analysis. *Front. Microbiol.* 14:1290246. doi: 10.3389/fmicb.2023.1290246

COPYRIGHT
© 2023 Xia, He, Zhang, Wang, He, Xiao and Yang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The causality between gut microbiome and anorexia nervosa: a Mendelian randomization analysis

Xuan Xia[1,2,3†], Shu-yang He[4†], Xiao-Lin Zhang[5,6], Decheng Wang[1,2,3], Qian He[7], Qing-Ao Xiao[1*] and Yong Yang[4*]

[1]Hubei Key Laboratory of Tumor Microenvironment and Immunotherapy, College of Basic Medical Science, China Three Gorges University, Yichang, China, [2]Institute of Infection and Inflammation, China Three Gorges University, Yichang, China, [3]Department of Physiology and Pathophysiology, College of Basic Medical Science, China Three Gorges University, Yichang, China, [4]Department of Critical Care Medicine, Yiling People's Hospital of Yichang City, Yichang, China, [5]Department of Interventional Radiology, The First College of Clinical Medical Science, China Three Gorges University, Yichang, China, [6]Yichang Central People's Hospital, Yichang, China, [7]Hunan Provincial Key Laboratory of Metabolic Bone Diseases, Department of Metabolism and Endocrinology, National Clinical Research Center for Metabolic Diseases, The Second Xiangya Hospital of Central South University, Changsha, China

Background and aim: Nutrient production by intestinal microbiota corresponds to regulate appetite while gut microbial composition was influenced by diet ingestion. However, the causal relationship between gut microbial taxa and anorexia nervosa (AN) remains unclear. Mendelian Randomization (MR) is a novel research method that effectively eliminates the interference of confounding factors and allows for the exploration of the direct causal effects between exposure and outcome. This study employs MR to explore the causal effect between AN and specific gut microbiome.

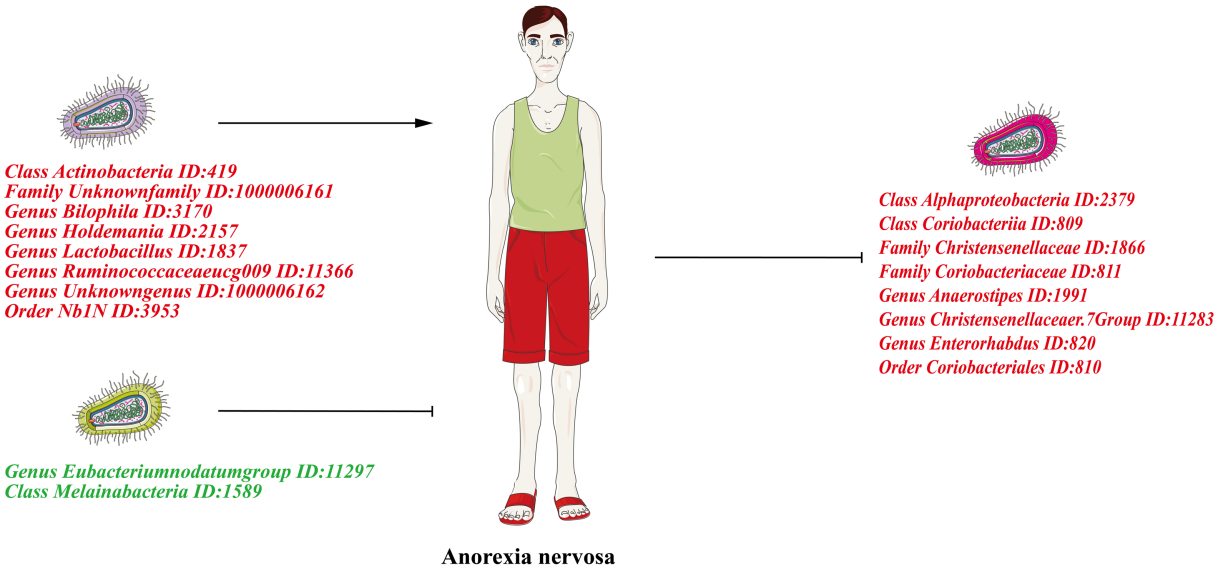
Methods: Large-scale Genome Wide Association Study (GWAS) data of AN and 211 gut microbes were obtained from the IEU open GWAS project and Mibiogen Consortium. Two-sample MR was performed to determine the causal relationship between gut microbiota and AN. Furthermore, a bi-directional MR analysis was to examine the direction of the causal relations. The Bonferroni correction test was used to adjust potential correlations among microbial taxa.

Result: In forward MR analysis, 10specific gut microbial taxa have an impact on the occurrence of AN (the *p* value of IVW <0.05). The high abundance of *Genus Eubacteriumnodatumgroup ID: 11297* (OR:0.78, 95% CI:0.62–0.98, p=0.035) and *Class Melainabacteria ID: 1589* (OR:0.72, 95% CI:0.51–0.99, p=0.045) may be considered protective factors for AN. But after Bonferroni correction, only *Class Actinobacteria ID:419* (OR:1.53, 95% CI:1.19–1.96, p=0.00089) remained significantly associated and high abundance of *Class Actinobacteria ID:419* considered as a risk factor for AN. In the reverse MR analysis, AN influences 8 gut microbial taxa with none-statistically significant associations after adjustment.

Conclusion: We identified a significant correlation between AN and 18 microbial taxa which have not been previously reported. Among them, 10 kinds of gut bacteria may affect the occurrence of AN, and the status of AN would affect 8 kinds of gut bacteria. After correction, the *Class Actinobacteria ID:419* continued to exert an influence on AN.

KEYWORDS

gut microbiome, Mendelian randomization, anorexia nervosa, genome-wide association study, causality



GRAPHICAL ABSTRACT
The left half of the figure shows the forward Mendelian randomization results showing the causal effect of gut microbiota on AN. The right half of the figure shows the reverse Mendelian randomization results showing the causal effect of AN on the gut microbiota. Risk factors are indicated in red font and protective factors in dark green font.

1. Introduction

Anorexia nervosa (AN) is one of the most serious psychiatric disorders, characterized by life-threatening very-low body weight, nutritional restrictions, and obviously occurs in females and adolescents (Treasure et al., 2015; Bulik et al., 2019; Seitz et al., 2019). Its prevalence in the population is estimated to be around 1% (Fan et al., 2023). Over the past decade, there has been a progressively increasing trend in the incidence of anorexia nervosa. This rising incidence has been observed in several countries such as the United Kingdom, Germany, and Japan (Holland et al., 2016; Bulik et al., 2019). The aggregate mortality is estimated to approximately 5.6% per decade, significantly surpassing that of the general population (Mitchell et al., 2020). However, AN suffers from efficient therapeutic methods and liability to relapse (Bulik et al., 2019). Previously researches displayed the incidence of AN accompanied by multiple dysfunctions, including endocrine alterations (Schorr and Miller, 2017), increased inflammation (Dai et al., 2013), and immune response (Seitz et al., 2019).

The human gastrointestinal tract harbors a multitude of microorganisms. Previous research has indicated that any disruption in the composition of the gut microbiota can lead to the onset of liver diseases (Tilg et al., 2022). These microorganisms can modulate individual behavior through various mechanisms, including metabolite, endocrine pathways (Albillos et al., 2020). The gut microbiota's involvement in the development of neurological disorders through the microbiota-gut-brain axis has been reported for some time (Schorr and Miller, 2017; Mossad et al., 2021; Drljaca et al., 2023). However, the specific role played by individual microbial groups in this process remains unclear. Recently, the crucial role of the gut microbiome in AN has been gradually unveiled. Nevertheless, exploring the connection between AN and the gut microbiota poses challenges owing to the susceptibility to various influencing factors. A recent meta-analysis has unveiled links between AN and numerous microbial taxa. However, numerous studies have generated conflicting outcomes regarding the precise relationships between particular microbial taxa and AN (Nikolova et al., 2021). The reasons for these divergent results are multifaceted. Firstly, there's the influence of confounding factors like environmental elements (such as mental status, sleep status, etc.). Secondly, the intricate interplay among gut microbiota complicates the causal effect between individual microbial groups and AN, making it difficult to reveal cause-and-effect relationship. Currently, there is also a lack of large-scale randomized controlled trials (RCTs) to substantiate this causal link.

Mendelian randomization (MR) is a genetic epidemiological approach that utilizes instrumental variables (IVs) which are highly correlated with the exposure of interest, to investigate causality and mitigate the impact of confounding factors (Emdin et al., 2017; Davies et al., 2018). This method capitalizes on the inherent property of Single Nucleotide Polymorphisms (SNPs) to be randomly assorted and distributed during gamete formation, rendering them unaffected by confounding factors following gametogenesis. Furthermore, the non-reversible nature of heredity helps in excluding the possibility of reverse causal effects (Bowden et al., 2015). In essence, MR, as an innovative methodology, offers the means to address confounding and reverse causality by leveraging genetic variants with close associations

Abbreviations: AN, Anorexia nervosa; CI, Confidence interval; GCAN, Genetic consortium for anorexia nervosa; GWAS, Genome-wide association study; IV, Instrumental variables; IVW, Inverse variance weighted; LD, Linkage disequilibrium; MR, Mendelian randomization; MR-PRESSO, Mendelian randomization pleiotropy RESidual sum and outlier; mbQTL, Microbiota quantitative trait loci; OR, Odds ratio; RCT, Randomized control trial; SNPs, Single nucleotide polymorphisms; WM, Weighted median; WTCCC3, Wellcome trust case control consortium 3.

to the exposure of interest in order to infer potential causal relationships with the outcome (Emdin et al., 2017; Davies et al., 2018).

In this study, we investigated the potential causal effect between 211 gut microbiota taxa and AN by MR study design. After correction, the *Class Actinobacteria ID:419* was found to exert an influence on AN. Our study provides a new sight into exploring the relationship between AN and gut microbiota.

2. Materials and methods

2.1. Study design

Two hundred and eleven gut microbiota taxa were selected as exposure and AN was defined as outcome for MR analysis. Then, the exposure and outcome were exchanged for reverse MR analysis. All MR analysis of this study was executed under three basic assumptions: (1) IVs must be strongly correlated with exposure; (2) IVs cannot be correlated with confounding factors; (3) IVs can only affect outcomes through exposure factors (Bowden and Holmes, 2019). The GWAS data we selected all originate from populations of European ancestry. These individuals are largely independent of each other. The flow-chart of this study was shown in Figure 1. Furthermore, we used the STROBE-MR (Strengthening the Reporting of Observational Studies in Epidemiology-Mendelian Randomization) checklist to explain our MR study (Skrivankova et al., 2021). The checklist shown in Supplementary Table S1.

2.2. GWAS data of gut microbiome

The large-scale GWAS summary data of gut microbiome were obtained from Mibiogen consortium, including 18,340 individuals from 24 cohorts (Kurilshikov et al., 2021). The consortium utilized standardized analytical pipelines for both microbiota phenotype and genotype, ensuring uniform data processing methods. This approach was employed to mitigate potential variations introduced by technical differences in generating microbiota data. This study used three different regions (V4: 10,413 samples, 13 cohorts, V3-V4: 4,211 samples, 6 cohorts and V1-V2: 3,716 samples, 5 cohorts) (V: hypervariable region sequencing for identifying bacterial taxa)of the 16S rRNA gene to analysis the composition of gut microbiota and identified genetic variants that influent the relative abundance of microbial taxa by use of microbiota Quantitative Trait loci (mbQTL) mapping (Kurilshikov et al., 2021). In the original study, the gut microbiota was categorized into 257 taxa at five taxonomic levels: Phylum, Class, Order, Family, and Genus. Finally, 211 taxa were defined, including 131 genera, 35 families, 20 orders, 16 classes, and 9 phyla. All data from this study are publicly accessible, which could acquire from the website (https://mibiogen.gcc.rug.nl/menu/main/home/).

2.3. GWAS data of AN

The GWAS summary data of AN were obtained from the IEU open GWAS project (v7.5.5-2023-08-09, $N = 42,348$, https://gwas.mrcieu.ac.uk/). The GWAS summary dataset encompassed 2,907 individuals diagnosed with AN across 14 countries, along with 14,860 ancestrally matched control subjects, constituting a part of the Genetic Consortium for Anorexia Nervosa (GCAN) and the Wellcome Trust Case Control Consortium 3 (WTCCC3) (Boraska et al., 2014). All individuals were of European ancestry and detailed information of ANwas available in the website (https://gwas.mrcieu.ac.uk/datasets/ieu-a-45/).

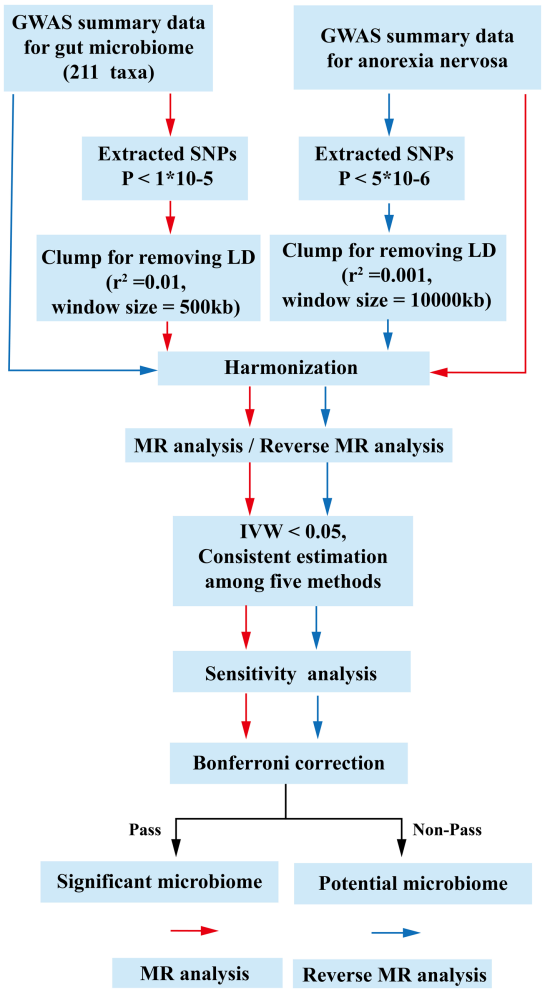


FIGURE 1
The flow chart of the study. The GWAS data of flora was used as exposure, and anorexia nervosa was used as outcomes for MR analysis. The instrumental variables of flora were extracted in the following way: (1) $p < 1 \times 10^{-5}$; (2) $r^2 = 0.01$, kb = 500. Five methods were used for MR analysis after harmonization. Floras with IVW estimates below 0.05, exhibiting consistent results across the five analytical methods, were deemed to hold potential association. Subsequently, an investigation into the presence of pleiotropy and heterogeneity among these potential floras was undertaken. Those floras displaying pleiotropic or heterogeneous characteristics were promptly eliminated. Meaningful bacterial groups were screened out. Then reverse MR analysis was performed, and different criteria were used for the screening of instrumental variables for AN ($p < 5 \times 10^{-6}$, $r^2 = 0.001$, kb = 10,000). All potential floras were corrected by the Bonferronitest. The floras passed the Bonferroni were considered as significant. MR, Mendelian randomization; LD, linkage disequilibrium; GWAS, Genome Wide Association Study; SNPs, single nucleotide polymorphisms; IVW, inverse variance weighting.

2.4. The selection of IVs

The criterion of selecting IVs as following: (1) SNPs, significantly associated with gut microbiota, were selected (the p value of SNPs $<1\times10^{-5}$) as the potential eligible IVs (Yu et al., 2023); (2) SNPs were clumped for excluding the effect of linkage disequilibrium ($r^2=0.01$, window size = 500 kb) (Xu et al., 2021); (3)palindromic alleles were removed. Then, For the reverse MR analysis, we filtered the IVs of AN. $p\leq 5\times10^{-6}$was chose as the criterion and clump were reset ($r^2=0.001$, window size = 10,000 kb) (Yu et al., 2023). Other criterions were the same as above. To avoid weak instrumental bias, the F statistics for each bacterial taxon was calculated by following equation (Xu et al., 2021):

$$F = R^2 \times \frac{n-1-k}{\left(1-R^2\right)\times k}$$

In this equation, R^2 is to explain exposure variance of the IVs, n is the sample size, and k is the number of IVs (Xu et al., 2021). According to previous study, F statistic ≥10 were considered that there is no weak instrument bias (Pierce et al., 2011).

2.5. MR analysis

Five methods (including Inverse variance weighted (IVW), MR Egger, Weighted median, Simple mode, and Weighted mode method) were used to estimate the causal effect of the gut microbiota on AN. Due to its assumption that all instrumental variables are valid, IVW is susceptible to the effects of instrumental variable pleiotropy and heterogeneity (Bowden et al., 2016). However, in the absence of these influences, IVW considered to be the most accurate method, even when the other four methods may not yield positive results (Long et al., 2023).

To establish the accuracy and seriousness, five distinct methods was employed to assess the causal impact of gut microbiota on AN, namely: (1) Inverse Variance Weighted (IVW); (2) MR Egger; (3) Weighted Median (WM); (4) Simple Mode; (5) Weighted Mode method. It's worth noting that, while the IVW method assumes the validity of all instrumental variables, it can be influenced by instrumental variable pleiotropy and heterogeneity. However, in scenarios devoid of these influences, IVW remains esteemed as the most precise technique (Wang et al., 2023). Thus, the result of MR analysis was mainly based on IVW (Burgess et al., 2013). The remaining four methods were considered supplementary to the IVW approach. Once instrumental variables (IVs) were harmonized, if the count of matched SNPs for a specific microbiota was <3, that microbiota was excluded due to lack of credibility in results.

A significance threshold for multiple testing was established at each taxonomic level (phylum, class, order, family, and genus). Bonferroni correction method was employed to adjust the p-values, mitigating the potential for false positives ($p<0.05/N$, N refers to the effective number of independent bacterial taxa at the specific taxonomic level). The significant p values were following: 0.00038 (131 Genera), 0.0014 (35 Families), 0.0025 (20 Orders), 0.0031 (16 Classes), and 0.0056 (9 Phyla). Subsequently, when the estimated value of five methods exhibited congruence and IVW yielded a value <0.05/N, the gut microbes were deemed to exhibit a statistically significant distinction. Microbial taxa with the p-values of IVW below 0.05, although not statistically significant after Bonferroni correction, are considered to have potential associations.

The significant microbiotas were tested for pleiotropy and heterogeneity to ensure the accuracy of the IVW results. MR-Egger Intercept Test and Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) global test were employed to detect horizontal pleiotropy (Long et al., 2023). MR-PRESSO could assess the overall horizontal pleiotropy of IVs and the abnormal SNPs which led to the pleiotropy (Verbanck et al., 2018). For assessing the presence of horizontal pleiotropy, p values greater than 0.05 for two methods were indicative of its absence. To gage the extent of heterogeneity, Cochran's Q test was employed, with p values above 0.05 indicating non-heterogeneity. Therefore, in our study, any microbiota with Cochran's Q test p-value remaining less than 0.05 were excluded from further analysis to ensure the reliable results of IVW method. Meanwhile, Leave-one-out analysis was employed to exclude the influence of single SNP.

2.6. Reverse MR analysis

To investigate whether AN exerted acausal influence on gut microbiomes, we additionally conducted a reverse MR analysis. In this procure, we employed SNPs strongly linked with AN as instrumental variables (IVs) to further probe the causal effect of AN on gut microbiome. The analytical approach was same with the MR analysis.

2.7. Data processing

All data processing and analysis were accomplished by R software (R.4.2.3; http://www.R-project.org). The R packages used in study is TwoSampleMR, MendelianRandomization, and MR-PRESSO.

3. Results

3.1. Instrumental variables for gut microbiome and AN

After strong correlation screening ($p<1\times10^{-5}$) and clump ($r^2=0.01$, window = 500 kb). SNPs associated with gut microbiota at difference levels were identified (Class: 231, Family: 509, Genus: 1740, Order: 289, Phylum: 126; Detail information shown in Supplementary Table S2). For AN, a threshold of 5×10^{-6} ($r^2=0.001$, window = 10,000 kb) adopted for selection. Finally, 7 IVs were filtered and detail information was displayed in Supplementary Table S3.

3.2. Causal effects of gut microbiota on AN

Under the condition of IVW < 0.05, 10 gut microbiotas were associated with AN (Figures 2, 3A). The result showed that high

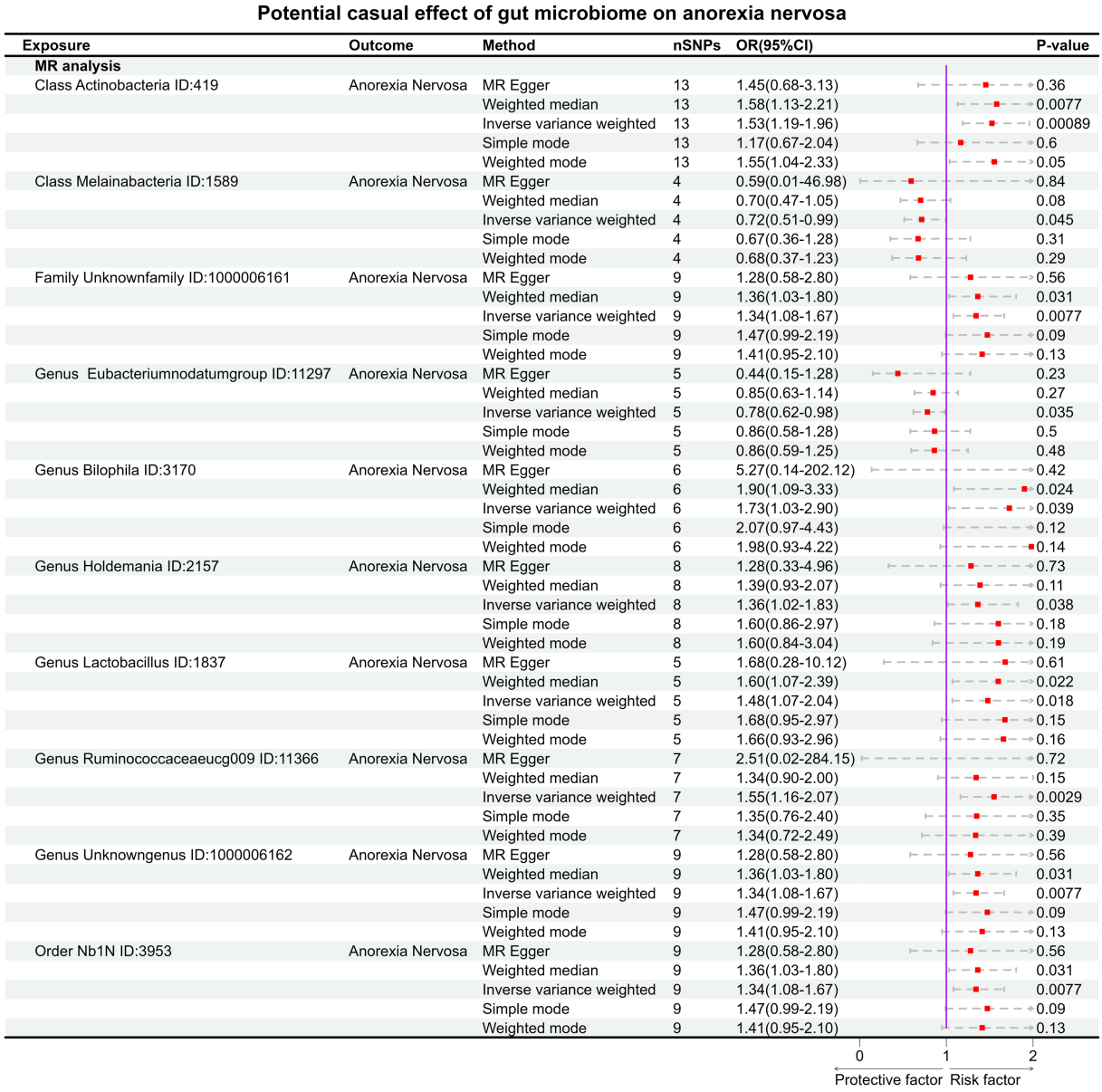


FIGURE 2
The result of MR between gut microbiome and anorexia nervosa by five methods.

abundancy of 8 microbiotas were the risk factors for the onset of AN, including *Class Actinobacteria ID:419* (OR:1.53, 95% CI:1.19–1.96, $p = 0.00089$), *Family Unknown family ID:1000006161* (OR:1.34, 95% CI:1.08–1.67, $p = 0.0077$), *Genus Bilophila ID:3170* (OR:1.73, 95% CI:1.03–2.90, $p = 0.039$), *Genus Holdemania ID:2157* (OR:1.36, 95% CI:1.02–1.83, $p = 0.038$), *Genus Lactobacillus ID:1837* (OR:1.48, 95% CI:1.07–2.04, $p = 0.018$), *Genus Ruminococcaceaeucg009 ID:11366* (OR:1.55, 95% CI:1.16–2.07, $p = 0.0029$), *Genus Unknowngenus ID:1000006162* (OR:1.34, 95% CI:1.08–1.67, $p = 0.0077$), *Order Nb1N ID:3953* (OR:1.34, 95% CI:1.08–1.67, $p = 0.0077$). Two microbiotas were the protective factors for AN, including *Genus EubacteriumnodatumgroupID:11297*(OR:0.78, 95%CI:0.62–0.98, $p = 0.035$), *Class Melainabacteria ID:1589* (OR:0.72, 95% CI:0.51–0.99, $p = 0.045$). After Bonferroni correction, only one microbiota was significance associated with AN (*Class Actinobacteria ID:419*).

3.3. Causal effects of AN on gut microbiota

In the reverse MR, the abundancy of eight gut microbial taxa were identified that are influenced by AN (Figures 3B, 4), including *Class Alphaproteobacteria ID:2379* (OR:1.02, 95% CI:1.00–1.03, $p = 0.0092$), *ClassCoriobacteriia ID:809* (OR:1.01, 95% CI:1.00–1.02, $p = 0.016$), *Family Christensenellaceae ID:1866* (OR:1.02, 95% CI:1.00–1.03, $p = 0.0056$), *Family Coriobacteriaceae ID:811* (OR:1.01, 95% CI:1.00–1.02, $p = 0.016$), *GenusAnaerostipesID:1991* (OR:1.01, 95% CI:1.00–1.02, $p = 0.035$), *Genus Christensenellaceaer.7Group ID:11283* (OR:1.02, 95% CI:1.00–1.03, $p = 0.032$), *Genus*

10.3389/fmicb.2023.1290246

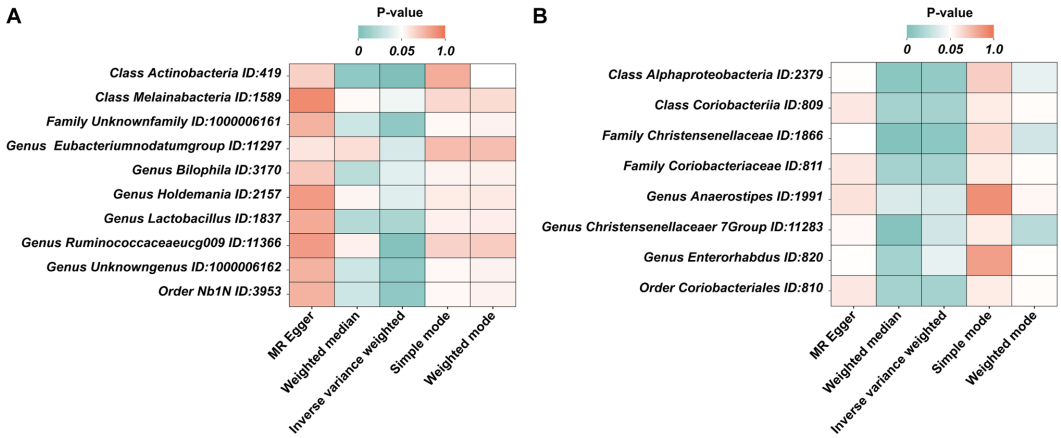


FIGURE 3
The heatmaps of five MR analysis methods. **(A)** The *p* value of five MR analysis (Forward); **(B)** The *p* value of five MR analysis (Reverse).

Potential casual effect of anorexia nervosa on gut microbiome

Exposure	Outcome	Method	nSNPs	OR(95%CI)	P-value
Reverse MR analysis					
Anorexia Nervosa	Class Alphaproteobacteria ID:2379	MR Egger	6	1.02(1.01-1.04)	0.06
		Weighted median	6	1.02(1.01-1.03)	0.0052
		Inverse variance weighted	6	1.02(1.00-1.03)	0.0092
		Simple mode	6	1.04(0.96-1.14)	0.37
		Weighted mode	6	1.02(1.01-1.04)	0.041
Anorexia Nervosa	Class Coriobacteriia ID:809	MR Egger	6	1.01(1.00-1.02)	0.21
		Weighted median	6	1.01(1.00-1.02)	0.016
		Inverse variance weighted	6	1.01(1.00-1.02)	0.016
		Simple mode	6	1.04(0.99-1.10)	0.18
		Weighted mode	6	1.01(1.00-1.02)	0.07
Anorexia Nervosa	Family Christensenellaceae ID:1866	MR Egger	6	1.02(1.01-1.03)	0.05
		Weighted median	6	1.02(1.01-1.03)	0.0022
		Inverse variance weighted	6	1.02(1.00-1.03)	0.0056
		Simple mode	6	1.04(0.97-1.11)	0.3
		Weighted mode	6	1.02(1.01-1.03)	0.032
Anorexia Nervosa	Family Coriobacteriaceae ID:811	MR Egger	6	1.01(1.00-1.02)	0.21
		Weighted median	6	1.01(1.00-1.02)	0.016
		Inverse variance weighted	6	1.01(1.00-1.02)	0.016
		Simple mode	6	1.04(0.99-1.10)	0.18
		Weighted mode	6	1.01(1.00-1.02)	0.07
Anorexia Nervosa	Genus Anaerostipes ID:1991	MR Egger	6	1.01(1.00-1.02)	0.24
		Weighted median	6	1.01(1.00-1.02)	0.036
		Inverse variance weighted	6	1.01(1.00-1.02)	0.035
		Simple mode	6	1.01(0.95-1.07)	0.8
		Weighted mode	6	1.01(1.00-1.02)	0.1
Anorexia Nervosa	Genus Christensenellaceaer 7Group ID:11283	MR Egger	6	1.02(1.00-1.04)	0.09
		Weighted median	6	1.02(1.01-1.03)	0.0037
		Inverse variance weighted	6	1.02(1.00-1.03)	0.032
		Simple mode	6	1.05(0.99-1.12)	0.18
		Weighted mode	6	1.02(1.01-1.03)	0.023
Anorexia Nervosa	Genus Enterorhabdus ID:820	MR Egger	6	1.03(1.01-1.05)	0.06
		Weighted median	6	1.02(1.00-1.04)	0.015
		Inverse variance weighted	6	1.02(1.00-1.04)	0.041
		Simple mode	6	1.02(0.93-1.12)	0.7
		Weighted mode	6	1.02(1.00-1.04)	0.06
Anorexia Nervosa	Order Coriobacteriales ID:810	MR Egger	6	1.01(1.00-1.02)	0.21
		Weighted median	6	1.01(1.00-1.02)	0.016
		Inverse variance weighted	6	1.01(1.00-1.02)	0.016
		Simple mode	6	1.04(0.99-1.10)	0.18
		Weighted mode	6	1.01(1.00-1.02)	0.07

FIGURE 4
The result of reverse MR analysis between gut microbiome and anorexia nervosa by five methods.

Enterorhabdus ID:820 (OR:1.02, 95% CI:1.00–1.04, $p = 0.041$), and *Order Coriobacteriales ID:810* (OR:1.01, 95% CI:1.00–1.02, $p = 0.016$). Under conditions of AN, these microbial taxa are suppressed and would display low abundancy. In essence, AN acted as a potential risk factor for these microbial communities. However, there was no significant microbiota after Bonferroni correction.

TABLE 1 The results of pleiotropy and heterogeneity in MR analysis.

Exposure	MR egger test	MR-PRESSO	Cochran's Q test	*F* value
Class Actinobacteria ID:419	0.899	0.657	0.586	31.143
Class Melainabacteria ID:1589	0.941	0.537	0.486	22.372
Family Unknown family ID:1000006161	0.902	0.867	0.875	24.548
Genus eubacterium nodatum group ID:11297	0.359	0.798	0.749	21.083
Genus Bilophila ID:3170	0.577	0.221	0.167	21.236
Genus Holdemania ID:2157	0.932	0.523	0.475	22.753
Genus Lactobacillus ID:1837	0.897	0.763	0.733	22.170
Genus Ruminococcaceaeucg009 ID:11366	0.849	0.569	0.514	22.163
Genus Unknowngenus ID:1000006162	0.902	0.867	0.875	24.548
Order Nb1N ID:3953	0.902	0.867	0.875	24.548

The outcome of this MR analysis is anorexia nervosa.

TABLE 2 The results of pleiotropy and heterogeneity in MR analysis.

Outcome	MR egger test	MR-PRESSO	Cochran's Q test	*F* value
Class Alphaproteobacteria ID:2379	0.495	0.567	0.573	1144.90
Class Coriobacteriia ID:809	0.491	0.592	0.766	1144.90
Family Christensenellaceae ID:1866	0.434	0.584	0.393	1144.90
Family Coriobacteriaceae ID:811	0.491	0.592	0.766	1144.90
Genus Anaerostipes ID:1991	0.600	0.687	0.898	1144.90
Genus Christensenellaceaer.7Group ID:11283	0.402	0.492	0.134	1144.90
Genus Enterorhabdus ID:820	0.205	0.486	0.354	1144.90
Order Coriobacteriales ID:810	0.491	0.592	0.766	1144.90

The exposure of this MR analysis is anorexia nervosa.

3.4. Sensitivity analysis of MR

MR PRESSO did not identify heterogeneity among the significant and potential microbiota (Tables 1, 2). Similarly, Cochran's Q test indicated the absence of heterogeneity across the studies (Tables 1, 2). Moreover, the Leave-one-out analysis revealed no significant differences in SNP effects. Furthermore, the MR Egger regression did not yield any evidence of horizontal pleiotropy ($p > 0.05$), and all F-statistical values exceeded 10 (Tables 1, 2). The MR analysis and Leave-one out analysis result of *Class Actinobacteria ID:419* was shown in the Figure 5.

4. Discussion

As early as the 20th century, it was discovered that probiotics could improve human mental well-being, such as fatigue, melancholia and the neurosis (Bested et al., 2013). The composition of gut microbiota in individuals with mental illnesses significantly differs from that of healthy individuals. Preclinical experiments have demonstrated that transplanting fecal material from individuals with mental disorders into the intestines of germ-free mice can markedly alter the behavior in mice (Kelly et al., 2016; Zheng et al., 2016; Li et al., 2019; Sharon et al., 2019; Zhu et al., 2020; Fan et al., 2023). Furthermore, previous researches had already demonstrated the role of gut microbiota in regulating appetite (Breton et al., 2016; Fetissov, 2017; Han et al., 2021). This process may depend on tryptophan (Dong et al., 2020). Thus, some researchers are also attempting to treat anorexia nervosa by inhibiting healthy individuals' feces (Wilson et al., 2023). Furthermore, a recent meta-analysis also indicates variations in the microbiota of individuals with mental disorders (Nikolova et al., 2021). This study incorporated 59 case-control studies, including 10 related to AN (involving 211 patients). It revealed alterations in 12 microbial taxa among AN, though the results pertaining to these taxa were not consistent across all studies (Nikolova et al., 2021). Current research faces challenges in obtaining consistent conclusions by experimental design that can eliminate the influence of confounding factors. The MR method ingeniously mitigates the impact of confounding factors, thereby yielding consistent results (Emdin et al., 2017).

To our knowledge, this is the first MR study to ascertain the relationship between AN and gut microbiome. We found that there was genetic liability to 18 gut microbiotas causally associated with AN. Our study also found some results were consistent with previously researches. For instance, a high abundance of *Genus Lactobacillus*, in our study, is a risk factor for AN. Interestingly, a recent study also observed that the abundance of several *Lactobacillus* species was increased in the Activity-Based Anorexia mouse model (Breton et al., 2021). In another study, an increased abundance of *Class Actinobacteria* was observed in patients of AN (Nikolova et al., 2021). Interestingly, a similar increase in *Class Actinobacteria* abundance was also found in a clinical trial involving

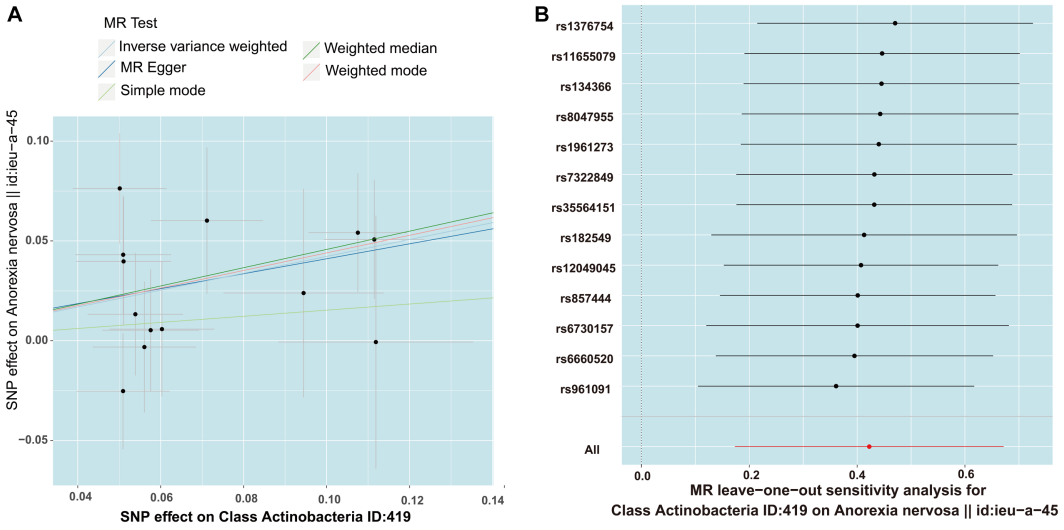


FIGURE 5
The result of MR between gut microbiome and anorexia nervosa by five methods. **(A)** In the scatter plot,estimated value of five MR tests were consistent. **(B)** The leave-one-out analysis validated that there is no significant differences in SNP effects between Class Actinobacteria ID:419 and anorexia nervosa.

obese patients, coinciding with weight reduction in overweight individuals (Chambers et al., 2019).

However, our study also deviated from several previous research findings. Another study highlighted variations in the composition of fecal microbiota between AN patients upon admission and at discharge, specifically involving *Genus Anaerostipes* and *Genus Christensenellaceaer.7Group*. In their study, the abundance of these two taxa decreased from admission to discharge. Our study, in contrast, revealed that the growth of *Genus Anaerostipes* and *Genus Christensenellaceaer.7Group* was likely inhibited under AN condition. We hypothesized that this increase might stem from complex interactions within the microbial communities. In addition, *Family Coriobacteriaceae ID:811*was reported increasing in the patients of AN. However, AN, in our study, acted as an inhibitory factor for the growth of *Family Coriobacteriaceae ID:811*. *Family Coriobacteriaceae ID:811*, should be decease under the condition of the AN. It's worth noting that our study also provided a clear conclusion for the conflicting results from prior research. For instance, a meta-analysis published in 2020 indicated a potential significant association between *Anaerostipes* and BMI (Di Lodovico et al., 2021). However, with an increase in studies, a meta-analysis in 2021 suggested inconsistent findings (Nikolova et al., 2021). In our study, we observed that *Genus Anaerostipes ID:1991* exhibited suppressed growth under condition of AN. One possible explanation for this difference could be that, while MR analysis eliminates the interference of various confounding factors, the inherent differences in the physical properties of the selected microbial samples themselves cannot be ignored. These differences may have led to variations in our study's conclusions compared to previous findings. Standardized sample selection and large-scale randomized controlled trials can help eliminate the potential interference caused by these differences.

Meanwhile, our study has several limitations. Firstly, constrained by GWAS data, we can accurately examine the relationship between the genus level or higher taxonomic levels and AN, but cannot extend the validation to more specific levels, such as species. Secondly, due to the utilization of publicly available GWAS data, there was an imbalance in the number of case and control groups within these cohorts, potentially increasing the likelihood of pleiotropy. Concurrently, the impact of genetic mutations on other pathways might also impact the interpretability of the results. Thirdly, the GWAS data we utilized are derived from individuals of European ancestry. Therefore, it remains uncertain whether our conclusions are applicable to other populations, such as East Asian or South Asian populations. Fourthly, since we were unable to obtain individual-level gender data from the GWAS data, we were unable to analyze AN patients of different genders and, therefore, explore the interaction between microbial taxa and AN patients of different genders. Lastly, the absence of pertinent GWAS data precluded us from conducting validation through replication samples.

In summary, our analysis delved into the causal connection between 211 gut microbiota and AN. For the first time, we have not only showed that 10 gut microbiotas acted as risk or protective factors for AN, but also revealed that 8 gut microbiotas were influenced under AN condition. This study offered novel insights into the causal relationship between AN and gut microbial taxa.

Data availability statement

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

Author contributions

XX: Data curation, Formal analysis, Funding acquisition, Investigation, Writing – original draft. S-yH: Data curation, Formal analysis, Writing – original draft. X-LZ: Methodology, Software, Supervision, Writing – review & editing. DW: Funding acquisition,

Methodology, Software, Supervision, Validation, Writing – review & editing. QH: Funding acquisition, Software, Validation, Writing – review & editing. Q-AX: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft. YY: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by Open Foundation of Hubei Key Laboratory of Tumor Microenvironment and Immunotherapy, China Three Gorges University (2023KZL012), National Natural Science Foundation of China (No. 31772709, 82100473), and Hubei Natural Science Foundation (2015CFB316) of China.

Acknowledgments

We acknowledge the participants and investigators of the IEU open GWAS project. We also thank the Mibiogen consortium. We thank for the help of Servier Medical Art. Parts of picture material in graphical abstract were drawn by using pictures from Servier Medical Art and we do some modified in microbes. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/license-s/by/3.0/). We thank for https://www.chiplot.online/ which helps us to draw the heatmap.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1290246/full#supplementary-material

References

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Bested, A. C., Logan, A. C., and Selhub, E. M. (2013). Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: part I - autointoxication revisited. *Gut Pathog.* 5:5. doi: 10.1186/1757-4749-5-5

Boraska, V., Franklin, C. S., Floyd, J. A., Thornton, L. M., Huckins, L. M., Southam, L., et al. (2014). A genome-wide association study of anorexia nervosa. *Mol. Psychiatry* 19, 1085–1094. doi: 10.1038/mp.2013.187

Bowden, J., Davey Smith, G., and Burgess, S. (2015). Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int. J. Epidemiol.* 44, 512–525. doi: 10.1093/ije/dyv080

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

Bowden, J., and Holmes, M. V. (2019). Meta-analysis and Mendelian randomization: a review. *Res. Synth. Methods* 10, 486–496. doi: 10.1002/jrsm.1346

Breton, J., Tennoune, N., Lucas, N., Francois, M., Legrand, R., Jacquemot, J., et al. (2016). Gut Commensal *E. coli* proteins activate host satiety pathways following nutrient-induced bacterial growth. *Cell Metab.* 23, 324–334. doi: 10.1016/j.cmet.2015.10.017

Breton, J., Tirelle, P., Hasanat, S., Pernot, A., L'Huillier, C., Do Rego, J. C., et al. (2021). Gut microbiota alteration in a mouse model of anorexia nervosa. *Clin. Nutr.* 40, 181–189. doi: 10.1016/j.clnu.2020.05.002

Bulik, C. M., Flatt, R., Abbaspour, A., and Carroll, I. (2019). Reconceptualizing anorexia nervosa. *Psychiatry Clin. Neurosci.* 73, 518–525. doi: 10.1111/pcn.12857

Burgess, S., Butterworth, A., and Thompson, S. G. (2013). Mendelian randomization analysis with multiple genetic variants using summarized data. *Genet. Epidemiol.* 37, 658–665. doi: 10.1002/gepi.21758

Chambers, E. S., Byrne, C. S., Morrison, D. J., Murphy, K. G., Preston, T., Tedford, C., et al. (2019). Dietary supplementation with inulin-propionate ester or inulin improves insulin sensitivity in adults with overweight and obesity with distinct effects on the gut microbiota, plasma metabolome and systemic inflammatory responses: a randomised cross-over trial. *Gut* 68, 1430–1438. doi: 10.1136/gutjnl-2019-318424

Dai, C., Zheng, C. Q., Meng, F. J., Zhou, Z., Sang, L. X., and Jiang, M. (2013). VSL#3 probiotics exerts the anti-inflammatory activity via PI3k/Akt and NF-kappaB pathway in rat model of DSS-induced colitis. *Mol. Cell. Biochem.* 374, 1–11. doi: 10.1007/s11010-012-1488-3

Davies, N. M., Holmes, M. V., and Davey Smith, G. (2018). Reading Mendelian randomisation studies: a guide, glossary, and checklist for clinicians. *BMJ* 362:k601. doi: 10.1136/bmj.k601

Di Lodovico, L., Mondot, S., Dore, J., Mack, I., Hanachi, M., and Gorwood, P. (2021). Anorexia nervosa and gut microbiota: a systematic review and quantitative synthesis of pooled microbiological data. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 106:110114. doi: 10.1016/j.pnpbp.2020.110114

Dong, F., Hao, F., Murray, I. A., Smith, P. B., Koo, I., Tindall, A. M., et al. (2020). Intestinal microbiota-derived tryptophan metabolites are predictive of ah receptor activity. *Gut Microbes* 12, 1788899–1788824. doi: 10.1080/19490976.2020.1788899

Drljaca, J., Milosevic, N., Milanovic, M., Abenavoli, L., and Milic, N. (2023). When the microbiome helps the brain-current evidence. *CNS Neurosci. Ther.* 29, 43–58. doi: 10.1111/cns.14076

Emdin, C. A., Khera, A. V., and Kathiresan, S. (2017). Mendelian Randomization. *JAMA* 318, 1925–1926. doi: 10.1001/jama.2017.17219

Fan, Y., Støving, R. K., Berreira Ibraim, S., Hyötyläinen, T., Thirion, F., Arora, T., et al. (2023). The gut microbiota contributes to the pathogenesis of anorexia nervosa in humans and mice. *Nat. Microbiol.* 8, 787–802. doi: 10.1038/s41564-023-01355-5

Fetissov, S. O. (2017). Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. *Nat. Rev. Endocrinol.* 13, 11–25. doi: 10.1038/nrendo.2016.150

Han, H., Yi, B., Zhong, R., Wang, M., Zhang, S., Ma, J., et al. (2021). From gut microbiota to host appetite: gut microbiota-derived metabolites as key regulators. *Microbiome* 9:162. doi: 10.1186/s40168-021-01093-y

Holland, J., Hall, N., Yeates, D. G., and Goldacre, M. (2016). Trends in hospital admission rates for anorexia nervosa in Oxford (1968-2011) and England (1990-2011): database studies. *J. R. Soc. Med.* 109, 59–66. doi: 10.1177/0141076815617651

Kelly, J. R., Borre, Y., C, O. B., Patterson, E., El Aidy, S., Deane, J., et al. (2016). Transferring the blues: depression-associated gut microbiota induces neurobehavioural changes in the rat. *J. Psychiatr. Res.* 82, 109–118. doi: 10.1016/j.jpsychires.2016.07.019

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

Li, N., Wang, Q., Wang, Y., Sun, A., Lin, Y., Jin, Y., et al. (2019). Fecal microbiota transplantation from chronic unpredictable mild stress mice donors affects anxiety-like and depression-like behavior in recipient mice via the gut microbiota-inflammation-brain axis. *Stress* 22, 592–602. doi: 10.1080/10253890.2019.1617267

Long, Y., Tang, L., Zhou, Y., Zhao, S., and Zhu, H. (2023). Causal relationship between gut microbiota and cancers: a two-sample Mendelian randomisation study. *BMC Med.* 21:66. doi: 10.1186/s12916-023-02761-6

Mitchell, J. E., Solomon, C. G., and Peterson, C. B. (2020). Anorexia Nervosa. *N. Engl. J. Med.* 382, 1343–1351. doi: 10.1056/NEJMcp1803175

Mossad, O., Nent, E., Woltemate, S., Folschweiller, S., Buescher, J. M., Schnepf, D., et al. (2021). Microbiota-dependent increase in delta-valerobetaine alters neuronal function and is responsible for age-related cognitive decline. *Nat. Aging* 1, 1127–1136. doi: 10.1038/s43587-021-00141-4

Nikolova, V. L., Smith, M. R. B., Hall, L. J., Cleare, A. J., Stone, J. M., and Young, A. H. (2021). Perturbations in gut microbiota composition in psychiatric disorders: a review and Meta-analysis. *JAMA Psychiatry* 78, 1343–1354. doi: 10.1001/jamapsychiatry.2021.2573

Pierce, B. L., Ahsan, H., and Vanderweele, T. J. (2011). Power and instrument strength requirements for Mendelian randomization studies using multiple genetic variants. *Int. J. Epidemiol.* 40, 740–752. doi: 10.1093/ije/dyq151

Schorr, M., and Miller, K. K. (2017). The endocrine manifestations of anorexia nervosa: mechanisms and management. *Nat. Rev. Endocrinol.* 13, 174–186. doi: 10.1038/nrendo.2016.175

Seitz, J., Belheouane, M., Schulz, N., Dempfle, A., Baines, J. F., and Herpertz-Dahlmann, B. (2019). The impact of starvation on the microbiome and gut-brain interaction in anorexia nervosa. *Front. Endocrinol. (Lausanne)* 10:41. doi: 10.3389/fendo.2019.00041

Sharon, G., Cruz, N. J., Kang, D. W., Gandal, M. J., Wang, B., Kim, Y. M., et al. (2019). Human gut microbiota from autism Spectrum disorder promote behavioral symptoms in mice. *Cells* 177:e1617, 1600–1618.e17. doi: 10.1016/j.cell.2019.05.004

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

Tilg, H., Adolph, T. E., and Trauner, M. (2022). Gut-liver axis: pathophysiological concepts and clinical implications. *Cell Metab.* 34, 1700–1718. doi: 10.1016/j.cmet.2022.09.017

Treasure, J., Zipfel, S., Micali, N., Wade, T., Stice, E., Claudino, A., et al. (2015). Anorexia nervosa. *Nat. Rev. Dis. Primers.* 1:15074. doi: 10.1038/nrdp.2015.74

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

Wang, S., Zhu, H., Pan, L., Zhang, M., Wan, X., Xu, H., et al. (2023). Systemic inflammatory regulators and risk of acute-on-chronic liver failure: a bidirectional mendelian-randomization study. *Front. Cell Dev. Biol.* 11:1125233. doi: 10.3389/fcell.2023.1125233

Wilson, B. C., Derraik, J. G. B., Albert, B. B., Leong, K. S. W., Tweedie-Cullen, R. Y., Creagh, C., et al. (2023). An open-label pilot trial of faecal microbiome transfer to restore the gut microbiome in anorexia nervosa: protocol. *BMJ Open* 13:e070616. doi: 10.1136/bmjopen-2022-070616

Xu, Q., Ni, J. J., Han, B. X., Yan, S. S., Wei, X. T., Feng, G. J., et al. (2021). Causal relationship between gut microbiota and autoimmune diseases: a two-sample Mendelian randomization study. *Front. Immunol.* 12:746998. doi: 10.3389/fimmu.2021.746998

Yu, H., Wan, X., Yang, M., Xie, J., Xu, K., Wang, J., et al. (2023). A large-scale causal analysis of gut microbiota and delirium: a Mendelian randomization study. *J. Affect. Disord.* 329, 64–71. doi: 10.1016/j.jad.2023.02.078

Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., et al. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Mol. Psychiatry* 21, 786–796. doi: 10.1038/mp.2016.44

Zhu, F., Guo, R., Wang, W., Ju, Y., Wang, Q., Ma, Q., et al. (2020). Transplantation of microbiota from drug-free patients with schizophrenia causes schizophrenia-like abnormal behaviors and dysregulated kynurenine metabolism in mice. *Mol. Psychiatry* 25, 2905–2918. doi: 10.1038/s41380-019-0475-4

frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 16 November 2023
DOI 10.3389/fmicb.2023.1281233

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Ahmed Gamal,
Case Western Reserve University, United States
Yingli Jing,
Capital Medical University, China

*CORRESPONDENCE
Bingjin Ji
✉ 570676078@qq.com
Anren Zhang
✉ an0124@163.com

†These authors have contributed equally to this work

RECEIVED 22 August 2023
ACCEPTED 31 October 2023
PUBLISHED 16 November 2023

CITATION
Xiang W, Xiang H, Wang J, Jiang Y, Pan C, Ji B and Zhang A (2023) Fecal microbiota transplantation: a novel strategy for treating Alzheimer's disease.
Front. Microbiol. 14:1281233.
doi: 10.3389/fmicb.2023.1281233

COPYRIGHT
© 2023 Xiang, Xiang, Wang, Jiang, Pan, Ji and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbiota transplantation: a novel strategy for treating Alzheimer's disease

Wu Xiang[1,2†], Han Xiang[3†], Junyu Wang[2†], Yiqin Jiang[1], Chuanhui Pan[1], Bingjin Ji[1*] and Anren Zhang[2*]

[1]Department of Rehabilitation, Beibei Traditional Chinese Medical Hospital, Chongqing, China, [2]Department of Rehabilitation Medicine, Shanghai Fourth People's Hospital Affiliated to Tongji University School of Medicine, Shanghai, China, [3]Department of Radiology, Daping Hospital, Army Medical University, Chongqing, China

Alzheimer's disease is a common neurological disorder, which has become one of the major factors affecting human health due to its serious impact on individuals, families and society. It has been confirmed that gut microbiota can affect the occurrence and development of Alzheimer's disease. Especially, fecal microbiota transplantation plays a positive role in the treatment of Alzheimer's disease. The mechanisms for improving Alzheimer's disease might include anti-inflammation and regulation of amyloid β-protein, synaptic plasticity, short-chain fatty acids, and histone acetylation. In this mini-review, the relationship between fecal microbiota transplantation and Alzheimer's disease was summarized. It is hoped that fecal microbiota transplantation would play a positive role in the prevention and treatment of Alzheimer's disease in the future.

KEYWORDS

fecal microbiota transplantation, gut microbiota, Alzheimer's disease, review, mechanism

1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease and the most common cause of dementia. The pathological features of AD mainly include deposition of amyloid β-protein (Aβ) (Duyckaerts et al., 2009; Bloom, 2014; Jouanne et al., 2017), neurofibrillary tangles caused by hyperphosphorylated tau protein (Duyckaerts et al., 2009; Bloom, 2014), and enhancement of neuroinflammation (Felsky et al., 2019). AD is usually characterized by cognitive deficits, as well as impairments in expressive speech, visuospatial processing, and executive function (Knopman et al., 2021). Epidemiology studies show that over 50 million people worldwide have been affected by AD (Hodson, 2018). According to the World Alzheimer's Disease Report, the number of people with dementia is expected to increase to 139 million by 2050 due to an aging population, among which AD accounting for approximately 60–80% (World Alzheimer Report, 2022).

The etiology of AD may be the result of the interaction between multiple factors, including age, genetic factors, family history, lifestyle actors, and environmental factors. For example, the percentage of AD patients increases dramatically with age. It has been found that 5% of people age 65 to 74, 13.1% of people age 75 to 84 and 33.3% of people age 85 and older suffer from AD (Rajan et al., 2021; Wang et al., 2021). Meanwhile, many genes can increase the risk of AD, among which apolipoprotein E4 is the greatest effect on risk of late-onset AD (Bellenguez et al., 2022). People who have or had a parent or sibling with AD

are more likely to develop the disorder (Loy et al., 2014). Genetic and non-genetic factors (e.g., diet and exercise) may play a role when disorders spread within families (Alzheimer's Association, 2023). In addition, gender (World Alzheimer Report, 2022), smoking (Jeong et al., 2023), education (Manly et al., 2022), traumatic head injury (Schneider et al., 2021), cardiovascular disease (Samieri et al., 2018), gut microbiota (Kim et al., 2020), and other heterogeneous factors (Abolhasani et al., 2023) have been reported to be related to AD. These studies show that AD poses an enormous threat to global health because of high incidence rate, lacking effective efficacious pharmacotherapy and poor prognosis. Therefore, it is necessary to develop new therapeutic approaches to solve this disease.

There are about 100 trillion microorganisms in intestine (Valdes et al., 2018), which play an important role in maintaining the balance of the human body. At the same time, due to their key role in the regulation of the central nervous system, the gut microbiota is named the human "second brain" (Ridaura and Belkaid, 2015). There is a gut-brain axis between gut microbiota and the central nervous system (Morais et al., 2021; Mayer et al., 2022). The gut-brain axis consists of bidirectional communication between the enteric nervous system and the central nervous system, including the immune system, tryptophan metabolism, the vagus nerve and the enteric nervous system (Cryan et al., 2019). Relevant studies have confirmed that gut microbiota can slow down the AD progression by regulating brain function (Dodiya et al., 2019; Elangovan et al., 2019; Fujii et al., 2019; Zhou et al., 2019; Hazan, 2020; Park et al., 2021; Jin et al., 2023).

The gut microbiota of patients with AD is disordered. Research shows that the diversity of α and β of gut microbiota was reduced in patients with AD compared to healthy people (Liu et al., 2019). The gut microbiota disturbance has also been demonstrated in animal models (Harach et al., 2017). Currently, it has become a new strategy in the treatment of AD by targeting regulation of gut microbiota. Prebiotics (Sun et al., 2019a), probiotics (Akbari et al., 2016), and antibiotics (Angelucci et al., 2019) are associated with improvement of prognosis in AD. However, fecal microbiota transplantation (FMT) may be more effective, which involve a broader range of microbiome modifications than prebiotics, probiotics, or antibiotics (Smits et al., 2013). It has been shown that the symptoms of AD can be improved by FMT. Compared with mice from the same background and conventional breeding, FMT can regulate the disturbance of gut microbiota and improve their cognitive function in AD mice (Yang, 2018; Sun et al., 2019b).

In this mini review, the current knowledge about the relationship between gut microbiota and AD has been introduced, including the relationship between AD and gut microbiota, as well as the possible mechanisms of the effects of FMT. In addition, the research progress in FMT for AD has also been summarized (**Table 1**). Finally, we emphasize that FMT may become a new therapy for the treatment of AD.

2. Alzheimer's disease and gut microbiota

As the largest microbiota in the human body, the human gut microbiota has over 1000 species. "Healthy microbiome" is defined as an ideal set of a healthy "functional core": a complement of metabolic and other molecular functions that are performed by the microbiome within a particular habitat (Shafquat et al., 2014). Such a core might need to be present as genetic potential, and it must include at least the housekeeping functions necessary for individual microbial life (Lloyd-Price et al., 2016). Meanwhile, a healthy microbiome may be characterized further by the resistance of a microbiome to stress and perturbation and its ability to recover to a healthy functional profile afterward (Bodelier, 2011; Backhed et al., 2012).

Several studies have shown that the gut microbiota of AD patients or model animals is disrupted. For example, compared with age matched wild-type mice, the abundance of Odoribacter and Helicobacter increased in AD mice, while Prevotella decreased (Shen et al., 2017). Another study found that the abundance of Verrucomicrobia and Proteobacteria increased in AD mice, while Ruminococcus and Butyricicoccus decreased (Zhang et al., 2017). In clinical studies, the abundance of Firmicutes and Actinobacteria decreased in AD patients, while Bacteroidetes increased. Meanwhile, the phylum with elevated abundance was negatively correlated with Aβ1-42/Aβ1-40, and positively correlated with P-tau and P-tau/Aβ1-42 (Vogt et al., 2017).

Previous studies have found that transplanting gut microbiota of AD patients or mice into germ-free mice can lead to significant cognitive deficits in object localization and recognition. In contrast, FMT of healthy patients or mice can reverse symptoms and pathologic manifestations in AD model animals. For example, it has been demonstrated that cognitive deficits caused by deposition of Aβ and neurofibrillary tangles can be improved by FMT from healthy mice donors (Kim et al., 2020). Meanwhile, FMT could improve cognitive function by reducing deposition of Aβ, attenuating glial cell hyperactivation and secondary neuroinflammation, and decreasing blood-brain barrier (BBB) permeability (Sun et al., 2019b; Kim et al., 2021). The above evidence suggest that gut microbiota is closely related to the occurrence and development of AD. Based on the existing evidence, it is suggested that improving cognitive function by regulating gut microbiota may provide new ideas for the prevention and treatment of AD.

3. Definition and process of FMT

Fecal microbiota transplantation is a technology that places stool from a healthy donor into another patient's gastrointestinal tract to change the recipient's gut microbiota, thereby gaining therapeutic benefits (Gupta and Khanna, 2017). The first records of FMT have been traced back to the fourth century China, where it was used in patients with severe diarrhea (Zhang et al., 2012). Studies have shown that FMT has a positive effect on various diseases, including Clostridium difficile infection (CDI) (Hamilton et al., 2012), inflammatory bowel disease (Paramsothy et al., 2017), metabolic syndrome (Mocanu et al., 2021), autoimmune disorders (Yang et al., 2023), and neurological disorders (Xu et al., 2015). It is worth noting that FMT is also beneficial for AD (Yang, 2018; Sun et al., 2019b).

The process of FMT is rigorous. First of all, strict donor screening tests of FMT are needed (Cammarota et al., 2017),

TABLE 1 Summary of the application of FMT in the intervention of AD.

References	Experimental model	FMT intervention	Duration of intervention	Results
Dodiya et al., 2019	Recipients: 7-week-old antibiotic-treated APPPS1-21 male mice ($n = 9$); donors: age-matched APP PS1-21 male mice ($n = 9$)	Gastric gavage	Once a day until sacrifice	Reduced cecum weight, increased abundance of Bacteroides, Prevotella, and S24-7, resulted in the restoration of diversity and restored Aβ amyloidosis and microglia morphology
Elangovan et al., 2019	Tg-FO: 32-week-old 5xFAD mice, age-matched WT donors ($n = 8$); Tg-FY: 32-week-old 5xFAD mice, 10-week-old WT donors ($n = 8$)	Oral gavage	7 consecutive days	Improved spatial and recognition memory, enhanced recognition memory and reduced cortical Aβ loading.
Fujii et al., 2019	Recipients: 4-week-old germ-free C57BL/6N mice ($n = 7$); donors: AD patient and age-matched healthy volunteers ($n = 7$)	Infusion	75 weeks	Reduced the levels of GABA, taurine, tryptophan, tyrosine, and valine, increased propionate, worsened cognitive function, reduced abundance of Verrucomicrobia and Proteobacteria
Sun et al., 2019b	Recipients: APP/PS1 mice ($n = 8$); donors: WT mice ($n = 8$)	Oral gavage	Once a day for 4 weeks	Reduced levels of Aβ, COX-2, CD11b, Aβ40, Aβ42 and phosphorylation of tau protein; increased PSD-95 and synapsin I expression, reversed the changes of gut microbiota and SCFAs.
Zhou et al., 2019	Recipients: male 8-week-old SD rats injected with aggregated $A\beta_{1-42}$ ($n = 6$); donors: same as recipients, treated with xanthoceraside or sham ($n = 6$)	Oral gavage	Once a day for 16 days	Reversed gut microbiota dysbiosis in AD animals, changed the abundances of several phyla and genus of bacterial.
Wang et al., 2020	Recipients: rats were treated with MnCl2 ($n = 10$); donors: SPF Sprague–Dawley male rats ($n = 6$)	Oral gavage	5 weeks	Reduced Aβ and Tau expression, downregulated NLRP3 and neuroinflammatory factors expression in the cerebral
Kim et al., 2020	Recipients: $ADLR^{APT}$ mice ($n = 12$); donors: WT mice ($n = 12$).	Oral gavage	Once a day for 16 weeks	Ameliorated the formation of Aβ plaques and neurofibrillary tangles, glial reactivity and cognitive impairment, reversed abnormalities genes expression.
Hazan, 2020	Recipients: an 82-year-old male with CDI and 5-year history of AD; donors: the patient's 85-year-old wife	Infusion	Single infusion	Improved mental acuity and affect, memory and mood, increased the MMSE scores.
Li et al., 2020	Recipients: young (~ 3 months) male SD rats; donors: aged (20 ~ 24 months) male SD rats	Gavage	once a day for 3 days, then twice a week for 2 months	Impaired cognitive behavior, changed synaptic structures and decreased dendritic spines, reduced BDNF, NMDA receptor NR1 subunit and synaptophysin expression, increased AGEs, RAGE, pro-inflammatory cytokines and oxidative stress expression.
Shen et al., 2020	FMT-AD: APP/PS1 mice with AD human donor ($n = 5$); FMT-AD-HP: APP/PS1 mice with healthy human donor ($n = 5$); Con-FMT-AD: WT mice with AD human donor ($n = 5$).	Oral gavage	28 days	FMT-AD: increased the expression of NLRP3, inflammatory factors, activated microglia. FMT-AD-HP: reduced the expression of NLRP3 and neuroinflammatory factors, suppressed the activation of microglia. Con-FMT-AD: increased the expression of NLRP3 and inflammatory factors.
D'Amato et al., 2020	Recipients: antibiotic treatment male C57BL/6 mice ($n = 12$); donors: adult (3 months) and aged (24 months) male C57BL/6 mice ($n = 12$)	Oral gavage	6 days	Impaired spatial learning and memory, reduced bacteria associated with SCFAs and CNS disorders, and acquired an ageing-like phenotype of microglia cell.
Kim et al., 2021	Recipients: 8-week-old C57-BL/6 ($n = 8$); donors: 9-month-old WT and 5xFAD mice ($n = 8$)	Oral gavage	5 consecutive days	Reduced the expression of adult hippocampal neurogenesis and brain-derived neurotrophic factor, increased p21 expression and pro-inflammatory cytokines.

(Continued)

TABLE 1 (Continued)

References	Experimental model	FMT intervention	Duration of intervention	Results
Wang et al., 2021	Recipients: 3-month-old SPF APP/PS1 mice (n = 4); donors: 16-month-old APP/PS1 mice (n = 4).	Oral gavage	7 consecutive days	Increased Aβ plaques, suppressed astrocyte activation
Park et al., 2021	Recipients: 90-year-old woman with CDI, 5-year history of AD; donors: a 27-year-old man with healthy	Colonoscopy	2 infusions 3 months apart	Improved cognitive function, changed the microbiota composition and short-chain fatty acids.
Zhang et al., 2022	Recipients: 8-month-old adult male C57BL/6N mice; donors: WT mice.	None	None	Improved the gut permeability, reduced the levels of fecal LCN2, lowered the levels of serum pro-inflammatory factors TNF-α and MCP-1, reversed the microglia activation and Aβ protein deposition.
Hang et al., 2022	Recipients: Tg mice (n = 6); donors: tumor-bearing mice or WT mice (n = 6)	Gavage	3 times a week for 1 month	Improved short-term memory level and cognitive ability, regulated Inflammatory factors in the plasma, decreased A plaques burden in the hippocampus and cortex, and reversed the metabolism of inorganic and organic salts in the intestinal flora.
Jin et al., 2023	Recipients: newly weaned WT mice (n = 8); donors: 12-month-old APP/PS1 mice (n = 8)	Gavage	3 times a week for 1 month	Increased gut BACE1 and Aβ42 levels.

SOD, superoxide dismutase; BDNF, brain derived neurotrophic factor; NT3, neurotrophin-3; NGF, nerve growth factor; GABA, γ-aminobutyric acid; MMSE, mini mental status examination; NMDA, *N*-methyl-D-aspartate; CNS, central nervous system; AGE, advanced glycation end products; RAGE, receptor for advanced glycation end products.

including a donor questionnaire, additional interview, standard donor screening protocols and the time between screening and donation. Secondly, patients undergoing FMT need support and education prior to treatment and antibiotics are avoided 12–48 h before fecal infusion (Blackburn et al., 2015). Finally, the current administration of stool by means include oral capsule, lower gastrointestinal route (via colonoscopy or retention enema) and upper gastrointestinal route (via nasogastric, nasojejunal, esophagogastroduodenoscopy, or nasoduodenal tube) (Wang et al., 2019). For example, a study showed that a non-significant difference in cure rate of recurrent CDI between upper and lower gastrointestinal routes of FMT (Youngster et al., 2014). However, another study found that the lower gastrointestinal route had higher clinical cure rate than the upper gastrointestinal route in CDI patients (Kassam et al., 2013). Furthermore, FMT via oral capsules had comparable results to delivery by colonoscopy in prevention of recurrent CDI (Kao et al., 2017). To sum up, there is no current strong evidence of the optimal FMT measure has been proved in clinical treatment, and it is recommended to select according to individual situation of patients.

4. Biological mechanisms of FMT to improve AD

More and more evidence showed that FMT may have potential for the prevention and treatment of AD. A randomized controlled trial suggested that the abundance of Proteobacteria and Verrucomicrobia decreased, while Bacteroidetes increased, in the FMT-treated mice. Meanwhile, the spatial learning ability and familiarity with novelty performed of FMT-treated mice better than AD model mice (Sun et al., 2019b). A randomized controlled trial suggested that the abundance of Proteobacteria and Verrucomicrobia decreased, while Bacteroidetes increased, in the FMT-treated mice. Meanwhile, the spatial learning ability and familiarity with novelty performed better than AD model mice (Sun et al., 2019b). Another study showed that FMT could reverse the disturbance of gut microbiota in AD mice and enhance the learning and memory ability (Yang, 2018). Numerous studies have shown that FMT can improve AD, which may be related to reducing the abundance of pathogenic bacteria, exerting the anti-inflammatory effects, decreasing the deposition of Aβ, regulating synaptic plasticity, increasing short chain fatty acids, and curbing histone acetylation.

4.1. Anti-inflammatory mechanisms

Inflammation plays an important role in the occurrence and development of many diseases, including metabolic disorders, immune system disorders, cardiovascular diseases and nervous system diseases.

The high expression of inflammatory factors can lead to metabolic disorders of neurotransmitters and disrupt the regulation and signaling mechanisms of behavior and cognition (Johnson et al., 2021). Research have found that the level of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) is significantly increased in the cerebrospinal fluid and peripheral blood of AD patients (Cattaneo et al., 2017). The symptoms of AD could be improved by inhibition of pro-inflammatory cytokines

and enhancement of anti-inflammatory cytokines. For example, the defect of exercise behavior induced by lipopolysaccharide improved and the number of activated microglia of nigra reduced while the IL-1 receptor antagonists was added to the brain of P70 newborn rats (Pang et al., 2015). Meanwhile, the cognitive and memory function of transgenic male mice with IL-6 knock-out was lower than wild-type control mice (Hryniewicz et al., 2007). A 6-month prospective, single-center, open study showed that the cognitive function of AD patients was improved by TNF-α inhibitor enalapril (Tobinick et al., 2006). In addition, FMT could increase the level of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and interleukin-22 (IL-22) (Kim et al., 2021).

Fecal microbiota transplantation can inhibit the level of pro-inflammatory cytokines and increase anti-inflammatory cytokines. For example, a study showed that manganese could induce the deposition of Aβ and production of tau protein, increasing inflammatory cytokines (e.g., IL-1β) and NLRP3 inflammasomes. FMT from healthy rat donors relieve neurotoxicity by reversing the above changes, but the composition of FMT is unclear (Wang et al., 2020). Another study found that FMT from AD mice donors led to impaired cognitive impairment, activated microglia, and increased NLRP3 and inflammatory factors (including IL-1β, IL-18, and TNF-α). The above changes could be reversed by FMT from healthy people donors, but the composition of FMT is unclear (Shen et al., 2020). In addition, the level of pro-inflammatory cytokines increased, such as TNF-α and monocyte chemoattractant protein-1 (MCP-1), in AD mice. Pearson correlation analysis showed that Ruminiclostridium_5 was positively correlated with inflammatory markers TNF-α and MCP-1. Nevertheless, FMT from wild type mice significantly decreased serum TNF-α and MCP-1 levels, which may be associated with reducing the abundance of Ruminiclostridium_5 (Zhang et al., 2022). Hang et al. (2022) showed that FMT treatment could increase the level of anti-inflammatory factors IL-2 and transforming growth factor-β (TGF-β) and reduce pro-inflammatory factors TNF-α and IL-1β in AD mice. The results may be associated with the increased Firmicutes and Prevotella and the decreased Bacteroidetes, Bacteroides and Sutterella. Yang (2018) found that the level of anti-inflammatory factors increased, such as IL-10, and the pro-inflammatory cytokines decreased, such as TNFα and IL-6, in the FMT-treated mice. The results may be associated with the increased Firmicutes, Bacteroidales, Verrucomicrobia, Clostridia, and Bacteroidia, and the decreased Erysipelotrichia, Clostridia, and Bacteroidia. Xu et al. (2020) showed that yeast β-glucans could increase beneficial bacteria and reduce pathogenic bacteria in the gut microbiota of AD mice, and reverse the increased IL-1β, IL-5, IL-6, and INF- γ and the decreased IL-10. Meanwhile, spearman correlation analyses showed that there were three negatively correlated (Oscillibacter, Butyricicoccus, and Mucispirillum) and two positively correlated (Lactobacillus and Bifidobacterium) with anti-inflammatory. Inversely, there were two negatively correlated (Lactobacillus and Bifidobacterium) and six positively correlated (Alistipes, Oscillibacter, Butyricicoccus, Rikenella, Mucispirillum, and Anaerotruncus) with pro-inflammatory. The above studies suggest that FMT may have a positive effect on cognitive function by regulating the gut microbiota, reducing the expression of pro-inflammatory cytokines, and increasing anti-inflammatory factors.

4.2. Regulating Aβ protein and synaptic plasticity

Previous studies have shown that excessive deposition of Aβ can accelerate the cascade of oxidative stress and neuroinflammation, and induce apoptosis of nerve cells. It has been reported that Aβ42 aggregation is the main cause of Aβ42 toxic protein, and the latter is the major reason of AD. The development of AD can be effectively prevented by inhibiting Aβ42 aggregation (Lansbury and Lashuel, 2006). Meanwhile, high level of Aβ was detected throughout the intestines of both mice and humans. The expression of Aβ42 increased in colons of mice after receiving the gut microbiota of aged APP/PS1 mice donors (Jin et al., 2023). Moreover, low density lipoprotein receptor-related protein 1 (LRP-1) overexpressing expression mice had reduced the level of Aβ in the blood and brain, which was associated with spatial learning, memory consolidation, and spatial recognition memory (Cheng et al., 2023). The above studies suggest a correlation between Aβ42 and AD.

It has been demonstrated that Aβ targets the modulation of synapses (Almeida et al., 2005), and impairment of synaptic plasticity is associated with cognitive decline in AD (D'Amato et al., 2020).

The definition of synaptic plasticity is the ability of synapses to adjust their function or shift shape in response to changes of internal and external environment. The time-dependent appearance of neurofibrillary tangles after deposition of Aβ can alter synaptic function, affect synaptic plasticity and cause synaptic loss in AD patients. Meanwhile, the memory and synaptic plasticity were damaged by oxidative stress induced by the imbalance between antioxidants and free radicals (Sehar et al., 2022). The change of synaptic plasticity in AD patients is characterized by synaptic dysfunction and impairment of synaptic morphology and structure, which regulated by neurotransmitters, synaptic plasticity related proteins and signaling pathways.

In recent years, the main target of synaptic plasticity mechanism studies is synapse-associated proteins, including growth-associated protein-43, synaptophysin, and postsynaptic density protein-95 (PSD-95). The synaptic function is inhibited by reduction of synapse-associated proteins. The expression of synaptic protein and PSD-95 decreased in AD mice. For example, the expression of PSD-95 in Tg2576 APP mutant neurons was lower than wild-type neurons (Almeida et al., 2005). In contrast, the synaptic plasticity enhanced by increasing the expression of PSD-95, thereby improving the reduction of synapse associated protein. Research showed that FMT from aged donors led to changed expression of proteins involved in synaptic plasticity and neurotransmission in young adult recipients. Meanwhile, four genera (Prevotellaceae, Faecalibaculum, Lachnospiraceae, and Ruminococcaceae) were found to be significantly differentially abundant compared to adult mice. There were three (Faecalibaculum, Lachnospiraceae, and Ruminococcaceae) were significantly correlated with proteins implicated in mitochondrial energy metabolism and neurotransmitter transport (D'Amato et al., 2020). Furthermore, FMT from aged donors led to significant changes in synaptic structure of young rats, which may be associated with reducing the abundance of Bacteroidetes, Prevotella, Bacteroides, and

Parabacteroides. However, FMT treatment could reduce the brain deposition of Aβ40 and Aβ42 and increase the expression of PSD-95 and synapsin I in AD model mice, which may be associated with increasing the abundance of Desulfovibrionaceae (Sun et al., 2019b). Therefore, we speculate that FMT may improve AD by reducing deposition of Aβ and inducing synapse recurrent (Li et al., 2020).

4.3. Regulating the short chain fatty acids and histone acetylation

Short chain fatty acids (SCFAs) are the main metabolites of the gut microbiota, including acetate, propionate, butyrate, pentanoate and Caproate. SCFAs can regulate human homeostasis and play critical roles in biological functions. Many studies have shown that SCFAs are closely related to the occurrence and development of AD. A study found that the acetate concentration decreased in the AD model drosophila induced by Aβ deposition (Kong et al., 2021). Meanwhile, the concentrations of propionate, butyrate, and isobutyric acid in AD model mice was lower than wild-type mice (Zheng et al., 2019). In clinical studies, the expression of SCFAs in AD patients was the lowest compared to healthy humans and patients with mild cognitive impairment (Wu et al., 2021). An increase in SCFAs can reduce deposition of Aβ and improve cognitive impairment in AD mice. For example, the reshaped gut microbiome and enhanced butyrate formation are highly associated with behavioral alteration and brain oxidative status, and SCFAs attenuated the behavioral disorders and Aβ accumulation in AD mice (Liu et al., 2021a). Furthermore, there was a strong correlation between the levels of increased SCFAs and reduced cognitive functions in AD mice (Liu et al., 2021b). These studies show that SCFAs play an important role in the occurrence and development of AD.

There is a closely relation between histone deacetylases (HDACs) and AD. In the hippocampus, HDAC1, HDAC2, and HDAC8 are reported to strengthen neuroinflammation associated with cognitive dysfunction, while HDAC3 decreases dendritic spine density and levels of proteins associated with synaptic plasticity (Yang et al., 2022). Meanwhile, it has been reported that the increased activity of HDAC6 related to memory impairment (Li et al., 2021). However, treatment with acetate was shown to reduce mRNA levels of HDAC2, HDAC5, HDAC7, and HDAC8 (Huang et al., 2021), which indicate that SCFAs can regulate the expression of HDACs. According to the report, SCFAs (such as butyrate) can inhibit the activity of histone deacetylase (Tan et al., 2014), reduce deposition of Aβ in neurons. Treatment with butyrate can improve cognitive functioning in AD mice by adding hippocampal acetylation and increasing the expression of genes associated with synaptic plasticity (Govindarajan et al., 2011). At the same time, butyrate can serve as a histone deacetylase inhibitor to enhance the acetylation of histones adjacent to the neurotrophic factor promoter, thereby improving memory (Barichello et al., 2015). Furthermore, acetyl CoA converted by acetate can serve as an acetyl donor for histone acetylation, thus altering the inflammatory signaling in the microglia of brains (Shi and Tu, 2015). The above studies suggest that SCFAs may improve AD by affecting histone acetylation.

Many studies showed that FMT treatment could improve AD by regulating gut microbiota and increasing the expression of SCFAs. For example, a study showed that FMT from aged donors led to impaired spatial learning and memory in young adult recipients, which may be associated with a strong reduction of bacteria associated with SCFAs production (Lachnospiraceae, Faecalibaculum, and Ruminococcaceae) (D'Amato et al., 2020). However, yeast β-glucans could reverse the increased Firmicutes, Oscillibacter, Mucispirillum, and Butyricicoccus, and the decreased Bacteroidetes, Lactobacillus, and Bifidobacterium in AD mice (Xu et al., 2020). Meanwhile, yeast β-glucans treatment was able to trigger the generation of SCFAs, which may be associated with increasing the abundance of Bacteroidetes, Lactobacillus and Bifidobacterium (Xu et al., 2020). Moreover, another study found that FMT could reverse the decreased Bacteroidetes in the Tg mice. Meanwhile, the level of butyrate was significantly increased in the FMT-treated mice, which may be associated with increasing the abundance of Bacteroidetes (Sun et al., 2019b). Therefore, we speculate that FMT may improve AD by increasing the inhibition of histone acetylation by SCFAs. Therefore, we speculate that FMT may improve AD by increasing the inhibition of histone acetylation by SCFAs.

5. Conclusion and future directions

Based on the above evidence, we conclude that FMT is beneficial for AD. AD has a serious impact on the health and quality of life of patients, and it is urgent to find low cost and few side effects intervention measures. However, there is relatively less research on the effects of FMT on AD, especially in clinical studies. So far, only three articles involved in FMT and AD, but no clinical trials have been published, among which one was terminated early due to the COVID-19 pandemic. Therefore, there is an urgent need for large-scale clinical trials to verify whether FMT can be used as a strategy for the treatment of AD. At the same time, seven gut microbiotas were defined as a good FMT, such as, Lachnospiraceae, Faecalibaculum, Ruminococcaceae, Bacteroidetes, Lactobacillus, Bifidobacterium, and Desulfovibrionaceae, which still needs to be further validation.

This mini-review summarized the evidence of FMT in the treatment of AD in recent years, which can bring significant benefits to the prevention and treatment of AD. FMT play a positive effect on AD through the anti-inflammatory effects, regulating deposition of Aβ, synaptic plasticity, SCFAs, and the histone acetylation. Our findings may provide evidence for fecal microbiota-related drug preparations as adjuvant therapy for the prevention and treatment of AD. In the future, the biological mechanism of improvement of AD of FMT still needs to be further clarified.

Author contributions

WX: Writing – original draft, Writing – review and editing. HX: Writing – original draft, Writing – review and editing. JW: Writing – original draft, Writing – review and editing. YJ: Writing – review and editing. CP: Writing – review and

editing. BJ: Conceptualization, Writing – review and editing. AZ: Conceptualization, Writing – review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the General Program of National Natural Science Foundation of China (81973927), the Key R&D Program of Sichuan Department of Science and Technology (2021YFS0133), the Special Project for Scientific Research of Traditional Chinese Medicine in Sichuan Province (2020), the Special Project of Traditional Chinese Medicine Scientific Research of Sichuan Provincial Administration of Traditional Chinese Medicine (2020LC0224), the Hospital Project of the General Hospital of the Western Theater (2019), and the Chongqing Beibei District Science and Technology Bureau Project (2022-18).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Abolhasani, E., Hachinski, V., Ghazaleh, N., Azarpazhooh, M. R., Mokhber, N., and Martin, J. (2023). Air pollution and incidence of dementia: A systematic review and meta-analysis. *Neurology* 100, e242–e254. doi: 10.1212/WNL.0000000000201419

Akbari, E., Asemi, Z., Kakhaki, R. D., Bahmani, F., Kouchaki, E., Tamtaji, O., et al. (2016). Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's Disease: A randomized, double-blind and controlled trial. *Front. Aging Neurosci* 8:256. doi: 10.3389/fnagi.2016.00256

Almeida, C. G., Tampellini, D., Takahashi, R. H., Greengard, P., Lin, M. T., Snyder, E. M., et al. (2005). Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. *Neurobiol. Dis.* 20, 187–198. doi: 10.1016/j.nbd.2005.02.008

Alzheimer's Association (2023). 2023 Alzheimer's disease facts and figures. *Alzheimers Dement.* 19, 1598–1695. doi: 10.1002/alz.13016

Angelucci, F., Cechova, K., Amlerova, J., and Hort, J. (2019). Antibiotics, gut microbiota, and Alzheimer's disease. *J. Neuroinflamm.* 16:108. doi: 10.1186/s12974-019-1494-4

Backhed, F., Fraser, C. M., Ringel, Y., Sanders, M. E., Sartor, R. B., Sherman, P. M., et al. (2012). Defining a healthy human gut microbiome: Current concepts, future directions, and clinical applications. *Cell. Host Microbe* 12, 611–622. doi: 10.1016/j.chom.2012.10.012

Barichello, T., Generoso, J. S., Simoes, L. R., Faller, C. J., Ceretta, R. A., Petronilho, F., et al. (2015). Sodium butyrate prevents memory impairment by re-establishing BDNF and GDNF expression in experimental pneumococcal meningitis. *Mol. Neurobiol.* 52, 734–740. doi: 10.1007/s12035-014-8914-3

Bellenguez, C., Küçükali, F., Jansen, I. E., Kleineidam, L., Moreno-Grau, S., Amin, N., et al. (2022). New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat. Genet.* 54, 412–436. doi: 10.1038/s41588-022-01024-z

Blackburn, L. M., Bales, A., Caldwell, M., Cordell, L., Hamilton, S., and Kreider, H. (2015). Fecal microbiota transplantation in patients with cancer undergoing treatment. *Clin. J. Oncol. Nurs.* 19, 111–114. doi: 10.1188/15.CJON.111-114

Bloom, G. S. (2014). Amyloid-β and tau: The trigger and bullet in Alzheimer disease pathogenesis. *JAMA Neurol.* 71, 505–508. doi: 10.1001/jamaneurol.2013.5847

Bodelier, P. L. (2011). Toward understanding, managing, and protecting microbial ecosystems. *Front. Microbiol.* 2:80. doi: 10.3389/fmicb.2011.0008

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., Kump, P., Satokari, R., et al. (2017). European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* 66, 569–580. doi: 10.1136/gutjnl-2016-313017

Cattaneo, A., Cattane, N., Galluzzi, S., Provasi, S., Lopizzo, N., Festari, C., et al. (2017). Association of brain amyloidosis with proinflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiol. Aging* 49, 60–68. doi: 10.1016/j.neurobiolaging.2016.08.019

Cheng, Y., He, C. Y., Tian, D. Y., Chen, S. H., Ren, J. R., Sun, H. L., et al. (2023). Physiological β-amyloid clearance by the liver and its therapeutic potential for Alzheimer's disease. *Acta Neuropathol.* 145, 717–731. doi: 10.1007/s00401-023-02559-z

Cryan, J. F., O'Riordan, K. J., Cowan, C. S. M., Sandhu, K. V., Bastiaanssen, T. F. S., Boehme, M., et al. (2019). The microbiota-gut-brain axis. *Physiol. Rev.* 99, 1877–2013. doi: 10.1152/physrev.00018.2018

D'Amato, A., Di Cesare Mannelli, L., Lucarini, E., Man, A. L., Le Gall, G., Branca, J. J. V., et al. (2020). Faecal microbiota transplant from aged donor mice affects spatial learning and memory via modulating hippocampal synaptic plasticity- and neurotransmission-related proteins in young recipients. *Microbiome* 8:140. doi: 10.1186/s40168-020-00914-w

Dodiya, H. B., Kuntz, T., Shaik, S. M., Baufeld, C., Leibowitz, J., Zhang, X., et al. (2019). Sex-specific effects of microbiome perturbations on cerebral Abeta amyloidosis and microglia phenotypes. *J. Exp. Med.* 216, 1542–1560. doi: 10.1084/jem.20182386

Duyckaerts, C., Delatour, B., and Potier, M. C. (2009). Classification and basic pathology of Alzheimer disease. *Acta Neuropathol.* 118, 5–36. doi: 10.1007/s00401-009-0532-1

Elangovan, S., Borody, T. J., and Holsinger, R. M. D. (2019). Fecal microbiota transplantation decreases amyloid load and improves cognition in Alzheimer's. *BioRxiv[Preprint]*. doi: 10.1101/687376

Felsky, D., Roostaei, T., Nho, K., Risacher, S. L., Bradshaw, E. M., Petyuk, V., et al. (2019). Neuropathological correlates and genetic architecture of microglial activation in elderly human brain. *Nat. Commun.* 10:409. doi: 10.1038/s41467-018-08279-3

Fujii, Y., Nguyen, T. T. T., Fujimura, Y., Kameya, N., Nakamura, S., Arakawa, K., et al. (2019). Fecal metabolite of a gnotobiotic mouse transplanted with gut microbiota from a patient with Alzheimer's disease. *Biosci. Biotechnol. Biochem* 83, 2144–2152.

Govindarajan, N., Agis-Balboa, R. C., Walter, J., and Sananbenesi, F. (2011). Fischer A Sodium butyrate improves memory function in an Alzheimer's disease mouse model when administered at an advanced stage of disease progression. *J. Alzheimers Dis.* 26:187197. doi: 10.3233/JAD-2011-110080

Gupta, A., and Khanna, S. (2017). Fecal microbiota transplantation. *JAMA* 318:102. doi: 10.1001/jama.2017.6466

Hamilton, M. J., Weingarden, A. R., Sadowsky, M. J., and Khoruts, A. (2012). Standardized frozen preparation for transplantation of fecal microbiota for recurrent *Clostridium difficile* infection. *Am. J. Gastroenterol.* 107, 761–767. doi: 10.1038/ajg.2011.482

Hang, Z., Cai, S., Lei, T., Zhang, X., Xiao, Z., Wang, D., et al. (2022). Transfer of tumor-bearing mice intestinal flora can ameliorate cognition in Alzheimer's Disease Mice. *J. Alzheimers Dis.* 86, 1287–1300. doi: 10.3233/JAD-215495

Harach, T., Marungruang, N., Duthilleul, N., Cheatham, V., Mc Coy, K. D., Frisoni, G., et al. (2017). Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. *Sci. Rep.* 7:41802. doi: 10.1038/srep41802

Hazan, S. (2020). Rapid improvement in Alzheimer's disease symptoms following fecal microbiota transplantation: A case report. *J. Int. Med. Res.* 48:0300060520925930. doi: 10.1177/030006052092593

Hodson, R. (2018). Alzheimer's disease. *Nature* 559:S1. doi: 10.1038/d41586-018-05717-6

Hryniewicz, A., Bialuk, I., Kamiński, K. A., and Winnicka, M. M. (2007). Impairment of recognition memory in interleukin-6 knock-out mice. *Eur. J. Pharmacol.* 577, 219–220.

Huang, W., Hu, W., Cai, L., Zeng, G., Fang, W., Dai, X., et al. (2021). Acetate supplementation produces antidepressant-like effect via enhanced histone acetylation. *J. Affect. Disord.* 281, 51–60. doi: 10.1016/j.jad.2020.11.121

Jeong, S. M., Park, J., Han, K., Yoo, J., Yoo, J. E., Lee, C. M., et al. (2023). Association of changes in smoking intensity with risk of dementia in Korea. *JAMA Netw. Open* 6:e2251506. doi: 10.1001/jamanetworkopen.2022.51506

Jin, J., Xu, Z., Zhang, L., Zhang, C., Zhao, X., Mao, Y., et al. (2023). Gut-derived β-amyloid: Likely a centerpiece of the gut-brain axis contributing to Alzheimer's pathogenesis. *Gut Microbes* 15:2167172. doi: 10.1080/19490976.2023.2167172

Johnson, D., Thurairajasingam, S., Letchumanan, V., Chan, K. G., and Lee, L. H. (2021). Exploring the role and potential of probiotics in the field of mental health: Major depressive disorder. *Nutrients* 13:1728. doi: 10.3390/nu13051728

Jouanne, M., Rault, S., and Voisin-Chiret, A. S. (2017). Tau protein aggregation in Alzheimer's disease: An attractive target for the development of novel therapeutic agents. *Eur. J. Med. Chem.* 139, 153–167. doi: 10.1016/j.ejmech.2017.07.070

Kao, D., Roach, B., Silva, M., Beck, P., Rioux, K., Kaplan, G. G., et al. (2017). Effect of oral capsule- vs colonoscopy-delivered fecal microbiota transplantation on recurrent *Clostridium difficile* infection: A randomized clinical trial. *JAMA* 318, 1985–1993. doi: 10.1001/jama.2017.17077

Kassam, Z., Lee, C. H., Yuan, Y., and Hunt, R. H. (2013). Fecal microbiota transplantation for *Clostridium difficile* infection: Systematic review and meta-analysis. *Am. J. Gastroenterol.* 108, 500–508. doi: 10.1038/ajg.2013.59

Kim, M. S., Kim, Y., Choi, H., Kim, W., Park, S., Lee, D., et al. (2020). Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model. *Gut* 69, 283–294. doi: 10.1136/gutjnl-2018-317431

Kim, N., Jeon, S. H., Ju, I. G., Gee, M. S., Do, J., Oh, M. S., et al. (2021). Transplantation of gut microbiota derived from Alzheimer's disease mouse model impairs memory function and neurogenesis in C57BL/6 mice. *Brain Behav. Immun.* 98, 357–365. doi: 10.1016/j.bbi.2021.09.002

Knopman, D. S., Amieva, H., Petersen, R. C., Chételat, G., Holtzman, D. M., Hyman, B. T., et al. (2021). Alzheimer disease. *Nat. Rev. Dis. Primers* 7:33. doi: 10.1038/s41572-021-00269-y

Kong, Y., Wang, L., and Jiang, B. (2021). The role of gut microbiota in aging and aging related neurodegenerative disorders: Insights from drosophila model. *Life* 11:855. doi: 10.3390/life11080855

Lansbury, P. T., and Lashuel, H. A. (2006). A century-old debate on protein aggregation and neurodegeneration enters the clinic. *Nature* 443, 774–779. doi: 10.1038/nature05290

Li, Y., Ning, L., Yin, Y., Wang, R., Zhang, Z., Hao, L., et al. (2020). Age-related shifts in gut microbiota contribute to cognitive decline in aged rats. *Aging* 12, 7801–7817. doi: 10.18632/aging.103093

Li, Y., Sang, S., Ren, W., Pei, Y., Bian, Y., Chen, Y., et al. (2021). Inhibition of Histone Deacetylase 6 (HDAC6) as a therapeutic strategy for Alzheimer's disease: A review (2010-2020). *Eur. J. Med. Chem.* 226:113874. doi: 10.1016/j.ejmech.2021.113874

Liu, P., Wu, L., Peng, G., Han, Y., Tang, R., Ge, J., et al. (2019). Altered microbiomes distinguish Alzheimer's disease from amnestic mild cognitive impairment and health in a Chinese cohort. *Brain Behav. Immun.* 80, 633–643. doi: 10.1016/j.bbi.2019.05.008

Liu, Q., Xi, Y., Wang, Q., Liu, J., Li, P., Meng, X., et al. (2021a). Mannan oligosaccharide attenuates cognitive and behavioral disorders in the 5xFAD Alzheimer's disease mouse model via regulating the gut microbiota-brain axis. *Brain Behav. Immun.* 95, 330–343. doi: 10.1016/j.bbi.2021.04.005

Liu, Q., Xie, T., Xi, Y., Li, L., Mo, F., Liu, X., et al. (2021b). Sesamol attenuates amyloid peptide accumulation and cognitive deficits in APP/PS1 Mice: The mediating role of the gut-brain axis. *J. Agric. Food. Chem.* 69, 12717–12729. doi: 10.1021/acs.jafc.1c04687

Lloyd-Price, J., Abu-Ali, G., and Huttenhower, C. (2016). The healthy human microbiome. *Genome. Med.* 8:51. doi: 10.1186/s13073-016-0307-y

Loy, C. T., Schofield, P. R., Turner, A. M., and Kwok, J. B. (2014). Genetics of dementia. *Lancet* 383, 828–840. doi: 10.1016/S0140-6736(13)60630-3

Manly, J. J., Jones, R. N., Langa, K. M., Ryan, L. H., Levine, D. A., McCammon, R., et al. (2022). Estimating the prevalence of dementia and mild cognitive impairment in the US: The 2016 health and retirement study harmonized cognitive assessment protocol project. *JAMA Neurol.* 79, 1242–1249. doi: 10.1001/jamaneurol.2022.3543

Mayer, E. A., Nance, K., and Chen, S. (2022). The gut-brain axis. *Annu. Rev. Med.* 73, 439–453. doi: 10.1146/annurev-med-042320-014032

Mocanu, V., Zhang, Z., Deehan, E. C., Kao, D. H., Hotte, N., Karmali, S., et al. (2021). Fecal microbial transplantation and fiber supplementation in patients with severe obesity and metabolic syndrome: A randomized double-blind, placebo-controlled phase 2 trial. *Nat. Med.* 27, 1272–1279. doi: 10.1038/s41591-021-01399-2

Morais, L. H., Schreiber, H. L. IV, and Mazmanian, S. K. (2021). The gut microbiota-brain axis in behaviour and brain disorders. *Nat. Rev. Microbiol.* 19, 241–255. doi: 10.1038/s41579-020-00460-0

Pang, Y., Tien, L. T., Zhu, H., Shen, J., Wright, C. F., Jones, T. K., et al. (2015). Interleukin-1 receptor antagonist reduces neonatal lipopolysaccharide-induced long-lasting neurobehavioral deficits and dopaminergic neuronal injury in adult rats. *Int. J. Mol. Sci.* 16, 8635–8654.

Paramsothy, S., Kamm, M. A., Kaakoush, N. O., Walsh, A. J., van den Bogaerde, J., Samuel, D., et al. (2017). Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: A randomised placebo-controlled trial. *Lancet* 389, 1218–1228. doi: 10.1016/S0140-6736(17)30182-4

Park, S. H., Lee, J. H., Shin, J., Kim, J. S., Cha, B., Lee, S., et al. (2021). Cognitive function improvement after fecal microbiota transplantation in Alzheimer's dementia patient: A case report. *Curr. Med. Res. Opin.* 37, 1739–1744. doi: 10.1080/03007995.2021.1957807

Rajan, K. B., Weuve, J., Barnes, L. L., McAninch, E. A., Wilson, R. S., and Evans, D. A. (2021). Population estimate of people with clinical Alzheimer's disease and mild cognitive impairment in the United States (2020-2060). *Alzheimers Dement.* 17, 1966–1975. doi: 10.1002/alz.12362

Ridaura, V., and Belkaid, Y. (2015). Gut microbiota: The link to your second brain. *Cell* 161, 193–194. doi: 10.1016/j.cell.2015.03.033

Samieri, C., Perier, M. C., Gaye, B., Proust-Lima, C., Helmer, C., Dartigues, J. F., et al. (2018). Association of cardiovascular health level in older age with cognitive decline and incident dementia. *JAMA* 320, 657–664. doi: 10.1001/jama.2018.11499

Schneider, A. L. C., Selvin, E., Latour, L., Turtzo, L. C., Coresh, J., Mosley, T., et al. (2021). Head injury and 25-year risk of dementia. *Alzheimers Dement.* 17, 1432–1441. doi: 10.1002/alz.12315

Sehar, U., Rawat, P., Reddy, A. P., Kopel, J., and Reddy, P. H. (2022). Amyloid beta in aging and Alzheimer's Disease. *Int. J. Mol. Sci.* 23:12924. doi: 10.3390/ijms232112924

Shafquat, A., Joice, R., Simmons, S. L., and Huttenhower, C. (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. *Trends Microbiol.* 22, 261–266. doi: 10.1016/j.tim.2014.01.011

Shen, H., Guan, Q., Zhang, X., Yuan, C., Tan, Z., Zhai, L., et al. (2020). New mechanism of neuroinflammation in Alzheimer's disease: The activation of NLRP3 inflammasome mediated by gut microbiota. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 100:109884. doi: 10.1016/j.pnpbp.2020.109884

Shen, L., Liu, L., and Ji, H. F. (2017). Alzheimer's disease histological and behavioral manifestations in transgenic mice correlate with specific gut microbiome state. *J. Alzheimers Dis.* 56, 385–390. doi: 10.3233/JAD-160884

Shi, L., and Tu, B. P. (2015). Acetyl-CoA and the regulation of metabolism: Mechanisms and consequences. *Curr. Opin. Cell Biol.* 33, 125–131. doi: 10.1016/j.ceb.2015.02.003

Smits, L. P., Bouter, K. E., de Vos, W. M., Borody, T. J., and Nieuwdorp, M. (2013). Therapeutic potential of fecal microbiota transplantation. *Gastroenterology* 145, 946–953. doi: 10.1053/j.gastro.2013.08.058

Sun, J., Liu, S., Ling, Z., Wang, F., Ling, Y., Gong, T., et al. (2019a). Fructooligosaccharides ameliorating cognitive deficits and neurodegeneration in APP/PS1 transgenic mice through modulating gut microbiota. *J. Agric. Food. Chem.* 67, 3006–3017. doi: 10.1021/acs.jafc.8b07313

Sun, J., Xu, J., Ling, Y., Wang, F., Gong, T., Yang, C., et al. (2019b). Fecal microbiota transplantation alleviated Alzheimer's disease-like pathogenesis in APP/PS1 transgenic mice. *Transl. Psychiatry.* 9:189. doi: 10.1038/s41398-019-0525-3

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., and Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Adv. Immunol.* 121, 91–119. doi: 10.1016/B978-0-12-800100-4.00003-9

Tobinick, E., Gross, H., Weinberger, A., and Cohen, H. (2006). TNF-alpha modulation for treatment of Alzheimer's disease: A 6-month pilot study. *Med. Gen. Med.* 8:25.

Valdes, A. M., Walter, J., Segal, E., and Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ* 361:k2179. doi: 10.1136/bmj.k2179

Vogt, N. M., Kerby, R. L., Dill-McFarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., et al. (2017). Gut microbiome alterations in Alzheimer's disease. *Sci. Rep.* 7:13537. doi: 10.1038/s41598-017-13601-y

Wang, H., Yang, F., Xin, R., Cui, D., He, J., Zhang, S., et al. (2020). The gut microbiota attenuate neuroinflammation in manganese exposure by inhibiting cerebral NLRP3 inflammasome. *Biomed. Pharmacother.* 129:110449.

Wang, J. W., Kuo, C. H., Kuo, F. C., Wang, Y. K., Hsu, W. H., Yu, F. J., et al. (2019). Fecal microbiota transplantation: Review and update. *J. Formos. Med. Assoc.* 118(Suppl. 1), S23–S31. doi: 10.1016/j.jfma.2018.08.011

Wang, M., Cao, J., Gong, C., Amakye, W. K., Yao, M., and Ren, J. (2021). Exploring the microbiota-Alzheimer's disease linkage using short-term antibiotic treatment followed by fecal microbiota transplantation. *Brain Behav. Immun.* 96, 227–238. doi: 10.1016/j.bbi.2021.06.00

World Alzheimer Report (2022). *Life after Diagnosis: Navigating Treatment, Care and Support*. London: Alzheimer's Disease International.

Wu, L., Han, Y., Zheng, Z., Peng, G., Liu, P., Yue, S., et al. (2021). Altered gut microbial metabolites in amnestic mild cognitive impairment and Alzheimer's disease: signals in host-microbe interplay. *Nutrients* 13:228. doi: 10.3390/nu13010228

Xu, M., Mo, X., Huang, H., Chen, X., Liu, H., Peng, Z., et al. (2020). Yeast β-glucan alleviates cognitive deficit by regulating gut microbiota and metabolites in Aβ1-42-induced AD-like Mice. *Int. J. Biol. Macromol.* 161, 258–270.

Xu, M. Q., Cao, H. L., Wang, W. Q., Wang, S., Cao, X. C., Yan, F., et al. (2015). Fecal microbiota transplantation broadening its application beyond intestinal disorders. *World J. Gastroenterol.* 21, 102–111. doi: 10.3748/wjg.v21.i1.102

Yang, L. (2018). *Effects of Fecal Microbiota Transplantation on Alzheimer'S Disease Mice and its Molecular Mechanisms*. Zhengzhou: Zhengzhou University.

Yang, L., Hao, J. R., Gao, Y., Yang, X., Shen, X. R., Wang, H. Y., et al. (2022). HDAC3 of dorsal hippocampus induces postoperative cognitive dysfunction in aged mice. *Behav. Brain Res.* 433:114002. doi: 10.1016/j.bbr.2022.114002

Yang, R., Chen, Z., and Cai, J. (2023). Fecal microbiota transplantation: Emerging applications in autoimmune diseases. *J. Autoimmun.* [Online ahead of print]. doi: 10.1016/j.jaut.2023.103038

Youngster, I., Sauk, J., Pindar, C., Wilson, R. G., Kaplan, J. L., Smith, M. B., et al. (2014). Fecal microbiota transplant for relapsing *Clostridium difficile* infection using a frozen inoculum from unrelated donors: A randomized, open-label, controlled pilot study. *Clin. Infect. Dis.* 58, 1515–1522. doi: 10.1093/cid/ciu135

Zhang, B., Chen, T., Cao, M., Yuan, C., Reiter, R. J., Zhao, Z., et al. (2022). Gut microbiota dysbiosis induced by decreasing endogenous melatonin mediates the pathogenesis of Alzheimer's Disease and obesity. *Front. Immunol.* 13:900132. doi: 10.3389/fimmu.2022.900132

Zhang, F., Luo, W., Shi, Y., Fan, Z., and Ji, G. (2012). Should we standardize the 1,700-year-old fecal microbiota transplantation? *Am. J. Gastroenterol.* 107:1755. doi: 10.1038/ajg.2012.251

Zhang, L., Wang, Y., Xiayu, X., Shi, C., Chen, W., Song, N., et al. (2017). Altered gut microbiota in a mouse model of Alzheimer's Disease. *J. Alzheimers Dis.* 60, 1241–1257. doi: 10.3233/JAD-170020

Zheng, J., Zheng, S. J., Cai, W. J., Yu, L., Yuan, B. F., and Feng, Y. Q. (2019). Stable isotope labeling combined with liquid chromatography-tandem mass spectrometry for comprehensive analysis of short-chain fatty acids. *Anal. Chim. Acta* 1070, 51–59. doi: 10.1016/j.aca.2019.04.021

Zhou, H., Tai, J., Xu, H., Lu, X., and Meng, D. (2019). Xanthoceraside could ameliorate Alzheimer's Disease symptoms of rats by affecting the gut microbiota composition and modulating the endogenous metabolite levels. *Front. Pharmacol.* 10:1035. doi: 10.3389/fphar.2019.01035

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 21 November 2023
DOI 10.3389/fmicb.2023.1299816

Check for updates

OPEN ACCESS

EDITED BY
Ludovico Abenavoli,
Magna Græcia University, Italy

REVIEWED BY
Giuseppe Guido Maria Scarlata,
University Magna Graecia of Catanzaro, Italy
Kenji Hashimoto,
Chiba University, Japan

*CORRESPONDENCE
Haiming Fang
✉ haimingfang@ahmu.edu.cn
Jiajia Wang
✉ wjj@ahmu.edu.cn

RECEIVED 09 October 2023
ACCEPTED 08 November 2023
PUBLISHED 21 November 2023

CITATION
Fang H, Yao T, Li W, Pan N, Xu H, Zhao Q, Su Y, Xiong K and Wang J (2023) Efficacy and safety of fecal microbiota transplantation for chronic insomnia in adults: a real world study. *Front. Microbiol.* 14:1299816. doi: 10.3389/fmicb.2023.1299816

COPYRIGHT
© 2023 Fang, Yao, Li, Pan, Xu, Zhao, Su, Xiong and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Efficacy and safety of fecal microbiota transplantation for chronic insomnia in adults: a real world study

Haiming Fang[1,2]*, Tingting Yao[1,2], Wanli Li[3], Na Pan[3], Hang Xu[4], Qian Zhao[1,2], Yuan Su[1,2], Kangwei Xiong[1,2] and Jiajia Wang[3]*

[1]Department of Gastroenterology, The Second Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China, [2]Center for Gut Microbiota Diagnosis and Treatment, The Second Affiliated Hospital of Anhui Medical University, Hefei, Anhui, China, [3]Department of Pharmacology, School of Basic Medical Sciences of Anhui Medical University, Hefei, Anhui, China, [4]School of Medicine, Anhui University of Science and Technology, Huainan, Anhui, China

Objective: To assess the efficacy and safety of fecal microbiota transplantation (FMT) for adult chronic insomnia.

Methods: Patients treated with FMT for chronic diseases were divided into chronic insomnia and non-insomnia group. The primary endpoint was the efficacy of FMT for insomnia 4 weeks after treatment, the secondary endpoints included the impacts of FMT on anxiety, depression, health-related quality of life, gut microbiota, and adverse events associated with FMT. Insomnia Severity Index (ISI) and Pittsburgh Sleep Quality Index (PSQI) were utilized to assess the efficacy of FMT on insomnia, self-rating anxiety/depression scale [Zung Self-Rating Anxiety Scale (SAS), Zung Self-Rating Depression Scale (SDS)] was employed to evaluate anxiety and depression. Quality of life was evaluated by SF-36. 16S rRNA sequencing was employed to analyze the gut microbiota and correlation analysis was performed.

Results: Forty patients met the inclusion criteria and seven were excluded. 33 patients were enrolled and stratified into chronic insomnia group ($N = 17$) and non-insomnia group ($N = 16$). Compared to baseline, FMT significantly ameliorated the ISI (17.31 ± 5.12 vs. 5.38 ± 5.99), PSQI (14.56 ± 2.13 vs. 6.63 ± 4.67), SAS (54.25 ± 8.90 vs. 43.68 ± 10.64) and SDS (57.43 ± 10.96 vs. 50.68 ± 15.27) score and quality of life of chronic insomnia patients. 76.47% (13/17) of insomnia patients achieved the primary endpoints. In chronic insomnia patients, the relative abundance of *Eggerthella* marked enhanced at baseline, while the relative abundance of *Lactobacillus, Bifidobacterium, Turicibacter, Anaerostipes*, and *Eisenbergiella* significantly increased after FMT treatment, the latter positive correlated with the efficacy of FMT. Encouragingly, FMT also improved the sleep quality of non-insomnia patients.

Conclusion: *Eggerthella* may potentially serve as a distinctive genus associated with chronic insomnia. FMT maybe a novel treatment option for adults with chronic insomnia and provide an alternative to traditional treatments for insomnia. The effects were positive correlated with the augmentation of probiotics, such as *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, and *Fusobacterium*.

KEYWORDS

chronic insomnia, brain-gut axis, fecal microbiota transplantation, gut microbiota, dysbiosis

Introduction

The physiological imperative of sleep for all living organisms is emphasized by the fact that adult humans spend average around one-third of their lives engaged in this essential activity. However, sleep disorders have emerged as a prevalent global public health concern in modern society (Han et al., 2022). The term insomnia refers to the unwelcome experience of difficulty in initiating or maintaining sleep, awakening during the night with difficulty returning to sleep, or early morning awakenings, resulting in impaired daytime functioning (Zhang et al., 2022). The prevalence of insomnia is considerably high, ranging from 6.6 to 12% in the general population, with its occurrence reported by 19 to 50% of adults (Koopman et al., 2020; Dos Reis et al., 2021). Nearly 50% of the elderly population suffer from chronic insomnia, which is characterized by fragmented sleep and early morning awakening (Reid et al., 2010; Ryden et al., 2019). The prevalence of this condition is higher in women than men, with a ratio ranging from 1.3 to 1.7, and it may manifest or worsen during hormonal fluctuations such as premenstrual, peripartum, and postmenopausal periods (Suh et al., 2018). Up to 40% of insomnia sufferers also experience comorbid psychiatric disorders, with depression and anxiety being the most prevalent (Roth, 2007). The occurrence of insomnia can be acute, intermittent, or chronic, making it one of the most prevalent forms of sleep disturbance. Insomnia is closely linked to the etiology and progression of a range of disorders, such as obesity, type II diabetes, and cardiovascular diseases (Liu et al., 2019).

The objective of treating insomnia is to enhance the patient's sleep experience and quality of life while minimizing any potential adverse effects. The current guidelines advocate a combination of pharmacological and non-pharmacological interventions, with cognitive behavioral therapy for insomnia (CBTI) being the preferred approach due to its proven efficacy, safety, and enduring benefits. Pharmacotherapy can be considered as an alternative in cases where CBTI fails to yield satisfactory outcomes (Sutton, 2021). Compared to no treatment or sleep hygiene advice, CBTI demonstrated superior efficacy in improving nocturnal sleep measures among adults with and without psychiatric and/or medical comorbidities, as well as those taking concomitant sleep medication (van Straten et al., 2018). However, each of these treatment modalities has limitations in clinical practice. While population surveys inquire about insomnia symptoms, sleep medicine trials and guidelines on insomnia treatment solely focus on individuals who meet the criteria for insomnia disorder. Therefore, these guidelines do not apply universally to all individuals experiencing symptoms of insomnia. Both CBTI and pharmacologic therapy yield only modest effects on objective sleep measures. The utilization of CBTI is further associated with increased access barriers in comparison to pharmacological treatments, which are commonly employed for the management of insomnia symptoms (Sutton, 2021). Hence, this underscores the imperative to develop innovative (non-pharmacological) treatment modalities for the management of chronic insomnia in adult individuals.

With the increasing comprehension of brain-gut axis, it is becoming increasingly clear that the relationship between host and microbiota is a key area of impact for populations (Cryan et al., 2020). It has been hypothesized that the gut microbiota may exert a significant impact on neurological disorders throughout the lifespan, encompassing early-onset conditions such as autism and attention deficit hyperactivity disorder (ADHD), adult-onset diseases like multiple sclerosis (MS) and major depressive disorder (MDD), as well as age-related afflictions such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Cox et al., 2019). The classification of psychiatric disorders is based on diagnostic categories encompassing a wide range of symptoms; however, patients with the same diagnosis often exhibit significant clinical. This clinical heterogeneity, coupled with the incomplete knowledge of the biological mechanisms of psychiatric disorders and their comorbidity with other conditions, have so far contributed towards the limited efficacy of current treatments. Multi-omics technologies (e.g., genomics, transcriptomics, proteomics, and lipidomics) provide a promising avenue for the identification of biomarkers in psychiatry, enabling patient stratification within a diagnosis, facilitating more targeted treatment options, and predicting treatment response (Sathyanarayanan et al., 2023). Furthermore, omic technologies also play pivotal roles in bacterial resistance reserach (Bongiorno et al., 2023) The gut microbiota has emerged as a promising therapeutic target for psychiatric disorders such as depression, Parkinson's disease, and dementia (Ho et al., 2021). Recently, a range of probiotics have been reported to possess effective stress-modulating and anxiolytic effects on stressed individuals by maintaining intestinal homeostasis, enhancing mucosal and systemic immunity, and regulating gut microbiota metabolism. The findings suggest that probiotics show potential as psychobiotics for mitigating psychiatric disorders, and they offer a promising non-pharmacological therapy for insomnia. For instance, *Lactobacillus*, a prominent bacterium in the gut microbiota, possesses the potential to modulate the central nervous system through the vagus nerve and exert an impact on sleep patterns. The utilization of probiotics for sleep and emotional disorders, such as insomnia, stress, anxiety, and depression, is gaining significant momentum (Irwin et al., 2020; Tian et al., 2022).

Fecal microbiota transplantation (FMT) commonly refers to the transfer of gut microbiota from healthy donors to patients with dysbiosis-related diseases, aiming to restore homeostasis of the gut microbiota and achieve treatment goals (Fang et al., 2021). FMT has demonstrated remarkable efficacy in treating recurrent *Clostridium difficile* infection, surpassing that of vancomycin in randomized controlled trials and exhibiting promising results for managing inflammatory bowel disease (IBD) in both pediatric and adult populations (Fang et al., 2018). Moreover, gut microbiota is involved in several mechanisms sustaining the model of the "gut-liver axis." Evaluation of changes in the gut microbiota composition in liver transplantation patients is essential for monitoring transplant success and potentially implement appropriate preventive measures (Abenavoli et al., 2023). FMT has shown preclinical and initial clinical promising results in non-alcoholic fatty liver disease treatment through re-modulation of microbial dysbiosis (Abenavoli et al., 2022). This highlights FMT as a potential therapeutic option with great promise (Fang et al., 2018). Germ-free mice receiving FMT from participants who underwent overnight sleep deprivation exhibited an increase in inflammatory states and enhanced intestinal barrier permeability, similar to that observed in specific pathogen-free (SPF) mice after sleep deprivation. These findings suggest that gut dysbiosis contributes to both peripheral and central inflammatory processes induced by sleep loss, highlighting the potential of manipulating the microbiota as a therapeutic intervention for mitigating the detrimental consequences of sleep deprivation (Wang et al., 2021). A pilot study

revealed that FMT had a beneficial impact on sleep quality as well as patients' mood of FMT, regardless of gastrointestinal symptom change in patients with irritable bowel syndrome (IBS), functional diarrhea (FD) or functional constipation (Kurokawa et al., 2018). However, it is important to note that the scope of above study was limited to investigating the potential enhancement of sleep quality specifically in patients with aforementioned intestinal diseases. Furthermore, the assessment relied solely on sleep-related sub-scores derived from the Hamilton Rating Scale for Depression (HAM-D). FMT could improve sleep quality among IBS patients experiencing poor sleep (Zhang Z. et al., 2023). However, to the best of our knowledge, there is a paucity of studies specifically investigating the efficacy and safety of FMT in individuals afflicted with chronic insomnia. The aim of our study was to investigate whether FMT could potentially ameliorate self-reported sleep quality, mood disturbances, daytime dysfunction and health-related quality of life in adults with chronic insomnia.

Methods

Patients and study design

The present study is an observational study conducted in a real-world setting, serving as a sub-analysis of an ongoing investigation into the efficacy and safety of FMT for refractory chronic diseases in adult patients. These disorders encompass recurrent *Clostridium difficile* infection (rCDI), irritable bowel syndrome (IBS), chronic functional constipation, ulcerative colitis (UC), or Parkinson's disease with constipation. The study employed a parallel pre-post design to investigate the impact of FMT on self-reported sleep quality, mood, quality of life, and gut microbiota composition at baseline and post-intervention (4 weeks after FMT). The study was conducted at the Department of Gastroenterology, the Second Affiliated Hospital of Anhui Medical University. Participants were enrolled from September 2020 to July 2022.

According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), the diagnostic criteria for insomnia necessitate the presence of at least one of the following symptoms: difficulty initiating sleep, difficulty maintaining sleep, or early morning awakening with an inability to return to sleep. Chronic insomnia is defined as experiencing any of these symptoms for a minimum of three nights per week over a period lasting 3 months or longer (Seow et al., 2018). The sleep quality was assessed using the Insomnia Severity Index (ISI) and Pittsburgh Sleep Quality Index (PSQI), which can be considered as objective assessment methods for subjective experiences (Besedovsky et al., 2019). The present study employed the ISI as a screening tool to identify clinical insomnia, whereby participants were classified into either the chronic insomnia group or control group based on their PSQI scores. Individuals who achieved a PSQI score of 11 or higher and had not undergone CBTI within the preceding 4 weeks were included in the chronic insomnia group, whereas those with a score below 11 were assigned to the control group representing typical sleep patterns. Patients with chronic insomnia who were pregnant, had a history of FMT treatment, had been exposed to probiotics or prebiotics within the past 4 weeks, exhibited evidence of infection such as cytomegalovirus or Epstein–Barr virus, or required parenteral antibiotics were excluded. The exclusion criteria also encompassed patients with comorbidities such as cardiovascular disease, pulmonary disease, cerebrovascular disease, a history of gastrointestinal malignancy or polyps, and those who had undergone abdominal surgery. Participants who were ineligible for endoscopy or had contraindications were also excluded from the study. The Ethics Committee of the Second Affiliated Hospital of Anhui Medical University granted approval for this study [No. YX2019-039(F2)] and [No. YX2019-040(F2)], and Trial registration: ChiCTR, ChiCTR2000030080 and ChiCTR1900027238. All patients provided written informed consent.

Donor-recipient matching model for donor selection and preparation of donor fecal microbiota solutions

Potential healthy stool donors were identified through a rigorous screening questionnaire, followed by a comprehensive medical interview and examination, as well as blood and stool testing to minimize the risk of disease transmission, in accordance with previously established protocols in our center (Fang et al., 2021). Bioinformatics analyzes were conducted, and the composition and stability of gut microbiota in stool samples obtained from eligible donors were dynamically monitored using metagenomics. Six representative microbial characteristics, namely richness, distance, beneficial taxa, harmful taxa, beneficial pathways and harmful pathways were employed as indicators for constructing a donor-recipient matching model using the Analytic Hierarchy Process (AHP) (Zhang B. et al., 2023). A minimum of 100 grams of fresh stool sample was required for each donation. The fresh stool (25%) should be homogenized with normal saline (60%) and pharmaceutical grade glycerol (15%), and then processed in an automatic stirring and separation machine. The specifications, appearance, quantity, and weight of all products were thoroughly examined before being immediately frozen at −80°C. Based on the donor-recipient matching model, a total of thirteen healthy donors were included, with an average age of 22.08 ± 2.29 (19–25) years old, comprising eight males and five females. The mean BMI was documented as 21.62 ± 1.36 (18.9–23.5) kg/m^2, while the health assessment indicated normal findings with no history of smoking or alcohol consumption and absence of oral or gut inflammation.

FMT procedure

The participants were given explicit instructions to abstain from consuming any other probiotic products or antibiotics throughout the entire duration of the study, and a light diet was recommended for 2–3 days prior to FMT. The participants received bowel preparation with polyethylene glycol electrolyte dissolved in 2 L of water, which is an isotonic lavage fluid for the entire intestine. This lavage fluid contains 125 mmol/L sodium ions, 10 mmol/L potassium ions, 20 mmol/L bicarbonate ions, 40 mmol/L sulfate ions, and 35 mmol/L chloride ions. The gastroscopy and colonoscopy procedures were conducted under propofol sedation, while the nasoduodenal graft tube was inserted below the descending portion of the duodenum 4–6 h prior to FMT. The participants underwent FMT treatments administered via the nasoduodenal route. Specifically, a total of 600 mL of donor fecal slurry was introduced into the gastrointestinal

tract through a naso-duodenal tube for three consecutive days (200 mL/d × 3d). Following completion of transplantation, all recipients were instructed to maintain a semi-recumbent position for at least 60 min without defecation. Stool specimens were collected at baseline (prior to FMT) and post-FMT (at least 4 weeks after treatment).

Assessments of the primary and secondary endpoints

The primary endpoints of this study were to evaluate the efficacy of FMT in improving sleep quality among patients with chronic insomnia, 4 weeks post-FMT. The secondary endpoints encompassed the evaluation of the impact of FMT on anxiety and depression, health-related quality of life, as well as any adverse events associated with FMT, which were documented throughout both the treatment and follow-up periods. Questionnaires were administered at baseline and post-treatment (4 weeks after FMT). The ISI and PSQI were employed to assess subjective sleep quality and severity of insomnia. The PSQI, a 19-item measure, was utilized to evaluate sleep quality and disturbances over a one-month period. From this assessment, seven sub-scores were derived: sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. Meanwhile, the Zung Self-Rating Anxiety Scale (SAS) and the Zung Self-Rating Depression Scale (SDS) were employed to evaluate participants' mental health status. They both had 20 questions, each with a score from 0 to 4. The total scores of 20 items were used as raw scores, and the index scores were derived from the raw scores multiplied by a factor of 1.25. According to the Chinese population, the SAS standard score cut-off value was established at 50 points, with scores ranging from 50 to 59 indicating mild anxiety, scores between 60 and 69 denoting moderate anxiety, and scores of 70 or higher representing severe anxiety. The SDS standard score cut-off value was set at 53 points, with scores ranging from 53 to 62 indicating mild depression, scores between 63 and 72 signifying moderate depression, and scores of 73 or higher reflecting severe depression. The severity of depression was determined by calculating the cumulative score of each item divided by 80. A score below 0.5 indicates the absence of depression, while a score ranging from 0.5 to 0.59 suggests mild to moderate depression, a score between 0.6 and 0.69 indicates moderate to severe depression, and a score above 0.7 is indicative of major depression. Health-related quality of life was evaluated using the SF-36 questionnaire.

Evaluation and analysis of the fecal microbiota through 16S rRNA sequencing

The donors provided fresh fecal samples, and patients' pre- and post-FMT samples were collected using a sterile collection spoon. Subsequently, the samples were preserved in 3 mL of solution and stored at −80°C for subsequent analysis. The gut microbiota was analyzed using 16S rRNA sequencing. The V3-V4 hypervariable region of the 16S rRNA gene was amplified through high-throughput sequencing on the Illumina MiSeq platform, and the raw sequencing data from these stool samples were processed into operational taxonomic units at a similarity level of 97%, following previously established protocols (Fang et al., 2021).

Statistical analyzes and visualization

A intention-to-treat analysis was conducted, wherein baseline demographic, medication, and disease parameters - including disease duration, severity, and extent - were presented using mean ± standard deviation ($\overline{x}$±s) or frequencies (percentages). The Chi-square test was utilized to compare categorical variables between groups, while the Student's t-test was employed to compare clinical response in both groups. The statistical analysis was conducted using SPSS Statistics v25.0, while the plotting was performed with GraphPad Prism 8. A significance level of $p < 0.05$ was considered.

The alpha diversity estimates were computed based on an evenly rarefied OTU abundance matrix, encompassing observed richness, species, and the Shannon, Simpson, ACE, and Chao1 indices using the R package vegan. Beta diversity was evaluated using the Bray-Curtis distance metric based on an evenly rarefied table of OTU abundances. LEfSe analysis was conducted to identify taxa exhibiting differential abundances across groups. Additionally, indicator analysis at the genus level was performed. To predict the functional content of metagenomics, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) was employed to forecast the presence of genes based on 16S data between groups. The metagenome predictions were conducted using the predicted metagenomes.py script. The significant difference analysis was performed using ANOVA. The results were visualized using a custom R script based on ggplot2, in accordance with previously reported protocols (Fang et al., 2021).

Results

Demographic characteristics of patients

The present study was conducted in a real-world setting. Forty patients met the inclusion criteria from September 2020 to July 2022, seven patients were excluded, including 3 who met the exclusion criteria and 4 who refused FMT therapy. Consequently, a total of 33 patients were enrolled in this study, including 25 females and 8 males. The disease spectrum encompasses a range of conditions including rCDI, IBS, chronic constipation, UC, and Parkinson's disease. The 33 patients were assigned to either the chronic insomnia group ($N = 17$) with an average age of 54.29 ± 19.76 years old or the typical sleep group ($N = 16$) with an average age of 56.81 ± 17.81 years old based on their PSQI scores. Interestingly, in this study, the prevalence rate of insomnia was found to be significantly higher among women compared to men, with a ratio of 15 to 2 (Table 1). There was no statistically significant disparity observed in terms of demographic characteristics and disease types between the two groups. The three consecutive days FMT treatment was administered to all 33 patients for their aforementioned diseases. The study flow chart illustrate the process of recruitment and group assignment (Figure 1), illustrating the baseline demographic features and clinical characteristics of patients with chronic insomnia.

Effects of FMT on sleep quality in chronic insomnia patients

In the chronic insomnia group, the patients exhibited symptoms such as difficulty initiating sleep, early morning awakening, reduced total sleep time, and daytime fatigue. The initial ISI score was 17.31 ± 5.12, while the baseline PSQI score was 14.56 ± 2.13. Following FMT treatment, there was a significant reduction in the PSQI score during the four-week follow-up period, averaging at 6.63 ± 4.67. FMT demonstrated a noteworthy improvement in PSQI scores compared to the baseline value ($p = 0.0041$). Additionally, Compared to the baseline value, the ISI value also demonstrated a significant decrease with an average of 5.38 ± 5.99 ($p = 0.0027$, Figure 2). 76.47% (13/17) of insomnia patients achieved the primary endpoints after undergoing FMT treatment.

TABLE 1 Demographic and clinical features of patient.

Accompanied symptoms	Chronic insomnia	Control group
Numbers	17	16
Female/Male	15/2	11/5
Age	54.29 ± 19.76	56.81 ± 17.81
Other diagnosis		
Chronic constipation	7	8
IBS	4	0
UC	1	2
Parkinson disease	2	2
rCDI	3	4
ISI score	17.31 ± 5.12	2.40 ± 2.47
PSQI score	14.56 ± 2.13	5.40 ± 2.58
FMT times	200 mL/d × 3d	200 mL/d × 3d
FMT route	Nasoduodenal	Nasoduodenal

According to the PSQI score sub-item, FMT demonstrated significant improvements in insomnia by reducing sleep onset latency, enhancing sleep efficiency, and subsequently alleviating symptoms of daytime fatigue (Figure 3).

Encouragingly, patients without insomnia who underwent FMT also reported improved sleep quality with reduced sleep onset latencies, decreased wake time after sleep onset, and increased sleep efficiency (data not shown).

Effects of FMT on anxiety and depression symptoms in chronic insomnia patients

In this study, we observed varying degrees of anxiety and depression symptoms among patients with insomnia, The average baseline scores for the SAS and SDS in these patients were 54.25 ± 8.90 and 57.43 ± 10.96, respectively. The patients with comorbid chronic insomnia and chronic diseases displayed mild levels of anxiety and depression. After FMT treatment, the mean SAS score decreased to 43.68 ± 10.64 and the mean SDS score decreased to 50.68 ± 15.27. Following FMT treatment, there was a significant reduction in self-reported scores for anxiety and

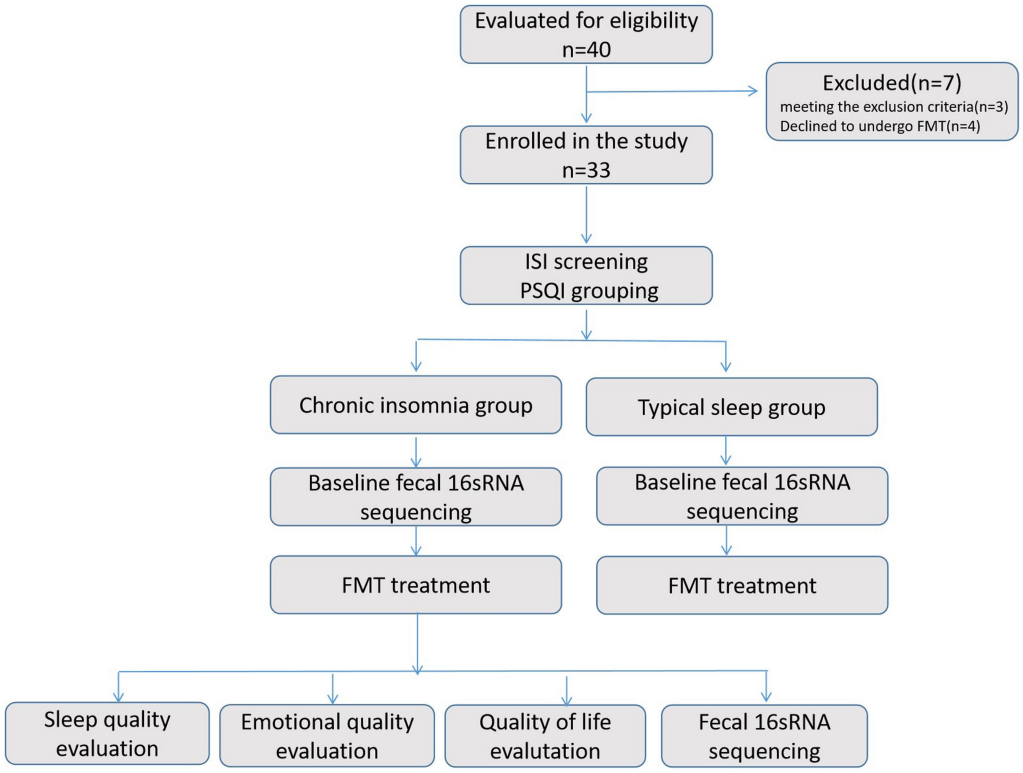


FIGURE 1
The study flow chart illustrates the process of recruitment and group assignment.

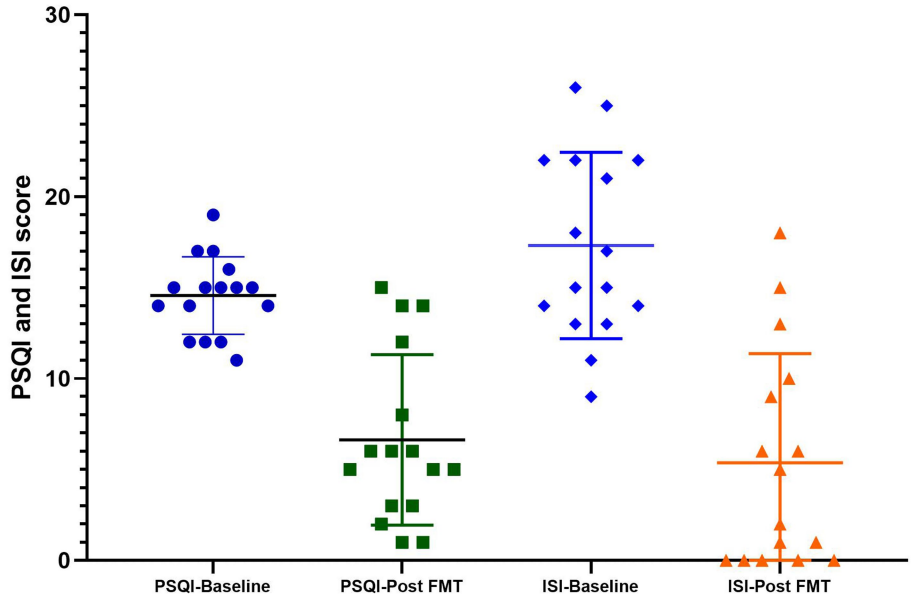


FIGURE 2
The PSQI and ISI scores at baseline and post FMT in the insomnia group. Compared to baseline, FMT significantly improved both PSQI ($p = 0.0041$) and ISI scores ($p = 0.0027$).

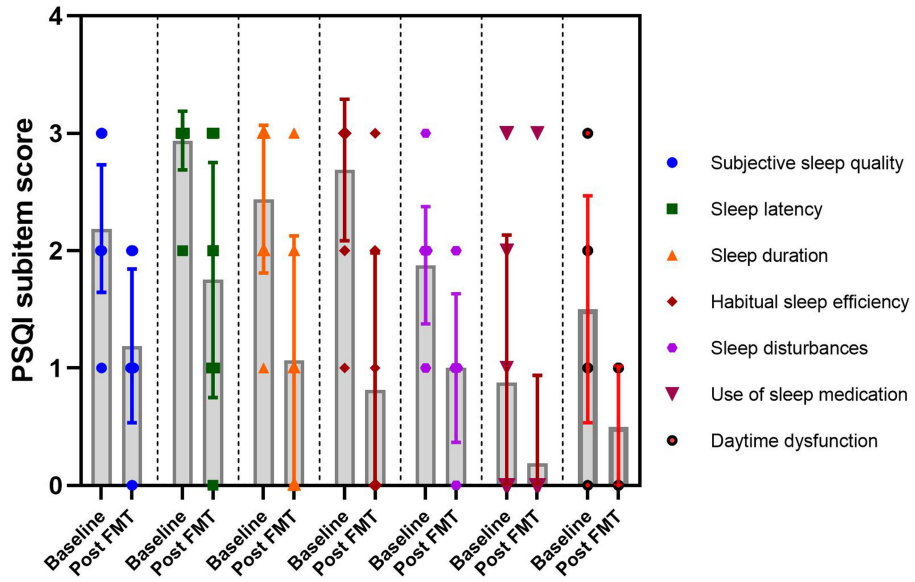


FIGURE 3
The PSQI sub-item score at baseline and post FMT in the insomnia group.

depression, indicating that FMT may effectively ameliorate psychiatric symptoms in patients with chronic diseases (Figure 4).

Effects of FMT on quality of life in chronic insomnia patients

The SF-36 is an 8-dimensional instrument specifically designed for the professional assessment of health-related quality of life (HRQoL). In order to investigate the impact of FMT on the quality of life in patients with chronic insomnia. We assessed the scores of the SF-36 questionnaire at baseline and 4 weeks after undergoing FMT treatment. As results, the FMT treatment demonstrated significant improvements in physical functioning, role-physical, body pain, general health, vitality, social functioning, role-emotional and mental health compared to the baseline (Figure 5), indicating FMT has the potential to enhance the quality of life for patients with chronic diseases.

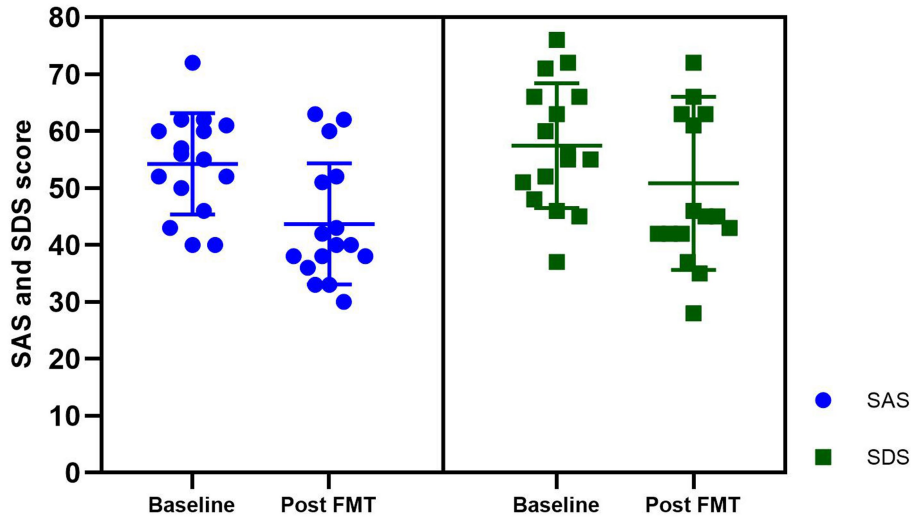


FIGURE 4
The anxiety and depression scores at baseline and post FMT in the insomnia group. SAS, Self-rating anxiety scale; SDS, Self-rating depression scale.

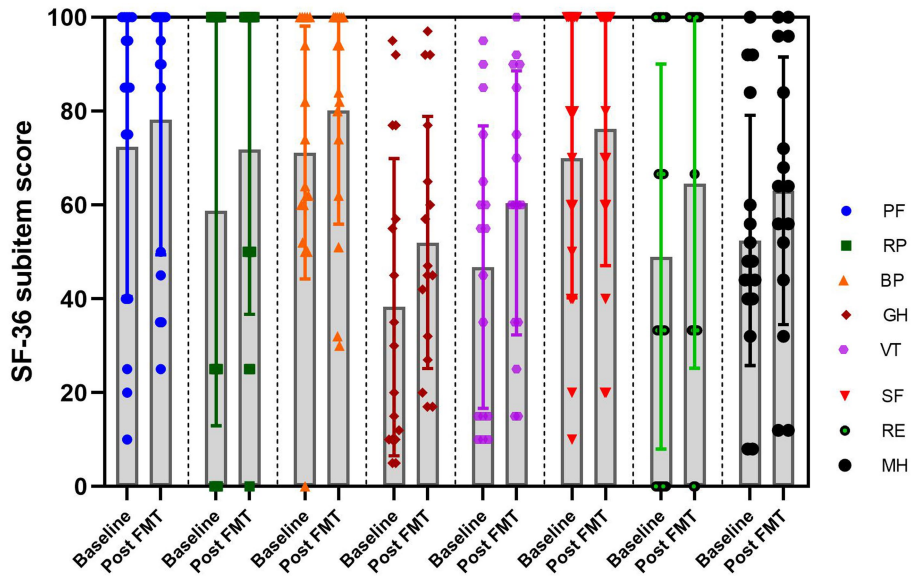


FIGURE 5
The plot of the SF-36 sub-item score at baseline and post FMT in the insomnia group. RF, Physical functioning; RP, Role-physical; BP, Body pain; GH, General health; VT, Vitality; SF, Social functioning; RE, Role-emotional; MH, Mental health.

Safety of FMT in the treatment of chronic insomnia

Adverse events were recorded after FMT and follow-up. All patients could tolerate FMT well, and no serious adverse events occurred. No FMT-related serious adverse events were reported during the study period.

Comparison of gut microbiota characteristics in patients with chronic diseases comorbid chronic insomnia or not

To investigate the potential variations in gut microbiota among individuals with chronic insomnia, the fecal microbiomes of the two groups at baseline were analyzed by sequencing the 16s rRNA gene. Based on the observed plateau in the rarefaction curve of the current sequencing data, it can be inferred that a substantial portion of diversity has already been captured across all samples. Compared to the non-insomnia group, the insomnia group did not exhibit a significant difference in the alpha and beta diversity index. The linear discriminant analysis effect size (LEfSe) was subsequently employed to identify differential microbial communities between the two groups. However, no statistically significant differences were observed at the phylum, class, or family levels, potentially due to the similarity in disease spectrum between the two groups. Interestingly, at the order level, there was a notable increase in the relative abundance of fecal *Rhizobiales* in the non-insomnia group; however, at the genus level, patients with chronic insomnia exhibited a significantly higher relative

abundance of *Eggerthella*. The reason for this can be attributed to the fact that both groups of patients had a similar range of diseases, except for the chronic insomnia symptoms.

PICRUSt algorithm was employed to predict the functional profiles of gut microbiota based on the predicted metagenome. Subsequently, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functions were categorized using PICRUSt. The findings from the PICRUSt analysis revealed significant reductions in styrene degradation and aminobenzoate degradation pathways among patients with chronic insomnia. The metabolic pathways, including riboflavin metabolism, cofactors and vitamins metabolism, nitrotoluene degradation, ethylbenzene degradation, chlorocyclohexane and chlorobenzene degradation, exhibit diversity. Although no statistically significant difference was observed (p value ranging from 0.0516 to 0.0616), this could be attributed to the limited number of patients enrolled in this study.

The regulatory effects of FMT on gut microbiota in patients with chronic insomnia

Based on the observed plateau in the rarefaction curve of the current sequencing data suggested that sequencing depth was enough to capture all bacterial species across all samples and sufficient for downstream analysis (Figure 6A). Venn diagram of gut microbial species among the groups of pre- and post-FMT therapy, as well as healthy donors was shown in Figure 6B. The alpha diversity indices such as observe and ACE showed significant difference between the healthy donors and the insomnia groups, while no notable disparity was observed in the diversity indexes (Shannon and Simpson, Figure 6C). This suggested that insomnia disorder may result in alteration of gut microbiota diversity. The β-diversity revealed substantial structural disparities between insomnia patients pre- and post-FMT therapy, as well as healthy donors. PCoA analysis unveiled a substantial divergence in the composition of gut microbiota between individuals with insomnia and healthy donors. Following FMT treatment, the dysbiosis of the gut microbiota was significantly ameliorated with no apparent separation from that observed among healthy donors (Figure 6D). Compared to healthy donors, patients with insomnia exhibited dysbiosis of gut microbiota, characterized by a significant increase in the relative abundance of *Howardella* at the general level. The ANOSIM analysis based on the Bray Curtis distance demonstrated a statistically significant disparity in gut microbiota composition between the baseline and post-FMT treatment groups ($R > 0$), although no significant differences were observed in terms of group contributions ($p = 0.1651$). The findings suggest that FMT effectively ameliorates dysbiosis in the gut microbiota of insomnia patients, resulting in a microbial community resembling that of healthy donors (Figure 6E).

The LEfSe analysis was employed to ascertain the dominant flora, with an LDA value >2 indicating a statistically significant difference in the healthy donor, chronic insomnia patients before and after FMT treatment. The results revealed a significant increase in the relative abundance of *Lactobacillus*, *Bifidobacterium, Prevotella_6*, and *Anaerofustis* at the genus level within the intestinal tracts of insomnia patients after FMT treatment (Figure 7B). Analysis on the difference of the gut microbiota at the phylum level among the healthy donors, pre and post FMT treatment group showed that *Actinobacteria, Fusobacteria* and *Cyanobacteria* displayed marked differences. The relative abundance of *Actinobacteria* in the gut was significantly increased, while *Cyanobacteria* showed a significant decrease in chronic insomnia patients who underwent FMT treatment (Figure 7C). The top 20 genera with significant differences in the intestinal flora at the genus level in chronic insomnia patients before and after FMT treatment was illustrated in Figure 7A. Additionally, Figure 7D presents a box diagram showcasing the top 10 genera with notable variations, we found that FMT treatment can significantly enhance the relative abundance of *Lactobacillus, Bifidobacterium, Turicibacter, Anaerostipes*, and *Eisenbergiella* in the gastrointestinal tract of patients with chronic insomnia.

To further elucidate the underlying mechanism of gut microbiota and metabolites in the treatment of insomnia, we conducted a comprehensive KEGG analysis. The PICRUSt analysis results revealed significant alterations in 40 KEGG pathways among insomnia patients before and after FMT treatment, as well as healthy donors, especially the D-Alanine metabolism, purine metabolism, purine metabolism, taurine and hypotaurine metabolism, DNA repair and recombination proteins, protein folding and associated processing, replication, recombination and repair proteinsribosome biogenesis, peptidoglycan biosynthesis aminoacyl-tRNA biosynthesis, translation proteins were marked different among the groups (Figure 7E).

Correlation analysis of different flora with sleep quality, mood and quality of life scores

The correlation analysis of the top 20 bacterial types with significant differences at the genus level and scores of sleep quality, mood, and quality of life in patients with chronic insomnia before and after FMT treatment revealed a negative correlation between *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, and *Fusobacterium*. Conversely, a positive correlation was observed with *Prevotella_6*, *Anaerofustis*, *Howardella*, *Lachnoanaerobaculum*, *Lachnospira*, *Roseburia*, *Neisseria,* and *Haemophilus*. Furthermore, *Bifidobacterium*, *Lactobacillus* and *Fusobacterium* exhibited negative correlations with ISI scores, PQSI total scores, PQSI subitem scores and SF-36 subitem scores in patients suffering from chronic insomnia (Figure 8).

Discussion

Sleep disorders have become an epidemic, whereas chronic sleep disturbances cause long-lasting gastrointestinal, psychiatric, and neurological problems. A total of 33 subjects diagnosed with chronic diseases were enrolled in this study, 51.5% (17/33) of them had comorbid chronic insomnia, and the prevalence rate of insomnia was found to be significantly higher among women compared to men. The high prevalence of chronic insomnia in this study is considered to be associated with the inclusion of patients suffering from chronic diseases (including rCDI, UC, IBS, chronic consitpaiton and Parkinson disease). Insomnia is comorbid with a range of psychiatric and inflammatory disorders as well as metabolic syndromes. Individuals with IBS have a twofold higher risk of comorbid depression, anxiety, and sleep disorders, conversely, patients

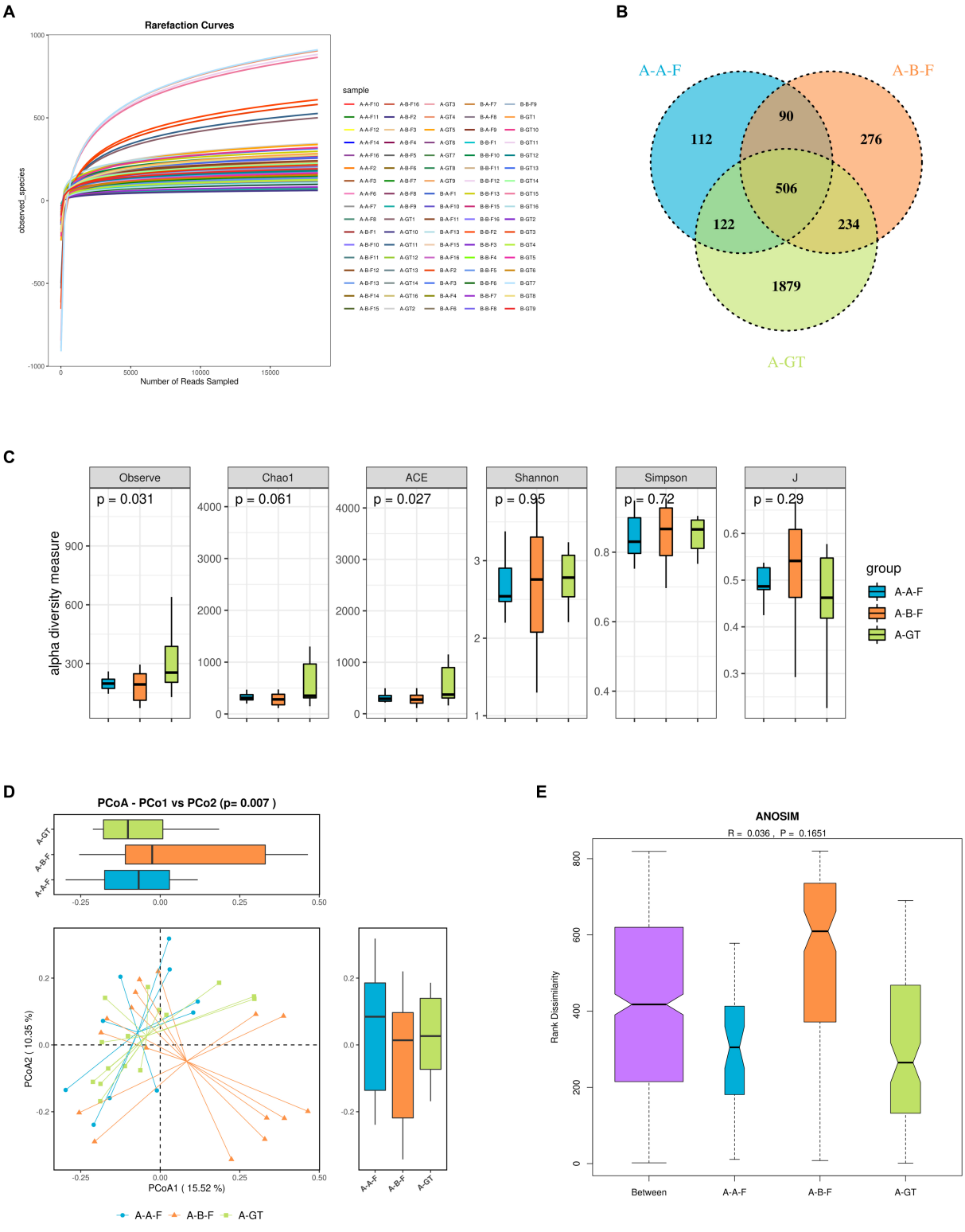
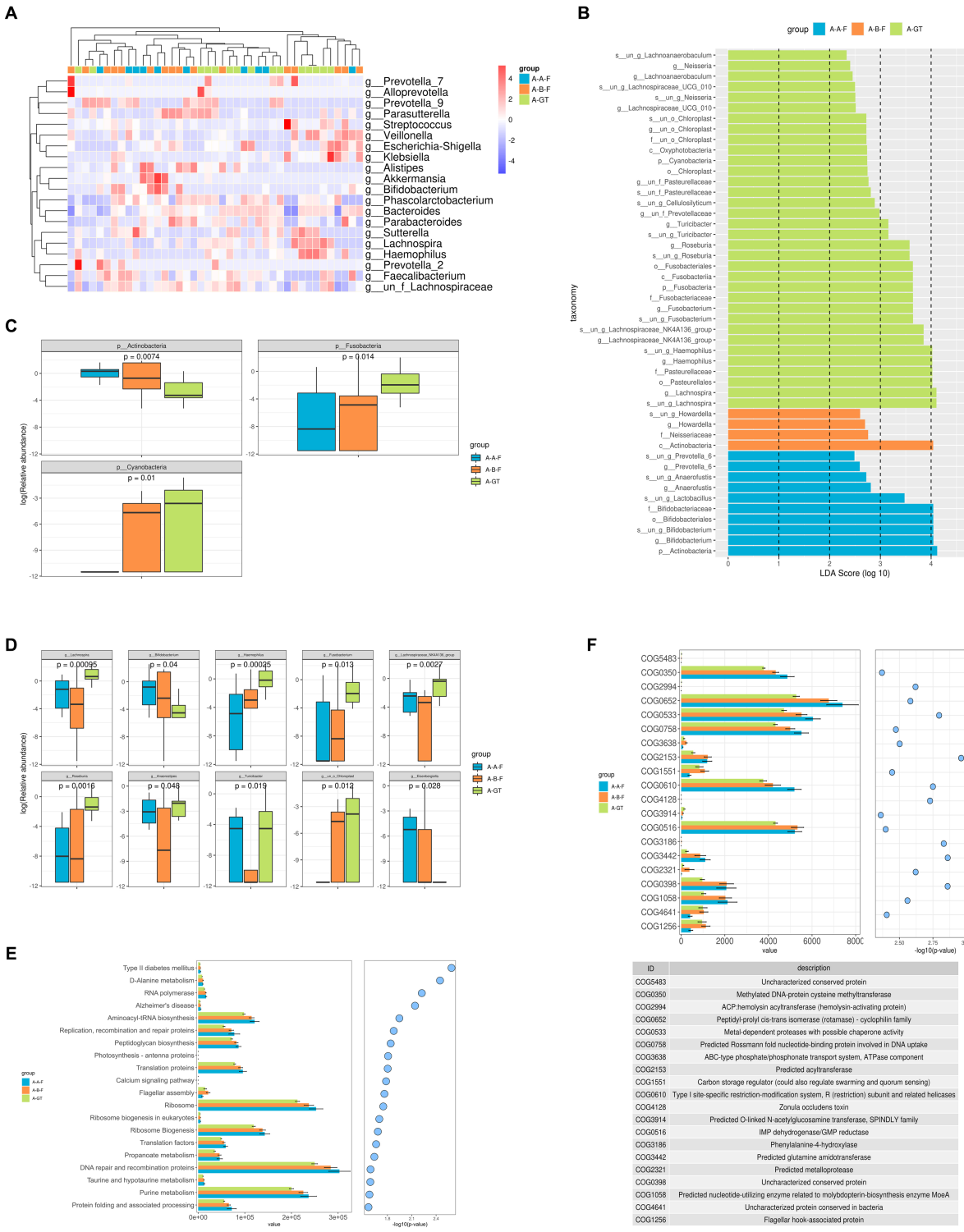


FIGURE 6
Stool microbiota composition analysis the healthy donors (marked as A-GT), chronic insomnia patients (marked as A-B-F) and patients post FMT treatment (marked as A-A-F). **(A)** The rarefaction curve of the current sequencing data. **(B)** Venn diagram indicating the number of differential OTUs in each group. **(C)** Alpha diversity index box chart, the abscissa represents sample grouping, and the ordinate is the alpha index. **(D)** Principal coordinate analysis (PCoA) of the gut microbiota among the healthy donors, chronic insomnia patients and patients post FMT treatment. The distance between the samples represents the similarity of the gut microbiota composition, and a closer distance indicates higher similarity. **(E)** Analysis of similarities (ANOSIM) among the healthy donors, chronic insomnia patients and patients post FMT treatment. A-GT: healthy donors, A-B-F: Patients with chronic insomnia before FMT intervention, A-A-F: Patients with chronic insomnia after FMT intervention.

10.3389/fmicb.2023.1299816



ID	description
COG5483	Uncharacterized conserved protein
COG0350	Methylated DNA-protein cysteine methyltransferase
COG2994	ACP:hemolysin acyltransferase (hemolysin-activating protein)
COG0652	Peptidyl-prolyl cis-trans isomerase (rotamase) - cyclophilin family
COG0533	Metal-dependent proteases with possible chaperone activity
COG0758	Predicted Rossmann fold nucleotide-binding protein involved in DNA uptake
COG3638	ABC-type phosphate/phosphonate transport system, ATPase component
COG2153	Predicted acyltransferase
COG1551	Carbon storage regulator (could also regulate swarming and quorum sensing)
COG0610	Type I site-specific restriction-modification system, R (restriction) subunit and related helicases
COG4128	Zonula occludens toxin
COG3914	Predicted O-linked N-acetylglucosamine transferase, SPINDLY family
COG0516	IMP dehydrogenase/GMP reductase
COG3186	Phenylalanine-4-hydroxylase
COG3442	Predicted glutamine amidotransferase
COG2321	Predicted metalloprotease
COG0398	Uncharacterized conserved protein
COG1058	Predicted nucleotide-utilizing enzyme related to molybdopterin-biosynthesis enzyme MoeA
COG4641	Uncharacterized protein conserved in bacteria
COG1256	Flagellar hook-associated protein

FIGURE 7
Histogram of taxonomic profiles of the gut microbiota among the healthy donors (marked as A-GT), chronic insomnia patients (marked as A-B-F) and patients post FMT treatment (marked as A-A-F). **(A)** Heat maps of different bacteria at genus level in the healthy donor, chronic insomnia patients before and after FMT treatment. **(B)** LDA scores of different species of gut microbiota in the healthy donor, chronic insomnia patients before and after FMT treatment. **(C)** Analysis on the difference of the gut microbiota at the phylum level. **(D)** Analysis on the difference of the gut microbiota at the genus level. **(E)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functions analyzes. **(F)** COG analyzes.

presenting with depression and anxiety are reported to have a heightened susceptibility to developing IBS (Liu et al., 2023). Idiopathic rapid eye movement sleep behavior disorder is a powerful early sign of Parkinson's disease (Postuma et al., 2019). The available evidences suggest that individuals with chronic lifelong diseases are at a heightened vulnerability to sleep disorders. The co-existence of

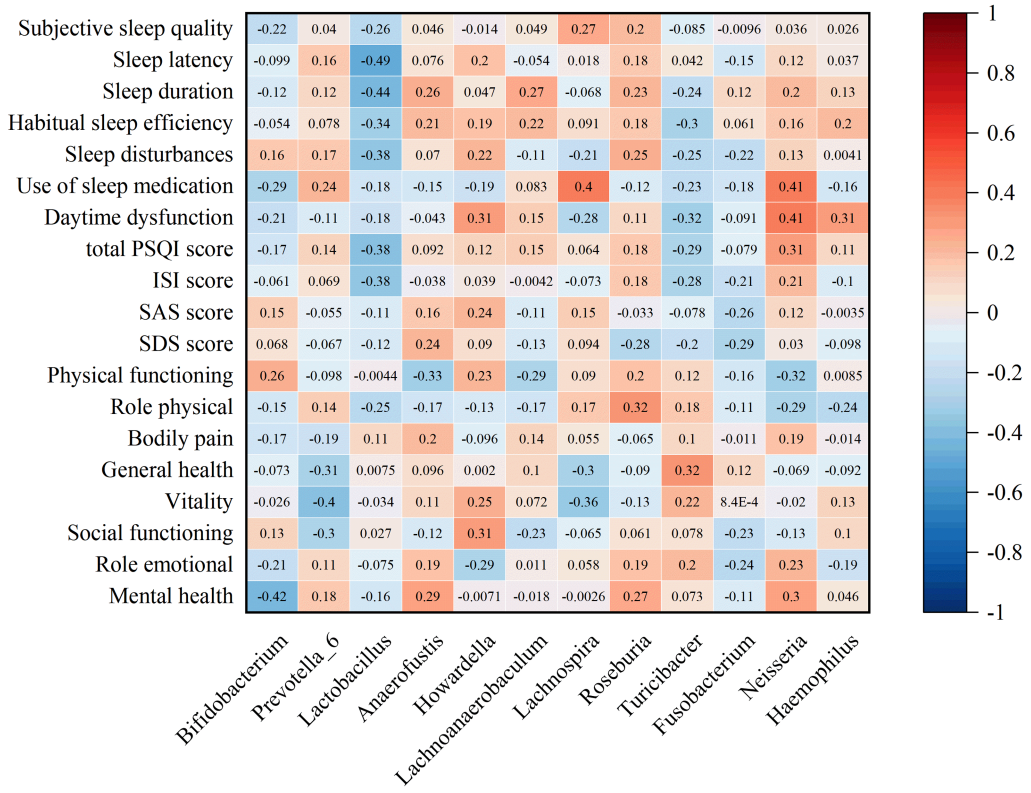


FIGURE 8
Correlation analysis of different species of gut microbiota in insomnia patients before and after FMT treatment.

chronic disease with psychiatric disorders can be a big burden, and the presence of comorbid depression exerts a more negative effect on health than depression or anxiety does alone (Moussavi et al., 2007). The growing prevalence of sleep disorders has raised the question of what governs sleep quality and how these factors can be manipulated to improve sleep (Sen et al., 2021).

Accumulated evidences suggest that the gut microbiota plays a crucial role in regulating human health, particularly metabolic, immunological, and neurobehavioral functions. The strong correlation between psychiatric disorders, including sleep, and dysbiosis of gut microbiota or alterations in intestinal metabolism has been extensively discussed, indicating an inherent cross-link between the gut microbiota and brain regions that has been referred to as the microbiota-gut-brain axis. In 1983, Rechtschaffen and colleagues conducted groundbreaking research in the fields of sleep, gut microbiota, and health. Their findings revealed a systemic deterioration in rats exposed to prolonged sleep deprivation (Rechtschaffen et al., 1983). Benedict and colleagues provide the inaugural published insights into the intricate interplay between sleep and gut microbiota in human subjects, thereby introducing a pioneering exploration of acute sleep restriction's impact on gut microbiota. This initiates a crucial discourse on future investigations of the gut microbiota in human sleep research (Benedict et al., 2016). A recent murine study revealed that mice with depleted gut bacteria using antibiotics exhibited significant sleep disturbances, characterized by spending less time in non-REM sleep during the light phase and spending more time in non-REM and REM sleep during the dark phase, leading to poorer quality of sleep (Ogawa et al., 2020). Disrupted sleep can also induce alterations in the composition of the gut microbiome. In human male adults, the sleep efficiency and total sleep time were positively correlated with the diversity of the gut microbiota (Smith et al., 2019). Compared to healthy controls, insomnia patients exhibit reduced microbial richness and diversity in the gut microbiota, as well as a depletion in anaerobic bacteria and short-chain fatty acid (SCFA)-producing bacteria. Moreover, there is an expansion of potential pathobionts present (Li et al., 2020). Despite the ongoing lack of understanding regarding the precise pathological mechanisms underlying insomnia, there is a widespread recognition that maintaining gut microbiota homeostasis plays a pivotal role in determining both the onset and severity of this disorder. Moreover, insomnia has been shown to exert profound structural and functional effects on the gut microbiota.

Here we investigate characteristics of fecal microbiome diversity and composition in patients with chronic bowel disease comorbid chronic insomnia or not. The analysis of gut microbiota characteristics in the two groups at baseline (before FMT treatment) revealed no significant differences at the phylum level. However, a statistically significant increase in the relative abundance of *Eggerthella* genus was observed among patients with chronic insomnia compared to individuals without insomnia. The observed dissimilarities at the phylum and genus levels between the two groups are hypothesized to be associated with their similar disease spectrum. The bacterium *Eggerthella* is a gram-positive, anaerobic, non-sporulating microorganism that typically resides in the human microbiota. However, emerging evidence suggests its potential as a significant human pathogen capable of causing life-threatening infections under

specific circumstances. Additionally, *Eggerthella* is significantly enriched in patients with various autoimmune diseases and exhibits an increased presence in individuals with inflammatory bowel disease (IBD) and exacerbates colitis models (Alexander et al., 2022). Interestingly, a recent meta-analysis of psychiatric disorders and gut microbiota showed that enriched levels of *Eggerthella* were consistently shared between major depressive disorder, anxiety, bipolar disorder, psychosis and schizophrenia, suggesting these disorders are characterized by an increase of pro-inflammatory genera (Nikolova et al., 2021). The bulk of earlier research into insomnia on gut microbiota has focused on animal models of sleep deprivation and intervention studies involving probiotics. To the best of our knowledge, our results represent the first evidence linking *Eggerthella* to chronic insomnia in humans, as revealed by a comprehensive real-world case analysis. However, sleep is a complex physiological process of the body, the initiation and maintenance of the sleep–wake cycle are regulated by both the central nervous system and peripheral tissues. The understanding of *Eggerthella's* pathogenic mechanism and treatment options still requires further enhancement. The acquisition of a substantial amount of clinical data is essential for a comprehensive exploration in this field.

The findings from the PICRUSt analysis revealed significant reductions in styrene degradation and aminobenzoate degradation pathways among patients with with refractory bowel disease comorbid chronic insomnia. Although no statistically significant differences were observed among the metabolic pathways, including riboflavin metabolism, cofactors and vitamins metabolism, nitrotoluene degradation, ethylbenzene degradation, chlorocyclohexane and chlorobenzene degradation; however, they did exhibit variations, which maybe due to the limited sample size. These findings may have implications for future investigations into the underlying mechanism of chronic insomnia, as the study included various disease types but shared a common comorbidity: chronic insomnia. For example, there were significant relationships between moderate/severe insomnia and the presence of malnutrition and risk of malnutrition (Soysal et al., 2019). The regression models showed that patients with generalized anxiety disorder displaying low levels of vitamin D demonstrate a poor subjective quality of sleep and heightened anxiety. A strong relationship between peripheral biomarkers of calcium homeostasis imbalance, insomnia, poor sleep quality, and anxiety symptomatology was underlined (Carbone et al., 2023).

Gaining insights into the correlation between sleep and gut microbiota may facilitate the development of targeted interventions aimed at enhancing sleep health by modulating the composition of gut microbiota. Gut microbiota-targeted interventions have been shown to exert a therapeutic effect on sleep disturbances. In 2013, Dinan and colleagues defined the term "psychobiotics" as a novel class of probiotics that suggest potential applications in treating psychiatric diseases (Dinan et al., 2013). Psychobiotics are efficacious in ameliorating neurodegenerative and neurodevelopmental disorders, including autism spectrum disorder (ASD), Parkinson's disease (PD) and Alzheimer's disease (AD), which led researchers to focus on a new area in neuroscience (Cheng et al., 2019). Evidence in preschool-aged children indicates a correlation between sleep duration and the diversity of gut microbiota, with children who have longer total nighttime sleep showing a higher relative abundance of *Bifidobacterium* (Wang et al., 2022). Importantly, studies conducted in adult humans have also demonstrated the efficacy of *Bifidobacterium*, whether administered as a standalone probiotic or in combination with other probiotics, in modulating mood (Pinto-Sanchez et al., 2017). Daily administration of *Lactobacillus plantarum* may lead to a decrease in depressive symptoms, fatigue level, cortical excitation, and an improvement in sleep quality during the deep sleep stage (Ho et al., 2021). In addition, lower *Bifidobacterium* and/or *Lactobacillus* counts were observed in subjects with major depressive disorder, probiotics composed of specific strains of genera *Lactobacillus* and *Bifidobacterium* have the potential to prevent and treat various psychiatric conditions such as depression and anxiety (Cheng et al., 2019).

FMT is a robust method to rebalance the gut microbiota ecosystem of recipient through the transfer of healthy donor-derived gut microbiota to patients. Engraftment of donor microbiota resulted in a long-lasting response in patients with recurrent *Clostridium difficile* infection and active ulcerative colitis (Weingarden et al., 2015; Fang et al., 2021). Furthermore, FMT is a well-tolerated, effective, and safe method as an add-on therapy in patients with mild-to-moderate ulcerative colitis (Tkach et al., 2023). In the present study, we demonstrated that FMT significantly enhanced sleep quality, alleviated anxiety and depression symptoms, and improved the quality of life in individuals with chronic insomnia as assessed through self-reported questionnaires. The ISI and PSQI score sub-item indicates that FMT significantly improves insomnia by reducing sleep onset latency, enhancing sleep efficiency, and subsequently alleviating symptoms of daytime fatigue. It is encouraging to note that patients without insomnia who underwent FMT also reported an improvement in their sleep quality. The application of FMT can also enhance the psychological well-being of patients with chronic insomnia, alleviating symptoms of anxiety and depression, while concurrently enhancing their quality of life. The analysis of gut microbiota before and after FMT treatment revealed a significant improvement in dysbiotic gut microbiota among patients with chronic insomnia who underwent FMT, leading to a composition resembling that of healthy donors, indicating a clear restoration in the dysbiosis of gut microbiota. The LEfSe analysis found that there was a significant increase in the relative abundance of *Lactobacillus*, *Bifidobacterium, Prevotella_6*, and *Anaerofustis* at the genus level within their gut microbiota. The correlation analysis revealed a significant positive correlation between the efficacy of FMT in treating insomnia and the presence of *Lactobacillus* and *Bifidobacterium*. The findings indicated that FMT exhibited the potential to enhance the abundance of probiotics in the gut of individuals suffering from chronic insomnia. The majority of these taxa have been linked with sleep-related neurochemicals in previous studies, such as serotonin, tryptophan and gamma-aminobutyric acid. For example, *Lactobacillus* and *Bifidobacterium* produce gamma-aminobutyric acid (GABA) and serotonin. In addition, strains of *Lactobacillus* produce acetylcholine (Cheng et al., 2019). Additionally, *Bacteroides* is a crucial taxon involved in the production of the SCFA propionate through the succinate pathway (Louis and Flint, 2017).

However, unlike probiotics, which only contain a limited number of bacterial species, FMT contains thousands of species native to the human gut. On the other hand, apart from alterations in the composition of the gut microbiota, disruptions were observed in both signaling pathways and metabolic functions among individuals with insomnia disorder. Mice with depleted gut bacteria displayed impaired sleep quality and decreased concentrations of serotonin in their gut

(Ogawa et al., 2020). Following FMT from sleep-fragmented mice, the recipients mimicked the donor's characteristics, including increased systemic white adipose tissue inflammation and altered insulin sensitivity (Poroyko et al., 2016). These suggest that FMT is likely to exert its therapeutic effects through multiple mechanisms. In the present study, PICRUSt supported the gut taxa composition, signaling pathways, and metabolic functions perturbed by insomnia disorder. There were significant alterations observed in 40 KEGG pathways among insomnia patients before and after FMT treatment, as well as healthy donors. Specifically, the D-alanine metabolism, purine metabolism, taurine and hypotaurine metabolism, DNA repair and recombination proteins, protein folding and associated processing, replication, recombination and repair proteins ribosome biogenesis, peptidoglycan biosynthesis aminoacyl-tRNA biosynthesis, translation proteins exhibited marked differences among the groups. This implies the potential mediating roles of neurotransmitters in connecting gut microbiota and sleep (Fung et al., 2019).

Our study demonstrates the following characteristics: firstly, it was conducted in a real-world setting and encompassed a diverse range of medical conditions, including rCDI, IBS, chronic constipation, UC, and Parkinson's disease with chronic constipation and all patients received a donor-recipient matching FMT tailored to these diseases. Additionally, a comparison was conducted between the gut microbiota characteristics of chronic disease patients with and without comorbid chronic insomnia at baseline. This makes it more applicable in clinical settings regarding FMT for the treatment of chronic insomnia. Secondly, there is a distinction in the definition of insomnia, using the ISI and PSQI for screening, grouping, and efficacy assessment. We define chronic insomnia as having a PSQI score equal to or exceeding 11, which provided evidence supporting the therapeutic potential of FMT in the treatment of chronic insomnia. Thirdly, we conducted an analysis on the characteristics of gut microbiota of chronic insomnia patients both before and after FMT treatment, as well as its correlation with treatment efficacy.

Of course, the current study is merely a limited sample size observational study. In addition, this study was primarily relied on self-reported questionnaires and lacked actigraphy sleep measures included total nighttime sleep (TST), sleep efficiency (SE), and wake-time after sleep onset (WASO). Therefore, it is imperative to conduct large-scale randomized controlled trials in conjunction with metagenomic and metabolomic to identify the effects of FMT on chronic insomnia. Although the potential benefits of FMT in various diseases, there exists a variation and complexity in procedural agreement among research groups for performing FMT, and the optimal approach to achieve engraftment after FMT remains unclear (Bokoliya et al., 2021). The adoption of the use of antibiotics prior to FMT and data remain controversial (Cammarota et al., 2017; Ng et al., 2020). As known, the host-microbiota encompasses a variety of microbial communications, including the oral, nasal, lung, gut, skin, bladder, and vaginal microbiota. Microbiota in one organ have the potential to influence those in another, and they can interact with the gut microbiota via inflammatory and immune system pathways (Hashimoto, 2023). The oral cavity serves as the primary entry point to the human body, and the oral microbiota represents the second-largest microbial community in humans, which may potentially contribute to the development and progression of psychiatric and neurodegenerative disorders, as well as autoimmune diseases. Patients with chronic insomnia harbored a significantly higher diversity of oral bacteria when compared to healthy controls. More importantly, the results revealed that the diversity and relative abundance of the bacterial community was significantly altered among different tongue coatings in patients but not in healthy individuals (Liu et al., 2020). Compared to the control group, insomniac patients exhibited a significantly higher abnormal low-frequency/high-frequency (LF/HF) ratio. Furthermore, linear discriminant analysis of oral microbial population revealed that the relative abundances of *Clostridia, Veillonella, Bacillus* and *Lachnospiraceae* were significantly higher in the insomniac patients than the control group (Park et al., 2022). The impact of various lifestyle and environmental factors on sleep is well-documented. Increasingly, evidence suggests that gut microbiota plays a significant role in regulating brain function and behavior (Sen et al., 2021). The present study included individuals with underlying chronic conditions who mainly reported no history of smoking or alcohol consumption, and their lifestyle and dietary practices aligned with widely promoted eating patterns prevalent in Anhui Province, China. Considering the exploratory nature of this study and the preliminary positive findings of FMT in managing adults chronic insomnia, it is imperative to incorporate the potential influence and significance of lifestyle factors and antibiotic pretreatment prior to FMT in subsequent randomized controlled trials (RCTs).

Conclusion

The present study represents the first real-world case analysis demonstrating that *Eggerthella* could potentially serve as a distinctive genus associated with chronic insomnia. FMT therapy not only improves sleep quality, but also enhances antidepressant and anxiolytic effects, as well as overall quality of life in patients with chronic insomnia and the effects was positive associated with the augmentation of probiotic microorganisms, such as *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, and *Fusobacterium*. Our preliminary empirical work supported the acceptability and feasibility of using FMT to be a novel treatment option for adults with chronic insomnia and provide an alternative to traditional treatments for insomnia., the mechanism may be correlated with reshaping the dysbiosis of gut microbiota and increasing the abundance of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*.

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/, PRJNA1020211.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Second Affiliated Hospital of Anhui Medical University. 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

HF: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. TY: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing. WL: Data curation, Formal analysis, Methodology, Resources, Validation, Visualization, Writing – review & editing. NP: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. HX: Data curation, Formal analysis, Methodology, Writing – review & editing. QZ: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. YS: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. KX: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. JW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by Anhui Provincial Natural Science Foundation (No. 2308085MH242), the Basic and Clinical Cooperative Research Promotion Program of Anhui Medical University (No. 021xkjT023) and Anhui Medical University Foundation Project (No. 2022xkj175).

Acknowledgments

The authors would like to acknowledge the TreatGut Company (Xiamen, China) for the technical assistance in16S rRNA sequencing and donor screening.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Abenavoli, L., Maurizi, V., Rinninella, E., Tack, J., di Berardino, A., Santori, P., et al. (2022). Fecal microbiota transplantation in NAFLD treatment. *Medicina (Kaunas)* 58:1559. doi: 10.3390/medicina58111559

Abenavoli, L., Scarlata, G. G. M., Paravati, M. R., Boccuto, L., Luzza, F., and Scarpellini, E. (2023). Gut microbiota and liver transplantation: immune mechanisms behind the rejection. *Biomedicine* 11:1792. doi: 10.3390/biomedicines11071792

Alexander, M., Ang, Q. Y., Nayak, R. R., Bustion, A. E., Sandy, M., Zhang, B., et al. (2022). Human gut bacterial metabolism drives Th17 activation and colitis. *Cell Host Microbe* 30, 17–30.e9. doi: 10.1016/j.chom.2021.11.001

Benedict, C., Vogel, H., Jonas, W., Woting, A., Blaut, M., Schürmann, A., et al. (2016). Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. *Mol Metab* 5, 1175–1186. doi: 10.1016/j.molmet.2016.10.003

Besedovsky, L., Lange, T., and Haack, M. (2019). The sleep-immune crosstalk in health and disease. *Physiol. Rev.* 99, 1325–1380. doi: 10.1152/physrev.00010.2018

Bokoliya, S. C., Dorsett, Y., Panier, H., and Zhou, Y. (2021). Procedures for Fecal microbiota transplantation in murine microbiome studies. *Front. Cell. Infect. Microbiol.* 11:711055. doi: 10.3389/fcimb.2021.711055

Bongiorno, D., Bivona, D. A., Cicino, C., Trecarichi, E. M., Russo, A., Marascio, N., et al. (2023). Omic insights into various ceftazidime-avibactam-resistant *Klebsiella pneumoniae* isolates from two southern Italian regions. *Front. Cell. Infect. Microbiol.* 12:1010979. doi: 10.3389/fcimb.2022.1010979

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., Kump, P., Satokari, R., et al. (2017). European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* 66, 569–580. doi: 10.1136/gutjnl-2016-313017

Carbone, E. A., Menculini, G., de Filippis, R., D'Angelo, M., de Fazio, P., Tortorella, A., et al. (2023). Sleep disturbances in generalized anxiety disorder: the role of calcium homeostasis imbalance. *Int. J. Environ. Res. Public Health* 20:4431. doi: 10.3390/ijerph20054431

Cheng, L. H., Liu, Y. W., Wu, C. C., Wang, S., and Tsai, Y. C. (2019). Psychobiotics in mental health, neurodegenerative and neurodevelopmental disorders. *J. Food Drug Anal.* 27, 632–648. doi: 10.1016/j.jfda.2019.01.002

Cox, L. M., Schafer, M. J., Sohn, J., Vincentini, J., Weiner, H. L., Ginsberg, S. D., et al. (2019). Calorie restriction slows age-related microbiota changes in an Alzheimer's disease model in female mice. *Sci. Rep.* 9:17904. doi: 10.1038/s41598-019-54187-x

Cryan, J. F., O'Riordan, K. J., Sandhu, K., Peterson, V., and Dinan, T. G. (2020). The gut microbiome in neurological disorders. *Lancet Neurol.* 19, 179–194. doi: 10.1016/S1474-4422(19)30356-4

Dinan, T. G., Stanton, C., and Cryan, J. F. (2013). Psychobiotics: a novel class of psychotropic. *Biol. Psychiatry* 74, 720–726. doi: 10.1016/j.biopsych.2013.05.001

Dos Reis, L. L., Terra Loyola, V., Leopoldino de Bortolli, C., Levy Andersen, M., Tufik, S., and Hachul, H. (2021). Effects of supplementation with lactobacillus probiotics on insomnia treatment. *Altern. Ther. Health Med.* 27, 178–184.

Fang, H., Fu, L., Li, X., Lu, C., Su, Y., Xiong, K., et al. (2021). Long-term efficacy and safety of monotherapy with a single fresh fecal microbiota transplant for recurrent active ulcerative colitis: a prospective randomized pilot study. *Microb. Cell Factories* 20:18. doi: 10.1186/s12934-021-01513-6

Fang, H., Fu, L., and Wang, J. (2018). Protocol for Fecal microbiota transplantation in inflammatory bowel disease: a systematic review and meta-analysis. *Biomed. Res. Int.* 2018, 8941340–8941311. doi: 10.1155/2018/8941340

Fung, T. C., Vuong, H. E., Luna, C. D. G., Pronovost, G. N., Aleksandrova, A. A., Riley, N. G., et al. (2019). Intestinal serotonin and fluoxetine exposure modulate bacterial colonization in the gut. *Nat. Microbiol.* 4, 2064–2073. doi: 10.1038/s41564-019-0540-4

Han, M., Yuan, S., and Zhang, J. (2022). The interplay between sleep and gut microbiota. *Brain Res. Bull.* 180, 131–146. doi: 10.1016/j.brainresbull.2021.12.016

Hashimoto, K. (2023). Emerging role of the host microbiome in neuropsychiatric disorders: overview and future directions. *Mol. Psychiatry*. doi: 10.1038/s41380-023-02287-6

Ho, Y. T., Tsai, Y. C., Kuo, T. B. J., and Yang, C. C. H. (2021). Effects of *Lactobacillus plantarum* PS128 on depressive symptoms and sleep quality in self-reported insomniacs: a randomized, double-blind, Placebo-Controlled Pilot Trial. *Nutrients* 13:2820. doi: 10.3390/nu13082820

Irwin, C., McCartney, D., Desbrow, B., and Khalesi, S. (2020). Effects of probiotics and paraprobiotics on subjective and objective sleep metrics: a systematic review and meta-analysis. *Eur. J. Clin. Nutr.* 74, 1536–1549. doi: 10.1038/s41430-020-0656-x

Koopman, A. D. M., Beulens, J. W., Dijkstra, T., Pouwer, F., Bremmer, M. A., van Straten, A., et al. (2020). Prevalence of insomnia (symptoms) in T2D and association with metabolic parameters and Glycemic control: meta-analysis. *J. Clin. Endocrinol. Metab.* 105, 614–643. doi: 10.1210/clinem/dgz065

Kurokawa, S., Kishimoto, T., Mizuno, S., Masaoka, T., Naganuma, M., Liang, K. C., et al. (2018). The effect of fecal microbiota transplantation on psychiatric symptoms among patients with irritable bowel syndrome, functional diarrhea and functional constipation: an open-label observational study. *J. Affect. Disord.* 235, 506–512. doi: 10.1016/j.jad.2018.04.038

Li, Y., Zhang, B., Zhou, Y., Wang, D., Liu, X., Li, L., et al. (2020). Gut microbiota changes and their relationship with inflammation in patients with acute and chronic insomnia. *Nat. Sci. Sleep* 12, 895–905. doi: 10.2147/NSS.S271927

Liu, B., Lin, W., Chen, S., Xiang, T., Yang, Y., Yin, Y., et al. (2019). Gut microbiota as an objective measurement for auxiliary diagnosis of insomnia disorder. *Front. Microbiol.* 10:1770. doi: 10.3389/fmicb.2019.01770

Liu, M., Wang, X., Wu, F., Dai, N., Chen, M., Yu, J., et al. (2020). Variations of Oral microbiome in chronic insomnia patients with different tongue features. *Am. J. Chin. Med.* 48, 923–944. doi: 10.1142/S0192415X20500445

Liu, M., Ye, Z., Wu, Q., Yang, S., Zhang, Y., Zhou, C., et al. (2023). Healthy sleep, mental health, genetic susceptibility, and risk of irritable bowel syndrome. *J. Affect. Disord.* 331, 25–32. doi: 10.1016/j.jad.2023.03.033

Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* 19, 29–41. doi: 10.1111/1462-2920.13589

Moussavi, S., Chatterji, S., Verdes, E., Tandon, A., Patel, V., and Ustun, B. (2007). Depression, chronic diseases, and decrements in health: results from the world health surveys. *Lancet* 370, 851–858. doi: 10.1016/S0140-6736(07)61415-9

Ng, S. C., Kamm, M. A., Yeoh, Y. K., Chan, P. K. S., Zuo, T., Tang, W., et al. (2020). Scientific frontiers in faecal microbiota transplantation: joint document of Asia-Pacific Association of Gastroenterology (APAGE) and Asia-Pacific Society for Digestive Endoscopy (APSDE). *Gut* 69, 83–91. doi: 10.1136/gutjnl-2019-319407

Nikolova, V. L., Smith, M. R. B., Hall, L. J., Cleare, A. J., Stone, J. M., and Young, A. H. (2021). Perturbations in gut microbiota composition in psychiatric disorders: a review and meta-analysis. *JAMA Psychiatry* 78, 1343–1354. doi: 10.1001/jamapsychiatry.2021.2573

Ogawa, Y., Miyoshi, C., Obana, N., Yajima, K., Hotta-Hirashima, N., Ikkyu, A., et al. (2020). Gut microbiota depletion by chronic antibiotic treatment alters the sleep/wake architecture and sleep EEG power spectra in mice. *Sci. Rep.* 10:19554. doi: 10.1038/s41598-020-76562-9

Park, S. H., Shin, N. R., Yang, M., Bose, S., Kwon, O., Nam, D. H., et al. (2022). A clinical study on the relationship among insomnia, tongue diagnosis, and Oral microbiome. *Am. J. Chin. Med.* 50, 773–797. doi: 10.1142/S0192415X2250032X

Pinto-Sanchez, M. I., Hall, G. B., Ghajar, K., Nardelli, A., Bolino, C., Lau, J. T., et al. (2017). Probiotic *Bifidobacterium longum* NCC3001 reduces depression scores and alters brain activity: a pilot study in patients with irritable bowel syndrome. *Gastroenterology* 153, 448–459.e8. doi: 10.1053/j.gastro.2017.05.003

Poroyko, V. A., Carreras, A., Khalyfa, A., Khalyfa, A. A., Leone, V., Peris, E., et al. (2016). Chronic sleep disruption alters gut microbiota, induces systemic and adipose tissue inflammation and insulin resistance in mice. *Sci. Rep.* 6:35405. doi: 10.1038/srep35405

Postuma, R. B., Iranzo, A., Hu, M., Högl, B., Boeve, B. F., Manni, R., et al. (2019). Risk and predictors of dementia and parkinsonism in idiopathic REM sleep behaviour disorder: a multicentre study. *Brain* 142, 744–759. doi: 10.1093/brain/awz030

Rechtschaffen, A., Gilliland, M. A., Bergmann, B. M., and Winter, J. B. (1983). Physiological correlates of prolonged sleep deprivation in rats. *Science* 221, 182–184. doi: 10.1126/science.6857280

Reid, K. J., Baron, K. G., Lu, B., Naylor, E., Wolfe, L., and Zee, P. C. (2010). Aerobic exercise improves self-reported sleep and quality of life in older adults with insomnia. *Sleep Med.* 11, 934–940. doi: 10.1016/j.sleep.2010.04.014

Roth, T. (2007). Insomnia: definition, prevalence, etiology, and consequences. *J. Clin. Sleep Med.* 3, S7–S10.

Ryden, A. M., Martin, J. L., Matsuwaka, S., Fung, C. H., Dzierzewski, J. M., Song, Y., et al. (2019). Insomnia disorder among older veterans: results of a postal survey. *J. Clin. Sleep Med.* 15, 543–551. doi: 10.5664/jcsm.7710

Sathyanarayanan, A., Mueller, T. T., Ali Moni, M., Schueler, K., ECNP TWG Network membersBaune, B. T., et al. (2023). Multi-omics data integration methods and their applications in psychiatric disorders. *Eur. Neuropsychopharmacol.* 69, 26–46. doi: 10.1016/j.euroneuro.2023.01.001

Sen, P., Molinero-Perez, A., O'Riordan, K. J., McCafferty, C. P., O'Halloran, K. D., and Cryan, J. F. (2021). Microbiota and sleep: awakening the gut feeling. *Trends Mol. Med.* 27, 935–945. doi: 10.1016/j.molmed.2021.07.004

Seow, L. S. E., Verma, S. K., Mok, Y. M., Kumar, S., Chang, S., Satghare, P., et al. (2018). Evaluating DSM-5 insomnia disorder and the treatment of sleep problems in a psychiatric population. *J. Clin. Sleep Med.* 14, 237–244. doi: 10.5664/jcsm.6942

Smith, R. P., Easson, C., Lyle, S. M., Kapoor, R., Donnelly, C. P., Davidson, E. J., et al. (2019). Gut microbiome diversity is associated with sleep physiology in humans. *PLoS One* 14:e0222394. doi: 10.1371/journal.pone.0222394

Soysal, P., Smith, L., Dokuzlar, O., and Isik, A. T. (2019). Relationship between nutritional status and insomnia severity in older adults. *J. Am. Med. Dir. Assoc.* 20, 1593–1598. doi: 10.1016/j.jamda.2019.03.030

Suh, S., Cho, N., and Zhang, J. (2018). Sex differences in insomnia: from epidemiology and Etiology to intervention. *Curr. Psychiatry Rep.* 20:69. doi: 10.1007/s11920-018-0940-9

Sutton, E. L. (2021). Insomnia. *Ann. Intern. Med.* 174:ITC33–ITC48. doi: 10.7326/AITC202103160

Tian, P., Chen, Y., Zhu, H., Wang, L., Qian, X., Zou, R., et al. (2022). *Bifidobacterium breve* CCFM1025 attenuates major depression disorder via regulating gut microbiome and tryptophan metabolism: a randomized clinical trial. *Brain Behav. Immun.* 100, 233–241. doi: 10.1016/j.bbi.2021.11.023

Tkach, S., Dorofeyev, A., Kuzenko, I., Falalyeyeva, T., Tsyryuk, O., Kovalchuk, O., et al. (2023). Efficacy and safety of fecal microbiota transplantation via colonoscopy as add-on therapy in patients with mild-to-moderate ulcerative colitis: a randomized clinical trial. *Front. Med. (Lausanne)* 9:1049849. doi: 10.3389/fmed.2022.1049849

van Straten, A., van der Zweerde, T., Kleiboer, A., Cuijpers, P., Morin, C. M., and Lancee, J. (2018). Cognitive and behavioral therapies in the treatment of insomnia: a meta-analysis. *Sleep Med. Rev.* 38, 3–16. doi: 10.1016/j.smrv.2017.02.001

Wang, Z., Chen, W. H., Li, S. X., He, Z. M., Zhu, W. L., Ji, Y. B., et al. (2021). Gut microbiota modulates the inflammatory response and cognitive impairment induced by sleep deprivation. *Mol. Psychiatry* 26, 6277–6292. doi: 10.1038/s41380-021-01113-1

Wang, Y., van de Wouw, M., Drogos, L., Vaghef-Mehrabani, E., Reimer, R. A., Tomfohr-Madsen, L., et al. (2022). Sleep and the gut microbiota in preschool-aged children. *Sleep* 45:zsac020. doi: 10.1093/sleep/zsac020

Weingarden, A., González, A., Vázquez-Baeza, Y., Weiss, S., Humphry, G., Berg-Lyons, D., et al. (2015). Dynamic changes in short- and long-term bacterial composition following fecal microbiota transplantation for recurrent *Clostridium difficile* infection. *Microbiome* 3:10. doi: 10.1186/s40168-015-0070-0

Zhang, Z., Li, Q., Zhang, S., Liu, Y., Lu, G., Wen, Q., et al. (2023). Washed microbiota transplantation targeting both gastrointestinal and extraintestinal symptoms in patients with irritable bowel syndrome. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 127:110839. doi: 10.1016/j.pnpbp.2023.110839

Zhang, T., Xie, X., Li, Q., Zhang, L., Chen, Y., Ji, G. J., et al. (2022). Hypogyrification in generalized anxiety disorder and associated with insomnia symptoms. Nat sci. *Sleep* 14:1009. Published 2022 May 25. doi: 10.2147/NSS.S358763

Zhang, B., Yang, L., Ning, H., Cao, M., Chen, Z., Chen, Q., et al. (2023). A matching strategy to guide donor selection for ulcerative colitis in Fecal microbiota transplantation: meta-analysis and analytic hierarchy process. *Microbiol. Spectr.* 11:e0215921. doi: 10.1128/spectrum.02159-21

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 30 November 2023
DOI 10.3389/fmicb.2023.1304232

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Yukiko Miyamoto,
University of California, San Diego, United States
Soonjae Hwang,
Gachon University, Republic of Korea
Xiaocang Cao,
Tianjin Medical University General Hospital, China

*CORRESPONDENCE
Wei Xu
✉ xu_wei111@126.com

†These authors have contributed equally to this work

RECEIVED 29 September 2023
ACCEPTED 13 November 2023
PUBLISHED 30 November 2023

CITATION
Zheng C, Zhong Y, Xie J, Wang Z, Zhang W, Pi Y, Zhang W, Liu L, Luo J and Xu W (2023) *Bacteroides acidifaciens* and its derived extracellular vesicles improve DSS-induced colitis.
Front. Microbiol. 14:1304232.
doi: 10.3389/fmicb.2023.1304232

COPYRIGHT
© 2023 Zheng, Zhong, Xie, Wang, Zhang, Pi, Zhang, Liu, Luo and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Bacteroides acidifaciens and its derived extracellular vesicles improve DSS-induced colitis

Cihua Zheng[1,2,3†], Yuchun Zhong[1†], Jian Xie[2†], Zhuoya Wang[2], Wenming Zhang[1], Yiming Pi[2], Wenjun Zhang[2], Li Liu[4], Jun Luo[2,3] and Wei Xu[1,3*]

[1]Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China, [2]Department of Rehabilitation Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China, [3]The Institute of Translational Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang University, Nanchang, Jiangxi, China, [4]Graduate School of Jiangxi University of Chinese Medicine, Nanchang, China

Introduction: "Probiotic therapy" to regulate gut microbiota and intervene in intestinal diseases such as inflammatory bowel disease (IBD) has become a research hotspot. *Bacteroides acidifaciens*, as a new generation of probiotics, has shown beneficial effects on various diseases.

Methods: In this study, we utilized a mouse colitis model induced by dextran sodium sulfate (DSS) to investigate how B. acidifaciens positively affects IBD. We evaluated the effects of *B. acidifaciens*, fecal microbiota transplantation, and bacterial extracellular vesicles (EVs) on DSS-induced colitis in mice. We monitored the phenotype of mouse colitis, detected serum inflammatory factors using ELISA, evaluated intestinal mucosal barrier function using Western blotting and tissue staining, evaluated gut microbiota using 16S rRNA sequencing, and analyzed differences in EVs protein composition derived from *B. acidifaciens* using proteomics to explore how *B. acidifaciens* has a positive impact on mouse colitis.

Results: We confirmed that *B. acidifaciens* has a protective effect on colitis, including alleviating the colitis phenotype, reducing inflammatory response, and improving intestinal barrier function, accompanied by an increase in the relative abundance of *B. acidifaciens* and Ruminococcus callidus but a decrease in the relative abundance of *B. fragilis*. Further fecal bacterial transplantation or fecal filtrate transplantation confirmed the protective effect of eosinophil-regulated gut microbiota and metabolites on DSS-induced colitis. Finally, we validated that EVs derived from *B. acidifaciens* contain rich functional proteins that can contribute to the relief of colitis.

Conclusion: Therefore, *B. acidifaciens* and its derived EVs can alleviate DSS-induced colitis by reducing mucosal damage to colon tissue, reducing inflammatory response, promoting mucosal barrier repair, restoring gut microbiota diversity, and restoring gut microbiota balance in mice. The results of this study provide a theoretical basis for the preclinical application of the new generation of probiotics.

KEYWORDS

inflammatory bowel disease, *Bacteroides acidifaciens*, extracellular vesicles, gut microbiota, intestinal mucosal barrier

1 Introduction

Inflammatory bowel disease (IBD) is a chronic disease with unclear pathogenesis and a repeated course and is mainly divided into Crohn's disease and ulcerative colitis (Jakubczyk et al., 2020; Santana et al., 2022). The basic characteristics of IBD are abdominal pain and diarrhea, which can be complicated with colon stenosis and obstruction and tissue fibrosis and even induce colon cancer (Ullman and Itzkowitz, 2011). Since 1950, the number of patients in the western world, represented by Europe and America, has gradually increased, and recent epidemiological surveys show that the number of patients in eastern countries has also begun to increase rapidly (Molodecky et al., 2012). IBD has become a global disease. At present, although there are many treatments for IBD, such as hormones, immunosuppressants, biological agents, small molecule drugs and surgical treatments, the therapeutic effects are limited due to the unclear disease mechanism (Rogler et al., 2021).

Although the etiology of IBD is unknown, it is recognized that IBD is influenced by heredity, environment and intestinal microbiota, and it is characterized by a host immune response, intestinal barrier destruction and intestinal microbial community changes (Kudelka et al., 2020). The intestinal microbiota plays an important role in maintaining the intestinal mucosal barrier, stabilizing intestinal immune function and regulating intestinal metabolism (Li et al., 2021). However, intestinal microbiota imbalance or gut microbiota displacement can lead to intestinal inflammation and even tumors (Deng et al., 2022). Studies have shown that compared with healthy people, the ecological imbalance of IBD is characterized by a decrease in bacterial diversity or a change in composition (Franzosa et al., 2018). Therefore, it has become a research hotspot to intervene in intestinal diseases such as IBD by regulating intestinal microbiota. For example, "probiotic therapy," in which enough live probiotics are supplemented to maintain the health of the host, has been widely used in the clinic and has achieved certain results (Fedorak et al., 2015; Tamaki et al., 2015).

Under physiological conditions, 90% of the intestinal microflora are composed of *Firmicutes* and *Bacteroides* (Eckburg et al., 2005). *Bacteroides acidifaciens* belongs to *Bacteroides*, a gram-negative anaerobic bacterium and one of the dominant symbiotic bacteria in the intestine (Momose et al., 2011). A study revealed that in a mouse model, *B. acidifaciens* has a protective effect on concanavalin A-induced liver injury (Wang et al., 2022). Nagahara et al. revealed that *B. acidifaciens* can increase the level of intestinal immunoglobulin A (IgA), protect the intestine from pathogen infection and relieve IBD (Nakajima et al., 2020). Another study showed that alginate oligosaccharide and its compound supplement may enhance immune function and improve intestinal microecological disorder by increasing the abundance of *B. acidifaciens* (Yan et al., 2023). Tamas Korcsmaros et al. revealed that bacterial extracellular vesicles (EVs) produced by the intestinal symbiotic bacteria *Bacteroides thetaiotaomicron* have potential therapeutic value in IBD (Gul et al., 2022). EVs are small, spherical double-layer structures (20∼400 nm) containing proteins, lipids, nucleic acids and some metabolites, which are secreted by gram-negative bacteria and some gram-positive bacteria (Kaparakis-Liaskos and Ferrero, 2015; Toyofuku et al., 2019). EVs are important cell communication carriers that participate in the exchange of biological information between prokaryotes and eukaryotes. EVs can pass through the aseptic mucous layer of the colon through different routes, enter the border intestinal epithelial cells, interact with mucosal immune cells and the intestinal vascular system, and promote its widespread and systemic spread (Chronopoulos and Kalluri, 2020; Liu J. H. et al., 2021; Chen et al., 2022). Previous studies have shown that EVs play an important role in regulating the intestinal microenvironment and host health (Díaz-Garrido et al., 2021; Liu L. et al., 2021). This indicates that bacterial EVs are a possible factor by which the intestinal microbiota alleviates diseases.

In this study, we used *B. acidifaciens* to interfere with dextran sulfate sodium (DSS)-induced colitis in mice, revealing its anti-inflammatory and barrier protection effects on enteritis mice. The effects of *B. acidifaciens*-mediated microorganisms and their metabolites on alleviating the symptoms of enteritis mice were confirmed by fecal bacteria transplantation (FMT) and aseptic fecal filtrate transplantation (FFT). Finally, we found that EVs of *B. acidifaciens* can inhibit inflammatory reactions, repair the intestinal mucosal barrier, and finally alleviate colitis in mice. Through proteomic analysis, it was confirmed that the functional proteins of *B. acidifaciens* and *B. acidifaciens*-EVs were different.

2 Materials and methods

2.1 Ethics statement

This study was approved by the Animal Experiment Ethics Committee of Nanchang University (NCULAE-20221031149).

2.2 Culture of bacteria

Bacteroides acidifaciens (BNCC353574, BeNa, Henan, China) was cultured on fastidious anaerobe broth (FAB, Cat# LA4550; Solarbio, Inc., China) or Columbia agar (Cat#HB8511; Qingdao Hope Bio-Technology, Co., Ltd., China) with 5% goat blood (Cat# TX0020; Solarbio, Inc., China) under anaerobic conditions at 37°C for 48–72 h (Wang et al., 2022). The bacterial concentration was calculated by counting the number of CFUs after plating on Columbia agar containing 5% goat blood.

2.3 Isolation and identification of EVs from *B. acidifaciens*

The conditioned medium containing *B. acidifaciens* was obtained as described above. The conditioned culture of *B. acidifaciens* was centrifuged at 15,000 × *g* at 4°C for 20 min and then filtered through a 0.45 μm sterile filter membrane to remove residual bacteria and debris. The filtered supernatant was centrifuged at 20,000 × *g* at 4°C for 40 min and then filtered through a 0.22 μm sterile filter membrane. The supernatant was

transferred to 100 kDa Amicon Ultra15 filters at a concentration of 300–400 times. The concentrated solution was centrifuged at 120,000 × *g* at 4°C for 2 h to remove the supernatant, washed with sterile PBS, and centrifugation was repeated once to obtain EVs (Ma et al., 2022). As described, EVs were purified by OptiPrep (Cat# D1556, Sigma Aldrich, USA) gradient density centrifugation. Nanoparticle tracking analysis (NTA) method was used to detect the particle size distribution of EVs, and transmission electron microscopy (TEM) was used to observe the morphology of EVs.

2.4 Animal experiments

In this study, male C57BL/6J mice aged 6–8 weeks and weighing approximately 22–25 g were purchased from Tianqin Biotechnology Co., Ltd (Changsha, Hunan, China). Mice were reared at 22 ± 2°C for 1 week under an alternating light/dark cycle every 12 h. One week later, the mouse colitis model was induced by 2.5% dextran sodium sulfate (molecular mass 36–50 kDa, MP Biologicals, Solon, OH, USA) in a free drink. To study the therapeutic effect of *B. acidifaciens* on DSS-induced colitis, 24 mice were divided into three groups (n = 8): C group: free access to drinking water and food; M group: 2.5% DSS in a free drink for 7 days and daily oral administration of 0.1 ml saline for 10 days; TBA group: 2.5% DSS in a free drink for 7 days and daily oral administration of 0.1 ml 1×10^9 CFU *B. acidifaciens* for 10 days.

To determine whether the therapeutic effect of *B. acidifaciens* on colitis works through metabolites, we performed FMT or FFT on DSS-induced mice (Wu et al., 2020). Twenty-four mice were treated with an antibiotic cocktail [ABX; metronidazole (1 g/L), neomycin sulfate (1 g/L), ampicillin (0.5 g/L), vancomycin (0.5 g/L)] for 7 days before fecal bacteria transplantation and then divided into 4 groups: M-FMT group: 2.5% DSS for 7 days and transplanted with fecal supernatant of the M group for 10 days; M-FFT group: 2.5% DSS for 7 days and transplanted with fecal supernatant filtrate of the M group for 10 days; BA-FMT group: 2.5% DSS for 7 days and transplanted with fecal supernatant of the *B. acidifaciens* treatment group for 10 days; BA-FFT group: 2.5% DSS for 7 days and transplanted with fecal supernatant filtrate of the *B. acidifaciens* treatment group for 10 days. After 7 days of ABX administration, 24 mouse fecal samples were anaerobic cultured on a Brain Heart Infusion Broth (BHI; Cat# HB8297-1; Qingdao Hope Bio-Technology, Co., Ltd., China) agar plate for 96 h to ensure the depletion of fecal microbiota. The feces of mice were collected and diluted with PBS (1:10), thoroughly homogenized, centrifuged at 500 × *g* for 1 min and collected the supernatant for FMT. The supernatant was filtered through a 0.22 μm filter membrane, and the filtrate was collected for FFT.

To study the therapeutic effect of EVs of *B. acidifaciens* on DSS-induced colitis, 24 mice were divided into 3 groups (n = 8): C group: free access to drinking water and food; M group: 2.5% DSS in a free drink for 7 days and daily oral administration of 0.1 ml saline for 10 days; TBA group: 2.5% DSS in a free drink for 7 days and daily oral administration of 0.1 ml EVs (50 μg/day) for 10 days. The weight of the mice was measured daily throughout the study. The disease activity index (DAI) evaluated the severity of colitis through weight loss, diarrhea of the stool, and the extent of blood in the feces (Ma et al., 2022).

2.5 H&E, AB/PAS, immunofluorescence and TUNEL staining

To evaluate the morphological changes in colon tissue in mice, the sample was fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5–6 μm thick sections, rehydrated with xylene and degraded ethanol for 3–5 min, washed with PBS, and then stained with hematoxylin-eosin (H&E). To evaluate the number of goblet cells in the colon, we also stained fixed colon tissue sections with Alcian blue/periodic acid-Schiff staining (AB/PAS). To assess the expression of the colon barrier proteins ZO-1 (Cat# 21773-1-AP; Proteintech Group, Inc.; 1:100, China) and Occludin (Cat# 27260-1-AP; Proteintech Group, Inc.; 1:100, China), the tissue sections were stained with immunofluorescence. To assess the apoptosis of colon tissue cells in mice, fixed colon sections were stained with a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit (Cat# T2196; Solarbio, Inc., China). Finally, the images were visualized by means of microscopy or fluorescence microscopy (Wu Z. et al., 2021).

2.6 Measurement of inflammatory cytokines and antioxidant indices in serum

To evaluate inflammation and oxidative stress in the serum of mice, the levels of the proinflammatory factors interleukin (IL)-1β (Cat# 2M-KMLJM211201m mouse; Camilo Biological, Nanjing, China), IL-6 (Cat# 2M-KMLJM219451m mouse; Camilo Biological, Nanjing, China), and tumor necrosis factor (TNF)-α (Cat# 2M-KMLJM220051m mouse; Camilo Biological, Nanjing, China) were determined according to the manufacturer's protocol.

2.7 Western blotting analysis

Mice colon tissue sample proteins were extracted using cell lysis buffer (Cat# R0020; Solarbio, Inc., China) supplemented with phenylmethanesulfonyl fluoride (PMSF; Cat# P0100; Solarbio, Inc., China) and protease inhibitor cocktail (Cat# HY-K0022; Med Chem Express, Inc., China). A bicinchoninic acid protein assay kit (BCA; Cat# PC0020; Solarbio, Inc., China) was used to determine the protein concentration of the samples. Then, the proteins were detached with 8–10% polyacrylamide resolving gels, transferred to polyvinylidene fluoride membranes (PVDF), and blocked with 5% skim milk for 90 min at RT (Xiao et al., 2023). Then, the membranes were coincubated with the following primary antibodies overnight at 4°C: rabbit anti-Zona occludens protein 1 (ZO-1; Cat# 21773-1-AP; Proteintech Group, Inc.; 1:5,000, China); rabbit anti-Occludin (Cat# 27260-1-AP; Proteintech Group, Inc.; 1:2,000, China); and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cat# 60004-1-Ig; Proteintech Group, Inc.; 1:5,000, China). After primary incubation, the membrane was washed with Tris-buffered-saline-Tween-20 (TBST) buffer three times for 10 min each and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Cat#SA00001-2; Proteintech

Group; 1: 5,000, China) or goat anti-mouse secondary antibody (Cat#SA00001-1; Proteintech Group; 1: 5,000, China) for 60 min at RT. Finally, the proteins were visualized using a super enhanced chemiluminescence reagent (ECL).

2.8 DNA extraction and 16S rRNA gene sequencing

For microbial DNA extraction, mouse feces samples were collected. Bacterial DNA was extracted from feces by the magnetic bead method combined with a genomic DNA kit (Cat# DP712-02; Tiangen Biotech Co., Ltd., China), and the purity and concentration of purified DNA were detected by spectrophotometry at 230 nm (A 230) and 260 nm (A 260). Then, the V4 region of the 16S ribosomal (r)RNA genes was amplified via primers (5'-AYTGGGYDTAAAGNG R-3'; 5'- TACNVGGGTATCTAATCC-3') in the sample, and the PCR products were sequenced with an Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA) (GenBank accession number PRJNA1009232) (Zheng et al., 2021).

2.9 Proteomic analysis

Three biological replicate samples of *B. acidifaciens* (BA1, BA2, and BA3) and *B. acidifaciens*-derived EVs (EV1, EV2, and EV3) were obtained, and the 4D-label-free quantitative proteomic analysis was performed. The protein extraction of BA and EV was as previously described and quantified using the BCA protein assay kit. Protein digestion by trypsin was performed according to filter-aided sample preparation procedure described by Matthias Mann. LC-MS/MS analysis was performed on a timsTOF Pro mass that was coupled to Nanoelute (Bruker Daltonics). The MS raw data for each sample were combined and searched using the MaxQuant 1.5.3.17 software for identification and quantitation analysis (Chen et al., 2022; Zheng et al., 2023).

2.10 Statistical analysis

The data are represented by the mean standard deviation (SD). GraphPad Prism 9.0 (San Diego, CA, USA) was used for statistical analyses. And statistical analyses of two groups was performed by unpaired two-tailed Student's *t*-test, multiple groups were performed by one-way ANOVA followed by Tukey's multiple comparison test. The adjusted $P < 0.05$ was considered statistically significant (Zheng et al., 2021).

3 Results

3.1 The intervention of *B. acidifaciens* alleviated DSS-induced colitis

To observe the relieving effect of *B. acidifaciens* on colitis in mice, we used 2.5% DSS to induce colitis in mice for 7 days and treated them with *B. acidifaciens* at the same time for 10 days (**Figure 1A**). The results showed that compared with the M group, *B. acidifaciens* significantly alleviated weight loss, reduced the DAI and reversed the shortening of the colon (**Figures 1B–D**). Histological analysis revealed that inflammatory cells infiltrated the colon, intestinal villi were shortened and thickened, and mucosa was damaged in response to DSS treatment, while the administration of *B. acidifaciens* alleviated the colonic inflammatory reaction (**Figure 1E**). To further assess the effect of *B. acidifaciens* on the systemic inflammatory response, we detected proinflammatory cytokines in the serum of mice. The serum concentrations of IL-1β, IL-6 and TNF-α in *B. acidifaciens*-treated mice were significantly decreased (**Figures 1F–H**). To determine the protective effect of *B. acidifaciens* on the intestinal barrier, we further detected the expression of barrier proteins by western blot and immunofluorescence staining and detected mucin-secreting goblet cells in the colonic epithelium by AB-PAS staining. The results showed that oral administration of *B. acidifaciens* significantly reversed the decrease in barrier proteins caused by DSS (**Figures 1I, J**) and maintained better mucus distribution (**Figure 1K**). Moreover, TUNEL analysis further revealed that DSS significantly increased the frequency of apoptotic cells in the colonic mucosa, but *B. acidifaciens* largely reversed this trend (**Figure 1L**).

3.2 *B. acidifaciens* regulated the gut microbiota composition

Next, we further explored the influence of *B. acidifaciens* on the composition of gut microbiota in DSS-treated mice via 16S rRNA gene sequencing. The α-diversity analysis showed that DSS treatment significantly reduced the diversity of the gut microbiota in mice, and the administration of *B. acidifaciens* promoted its gradual recovery (**Figures 2A–C**). Principal coordinate analysis (PCoA) confirmed that DSS caused the gut microbiota to be located far away from the C group, while *B. acidifaciens* caused the TBA and C groups to be close to each other (**Figure 2D**). In addition, the Venn diagram showed (**Figure 2E**) that there were 1,399, 801, and 1,102 OTUs in the C, M and TBA groups, and the percentages of common OTUs in each group were 19.30% (270/1,399), 33.71% (270/801) and 24.50% (270/1,102), respectively.

Next, we further explored the gut microbiota composition and intestinal disease-related bacteria at the phylum, genus and species levels. At the phylum level, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were predominant phyla in the fecal microbiota (**Figures 2F, I–L**). Moreover, the results showed that colitis mice showed a low relative abundance of *Bacteroides*, and the administration of *B. acidifaciens* reversed the relative abundance of *Bacteroides* (**Figure 2I**). At the genus level, the fecal microbiota was dominated by *Bacteroides*, *Paraprevotella*, and *Parabacteroides* (**Figure 2G**). Further analysis showed that the administration of *B. acidifaciens* significantly increased the relative abundance of *Bacteroides*, *Paraprevotella*, and *Prevotella* while decreasing the relative abundance of *Parabacteroides* and *Streptococcus* (**Figures 2M–Q**). The results of species level analysis showed that the administration of *B. acidifaciens* sharply increased the relative abundance of *B. acidifaciens* and *Ruminococcus callidus*

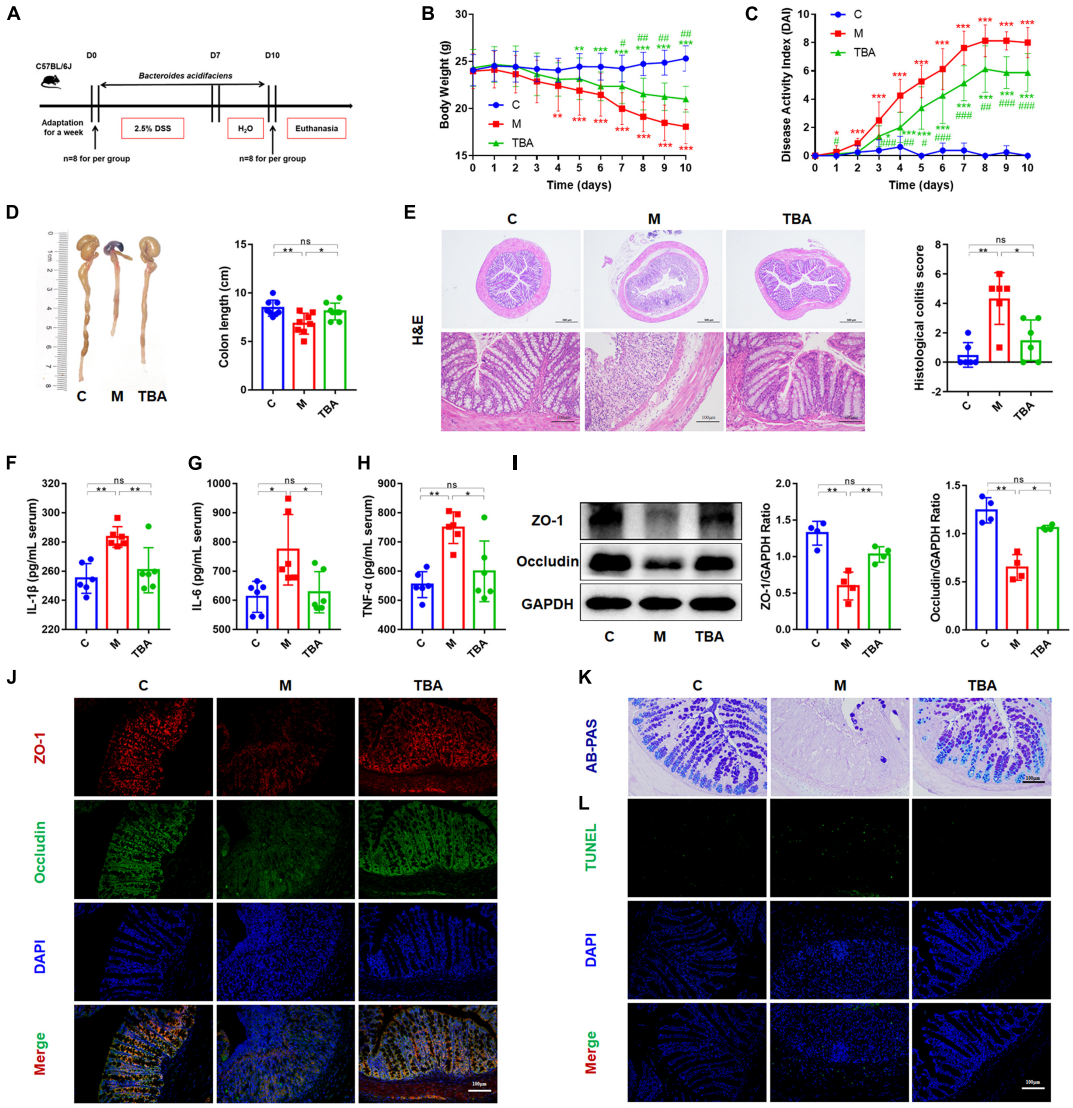


FIGURE 1
Bacteroides acidifaciens alleviated DSS-induced colitis in mice. **(A)** Experimental design. **(B)** Mouse body weight. **(C)** DAI score. **(D)** Colon length. **(E)** H&E staining and pathological score of colon sections. **(F–H)** Serum IL-1β, IL-6 and TNF-α levels. **(I)** The expression of tight junction proteins (ZO-1, Occludin) in mouse colon tissue. **(J)** Immunofluorescence was used to evaluate the expression of tight junction proteins (ZO-1, Occludin) in colonic sections. **(K)** Representative images of the Alcian blue-stained inner mucus layer of colonic sections. **(L)** Representative fluorescent pictures of TUNEL staining of colonic sections. C, control group; M, DSS group; TBA, DSS mice orally administered *B. acidifaciens* group. C, control group; M, DSS group; TBA, DSS mice orally administered *B. acidifaciens* group. Scale bar, 100 μm. Data are presented as the mean ± SD. *$P < 0.05$ versus the C group, # $P < 0.05$ versus the M group. ns, $P > 0.05$; */# $P < 0.05$; **/## $P < 0.01$; ***/### $P < 0.001$.

but slightly decreased the relative abundance of *Bacteroides fragilis* (Figures 2H, R–T). However, there was no significant difference in the relative abundance of *B. fragilis* between the TBA group and the other two groups.

3.3 *B. acidifaciens*-changed gut microbiota and bacterial metabolites contributed to the alleviation of colitis

We further performed FMT and FFT to determine the role of gut microbiota and bacterial metabolites in the remission of colitis mediated by *B. acidifaciens* (Figure 3A). The results showed that compared with the FMT or FFT of the M group mice, the FMT and FFT of the donor mice treated with *B. acidifaciens* reduced the weight loss and DAI score and shortened the colon length (Figures 3B–D). Histological analysis showed that FMT or FFT of *B. acidifaciens*-treated donor mice significantly alleviated the infiltration of inflammatory cells into the colon, shortened and thickened villi, and damaged mucosa, thus reducing the histological score (Figure 3E). In addition, the FMT of *B. acidifaciens*-treated donor mice was helpful to reduce the level of serum inflammation, which was proven by the significantly reduced levels of IL-1β, IL-6 and TNF-α (Figures 3F–H). Furthermore, FMT and FFT of *B. acidifaciens*-treated donor mice significantly increased the expression of the barrier proteins ZO-1 and Occludin

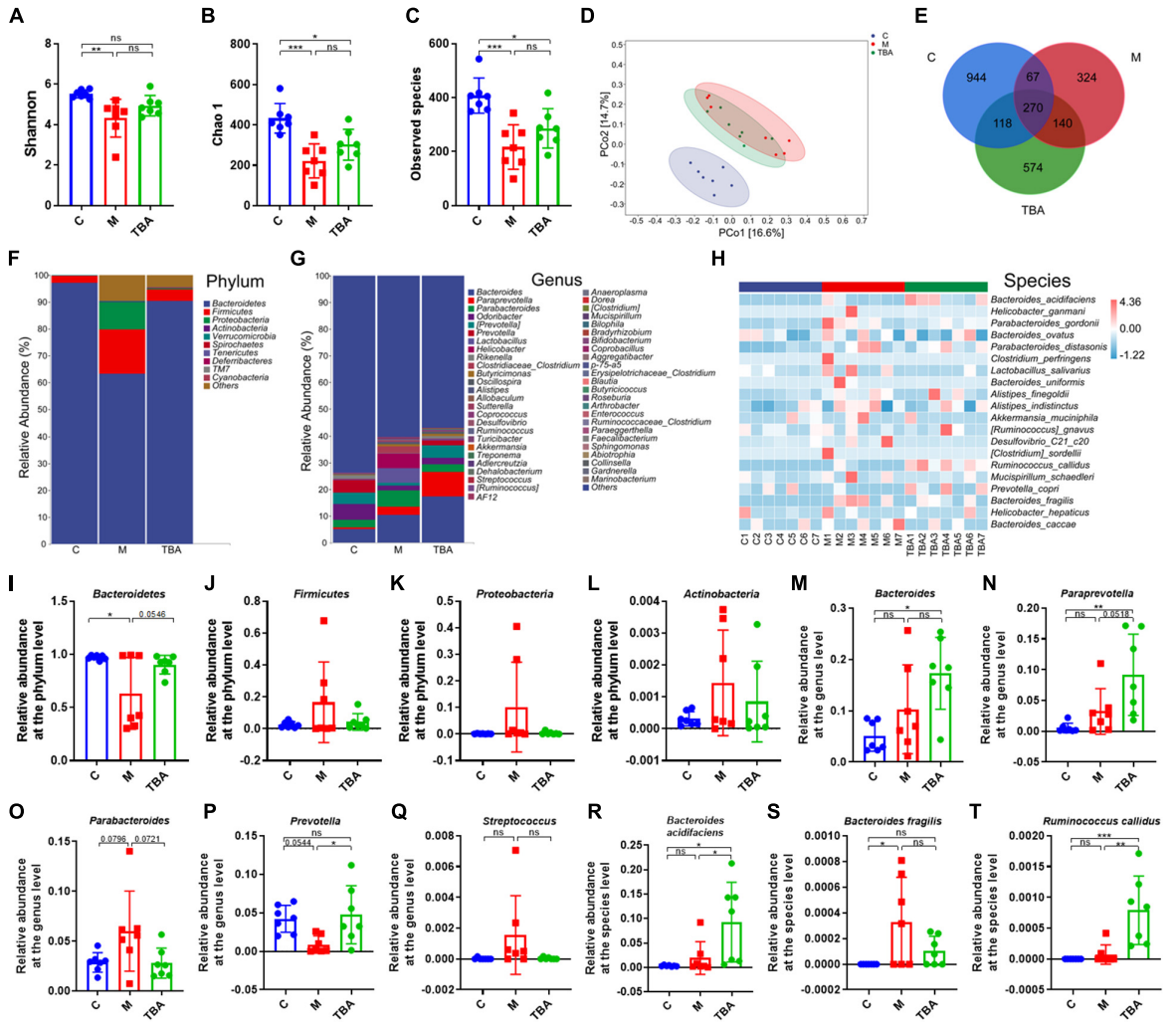


FIGURE 2
Bacteroides acidifaciens altered gut microbiota diversity in DSS-induced colitis mice. **(A)** Shannon index. **(B)** Chao 1 index. **(C)** Observed species. **(D)** PCoA of the β diversity index. **(E)** Venn diagram. **(F–H)** Relative abundance of the identified fecal microbiota at the **(F)** phylum, **(G)** genus, and **(H)** species levels as per 16S rRNA gene sequencing. **(I)** *Bacteroidetes*. **(J)** *Firmicutes*. **(K)** *Proteobacteria*. **(L)** *Actinobacteria*. **(M)** *Bacteroides*. **(N)** *Paraprevotella*. **(O)** *Parabacteroides*. **(P)** *Prevotella*. **(Q)** *Streptococcus*. **(R)** *B. acidifaciens*. **(S)** *Bacteroides fragilis*. **(T)** *Ruminococcus callidus*. C, control group; M, DSS group; TBA, DSS mice orally administered *B. acidifaciens* group. Data are presented as the mean ± SD. $n = 7$. $^*P < 0.05$ versus the C group. ns, $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(Figures 3I, J) and maintained the distribution of intestinal mucus (Figure 3K). TUNEL analysis further revealed that FMT or FFT of *B. acidifaciens*-treated donor mice significantly reduced cell apoptosis in colon mucosa (Figure 3L). Collectively, *B. acidifaciens*-changed gut microbiota and metabolites were able to alleviate DSS-induced colitis.

3.4 *B. acidifaciens*-EV administration alleviated DSS-induced colitis

Furthermore, we isolated and purified bacterial EVs to explore whether the EVs of *B. acidifaciens* alleviated DSS-induced colitis (Figure 4A). Transmission electron microscopy of EVs showed saucer-like vesicle morphology (Figure 4B). The NTA results showed that the diameter of the EVs was approximately 129 nm (Figure 4C). Furthermore, we explored whether *B. acidifaciens* EVs had a therapeutic effect on DSS-induced colitis. Compared with the M group, the administration of *B. acidifaciens* or its EVs resulted in lower body weight loss, DAI score, and shortened colon length (Figures 4D–F). In addition, we observed that the infiltration of inflammatory cells, tissue damage and histological score in the colon decreased after the administration of *B. acidifaciens* EVs (Figures 4G, H). Moreover, the levels of IL-1β, IL-6, and TNF-a were significantly reduced in the TBA and TEV groups (Figures 4I–K). Consistently, western blot and immunofluorescence analysis of ZO-1 and Occludin showed that *B. acidifaciens* or EV treatment weakened the epithelial tight junction protein barrier damaged by DSS (Figures 4L, M). The administration of *B. acidifaciens* or EVs also significantly enhanced the integrity of the mucus barrier, showing more mucin-positive coverage (Figure 4N). TUNEL analysis revealed that *B. acidifaciens* or EVs significantly reduced cell apoptosis in the colonic mucosa (Figure 4O). Notably, compared with the

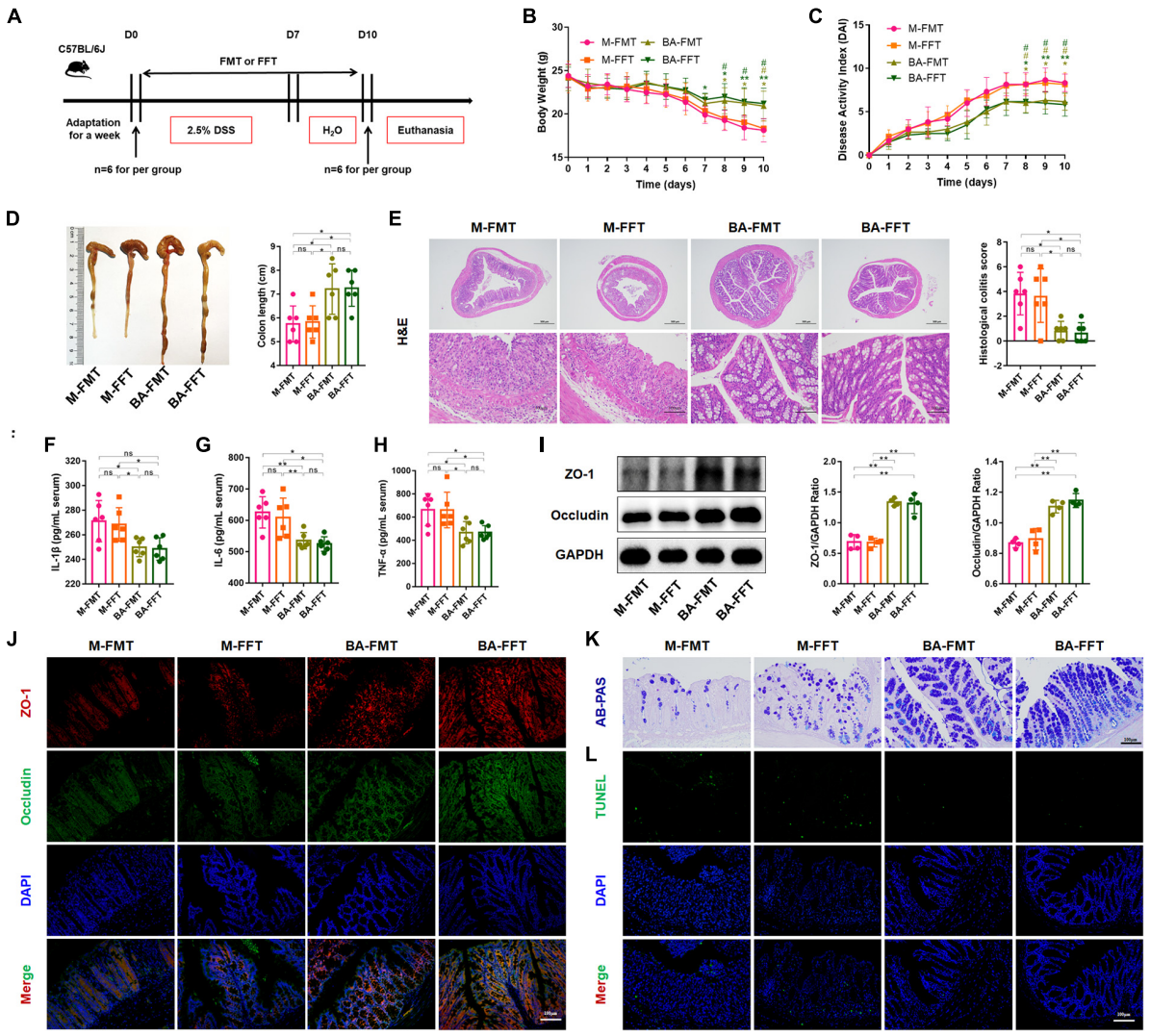


FIGURE 3
Bacteroides acidifaciens changed the gut microbiota, and metabolites contributed to alleviating colitis. **(A)** Experimental design. **(B)** Mouse body weight. **(C)** DAI score. **(D)** Colon length. **(E)** H&E staining and pathological score of colon sections. **(F–H)** Serum IL-1β, IL-6 and TNF-α levels. **(I)** The expression of tight junction proteins (ZO-1, Occludin) in mouse colon tissue. **(J)** Immunofluorescence representative images of tight junction proteins (ZO-1, Occludin) in colonic sections. **(K)** Representative images of the Alcian blue-stained inner mucus layer of colonic sections. **(L)** Representative fluorescent pictures of TUNEL staining of colonic sections. M-FMT: transplanted with fecal supernatant of the M group; M-FFT: transplanted with fecal supernatant filtrate of the M group; BA-FMT: transplanted with fecal supernatant of the TBA group; BA-FFT: transplanted with fecal supernatant filtrate of the TBA group. Scale bar, 100 μm. Data are presented as the mean ± SD. *$P < 0.05$ versus the M-FMT group, # $P < 0.05$ versus the M-FFT group. ns, $P > 0.05$; */# $P < 0.05$; **$P < 0.01$.

treatment of *B. acidifaciens*, EV administration can significantly alleviate the symptoms of acute colitis.

3.5 Proteomic analysis of *B. acidifaciens*-EVs and *B. acidifaciens*

Proteins in *B. acidifaciens* EVs or *B. acidifaciens* were identified and quantified using 4D- label-free proteomic analysis. A total of 1,990 proteins were identified in *B. acidifaciens*-EVs and *B. acidifaciens*, of which 1,978 were quantified (Figure 5A). Venn diagram analysis showed that there were fewer unique differential proteins identified in EVs compared to *B. acidifaciens* (Figure 5B and Supplementary Table 1). Furthermore, we found that *B. acidifaciens* EVs resulted in 277 upregulated differentially expressed proteins (DEPs) and 788 downregulated DEPs (Figure 5C). As shown in Figure 5D, 61.02% of them were from cytoplasmic proteins, and 13.23% of them were annotated as outer membrane proteins. The subcellular localizations of 10.77% of the proteins were periplasmic, and the remaining proteins were from the inner membrane (8.30%) or extracellular (6.68%) (Figure 5D). To further identify biological alterations in *B. acidifaciens* EVs, the DEPs were classified into three categories based on gene ontology (GO) analysis: biological process (BP), molecular function (MF), cellular component (CC), and the top 20 terms were displayed in Figure 5E. The maximum proportion of DEPs was concentrated in bacterial biological processes, including cellular processes and cellular metabolic processes. Kyoto

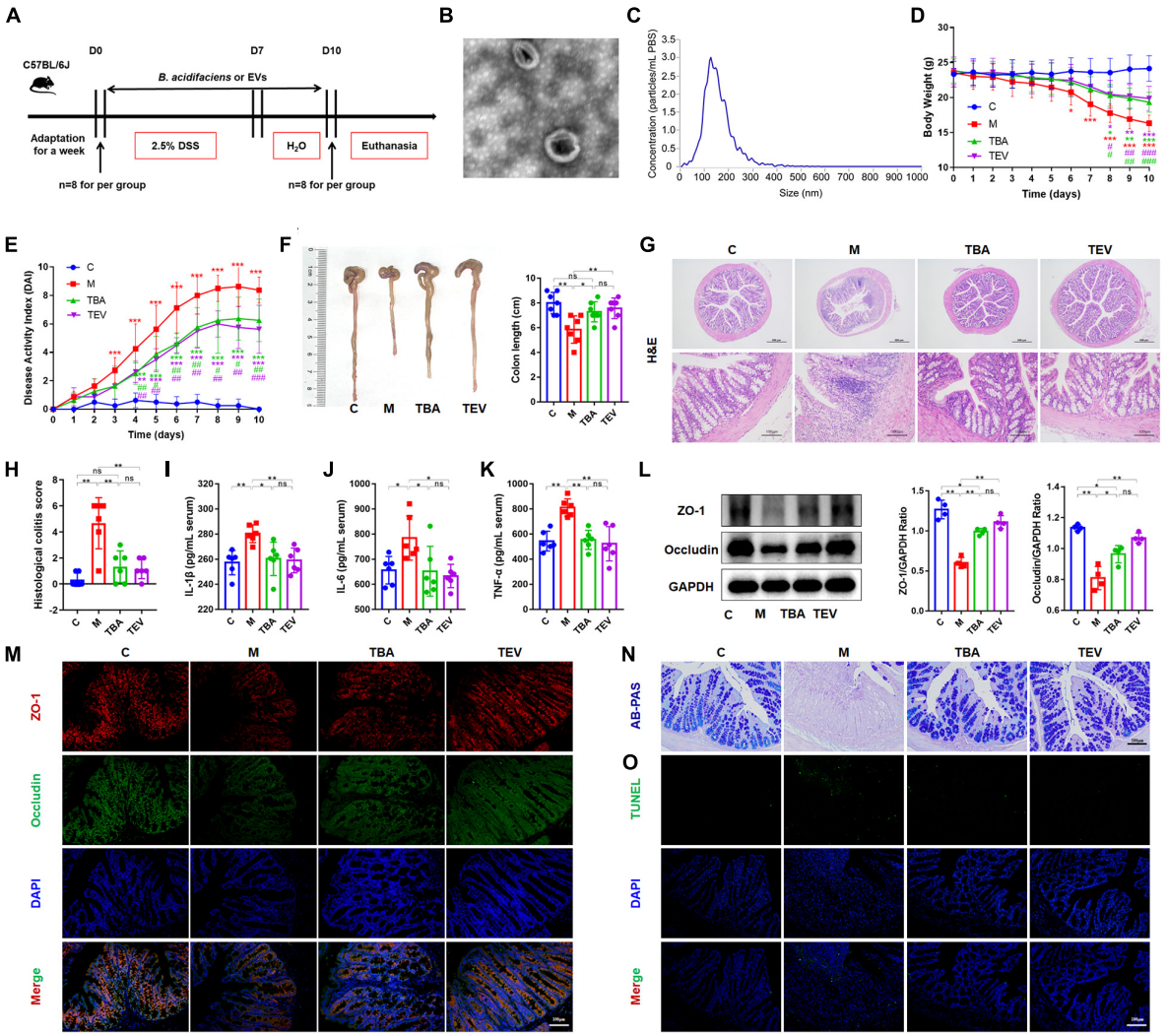


FIGURE 4
Bacteroides acidifaciens-EVs had a relieving effect on DSS-induced colitis. **(A)** Experimental design. **(B)** Observation of *B. acidifaciens*-EV morphology by means of TEM. Scale bar, 50 nm. **(C)** Observation of *B. acidifaciens*-EV size distribution analyzed by NTA. **(D)** Mouse body weight. **(E)** DAI score. **(F)** Colon length. **(G,H)** H&E staining and pathological score of colon sections. Scale bar, 100 μm. **(I–K)** Serum IL-1β, IL-6 and TNF-α levels. **(L)** The expression of tight junction proteins (ZO-1, Occludin) in mouse colon tissue. **(M)** Immunofluorescence representative images of tight junction proteins (ZO-1, Occludin) in colonic sections. **(N)** Representative images of the Alcian blue-stained inner mucus layer of colonic sections. **(O)** Representative fluorescent pictures of TUNEL staining of colonic sections. C, control group; M, DSS group; TBA, DSS mice orally administered *B. acidifaciens* group; TEV, DSS mice orally administered *B. acidifaciens*-EVs group. Data are presented as the mean ± SD. **P* < 0.05 versus the C group, # *P* < 0.05 versus the M group. ns, *P* > 0.05; */# *P* < 0.05; **/## *P* < 0.01; ***/### *P* < 0.001.

Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that the upregulated proteins in EVs compared with *B. acidifaciens* were involved in the biosynthesis of various antibiotics, apoptosis, and bacterial chemotaxis. Moreover, EVs downregulated proteins related to biosynthesis and metabolism, including lipopolysaccharide biosynthesis (Figure 5F). Thus, EVs underwent significant changes in protein composition compared to *B. acidifaciens*, and ultimately altering the biological functions.

4 Discussion

In this study, we explored whether *B. acidifaciens*, a specific intestinal symbiotic bacterium, can alleviate the symptoms of DSS-induced colitis by reducing the inflammatory reaction and repairing the intestinal mucosal barrier. In addition, through fecal transplantation and fecal filtrate transplantation from *B. acidifaciens*-treated mice, we confirmed that the gut microbiota and metabolites regulated by *B. acidifaciens* may be the reason the symptoms of colitis are alleviated. Finally, we proved that *B. acidifaciens* EVs can also alleviate the symptoms of colitis by reducing inflammatory reactions and repairing the intestinal mucosal barrier (Figure 6).

A previous study reported that oral administration of 3% DSS for 5 days can reduce EVs from *B. acidifaciens* and *Akkermansia muciniphila* (Paredes-Sabja et al., 2013). Another study revealed that the protective effect of sulfated polysaccharides from *Gracilaria lemaneiformis* on DSS-induced colitis in mice was closely related to probiotics such as *Alistipes* and *B. acidifaciens* (Han et al., 2021). To explore whether oral administration of *B. acidifaciens*

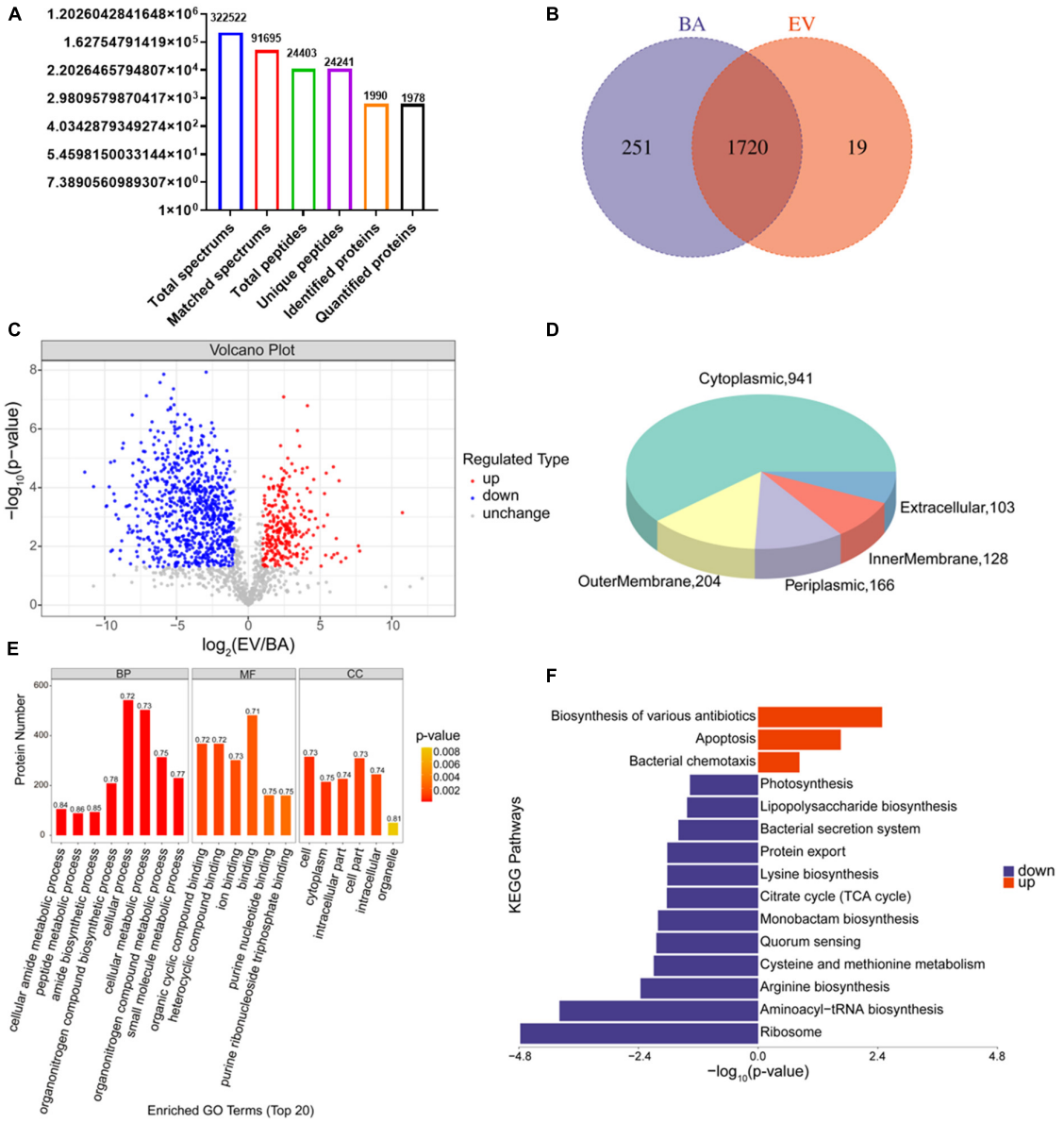


FIGURE 5
Proteomic analysis of *B. acidifaciens* -EVs and *B. acidifaciens*. **(A)** Summary of tandem mass spectrometry (MS/MS) database search results for *B. acidifaciens*-EVs and *B. acidifaciens*. **(B)** Venn diagram. **(C)** Volcano plot showing the number of proteins differentially expressed between *B. acidifaciens*-EVs and *B. acidifaciens*, with a cutoff of $P < 0.05$ and | fold change| > 2. **(D)** Subcellular localization pie chart of differentially expressed proteins in *B. acidifaciens*-EVs and *B. acidifaciens*. **(E)** GO classification (biological process, molecular function, and cellular components) of all differentially expressed proteins in *B. acidifaciens*-EVs and the top 20 enriched GO terms. **(F)** KEGG pathway enrichment analysis of the differentially expressed proteins in *B. acidifaciens*-EVs and *B. acidifaciens*. BA, *B. acidifaciens*; EV, *B. acidifaciens*-EVs. $n = 3$.

can alleviate colitis, acute colitis in mice was induced by 2.5% DSS and then treated with oral *B. acidifaciens*. Consistent with previous studies, we confirmed that the therapeutic application of oral *B. acidifaciens* can alleviate DSS-induced colitis, as shown by the DAI score, colon length and histological damage (Hu et al., 2020; Dong et al., 2022; Sharma et al., 2023). Similarly, oral *B. acidifaciens* also alleviated colonic inflammation, as exemplified by the decline in inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the serum of DSS-treated mice (Hao et al., 2021; Zhao et al., 2021; Li X. et al., 2022). Furthermore, oral administration of *B. acidifaciens* alleviated DSS-induced damage to integrity and barrier function, as evidenced by the increased colonic mucus as well as the enhanced tight junctions of the colonic mucosa, in agreement with previous studies (Cui et al., 2021; Wu H. et al., 2021). Moreover, DSS-induced colitis can lead to apoptosis of colon tissue cells, while oral administration of *B. acidifaciens* reduced this phenomenon (Qu et al., 2017; Li et al., 2019; Kim et al., 2023).

Furthermore, we investigated the differences between the feces of mice orally administered *B. acidifaciens* (TBA) and the other two groups (C and M). The results of α-diversity analysis showed that the DSS-induced group significantly reduced the microbial diversity of mouse feces, while supplementation with *B. acidifaciens* reversed this result (Zhang et al., 2019; Huang et al., 2022). PCoA revealed differences in the microbial community structure

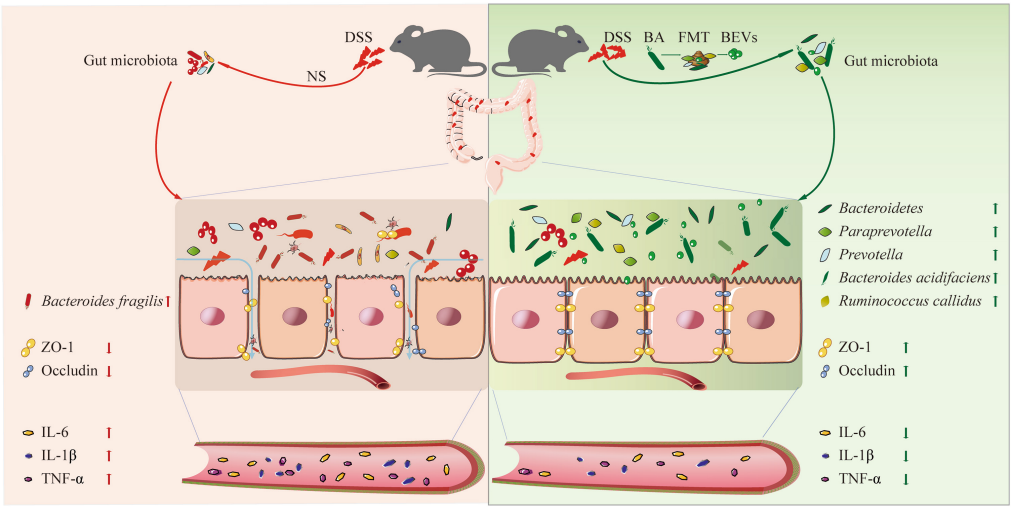


FIGURE 6
A schematic diagram summarizing the main findings of this study.

among the C, M and TBA groups, with the microbial community structure of the TBA group gradually approaching that of the C group. Further analysis of the gut microbiota composition of mice treated with *B. acidifaciens* showed a significant increase in the abundance of *Bacteroides*, *Paraprevotella*, and *Prevotella*. A previous study revealed a decrease in the metabolites of *Bacteroides* and SCFAs in IBD patients (Deleu et al., 2021; Guo et al., 2021; Zhang et al., 2022). The *Paraprevotella* genus has been proven to have anti-inflammatory effects (Renson et al., 2020). *Lachnospira* and *Paraprevotella* are symbiotic bacteria that may produce short-chain fatty acids (such as succinic acid or acetic acid) in the gastrointestinal tract (Hattori et al., 2020). In addition, *Paraprevotella* can accumulate at the healing site of the intestinal mucosa (Feng et al., 2021). Another study reported that the establishment of a UC model can reduce the proportion of *Prevotella* species, while using drugs to alleviate enteritis can restore their relative abundance (Liu et al., 2020). Specific species of the *Prevotella* genus can successfully inhibit inflammatory demyelinating disease and multiple sclerosis by inducing Tregs and regulating cytokines (Mangalam et al., 2017; Shahi et al., 2019). Moreover, supplementation with *B. acidifaciens* also reduced the relative abundance of *Parabacteroides* and *Streptococcus* (Li Q. et al., 2022; Wu et al., 2023). Fabio Cominelli et al. reported that *Parabacteroides distasonis* opportunistically colonizes the gut niche of susceptible CD patients and exerts a depressive effect (Gomez-Nguyen et al., 2021). At the species level, supplementation with *B. acidifaciens* significantly reduced the relative abundance of *B. fragilis*. Toshifumi Hibi et al. revealed that *B. fragilis* was the main bacterial species in the feces of UC patients (Takaishi et al., 2008). In the present study, oral administration of *B. acidifaciens* significantly increased the relative abundance of *Ruminococcus callidus*, which was negatively correlated with intestinal symptoms, especially discomfort (Hiel et al., 2019).

Previous studies have reported that disease treatment can be achieved by transplanting functional microflora (FMT) from healthy human feces into the gastrointestinal tract of patients with ulcerative colitis and rebuilding a new gut microbiota (Moayyedi et al., 2015; Sokol et al., 2020). Similarly, transplanting fecal filtrate (FFT) from treatment group mice to enteritis mice can alleviate the colitis phenotype by eliminating ecological imbalances (Wu Z. et al., 2021; Ma et al., 2022). To verify the effects of microbiota and metabolites mediated by *B. acidifaciens*, we performed FMT and FFT. Consistent with the treatment with *B. acidifaciens*, BA-FMT was more effective than M-FMT in alleviating the phenotype of colitis in mice, reducing inflammatory reactions and repairing the intestinal mucosal barrier (Dong et al., 2021). In addition, the treatment of FFT in donors with *B. acidifaciens* also alleviated DSS-induced colitis (Wu Z. et al., 2021). We demonstrated that the gut microbiota and its metabolites induced by *B. acidifaciens* played a key role in alleviating DSS-induced experimental colitis.

Bacterial EVs are an important bridge for information exchange between gut microbiota and host cells (Toyofuku et al., 2019). EVs selectively carry various molecules (such as nucleic acids and proteins) from their bacteria, which may regulate host signaling pathways and various cellular processes (Toyofuku et al., 2019; Díaz-Garrido et al., 2021). To explore the interaction between *B. acidifaciens* and the host, we further investigated the role of EVs derived from *B. acidifaciens* in preventing colitis. As expected, *B. acidifaciens* and its derived EVs improved colitis symptoms, including colitis phenotype and histological scores (Hao et al., 2021; Tong et al., 2021). Consistent with previous studies, EVs treatment also reduced the expression of proinflammatory genes, such as IL-1β, IL-6, and TNF-α (Hao et al., 2021; Tong et al., 2021). Moreover, EVs enhance the epithelial barrier by regulating the expression of the TJ proteins Occludin and ZO-1, stabilizing the distribution of intestinal mucin proteins, and reducing intestinal tissue cell apoptosis (Kang et al., 2020). In summary, the above results emphasize the crucial role of probiotic-derived EVs in regulating DSS-induced colitis.

Compared to bacteria themselves, EVs derived from bacteria are rich in various functional proteins that can directly act on receptor cells and perform corresponding functions (Brown et al., 2015). Previous studies have shown that compared to *Lactobacillus animalis*, *L. animalis* EVs may be able to

transfer some pro-angiogenic, pro-osteogenic, and anti-apoptotic proteins to endothelial and bone cells to exert bone protective effects (Chen et al., 2022). Another study reported that signal exchange between the microbiota and host bone can effectively alleviate osteoporosis by transferring *Akkermansia muciniphila* functional EVs from bacteria to bone cells (Liu J. H. et al., 2021). Therefore, we further utilized proteomics analysis to investigate the differential expression of extracellular vesicular proteins derived from *B. acidifaciens*. Bacteria have evolved unique ways of killing each other, so many of our most powerful antibiotics are derived from the bacteria themselves (Niehus et al., 2021). Our research findings suggested that *B. acidifaciens*-EVs have enriched biosynthesis of various antimicrobial-related proteins, which may be their potential mechanism for inhibiting the growth of other bacteria (Domínguez Rubio et al., 2022; Ma et al., 2023). Lipopolysaccharides are components of the outer wall of gram-negative bacterial cells and are endotoxins that can exert their toxic effects by acting on animal cells (Wang and Quinn, 2010). Additionally, we observed that *B. acidifaciens*-EVs also downregulated biosynthesis- and metabolism-related proteins, including lipopolysaccharide biosynthesis, which may be an indirect mechanism of the action of eosinophils on DSS-induced colitis, which still requires future exploration.

5 Conclusion

In summary, *B. acidifaciens* and its derived EVs can alleviate DSS-induced colitis by reducing mucosal damage to colon tissue, reducing inflammatory response, promoting mucosal barrier repair, restoring gut microbiota diversity, and restoring gut microbiota balance in mice. In this study, we clarified the effect of *B. acidifaciens* on DSS-induced colitis and explored its mechanism of action through its derived EVs. And through proteomics, it was found that there are differences in the proteomics of *B. acidifaciens* and its EVs. However, how EVs communicates with the host and participates in host regulation may be the key to the treatment of colitis with *B. acidifaciens*, and further exploration is needed. The results of this study provide a theoretical basis for the preclinical application of the new generation of probiotics.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI–PRJNA1009232.

Ethics statement

The animal study was approved by the Animal Experiment Ethics Committee of Nanchang University (NCULAE-202210311149). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CZ: Methodology, Visualization, Writing – original draft. YZ: Methodology, Software, Visualization, Writing – original draft. JX: Data curation, Methodology, Software, Writing – original draft. ZW: Methodology, Software, Writing – original draft. WenmZ: Methodology, Visualization, Writing – original draft. YP: Methodology, Writing – original draft. WenjZ: Visualization, Writing – original draft. LL: Methodology, Writing – original draft. JL: Conceptualization, Funding acquisition, Writing – original draft. WX: Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing.

Funding

The authors declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by the National Natural Science Foundation of China grant 81860435 (WX), The Second Affiliated Hospital of Nanchang University Funding Program 2022efyA01 (WX), Jiangxi Provincial Department of Science and Technology Project 20224ABC03A02 (JL), and The Second Affiliated Hospital of Nanchang University Funding Program 2022efyB03 (JL).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1304232/full#supplementary-material

SUPPLEMENTARY TABLE 1
The detailed list of differential proteins identified in EVs compared to *B. acidifaciens*.

References

Brown, L., Wolf, J. M., Prados-Rosales, R., and Casadevall, A. (2015). Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat. Rev. Microbiol.* 13, 620–630. doi: 10.1038/nrmicro3480

Chen, C. Y., Rao, S. S., Yue, T., Tan, Y. J., Yin, H., Chen, L. J., et al. (2022). Glucocorticoid-induced loss of beneficial gut bacterial extracellular vesicles is associated with the pathogenesis of osteonecrosis. *Sci. Adv.* 8:eabg8335. doi: 10.1126/sciadv.abg8335

Chronopoulos, A., and Kalluri, R. (2020). Emerging role of bacterial extracellular vesicles in cancer. *Oncogene* 39, 6951–6960. doi: 10.1038/s41388-020-01509-3

Cui, L., Guan, X., Ding, W., Luo, Y., Wang, W., Bu, W., et al. (2021). *Scutellaria baicalensis* Georgi polysaccharide ameliorates DSS-induced ulcerative colitis by improving intestinal barrier function and modulating gut microbiota. *Int. J. Biol. Macromol.* 166, 1035–1045. doi: 10.1016/j.ijbiomac.2020.10.259

Deleu, S., Machiels, K., Raes, J., Verbeke, K., and Vermeire, S. (2021). Short chain fatty acids and its producing organisms: an overlooked therapy for IBD? *EBioMedicine* 66:103293. doi: 10.1016/j.ebiom.2021.103293

Deng, J., Zhao, L., Yuan, X., Li, Y., Shi, J., Zhang, H., et al. (2022). Pre-administration of berberine exerts chemopreventive effects in AOM/DSS-induced colitis-associated carcinogenesis mice via modulating inflammation and intestinal microbiota. *Nutrients* 14, 726–747. doi: 10.3390/nu14040726

Díaz-Garrido, N., Badia, J., and Baldomà, L. (2021). Microbiota-derived extracellular vesicles in interkingdom communication in the gut. *J. Extracell. Vesicles* 10:e12161. doi: 10.1002/jev2.12161

Domínguez Rubio, A. P., D'Antoni, C. L., Piuri, M., and Pérez, O. E. (2022). Probiotics, their extracellular vesicles and infectious diseases. *Front. Microbiol.* 13:864720. doi: 10.3389/fmicb.2022.864720

Dong, F., Xiao, F., Li, X., Li, Y., Wang, X., Yu, G., et al. (2022). *Pediococcus pentosaceus* CECT 8330 protects DSS-induced colitis and regulates the intestinal microbiota and immune responses in mice. *J. Transl. Med.* 20, 33–49. doi: 10.1186/s12967-022-03235-8

Dong, S., Zhu, M., Wang, K., Zhao, X., Hu, L., Jing, W., et al. (2021). Dihydromyricetin improves DSS-induced colitis in mice via modulation of fecal-bacteria-related bile acid metabolism. *Pharmacol. Res.* 171:105767. doi: 10.1016/j.phrs.2021.105767

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi: 10.1126/science.1110591

Fedorak, R. N., Feagan, B. G., Hotte, N., Leddin, D., Dieleman, L. A., Petrunia, D. M., et al. (2015). The probiotic VSL#3 has anti-inflammatory effects and could reduce endoscopic recurrence after surgery for crohn's disease. *Clin. Gastroenterol. Hepatol.* 13, 928–935. doi: 10.1016/j.cgh.2014.10.031

Feng, Z., Peng, S., Wu, Z., Jiao, L., Xu, S., Wu, Y., et al. (2021). Ramulus mori polysaccharide-loaded PLGA nanoparticles and their anti-inflammatory effects in vivo. *Int. J. Biol. Macromol.* 182, 2024–2036. doi: 10.1016/j.ijbiomac.2021.05.200

Franzosa, E. A., Sirota-Madi, A., Avila-Pacheco, J., Fornelos, N., Haiser, H. J., Reinker, S., et al. (2018). Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat. Microbiol.* 4, 293–305. doi: 10.1038/s41564-018-0306-4

Gomez-Nguyen, A., Basson, A. R., Dark-Fleury, L., Hsu, K., Osme, A., Menghini, P., et al. (2021). Para*bacteroides* distasonis induces depressive-like behavior in a mouse model of Crohn's disease. *Brain Behav. Immun.* 98, 245–250. doi: 10.1016/j.bbi.2021.08.218

Gul, L., Modos, D., Fonseca, S., Madgwick, M., Thomas, J. P., Sudhakar, P., et al. (2022). Extracellular vesicles produced by the human commensal gut bacterium *Bacteroides* thetaiotaomicron affect host immune pathways in a cell-type specific manner that are altered in inflammatory bowel disease. *J. Extracell. Vesicles* 11:e12189. doi: 10.1002/jev2.12189

Guo, C., Wang, Y., Zhang, S., Zhang, X., Du, Z., Li, M., et al. (2021). *Crataegus pinnatifida* polysaccharide alleviates colitis via modulation of gut microbiota and SCFAs metabolism. *Int. J. Biol. Macromol.* 181, 357–368. doi: 10.1016/j.ijbiomac.2021.03.137

Han, R., Ma, Y., Xiao, J., You, L., Pedisić, S., and Liao, L. (2021). The possible mechanism of the protective effect of a sulfated polysaccharide from *Gracilaria Lemaneiformis* against colitis induced by dextran sulfate sodium in mice. *Food Chem. Toxicol.* 149:112001. doi: 10.1016/j.fct.2021.112001

Hao, H., Zhang, X., Tong, L., Liu, Q., Liang, X., Bu, Y., et al. (2021). Effect of extracellular vesicles derived from *Lactobacillus plantarum* Q7 on gut microbiota and ulcerative colitis in mice. *Front. Immunol.* 12:777147. doi: 10.3389/fimmu.2021.777147

Hattori, S., Nakamura, M., Yamamura, T., Maeda, K., Sawada, T., Mizutani, Y., et al. (2020). The microbiome can predict mucosal healing in small intestine in patients with Crohn's disease. *J. Gastroenterol.* 55, 1138–1149. doi: 10.1007/s00535-020-01728-1

Hiel, S., Bindels, L. B., Pachikian, B. D., Kalala, G., Broers, V., Zamariola, G., et al. (2019). Effects of a diet based on inulin-rich vegetables on gut health and nutritional behavior in healthy humans. *Am. J. Clin. Nutr.* 109, 1683–1695. doi: 10.1093/ajcn/nqz001

Hu, T., Wang, H., Xiang, C., Mu, J., and Zhao, X. (2020). Preventive effect of *Lactobacillus acidophilus* XY27 on DSS-induced ulcerative colitis in mice. *Drug Design Dev. Ther.* 14, 5645–5657. doi: 10.2147/dddt.S284422

Huang, S., Hu, S., Liu, S., Tang, B., Liu, Y., Tang, L., et al. (2022). Lithium carbonate alleviates colon inflammation through modulating gut microbiota and Treg cells in a GPR43-dependent manner. *Pharmacol. Res.* 175:105992. doi: 10.1016/j.phrs.2021.105992

Jakubczyk, D., Leszczynska, K., and Gorska, S. (2020). The effectiveness of probiotics in the treatment of inflammatory bowel disease (IBD)-a critical review. *Nutrients* 12:1973. doi: 10.3390/nu12071973

Kang, E. A., Choi, H.-I., Hong, S. W., Kang, S., Jegal, H.-Y., Choi, E. W., et al. (2020). Extracellular vesicles derived from kefir grain *Lactobacillus* ameliorate intestinal inflammation via regulation of proinflammatory pathway and tight junction integrity. *Biomedicines* 8, 522–533. doi: 10.3390/biomedicines8110522

Kaparakis-Liaskos, M., and Ferrero, R. L. (2015). Immune modulation by bacterial outer membrane vesicles. *Nat. Rev. Immunol.* 15, 375–387. doi: 10.1038/nri3837

Kim, K.-Y., Son, J. D., Hwang, S.-J., Lee, J. K., Park, J. Y., Park, K. I., et al. (2023). Fermented glutinous rice extract mitigates DSS-induced ulcerative colitis by alleviating intestinal barrier function and improving gut microbiota and inflammation. *Antioxidants* 12, 336–351. doi: 10.3390/antiox12020336

Kudelka, M. R., Stowell, S. R., Cummings, R. D., and Neish, A. S. (2020). Intestinal epithelial glycosylation in homeostasis and gut microbiota interactions in IBD. *Nat. Rev. Gastroenterol. Hepatol.* 17, 597–617. doi: 10.1038/s41575-020-0331-7

Li, G., Lin, J., Zhang, C., Gao, H., Lu, H., Gao, X., et al. (2021). Microbiota metabolite butyrate constrains neutrophil functions and ameliorates mucosal inflammation in inflammatory bowel disease. *Gut Microbes* 13:e1968257. doi: 10.1080/19490976.2021.1968257

Li, H., Shen, L., Lv, T., Wang, R., Zhang, N., Peng, H., et al. (2019). Salidroside attenuates dextran sulfate sodium-induced colitis in mice via SIRT1/FoxOs signaling pathway. *Eur. J. Pharmacol.* 861:172591. doi: 10.1016/j.ejphar.2019.172591

Li, Q., Wu, W., Fang, X., Chen, H., Han, Y., Liu, R., et al. (2022). Structural characterization of a polysaccharide from bamboo (*Phyllostachys edulis*) shoot and its prevention effect on colitis mouse. *Food Chem.* 387:132807. doi: 10.1016/j.foodchem.2022.132807

Li, X., Wu, X., Wang, Q., Xu, W., Zhao, Q., Xu, N., et al. (2022). Sanguinarine ameliorates DSS induced ulcerative colitis by inhibiting NLRP3 inflammasome activation and modulating intestinal microbiota in C57BL/6 mice. *Phytomedicine* 104:154321. doi: 10.1016/j.phymed.2022.154321

Liu, B., Piao, X., Niu, W., Zhang, Q., Ma, C., Wu, T., et al. (2020). Kuijieyuan decoction improved intestinal barrier injury of ulcerative colitis by affecting TLR4-dependent PI3K/AKT/NF-κB oxidative and inflammatory signaling and gut microbiota. *Front. Pharmacol.* 11:1036. doi: 10.3389/fphar.2020.01036

Liu, J. H., Chen, C. Y., Liu, Z. Z., Luo, Z. W., Rao, S. S., Jin, L., et al. (2021). Extracellular vesicles from child gut microbiota enter into bone to preserve bone mass and strength. *Adv. Sci. (Weinh)* 8:2004831. doi: 10.1002/advs.202004831

Liu, L., Liang, L., Yang, C., Zhou, Y., and Chen, Y. (2021). Extracellular vesicles of *Fusobacterium nucleatum* compromise intestinal barrier through targeting RIPK1-mediated cell death pathway. *Gut Microbes* 13:e1902718. doi: 10.1080/19490976.2021.1902718

Ma, L., Lyu, W., Song, Y., Chen, K., Lv, L., Yang, H., et al. (2023). Anti-inflammatory effect of *Clostridium butyricum*-derived extracellular vesicles in ulcerative colitis: impact on host microRNAs expressions and gut microbiome profiles. *Mol. Nutr. Food Res.* 67:e2200884. doi: 10.1002/mnfr.202200884

Ma, L., Shen, Q., Lyu, W., Lv, L., Wang, W., Yu, M., et al. (2022). *Clostridium butyricum* and its derived extracellular vesicles modulate gut homeostasis and ameliorate acute experimental colitis. *Microbiol. Spectr.* 10, 1–21. doi: 10.1128/spectrum.01368-22

Mangalam, A., Shahi, S. K., Luckey, D., Karau, M., Marietta, E., Luo, N., et al. (2017). Human gut-derived commensal bacteria suppress CNS inflammatory and demyelinating disease. *Cell Rep.* 20, 1269–1277. doi: 10.1016/j.celrep.2017.07.031

Moayyedi, P., Surette, M. G., Kim, P. T., Libertucci, J., Wolfe, M., Onischi, C., et al. (2015). Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology* 149, 102–109. doi: 10.1053/j.gastro.2015.04.001

Molodecky, N. A., Soon, I. S., Rabi, D. M., Ghali, W. A., Ferris, M., Chernoff, G., et al. (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 142, 46–54. doi: 10.1053/j.gastro.2011.10.001

Momose, Y., Park, S. H., Miyamoto, Y., and Itoh, K. (2011). Design of species-specific oligonucleotide probes for the detection of *Bacteroides* and Para*bacteroides* by fluorescence in situ hybridization and their application to the analysis of mouse caecal *Bacteroides*-Para*bacteroides* microbiota. *J. Appl. Microbiol.* 111, 176–184. doi: 10.1111/j.1365-2672.2011.05039.x

Nakajima, A., Sasaki, T., Itoh, K., Kitahara, T., Takema, Y., Hiramatsu, K., et al. (2020). A soluble fiber diet increases *Bacteroides fragilis* group abundance and immunoglobulin a production in the gut. *Appl. Environ. Microbiol.* 86:e00405-20. doi: 10.1128/aem.00405-20

Niehus, R., Oliveira, N. M., Li, A., Fletcher, A. G., and Foster, K. R. (2021). The evolution of strategy in bacterial warfare via the regulation of bacteriocins and antibiotics. *ELife* 10:e69756. doi: 10.7554/eLife.69756

Paredes-Sabja, D., Kang, C.-S., Ban, M., Choi, E.-J., Moon, H.-G., Jeon, J.-S., et al. (2013). Extracellular vesicles derived from gut microbiota, especially *Akkermansia muciniphila*, protect the progression of dextran sulfate sodium-induced colitis. *PLoS One* 8:e76520. doi: 10.1371/journal.pone.0076520

Qu, C., Yuan, Z.-W., Yu, X.-T., Huang, Y.-F., Yang, G.-H., Chen, J.-N., et al. (2017). Patchouli alcohol ameliorates dextran sodium sulfate-induced experimental colitis and suppresses tryptophan catabolism. *Pharmacol. Res.* 121, 70–82. doi: 10.1016/j.phrs.2017.04.017

Renson, A., Mullan Harris, K., Dowd, J. B., Gaydosh, L., McQueen, M. B., Krauter, K. S., et al. (2020). Early signs of gut microbiome aging: biomarkers of inflammation, metabolism, and macromolecular damage in young adulthood. *J. Gerontol. A Biol. Sci. Med. Sci.* 75, 1258–1266. doi: 10.1093/gerona/glaa122

Rogler, G., Singh, A., Kavanaugh, A., and Rubin, D. T. (2021). Extraintestinal manifestations of inflammatory bowel disease: current concepts, treatment, and implications for disease management. *Gastroenterology* 161, 1118–1132. doi: 10.1053/j.gastro.2021.07.042

Santana, P. T., Rosas, S. L. B., Ribeiro, B. E., Marinho, Y., and de Souza, H. S. P. (2022). Dysbiosis in inflammatory bowel disease: pathogenic role and potential therapeutic targets. *Int. J. Mol. Sci.* 23, 3464–3489. doi: 10.3390/ijms23073464

Shahi, S. K., Freedman, S. N., Murra, A. C., Zarei, K., Sompallae, R., Gibson-Corley, K. N., et al. (2019). *Prevotella histicola*, a human gut commensal, is as potent as COPAXONE(R) in an animal model of multiple sclerosis. *Front. Immunol.* 10:462. doi: 10.3389/fimmu.2019.00462

Sharma, S., Bhatia, R., Devi, K., Rawat, A., Singh, S., Bhadada, S. K., et al. (2023). A synbiotic combination of *Bifidobacterium longum* Bif10 and *Bifidobacterium breve* Bif11, isomaltooligosaccharides and finger millet arabinoxylan prevents dextran sodium sulphate induced ulcerative colitis in mice. *Int. J. Biol. Macromol.* 231:123326. doi: 10.1016/j.ijbiomac.2023.123326

Sokol, H., Landman, C., Seksik, P., Berard, L., Montil, M., Nion-Larmurier, I., et al. (2020). Fecal microbiota transplantation to maintain remission in Crohn's disease: a pilot randomized controlled study. *Microbiome* 8, 12–26. doi: 10.1186/s40168-020-0792-5

Takaishi, H., Matsuki, T., Nakazawa, A., Takada, T., Kado, S., Asahara, T., et al. (2008). Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. *Int. J. Med. Microbiol.* 298, 463–472. doi: 10.1016/j.ijmm.2007.07.016

Tamaki, H., Shibatoge, M., and Nakase, H. (2015). Efficacy of probiotic treatment with *Bifidobacterium longum* 536 for induction of remission in active ulcerative colitis: a randomized, double-blinded, placebo-controlled multicenter trial. *J. Crohns Colitis* 9, S347–S347. doi: 10.1111/den.12553

Tong, L., Zhang, X., Hao, H., Liu, Q., Zhou, Z., Liang, X., et al. (2021). *Lactobacillus rhamnosus* GG derived extracellular vesicles modulate gut microbiota and attenuate inflammatory in DSS-induced colitis mice. *Nutrients* 13, 3319–3335. doi: 10.3390/nu13103319

Toyofuku, M., Nomura, N., and Eberl, L. (2019). Types and origins of bacterial membrane vesicles. *Nat. Rev. Microbiol.* 17, 13–24. doi: 10.1038/s41579-018-0112-2

Ullman, T. A., and Itzkowitz, S. H. (2011). Intestinal inflammation and cancer. *Gastroenterology* 140, 1807–1816.e1801. doi: 10.1053/j.gastro.2011.01.057

Wang, H., Wang, Q., Yang, C., Guo, M., Cui, X., Jing, Z., et al. (2022). *Bacteroides acidifaciens* in the gut plays a protective role against CD95-mediated liver injury. *Gut Microbes* 14:e2027853. doi: 10.1080/19490976.2022.2027853

Wang, X., and Quinn, P. J. (2010). Lipopolysaccharide: biosynthetic pathway and structure modification. *Prog. Lipid Res.* 49, 97–107. doi: 10.1016/j.plipres.2009.06.002

Wu, H., Chen, Q.-Y., Wang, W.-Z., Chu, S., Liu, X.-X., Liu, Y.-J., et al. (2021). Compound sophorae decoction enhances intestinal barrier function of dextran sodium sulfate induced colitis via regulating notch signaling pathway in mice. *Biomed. Pharmacother.* 133:110937. doi: 10.1016/j.biopha.2020.110937

Wu, J., Wei, Z., Cheng, P., Qian, C., Xu, F., Yang, Y., et al. (2020). Rhein modulates host purine metabolism in intestine through gut microbiota and ameliorates experimental colitis. *Theranostics* 10, 10665–10679. doi: 10.7150/thno.43528

Wu, Y., Ran, L., Yang, Y., Gao, X., Peng, M., Liu, S., et al. (2023). Deferasirox alleviates DSS-induced ulcerative colitis in mice by inhibiting ferroptosis and improving intestinal microbiota. *Life Sci.* 314:121312. doi: 10.1016/j.lfs.2022.121312

Wu, Z., Huang, S., Li, T., Li, N., Han, D., Zhang, B., et al. (2021). Gut microbiota from green tea polyphenol-dosed mice improves intestinal epithelial homeostasis and ameliorates experimental colitis. *Microbiome* 9, 184–206. doi: 10.1186/s40168-021-01115-9

Xiao, H., Wei, C., Liu, H., Li, Z., Zheng, C., and Luo, J. (2023). Lentinan alleviates sciatic nerve injury by promoting autophagy to remove myelin fragments. *Phytother. Res.* 2023, 1–17. doi: 10.1002/ptr.7862

Yan, S., Zhu, Y., Li, L., Qin, S., Yuan, J., Chang, X., et al. (2023). Alginate oligosaccharide ameliorates azithromycin-induced gut microbiota disorder via *Bacteroides acidifaciens*-FAHFAs and *Bacteroides*-TCA cycle axes. *Food Funct.* 14, 427–444. doi: 10.1039/d2fo02812c

Zhang, W., Cheng, C., Han, Q., Chen, Y., Guo, J., Wu, Q., et al. (2019). Flos *Abelmoschus manihot* extract attenuates DSS-induced colitis by regulating gut microbiota and Th17/Treg balance. *Biomed. Pharmacother.* 117:109162. doi: 10.1016/j.biopha.2019.109162

Zhang, Z., Zhang, H., Chen, T., Shi, L., Wang, D., and Tang, D. (2022). Regulatory role of short-chain fatty acids in inflammatory bowel disease. *Cell Commun. Signal.* 20, 64–74. doi: 10.1186/s12964-022-00869-5

Zhao, Y., Luan, H., Jiang, H., Xu, Y., Wu, X., Zhang, Y., et al. (2021). Gegen Qinlian decoction relieved DSS-induced ulcerative colitis in mice by modulating Th17/Treg cell homeostasis via suppressing IL-6/JAK2/STAT3 signaling. *Phytomedicine* 84:153519. doi: 10.1016/j.phymed.2021.153519

Zheng, C., Chen, T., Lu, J., Wei, K., Tian, H., Liu, W., et al. (2021). Adjuvant treatment and molecular mechanism of probiotic compounds in patients with gastric cancer after gastrectomy. *Food Funct.* 12, 6294–6308. doi: 10.1039/d1fo01375k

Zheng, C., Zhong, Y., Zhang, W., Wang, Z., Xiao, H., Zhang, W., et al. (2023). Chlorogenic acid ameliorates post-infectious irritable bowel syndrome by regulating extracellular vesicles of gut microbes. *Adv. Sci.* 10:2302798. doi: 10.1002/advs.202302798

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 08 January 2024
DOI 10.3389/fmicb.2023.1332230

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Muhammad Akbar Shahid,
Bahauddin Zakariya University, Pakistan
Waqas Ahmed,
The University of Tennessee, Knoxville, United States

*CORRESPONDENCE
Hai Xiang
xh@fosu.edu.cn
Hua Li
okhuali@fosu.edu.cn

†These authors have contributed equally to this work

RECEIVED 02 November 2023
ACCEPTED 18 December 2023
PUBLISHED 08 January 2024

CITATION
Song J, Luo C, Liu Z, Liu J, Xie L, Zhang X, Xie Z, Li X, Ma Z, Ding J, Li H and Xiang H (2024) Early fecal microbiota transplantation from high abdominal fat chickens affects recipient cecal microbiome and metabolism.
Front. Microbiol. 14:1332230.
doi: 10.3389/fmicb.2023.1332230

COPYRIGHT
© 2024 Song, Luo, Liu, Liu, Xie, Zhang, Xie, Li, Ma, Ding, Li and Xiang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Early fecal microbiota transplantation from high abdominal fat chickens affects recipient cecal microbiome and metabolism

Jiani Song[1†], Chaowei Luo[1†], Zhijie Liu[1], Jingshou Liu[2], Li Xie[1], Xing Zhang[1], Zhuojun Xie[1], Xiangkun Li[1], Zheng Ma[1], Jinlong Ding[2], Hua Li[1,2]* and Hai Xiang[1]*

[1]Guangdong Provincial Key Laboratory of Animal Molecular Design and Precise Breeding, School of Life Science and Engineering, Foshan University, Foshan, China, [2]Guangdong Tinoo's Foods Group Co., Ltd., Guangdong, China

Abdominal fat deposition (AFD) in chickens is closely related to the gut microecological balance. In this study, the gut microbiota from high-AFD chickens was transplanted into the same strain of 0-day-old chicks via fecal microbiota transplantation (FMT). The FTM from chickens with high AFD had no obvious effects on growth traits, adult body weight, carcass weight, abdominal fat weight, and abdominal fat percentage, but did reduce the coefficient of variation of AFD traits. FMT significantly decreased cecal microbiome richness, changed the microbiota structure, and regulated the biological functions associated with energy metabolism and fat synthesis. Additionally, the cecal metabolite composition and metabolic function of FMT recipient chickens were also significantly altered from those of the controls. Transplantation of high-AFD chicken gut microbiota promoted fatty acid elongation and biosynthesis and reduced the metabolism of vitamins, steroids, and carbohydrates in the cecum. These findings provide insights into the mechanisms by which chicken gut microbiota affect host metabolic profiles and fat deposition.

KEYWORDS

gut microbiota, cecal microbiome, chickens, fecal microbiota transplantation, fatty acids

1 Introduction

With the development of breeding technology and nutrition level improvement, the growth and metabolic capacity of modern broiler chickens have rapidly improved but are accompanied by a significant increase in fat deposition that leads to a large amount of body fat deposition in broilers, especially in high-quality chickens (Abdalla et al., 2018). Studies have found that excessive deposition of abdominal fat not only affects the flavor components (Anthony, 1998; Dransfield and Sosnicki, 1999; Zhao et al., 2023) and reduces the taste of chicken, but also seriously reduces the slaughter rate of the fresh market population, reduces feeding efficiency, and increases environmental pollution through the production of large amounts of animal fat waste (Yan et al., 2015; Jiang et al., 2017). Therefore, exploring the

regulatory mechanisms and factors influencing fat deposition in chickens is a research hotspot in this field.

Fat deposition in chickens is a physiological and biochemical process that is regulated by multiple factors that are affected by the body's fat synthesis, catabolism, and transporter capacity, as well as linked to factors such as genetic regulation, nutritional level, and feeding mode (Chen et al., 2019; Xiang et al., 2021a; Nematbakhsh et al., 2021; Liu et al., 2023). Previous studies have shown that the genomes of gut microorganisms contain functional genes related to fat metabolism that are closely related to the host's glucose metabolism, short-chain fatty acids (SCFAs) metabolism, and amino acid metabolism (Gill et al., 2006; Yan et al., 2018; Liu et al., 2022). The gut microbiome provides the energy needed for animal metabolism and fatty acids produced during lipid metabolism can promote fat deposition in intestinal epithelial cells (de Moreno de LeBlanc et al., 2018). Therefore, chicken fat deposition is closely related to the intestinal microecological balance (Ding et al., 2016). Fecal microbiota transplantation (FMT) has been shown to alter the diversity and species of intestinal flora by reconstructing the microecology of the host's intestinal commensal bacteria, thereby modulating the nutrient absorption and metabolism of the host and altering the phenotypic traits of the host (Bäckhed et al., 2004; Ridaura et al., 2013; Qiao et al., 2014; Fu et al., 2023a). Previous studies have shown that FMT increases the abundance of probiotics such as *Lactobacillus* and the concentration of SCFAs enhances glycolipid metabolism and promotes the growth and development of chickens (Ma et al., 2023). However, there is a lack of studies on the effects of microbiota transplantation on the structure and metabolism of chicken intestinal microbiota and abdominal fat deposition.

To evaluate the effects of early high-fat microbiota transplantation on chicken abdominal fat deposition, intestinal microbiota was isolated from high AFD Qingyuan partridge chickens and transplanted into the same strain of Qingyuan partridge chicks at 0 days of age. The growth and development characteristics and abdominal fat deposition level of the recipient chickens were tracked and compared with those of the control group, and the profiles of 16S rRNA gene and metabolomics were used to evaluate the effects on the intestinal microbiota community and metabolic function.

2 Materials and methods

2.1 Chickens and sample collection

One hundred hens with similar body weights were randomly selected from the same batch of 140-day-old Qingyuan partridge chickens and raised under the same conditions. Traits such as body weight, body size, and abdominal fat weight were measured and the cecal contents were collected. By calculating the abdominal fat rate of each chicken, the cecal contents of the top 10 abdominal fat rate chickens with similar body weights were selected for bacterial suspension preparation as previously described (Yan et al., 2021). Briefly, 2 g of cecal content from each high abdominal fat chicken was mixed in equal amounts in 2 L 0.9% saline. Once the content was fully dissolved, it was incubated on ice for 10 min, and the supernatant was aspirated and filtered through medical sterile gauze and stored in sterilized EP tubes at −20°C.

Two hundred and forty 0-day-old chicks from the same batch of Qingyuan partridge hens were randomly selected and divided into two groups. One group was fed 1 mL of high-fat chicken bacterial suspension every day from the age of 0–9 days (GG), while the other group was fed saline under the same feeding conditions (EE). Both groups were then raised under identical conditions for 140 days.

2.2 Production performance and carcass character measurement

Each individual chicken was weighed weekly from 58 days old. Body slant length, keel length, crown height, shank length, shank circumference, chest width, chest depth, pelvic width, and waist circumference were measured about every 2 weeks for each individual chicken starting at 58 days. At 140 days, the slaughter weight and abdominal fat weight (AFW) were recorded, and abdominal fat percentage (AFP) was calculated.

2.3 Cecal content collection and 16S rRNA gene sequencing

After the chickens were slaughtered at 140 days, the contents of the cecum were immediately frozen in liquid nitrogen and stored at −80°C. Forty GG and EE individuals with similar body weights were selected for further analysis and the DNA of their cecal content was extracted using the QIAamp Fast DNA Stool Mini Kit. PCR amplification was performed using the universal amplification primer 341F/806R for the 16S rRNA gene V3-V4 region, and the PCR products were purified and used to construct high-throughput sequencing libraries using the TruSeq DNA PCR-Free Sample Preparation Kit, following the manufacturer's protocols. Qualified libraries were subjected to high-throughput sequencing using PE250 on an Illumina NovaSeq 6,000 platform.

2.4 16S rRNA gene sequencing data processing

The sequenced data were processed using QIIME2 software (Bolyen et al., 2019). After filtering out the low-quality sequences, all the remaining high-quality reads were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold. The OTU abundance and taxonomic classification from phylum to species were determined for each sample. The α-diversity metrics, including Shannon, Simpson, and Good's Coverage indices, were calculated using QIIME2 software. A principal coordinate analysis (PCoA) plot based on Bray–Curtis dissimilarity was used to measure β-diversity. Differential analyses of taxa were performed using LEfSe analysis (Segata et al., 2011), and LDA scores>2.5 and $p < 0.05$ were selected as thresholds. Pearson's correlation between differential microbes and AFW and AFP were then calculated. Microbial functions were predicted using PICRUSt (Langille et al., 2013), and principal component analysis (PCA) and DunnTest statistical analyses of the differential metabolic pathways ($p < 0.05$) between the groups were performed.

2.5 Metabolite profiling of cecal contents

Metabolite profiling of cecal contents was performed using UHPLC-Q/TOF-MS for 15 individuals with similar body weights from the GG and EE groups. Several quality control (QC) samples were prepared to monitor repeatability. The metabolites were separated using ultra-high performance liquid chromatography (Vanquish UHPLC, Thermo Fisher) and data was acquired using the Q Exactive™ HF platform (Thermo Fisher). Positive (ESI+) and negative (ESI-) modes were used to acquire MS data for alkali and acidic metabolites, respectively. The raw data were converted into the MzXML format using ProteoWizard software (Chambers et al., 2012). Peak identification, filtration, and alignment were performed using the XCMS package in R. A data matrix including mass-to-charge ratio (m/z), retention time, and intensity was constructed to identify the precursor molecules in the ESI+/− modes.

2.6 Differential metabolites identification and functional analysis

Subsequently, multivariate statistical analyses, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA) were conducted using the R package MetaboAnalystR. To construct a sample classifier for each group, support vector machines (SVM) and bagging models were applied to select the characteristic metabolites with the highest contribution to group differences. The prediction effect of the SVM model was represented by a ROC curve, which was depicted using the pROC package v2.0.1, in R. Metabolites with variable importance in projection (VIP) > 2, fold change >2, and *P*-adjust <0.05 were considered differential metabolites. The KEGG database was used to analyze the functional characteristics and classifications of the differential metabolites. The KEGG enrichment analysis was conducted using a t-test, and only pathways with $p < 0.05$ were considered significant.

2.7 Statistical analyses

All values were displayed as the mean ± SD. Significance tests for differences between groups were performed by one-way analysis of variance (ANOVA) for phenotypic data using SPSS 23 software. Significance tests for microbial diversity, differences in flora abundance, and differences in the degree of functional enrichment between groups were performed using R software based on ANOVA and *t*-tests, respectively. The *p*-values were adjusted by the false discovery rate (FDR) using the Benjamini–Hochberg method with the p.adjust function in R. All values with $p < 0.05$ indicated a significant difference, and $p < 0.01$ indicated a highly significant difference.

3 Results

3.1 Growth and abdominal fat deposition in FMT chickens

Considerable coefficients of variation (CV) were observed for both AFW and AFP in the GG and EE groups (Table 1). However, their body weight and carcass weight were similar.

After transplantation of the high-fat chicken cecal bacterial suspension, the GG group consistently had slightly higher body weight than the EE group at 58–140 days (Supplementary Figure S1). No significant differences were observed in the body size traits between the two groups ($p > 0.05$; Supplementary Table S1). At 140 days, body weight, carcass weight, abdominal fat weight, and abdominal fat percentage were slightly higher in the GG group than in the EE group ($p > 0.05$); however, the CV for these traits in the GG group was lower than that in the EE group (Table 1). In particular, the variation in AFW (30.74% vs. 40.62) and AFP (29.23% vs. 38.79%) in the GG group decreased more significantly than that in the EE group (Table 1).

3.2 The effects of FMT on chicken cecal microbial diversity

An average of 104,621.2 high-quality reads per sample was obtained from the GG group, and there were 94,638.6 high-quality reads per sample from the EE group. The rarefaction curve of the observed OTUs for all samples plateaued, with an average Good's coverage index of 0.9997 per sample (0.9991–0.9999), suggesting sufficient sequencing depth. A total of 1844 OTUs were identified for all samples, and similar taxonomic classification proportions were annotated for GG and EE individuals. Alpha diversity, including Shannon, Simpson, and Good's coverage indices, suggested that early high-fat microbiota transplantation had significant negative effects on the chicken gut microbiome abundance (Supplementary Table S2). Beta-diversity analyses including Anosim, Permanova, and Permdisprevealed, also showed that different gut microbial communities in chickens with different AFD levels (Supplementary Table S3). The PCoA using the Bray Curtis test demonstrated a distinct separation between the GG and EE groups (Figure 1A), indicating a different gut microbial composition of early high-fat FMT chickens compared to control individuals.

3.3 The effects of FMT on chicken cecal microbiome taxa

The relative abundance of microbes was compared between GG and EE chickens to evaluate the effects of FMT on the chicken gut microbial composition. Among the top 20 dominant phyla in the GG and EE groups, *Bacteroidetes* and *Firmicutes* were the dominant taxa in both groups (Figure 1B), with a *Firmicutes*/*Bacteroidetes* ratio of 0.75 in the GG group, which was higher than that of 0.69 in the EE group. At the phylum level, the relative abundance of *Actinobacteria* and *Euryarchaeota* was higher in the GG group than in the EE group, whereas *Fusobacteria* and *Tenericutes* were less abundant in the GG group than in the EE group. Consistent with the results of phylum comparison, the dominant genera in both groups were *Bacteroides* and *Unspecified_Bacteroidales*, which were less abundant in the GG group than in the EE group (Figure 1C).

The LEfSe analysis of the taxonomic profiles clearly showed that the phyla *Actinobacteria* and *Euryarchaeota* and 34 genera, including *Methanobrevibacter*, *Desulfovibrio*, *Faecalibacterium*, *Blautia*, *Olsenella*, *Slackia,* and *Akkermansia*, as well as eight species, including *Bacteroides barnesiae*, *Bacteroides coprophilus*, *Subdoligranulum variabile*, *Clostridium celatum,* and *Clostridium*

TABLE 1 The descriptive statistics of growth traits and abdominal fat deposition of the donor and recipient chickens.

Phenotypic traits	GG ($n = 120$)		EE ($n = 120$)	
	Mean ± standard deviation	CV(%)	Mean ± standard deviation	CV(%)
BW (g)	1513.51 ± 144.70	9.56	1494.73 ± 171.94	11.5
CW (g)	1343.05 ± 138.14	10.29	1326.13 ± 160.19	12.08
AFW (g)	56.37 ± 17.33	30.74	52.65 ± 21.39	40.62
AFP (%)	3.72 ± 1.09	29.23	3.50 ± 1.36	38.79

BW, body weight; CW, carcass weight; AFW, abdominal fat weight; AFP, abdominal fat percentage; CV, coefficient of variation.

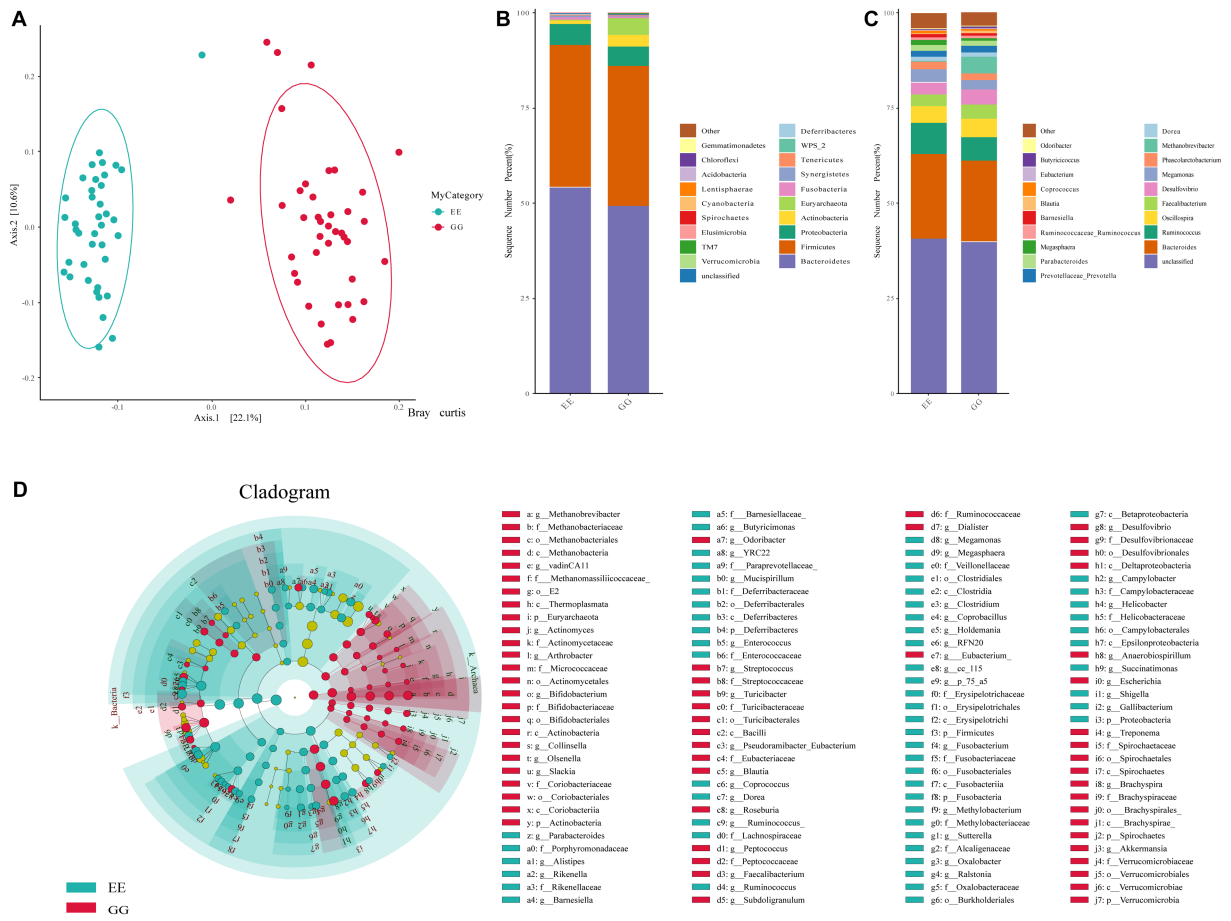


FIGURE 1
Gut microbial diversity and community between GG and EE chickens. **(A)** The PCoA analysis based on Bray Curtis statistic. **(B)** The comparison of the top 20 abundant phyla between GG and EE groups. **(C)** The comparison of the top 20 abundant genera between GG and EE groups. **(D)** Differential gut microorganism identified using LEfSe analysis.

ruminantium were significantly more abundant in the GG group than the EE group (Figure 1D and Supplementary Table S4). Additionally, six phyla (*Bacteroidetes*, *Lentisphaerae*, *Fusobacteria*, *Cyanobacteria*, *Tenericutes,* and *Elusimicrobia*) and 31 genera, including *Ruminococcus*, *Megamonas*, *Parabacteroides*m, *Methylobacteriaceae*, *Succinatimonas*, *Rikenella*, *Fusobacterium*; and 14 species, including *Anaerobiospirillum thomasii*, *Bacteroides plebeius*, *Megamonas hypermegale*, *Helicobacter equorum*, *Barnesiella viscericola,* and *Mucispirillum schaedleri* were significantly more abundant in the EE group than the GG group (Figure 1D and Supplementary Table S4).

Pearson's correlation between the abundance of differential microbiota, AFW, and AFP showed that the abundance of the genus *Parabacteroides* decreased with an increase in AFW ($r = -0.234$, $p < 0.05$) and AFP ($r = -0.231$, $p < 0.05$), whereas the abundance of the genus *Clostridium* increased with AFW ($r = 0.265$, $p < 0.05$) and AFP ($r = 0.263$, $p < 0.05$) increased (Supplementary Figure S2). At the species level, the abundance of *A. thomasii* was negatively related to

chicken AFW ($r = -0.206$, $p < 0.05$) and AFP ($r = -0.242$, $p < 0.05$) (Supplementary Figure S2).

3.4 Function capacities of the differential cecal microbes

The PCA of the KEGG pathways for differential microbiota showed significant differentiation between the GG and EE groups, suggesting that early microbiota transplantation from high-fat chicken donors led to significant changes in the functional capacities of the recipient chicken gut microbiome community (Figure 2A). The differential functional pathways between the GG and EE groups were annotated to the L2 level pathways, and the results showed that the differential functional pathways were mainly related to 10 L2 level pathways of the catalog metabolism, including global and overview maps, amino acid metabolism, chemical structure transformation maps, lipid metabolisms, carbohydrate metabolism, biosynthesis of other secondary metabolites, and so on (Figure 2B and Supplementary Table S5). Specifically, 42 KEGG pathways were differentially expressed ($p < 0.05$) between the GG and EE chickens (Figure 2C), among which 19 pathways were upregulated in the GG group, including the biosynthesis of amino acids, D-glutamine and D-glutamate metabolism, glycerophospholipid metabolism, methane metabolism, metabolic pathways, primary bile acid biosynthesis, urine metabolism, and pyrimidine metabolism. There were 23 metabolic pathways that were down-regulated in the GG group, including alpha-linolenic acid metabolism, linoleic acid metabolism, biosynthesis of terpenoids and steroids, carotenoid biosynthesis, and fatty acid metabolism.

3.5 Early FMT of chicken cecal content alters metabolite composition

The signal drift recorded by the QC samples was stable, and all samples were closely clustered (Supplementary Figure S3), suggesting high repeatability of the experiment. A comparison of the dominant metabolites between the GG and EE groups showed similar metabolite compositions between these two groups. In the positive ion mode, the top 10 metabolites in both groups were oleamide, L-phenylalanine, trans-3-indoleacrylic acid, xanthine, l-esterearic acid, 9-oxo-ODE, 2-oxindole, nicotinic acid, stearamide, and hexadecanamide (Figure 3A). The top 10 metabolites in negative

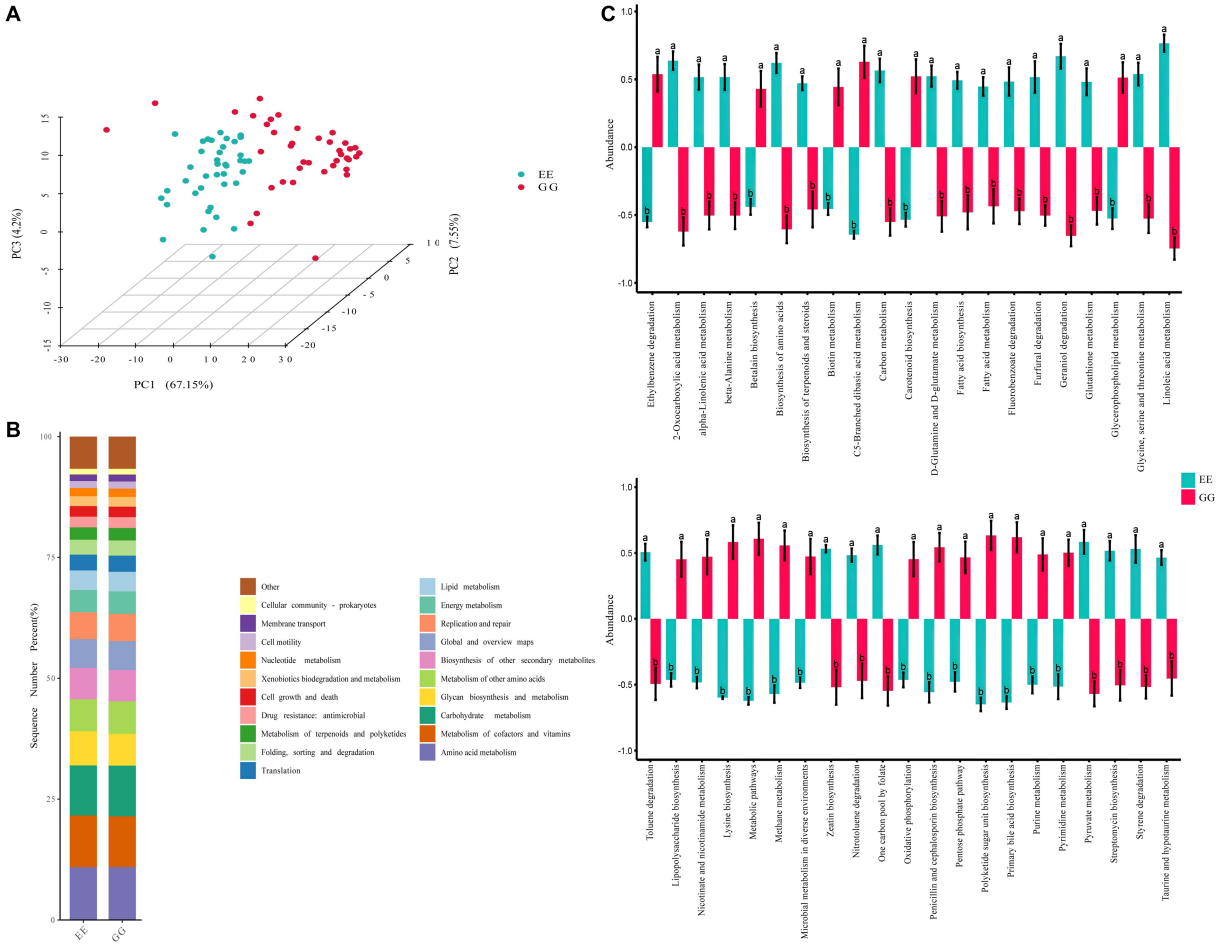


FIGURE 2
Predicted pathways of the differential microbiota in GG and EE chickens. **(A)** Principal component analysis (PCA) based on KEGG pathway enrichment. **(B)** The catalog of differential enriched KEGG pathways. **(C)** Statistically different metabolism pathways between GG and EE groups. Different letters indicates $P < 0.05$.

ion mode were prostaglandin D2, hydrocinnamic acid, 16-hydroxyhexadecanoic acid, tridecylic acid, stearic acid, arachidonic acid, lauric acid ethyl ester, elaidic acid, ethyl myristate, and pentadecanoic acid (Figure 3B). However, the PLS-DA results showed that individuals in the GG and EE groups were distributed in significantly different regions (Figures 3C,D), suggesting that the metabolite compositions of the cecal content of the two groups were significantly different. The clustering heatmap of all metabolites showed that there were a large number of metabolites that differed between the GG and EE groups in both the positive and negative ion modes (Supplementary Figure S4). Furthermore, an SVM classifier was used to evaluate the diagnostic value of the differential metabolites between the GG and EE groups. Among the top 15 characterized metabolites in the positive ion mode, 2-amino-1,3,4-octadecanetriol, +/−56- +/−56-EET ethanolamide, YMK, coniferin, 9-KODE, 1-methylxanthine, *N*-morpholinocarbonyl-4-morpholinosulfonylbenzamide, VLK, and 3-hydroxy-3, 4-bis4-hydroxy- 3-methoxyphenylmethyloxolan-2-one were more abundant in the GG group, whereas shikonin, 3-acetoxyurs-12-en-23-oic acid, nitrosoheptamethyleneimine, (6 └)17(18)-EpETE, and 13,14-dihydro-15-keto prostaglandin A2 were more abundant in the EE group (Supplementary Figure S5). Among the top 15 characterized metabolites in the negative ion mode, cer-NS d181/160, 5-tert-butyl-2-methyl-*N*-5-methyl-3-isoxazolyl-3-furamide, tetradecanedioic acid, N-5-trifluoromethyl-2-pyridinylbenzenesulfonamide, 3-phenoxybenzoic acid, and dopamine HCl were higher in the GG group, while PE130/150, 3-hydroxybutyric acid, lysoPC 204, O1-4-chlorobenzoyl-4-nitrobenzene-1-carbohydroximamide, β-D-glucopyranuronic acid, cyclocytidine, 4-hydroxy-3-methylbenzoic acid, LPA 221, and 2,6-dihydroxybenzoic acid were higher in the EE group (Supplementary Figure S6). Group predictions using the screened characterized metabolites in both modes complied with an area under the receiver operating curve (AUC) of 0.932 (Supplementary Figure S5) and 0.952 (Supplementary Figure S6), further suggesting that the metabolic compositions were different between the GG and EE chickens.

3.6 Differential metabolites and functions affected by early FMT

The OPLS-DA analysis of metabolite expression was conducted to screen for important characterized metabolites, followed by an ANOVA significance test. The results showed that there were 24 significantly different important metabolites in the positive ion mode, including morpholine carbonyl-4-morpholine sulfonyl benzoamide, VLK, GLK, 2-amino-1.3.4-octadecanetriol, N-desmethyl sildenafil were significantly higher in the GG group; while 3-acetoxyurs-12-en-23-oic-acid, 2-{[4-(tert-butyl)benzyl]thio}-4-methylquinoline-3-carbonitrile, metanenhrine L-threonine, 13,14-dihydro-15-keto-prostaglandin A2, shikonin, L-saccharopine, DL-carnitine, deoxyinosine, and homo-gamma-linolenic acid (C20:3) were significantly higher in the EE group (Table 2). Five significant and important metabolites were present in the negative ion mode, including cer-NS (d18:1/16:0) and N-[5-(trifluoromethyl)2- pyridinyl] benzenesulfonamide, which were significantly higher in the GG group than in the EE group, and 3-hydroxybutyric acid, O1-(4-chlorobenzoyl)-4-nitrobenzene-1-carbohydroximami, and cyclocytidine, which were significantly lower in the GG group than in the EE group (Table 2).

Biological function analysis showed that peptide function was higher, while vitamins and cofactors, nucleic acids, steroids, and carbohydrates were lower in the positive ion mode metabolites identified in the GG group (Figure 4A). We also found that lipid function was higher, and steroids, hormones, and transmitters were lower for negative ion mode metabolites in the GG group (Figure 4B). KEGG pathway enrichment of the differential metabolites between the GG and EE groups showed that positive ion mode differential metabolites were enriched in arginine and proline metabolism, histidine metabolism, alanine, aspartate, and glutamate metabolism; thiamine metabolism, nicotinate and nicotinamide metabolism; fatty acid elongation in the mitochondria, fatty acid biosynthesis, fatty acid metabolism, and 23 other differential enrichment pathways (Figure 4C). Nine metabolic pathways were enriched in negative-ion mode differential metabolites, including primary bile acid biosynthesis, taurine and hypotaurine metabolism, terpenoid backbone biosynthesis, galactose metabolism, nicotinate and nicotinamide metabolism, arginine and proline metabolism, and starch and sucrose metabolism (Figure 4D).

4 Discussion

Abdominal fat deposition is important for improving chicken production efficiency and meat flavor. The Qingyuan partridge chicken is a typical local breed of slow and high-quality yellow-feathered broilers that are usually slaughtered and marketed when they reach sexual maturity at 140 days of age. Therefore, excessive abdominal fat deposition is a serious problem in Qingyuan partridge chicken production and was confirmed by the high AFW and AFP of both donor and recipient chickens in this study. Early microbiota transplantation from high-fat chickens did not affect growth traits, body weight, carcass weight, and body size of recipient Qingyuan partridge chickens but did greatly reduce the coefficient of variation of abdominal fat deposition traits, providing evidence that a change in the bacterial colony structure is important in the regulation of the abdominal fat deposition capacity of chickens.

Extensive studies have shown that the abundance and structure of gut microbiota are closely related to abdominal fat deposition in chickens (Ding et al., 2016; Wen et al., 2019). Based on our previous studies that reported the involvement of gut microbiota in the regulation of abdominal fat deposition in Qingyuan partridge chickens (Xiang et al., 2021b), the present study further showed that early microbiota transplantation from high-fat chickens significantly reduced the diversity of the microbiota in recipient chickens, which is consistent with the hypothesis that gut microbial abundance is negatively correlated with the level of abdominal fat deposition in chickens.

Actinobacteria and *Euryarchaeota* were the dominant cecum microbes at the phylum level; their abundance increased in recipient chickens after transplantation, whereas that of *Bacteroidetes*, *Lentisphaerae*, *Fusobacteria*, *Tenericutes*, and *Elusimicrobia* decreased. Previous studies reported that the biological functions of *Actinobacteria* and *Euryarchaeota* promoted fat deposition in chickens (Bäckhed et al., 2004). Torok et al. (2011) found that *Firmicutes*, *Bacteroidetes,* and other dominant taxa were correlated

10.3389/fmicb.2023.1332230

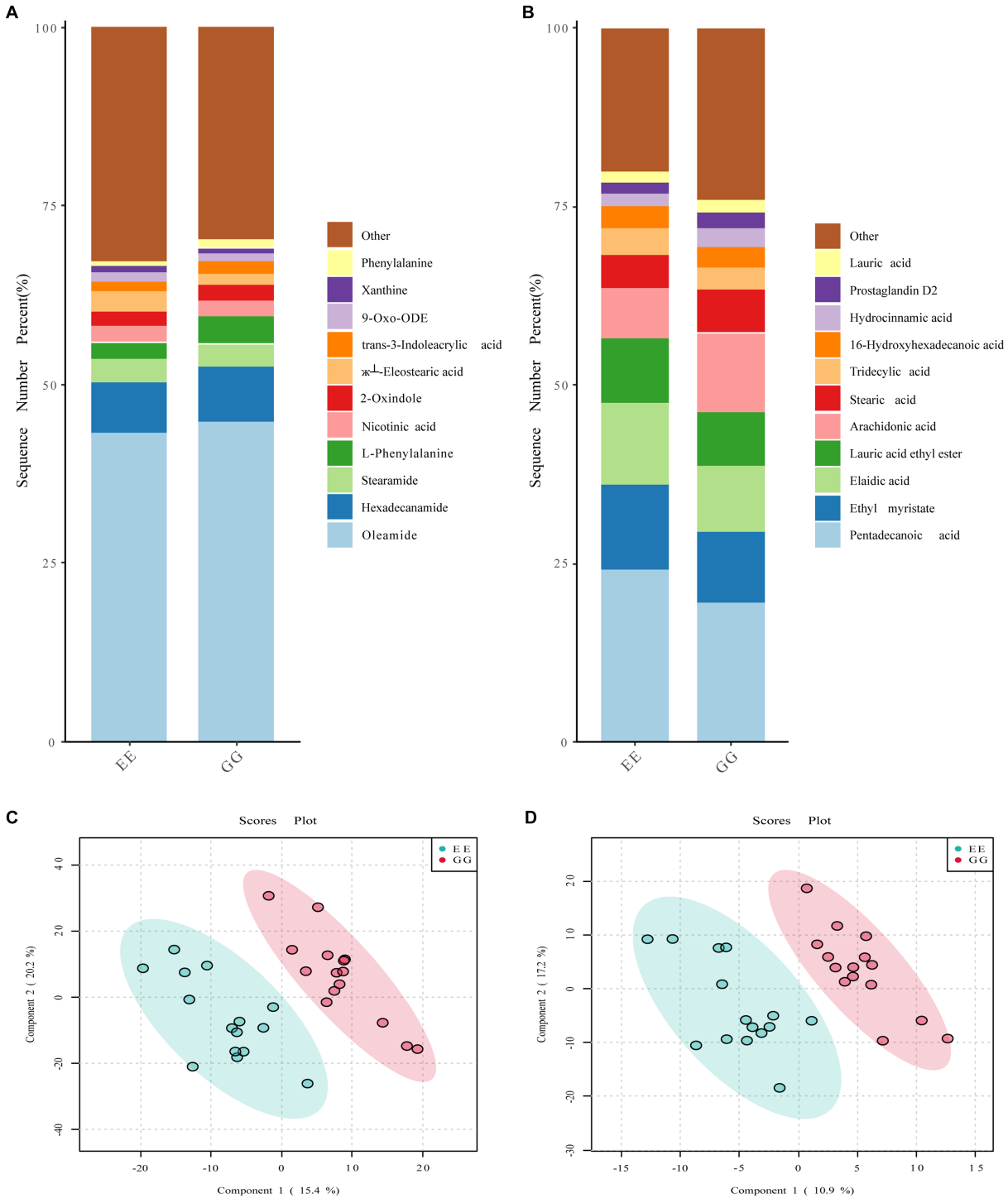


FIGURE 3
Metabolic profiles affected by the early transplantation of the high-fat chicken gut microbiota. The comparison of the top 10 metabolites identified in **(A)** positive ion mode and **(B)** negative ion mode between GG and EE groups. The PLS-DA score plots of **(C)** positive ion mode and **(D)** negative ion mode metabolites.

with broiler production performance, while Ding et al. (2016) reported that the abundance of *Fusobacteria* and their related genera and species in the feces and gut content of high-AFD chickens was lower than that of low-AFD chickens. In the gut biota of both obese and obese mice, the abundance of *Bacteroidetes* was significantly lower and the diversity of the biota was significantly reduced when compared with that of the normal group (Ley et al., 2006). Additionally, the relative abundance of *Firmicutes* and *Proteobacteria* increased and the abundance of *Bacteroidetes* decreased in the intestinal tract of pups after FMT (Tuniyazi et al., 2022).

In the present study, early microbiota transplantation from high-fat chickens increased the abundance of 34 genera in Qingyuan partridge chicken recipients, including *Methanobrevibacter*, *Desulfovibrio*, *Faecalibacterium*, *Blautia*, *Olsenella,* and *Slackia*. A

TABLE 2 Differential metabolites affected by the early transplantation of high-fat chicken gut microbiota.

Ion mode	Name of metabolite	VIP	*P*-value	FDR
Positive ion mode	2-{[4-(tert-butyl)benzyl] thio}-4-methylquinoline-3-carbonitrile	2.8165	0.0000	0.0073
	3-Acetoxyurs-12-en-23-oic acid	2.7475	0.0000	0.0073
	N-(morpholinocarbonyl)-4-(morpholinosulfonyl) benzamide	2.8048	0.0000	0.0077
	13,14-dihydro-15-keto Prostaglandin A2	2.5734	0.0001	0.0148
	L-Threonine	2.5471	0.0001	0.0148
	Metanephrine	2.5795	0.0001	0.0148
	VLK	2.5679	0.0001	0.0148
	GLK	2.5011	0.0001	0.016
	Shikonin	2.5133	0.0001	0.016
	2-Amino-1,3,4-octadecanetriol	2.3726	0.0002	0.0174
	L-Saccharopine	2.4868	0.0002	0.0174
	DL-Carnitine	2.4436	0.0003	0.024
	Deoxyinosine	2.4257	0.0005	0.0367
	Homo-Gamma-Linolenic Acid (C20:3)	2.3478	0.0005	0.0367
	N-Desmethyl sildenafil	2.3057	0.0005	0.0367
	Thiamine monophosphate	2.3413	0.0006	0.0367
	YMK	2.2882	0.0006	0.0367
	L-Dopa	2.2825	0.0007	0.0371
	N-Acetyl-L-glutamic acid	2.2775	0.0006	0.0371
	2-benzylideneindan-1-one	2.2675	0.0008	0.0394
	Dl-3-Hydroxynorvaline	2.2997	0.0008	0.0394
	L-(+)-Citrulline	2.2992	0.0008	0.0394
	SPK	2.2439	0.0008	0.0394
	Melanin	2.2165	0.0011	0.049
Negative ion mode	3-Hydroxybutyric acid	2.9243	0	0.01
	O1-(4-chlorobenzoyl)-4-nitrobenzene-1-carbohydroximamide	2.9571	0	0.01
	Cer-NS(d18:1/16:0)	2.7258	0.0001	0.0179
	N-[5-(trifluoromethyl)-2-pyridinyl] benzenesulfonamide	2.8169	0.0001	0.0179
	Cyclocytidine	2.5086	0.0004	0.0377

positive correlation between *Methanobrevibacter* and body weight in children has been reported (Mbakwa et al., 2015), and we also found that the abundance of both *Methanobrevibacter* and *Mucispirillum schaedleri* were significantly correlated with chicken fat deposition (Wen et al., 2019). A previous study reported that a high-fat diet in normal mice resulted in weight gain, and susceptibility to obesity was closely related to *Desulfovibrio* and other gut microbiota, which may be involved in lipid metabolism disorders (Hong et al., 2021). Oral application of a complex polysaccharide to a high-fat diet for 6 weeks increased the abundance of *Bifidobacteraceae* and significantly decreased *Desulfovibrio* abundance in the guts of mice, suggesting the facilitation of fat deposition in *Desulfovibrio* (Sha et al., 2018). Both *Olsenella* and *Slackia* were more abundant in fat Tiannong partridge chickens than in lean ones, which were suggested to possibly enhance energy capture and positively contribute to fat deposition (Xiang et al., 2021b). In addition, *Clostridium* was positively associated with both somatic adipogenesis and visceral fat deposition in children (Awadel-Kariem et al., 2010), which is consistent with the genus *Clostridium* and its subordinate species, *C. spiroforme* and *C. celatum*, which were enriched with an increase in AFW and AFP, and a significantly higher abundance of *C. celatum* in the GG group after FMT. Moreover, the abundance of the genus *Parabacteroides* decreased as AFW and AFP increased, and then decreased in abundance after FMT, consistent with our previous finding that *Parabacteroides* and its species *Parabacteroides distasonis* were more enriched in lean than fat chickens (Xiang et al., 2021b). Previous studies reported that *P. distasonis* alleviates obesity and metabolic dysfunction by producing succinate and secondary bile acids (Wang et al., 2019).

Our metabolomic analyses revealed that alterations in the gut microbiota regulate fat deposition by affecting host metabolic profiles. Early transplantation of high-fat chicken gut microbiota changed the metabolite concentrations of many metabolites in the cecum content of Qingyuan partridge chickens. Under conditions of oxygen radical catalysis and a series of chemical reactions, arachidonic acid produces substances similar in structure to prostaglandins (Morrow et al., 1990). Saturated fatty acids are the main components of lipids and a variety of saturated fatty acids, such as lauric acid, molluscic acid, and stearic acid, are commonly found in agricultural animal fats. Unsaturated fatty acids are essential fatty acids for the host, and arachidonic acid converted from oleic acid is present mainly in the cell membrane (Xu, 1995). More arachidonic acid was observed in the recipients of high-fat chicken gut microbiota transplantation, which may be due to the fact that arachidonic acid alters the permeability of cell membranes and promotes chicken fat deposition (Zhou et al., 2021). Obese individuals can obtain more energy from food than lean individuals, potentially because of differences in their gut microbiome composition, and obese individuals may further stimulate adipogenesis after energy acquisition (Turnbaugh et al., 2006). These results, combined with our findings, suggest that the compounds produced by the gut microbiota during the regulation of unsaturated fatty acid concentration and amino acid metabolism affect lipid metabolism to increase abdominal fat deposition in chickens. Early FMT from the high-fat chickens also resulted in the differential accumulation of metabolites such as 2-amino-1,3,4-octadecanetriol, which has been shown to stimulate the synthesis of leptin in adipocytes and reduce excess subcutaneous fat when combined with lipolytic mixtures. Shikonin improves the reaction of oxygen free radicals with polyunsaturated fatty acids to form lipid peroxide by promoting neutrophil apoptosis (Zhang et al., 2020), while in 3 T3-L1 adipocytes, shikonin can inhibit the accumulation of triglycerides and the formation and deposition of fats, suggesting that shikonin has an anti-obesity effect (Lee et al., 2010). DL-carnitine is an amino acid-like compound that can promote the conversion of fat into energy in the

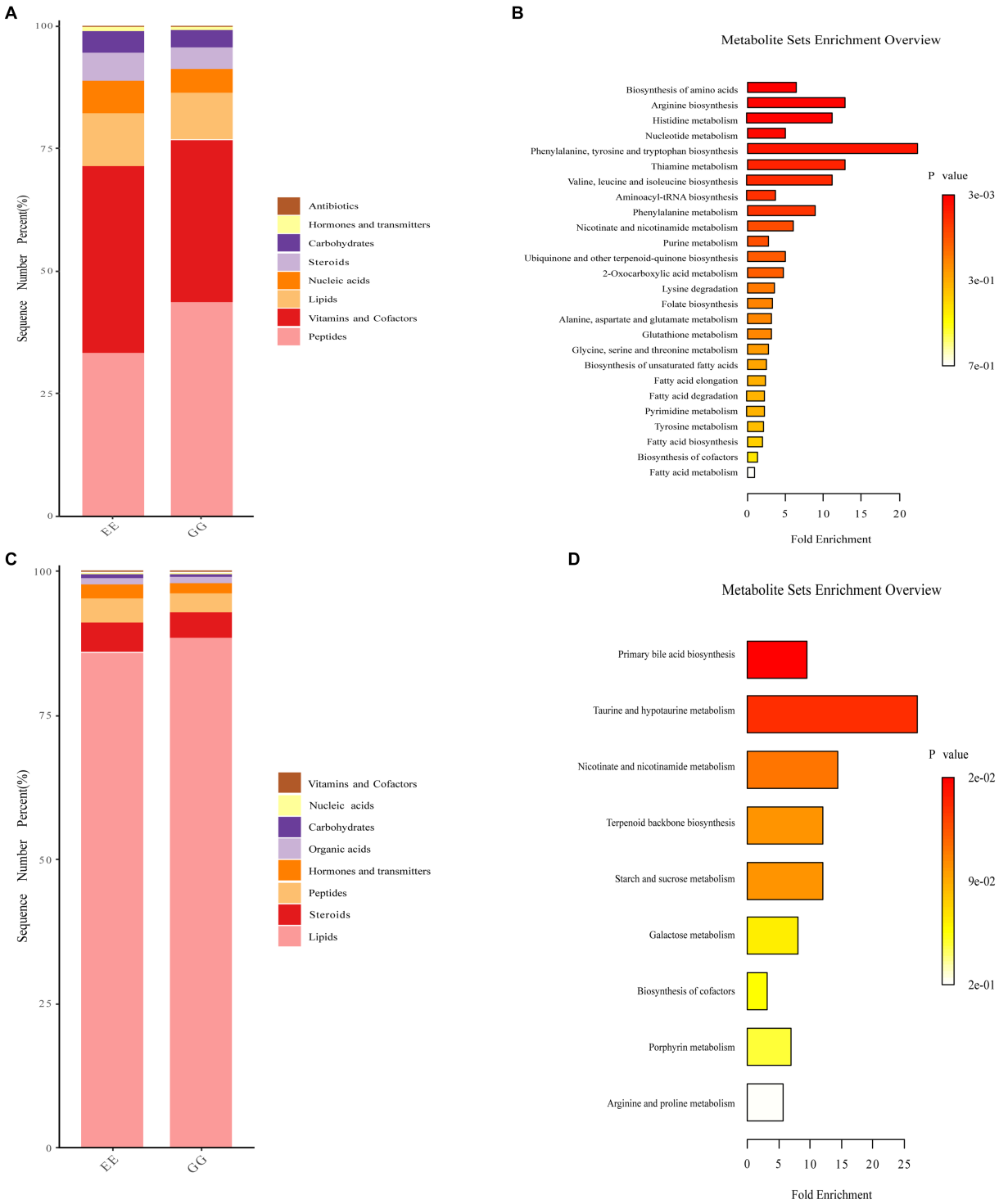


FIGURE 4
Functional annotation of differential metabolites in GG and EE chickens. Accumulation diagram of differential biological roles of metabolites enriched in **(A)** positive ion mode and **(B)** and negative ion mode mass spectrometry analysis. The differential pathways enriched by **(C)** positive ion mode and **(D)** negative ion mode metabolites.

body. It also has various physiological functions, such as oxidizing and decomposing fat, weight loss, and anti-fatigue, and is widely used as a food additive and nutritional supplement for humans and animals (Tao et al., 1981; Visser et al., 1986).

The gut microbiota and its metabolites can influence host gene expression, immune system development, and maintenance of intestinal physiological functions and, thus, participate in host lipid metabolism (Jing et al., 2022; Chen et al., 2023; Feng et al., 2023). Our previous study showed that multiple microbial taxa can regulate lipid biosynthesis and energy metabolism in the Qingyuan partridge chicken by producing secondary metabolites that enhance energy capture, promote fat deposition, or alleviate obesity (Xiang et al., 2021b). In the present study,

the functional pathways of the differential microbial taxa between the GG and EE groups were primarily metabolic pathways. Early transplantation of high-fat gut microbiota upregulated the pathways of amino acid biosynthesis, primary bile acid biosynthesis, methane metabolism, and metabolic pathways, while the pathways of alpha linolenic acid metabolism, linoleic acid metabolism, and biosynthesis of terpenoids and steroids were downregulated. These findings are consistent with the results of the transplantation of fresh bacterial suspensions from adult high-fat poodles into the intestines of puppies (Tuniyazi et al., 2022). Both FMT research on dogs and Qingyuan partridge chickens demonstrated that early transplantation of high-fat gut microbiota enhanced a variety of metabolic processes, such as carbohydrate metabolism, lipid metabolism, and energy metabolism, as well as other metabolic processes of gut microbial metabolites (Cai et al., 2020). The present study also found that transplantation of high-fat gut microbiota had a significant effect on metabolite functions in the cecum of Qingyuan partridge chickens. Early transplantation of high-fat chicken gut microbiota significantly promoted lipid and peptide functions and reduced the biological functions related to vitamins and cofactors, nucleic acids, steroids, carbohydrates, hormones, and transmitters. These were consistent with previous studies that tryptophan a proper adding of tryptophan to the basic diet of broilers and laying hens can effectively affect abdominal fat deposition in chickens (Rosa et al., 2001; Fatufe et al., 2005) and therefore tryptophan is a common additive used in livestock farming to improve intestinal epithelial barrier through its metabolites (Liikonen et al., 2023; Fu et al., 2023b). L-phenylalanine, threonine, and tyrosine are metabolized to phenolic compounds and terpenoids by microbial activities, whereas amino acids such as proline, leucine, and isoleucine can be converted to fatty acids by further biochemical reactions (Nicholson et al., 2012).

5 Conclusion

Early transplantation of high-fat chicken gut microbiota did not significantly alter the growth, development, or carcass traits of recipient chickens, but did reduce the coefficient of variation of chicken abdominal fat deposition traits. Transplantation resulted in a significant decrease in the abundance and changes in the structure of the cecum microbiome and significantly altered the metabolite composition of chicken cecum contents by regulating the biological functions associated with energy metabolism and fat synthesis.

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.cncb.ac.cn/, CRA013128; https://www.cncb.ac.cn/, OMIX005134.

Ethics statement

The animal studies were approved by Laboratory Animal Welfare and Animal Experimental Ethical Inspection board of Foshan University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JS: Writing – original draft, Data curation, Formal analysis, Investigation, Validation, Visualization. CL: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology, Visualization. ZL: Writing – original draft, Data curation, Formal analysis, Investigation. JL: Investigation, Resources, Supervision, Writing – review & editing. LX: Writing – original draft, Data curation, Investigation, Methodology. XZ: Writing – original draft, Formal analysis, Investigation, Visualization. ZX: Writing – original draft, Data curation, Investigation, Visualization. XL: Writing – original draft, Data curation, Investigation. ZM: Data curation, Formal analysis, Methodology, Writing – review & editing. JD: Investigation, Methodology, Resources, Writing – review & editing. HL: Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision. HX: Writing – review & editing, Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was funded by the Major Projects in Agricultural Biology Breeding (2023ZD04064), the Science and Technology Foundation of Qingyuan City (2021SXJM01) and the National Natural Science Foundations of China (32002156 and 32102538). The funding bodies contributed nothing to the study design, data analyses, data interpretation, or manuscript preparation.

Conflict of interest

JL, JD, and HL were employed by the company Guangdong Tinoo's Foods Group 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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1332230/full#supplementary-material

SUPPLEMENTARY FIGURE S1
The accumulating curve of body weight of GG and EE chickens.

SUPPLEMENTARY FIGURE S2
The Pearson's correlation of the abundance of different enriched gut microorganism and the AFD of Qingyuan partridge chickens.

SUPPLEMENTARY FIGURE S3
Quality control of metabolites detection under positive **(A)** and negative **(B)** ion mode.

SUPPLEMENTARY FIGURE S4
The clustering heatmap of positive **(A)** and negative ion mode **(B)** metabolites.

SUPPLEMENTARY FIGURE S5
The SVM classifier of positive ion metabolites.

SUPPLEMENTARY FIGURE S6
The SVM classifier of negative ion metabolites.

References

Abdalla, B. A., Chen, J., Nie, Q., and Zhang, X. (2018). Genomic insights into the multiple factors controlling abdominal fat deposition in a chicken model. *Front. Genet.* 9:262. doi: 10.3389/fgene.2018.00262

Anthony, N. B. (1998). A review of genetic practices in poultry: efforts to improve meat quality. *J. Muscle Foods* 9, 25–33. doi: 10.1111/J.1745-4573.1998.TB00641.X

Awadel-Kariem, F., Patel, P., Kapoor, J., Brazier, J., and Goldstein, E. (2010). First report of *Parabacteroides goldsteinii* bacteraemia in a patient with complicated intra-abdominal infection. *Anaerobe* 16, 223–225. doi: 10.1016/j.anaerobe.2010.01.001

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A. D., et al. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. U. S. A.* 101, 15718–15723. doi: 10.1073/PNAS.0407076101

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Cai, W., Xu, J.-X., Li, G., Liu, T.-H., Guo, X., Wang, H., et al. (2020). Ethanol extract of propolis prevents high-fat diet-induced insulin resistance and obesity in association with modulation of gut microbiota in mice. *Food Res. Int.* 130:108939. doi: 10.1016/j.foodres.2019.108939

Chambers, M., Maclean, B. X., Burke, R., Amodei, D., Ruderman, D. L., Neumann, S., et al. (2012). A cross-platform toolkit for mass spectrometry and proteomics. *Nat. Biotechnol.* 30, 918–920. doi: 10.1038/nbt.2377

Chen, Y., Akhtar, M., Ma, Z., Hu, T., Liu, Q., Pan, H., et al. (2023). Chicken cecal microbiota reduces abdominal fat deposition by regulating fat metabolism. *NPJ Biofilms Microbiomes* 9:28. doi: 10.1038/s41522-023-00390-8

Chen, S., Xiang, H., Zhang, H., Zhu, X., Wang, D., Wang, J., et al. (2019). Rearing system causes changes of behavior, microbiome, and gene expression of chickens. *Poult. Sci.* 98, 3365–3376. doi: 10.3382/ps/pez140

de Moreno de LeBlanc, A., Levit, R., de Giori, G. S., and LeBlanc, J. G. (2018). Vitamin producing lactic acid Bacteria as complementary treatments for intestinal inflammation. *Anti-Inflamm Anti-allergy Agents Medic. Chem.* 17, 50–56. doi: 10.2174/1871523017666180502170659

Ding, J., Zhao, L., Wang, L., Zhao, W., Zhai, Z., Leng, Ł., et al. (2016). Divergent selection-induced obesity alters the composition and functional pathways of chicken gut microbiota. *Genetics Select. Evol.* 48:93. doi: 10.1186/s12711-016-0270-5

Dransfield, E., and Sosnicki, A. A. (1999). Relationship between muscle growth and poultry meat quality. *Poult. Sci.* 78, 743–746. doi: 10.1093/PS/78.5.743

Fatufe, A. A., Hirche, F., and Rodehutscord, M. (2005). Estimates of individual factors of the tryptophan requirement based on protein and tryptophan accretion responses to increasing tryptophan supply in broiler chickens 8–21 days of age. *Arch. Anim. Nutr.* 59, 181–190. doi: 10.1080/17450390500147925

Feng, Y., Zhang, M., Liu, Y., Yang, X., Wei, F., Jin, X., et al. (2023). Quantitative microbiome profiling reveals the developmental trajectory of the chicken gut microbiota and its connection to host metabolism. *iMeta*. 2:e105. doi: 10.1002/imt2.105

Fu, Y., Hu, J., Erasmus, M. A., Zhang, H., Johnson, T. A., and Cheng, H. (2023a). Cecal microbiota transplantation: unique influence of cecal microbiota from divergently selected inbred donor lines on cecal microbial profile, serotonergic activity, and aggressive behavior of recipient chickens. *J. Anim. Sci. Biotechnol.* 14:66. doi: 10.1186/s40104-023-00866-9

Fu, Y., Lyu, J., and Wang, S. (2023b). The role of intestinal microbes on intestinal barrier function and host immunity from a metabolite perspective. *Front. Immunol.* 14:1277102. doi: 10.3389/fimmu.2023.1277102

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., et al. (2006). Metagenomic analysis of the human distal gut microbiome. *Science* 312, 1355–1359. doi: 10.1126/science.1124234

Hong, Y., Sheng, L., Zhong, J., Tao, X., Zhu, W., Ma, J., et al. (2021). *Desulfovibrio vulgaris*, a potent acetic acid-producing bacterium, attenuates nonalcoholic fatty liver disease in mice. *Gut Microbes* 13, 1–20. doi: 10.1080/19490976.2021.1930874

Jiang, M., Fan, W., Xing, S., Wang, J., Li, P., Liu, R. R., et al. (2017). Effects of balanced selection for intramuscular fat and abdominal fat percentage and estimates of genetic parameters. *Poult. Sci.* 96, 282–287. doi: 10.3382/ps/pew334

Jing, Y., Yuan, Y., Monson, M. S., Wang, P., Mu, F., Zhang, Q., et al. (2022). Multi-omics association reveals the effects of intestinal microbiome–host interactions on fat deposition in broilers. *Front. Microbiol.* 12:815538. doi: 10.3389/fmicb.2021.815538

Langille, M. G. I., Zaneveld, J. R., Caporaso, J. G., Mcdonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lee, H., Kang, R., and Yoon, Y. (2010). Shikonin inhibits fat accumulation in 3T3-L1 adipocytes. *Phytother. Res.* 24, 344–351. doi: 10.1002/ptr.2942

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Microbial ecology: human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Liikonen, V., Gómez-Gallego, C., and Kolehmainen, M. (2023). The effects of whole grain cereals on tryptophan metabolism and intestinal barrier function: underlying factors of health impact. *Proc. Nutr. Soc.*, 1–13. doi: 10.1017/s0029665123003671

Liu, X., Wang, C., Wang, Y., Wang, C., Sun, X., Zhu, Y., et al. (2023). Age-associated changes in the growth development of abdominal fat and their correlations with cecal gut microbiota in broiler chickens. *Poult. Sci.* 102:102900. doi: 10.1016/j.psj.2023.102900

Liu, J., Wang, J., Zhou, Y., Han, H., Liu, W., Li, D., et al. (2022). Integrated omics analysis reveals differences in gut microbiota and gut-host metabolite profiles between obese and lean chickens. *Poult. Sci.* 101:102165. doi: 10.1016/j.psj.2022.102165

Ma, Z., Akhtar, M., Pan, H., Liu, Q., Chen, Y., Zhou, X., et al. (2023). Fecal microbiota transplantation improves chicken growth performance by balancing jejunal Th17/Treg cells. *Microbiome* 11:137. doi: 10.1186/s40168-023-01569-z

Mbakwa, C. A., Penders, J., Savelkoul, P. H. M., Thijs, C., Dagnelie, P. C., Mommers, M., et al. (2015). Gut colonization with methanobrevibacter smithii is associated with childhood weight development. *Obesity* 23, 2508–2516. doi: 10.1002/oby.21266

Morrow, J. D., Harris, T. M., and Roberts, L. J. (1990). Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. *Anal. Biochem.* 184, 1–10. doi: 10.1016/0003-2697(90)90002-Q

Nematbakhsh, S., Pei Pei, C., Selamat, J., Nordin, N. F. H., Idris, L. H., and Abdull Razis, A. F. (2021). Molecular regulation of lipogenesis, Adipogenesis and fat deposition in chicken. *Genes* 12:414. doi: 10.3390/genes12030414

Nicholson, J. K., Holmes, E., Kinross, J. M., Burcelin, R., Gibson, G. R., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Qiao, Y., Sun, J., Xia, S., Tang, X., Shi, Y.-H., and Le, G. (2014). Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. *Food Funct.* 5, 1241–1249. doi: 10.1039/c3fo60630a

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341:1241214. doi: 10.1126/science.1241214

Rosa, A. P., Pesti, G. M., Edwards, H. M., and Bakalli, R. (2001). Tryptophan requirements of different broiler genotypes. *Poult. Sci.* 80, 1718–1722. doi: 10.1093/ps/80.12.1718

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

Sha, J., Zhang, J., Sui, B., and Zhang, C. (2018). Effects of aerobic exercise and polysaccharide compounds interventions on gut microbiota of the high fat diet induced obese rats. *Chin. J. Sports Med.* 37, 328–336. doi: 10.16038/j.1000-6710.2018.04.009

Tao, R. C., Peck, G., and Yoshimura, N. N. (1981). Effect of carnitine on liver fat and nitrogen balance in intravenously fed growing rats. *J. Nutr.* 111, 171–177. doi: 10.1093/JN/111.1.171

Torok, V. A., Hughes, R. J., Mikkelsen, L. L., Perez-Maldonado, R., Balding, K., Macalpine, R., et al. (2011). Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Appl. Environ. Microbiol.* 77, 5868–5878. doi: 10.1128/AEM.00165-11

Tuniyazi, M., Hu, X., Fu, Y., and Zhang, N.-S. (2022). Canine fecal microbiota transplantation: current application and possible mechanisms. *Vet. Sci.* 9:396. doi: 10.3390/vetsci9080396

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V. J., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Visser, M., Scholte, H. R., Schutgens, R. B. H., Bolhuis, P. A., Luyt-Houwen, I. E. M., Vaandrager-Verduin, M. H. M., et al. (1986). Riboflavin-responsive lipid-storage myopathy and glutaric aciduria type II of early adult onset. *Neurology* 36, 367–372. doi: 10.1212/WNL.36.3.367

Wang, K., Liao, M., Zhou, N., Bao, L., Ma, K.-Y., Zheng, Z., et al. (2019). *Parabacteroides distasonis* alleviates obesity and metabolic dysfunctions via production of succinate and secondary bile acids. *Cell Rep.* 26, 222–235.e5. doi: 10.1016/j.celrep.2018.12.028

Wen, C., Yan, W., Sun, C., Ji, C.-L., Zhou, Q., Zhang, D., et al. (2019). The gut microbiota is largely independent of host genetics in regulating fat deposition in chickens. *ISME J.* 13, 1422–1436. doi: 10.1038/s41396-019-0367-2

Xiang, H., Chen, S., Zhang, H., Zhu, X., Wang, D., Liu, H., et al. (2021a). Removal of roosters alters the domestic phenotype and microbial and genetic profile of hens. *Sci. China Life Sci.* 64, 1964–1976. doi: 10.1007/s11427-020-1770-1

Xiang, H., Gan, J., Zeng, D., Li, J., Yu, H., Zhao, H., et al. (2021b). Specific microbial taxa and functional capacity contribute to chicken abdominal fat deposition. *Front. Microbiol.* 12:643025. doi: 10.3389/fmicb.2021.643025

Xu, T. (1995). Production of eicosapentaenoic acid and docosahexaenoic acid by biotechnology. *Food Fermentation Industries* 1, 56–65. (in Chinese).

Yan, S., Wei, P., Chen, Q., Chen, X., Wang, S.-C., Li, J.-R., et al. (2018). Functional and structural characterization of a β-glucosidase involved in saponin metabolism from intestinal bacteria. *Biochem. Biophys. Res. Commun.* 496, 1349–1356. doi: 10.1016/j.bbrc.2018.02.018

Yan, C., Xiao, J., Li, Z., Liu, H., Zhao, X., Liu, J., et al. (2021). Exogenous fecal microbial transplantation alters fearfulness, intestinal morphology, and gut microbiota in broilers. *Front. Vet. Sci.* 8:706987. doi: 10.3389/fvets.2021.706987

Yan, H., Zheng, P., Yu, B., Yu, J., Mao, X., He, J., et al. (2015). Postnatal high-fat diet enhances ectopic fat deposition in pigs with intrauterine growth retardation. *Eur. J. Nutr.* 56, 483–490. doi: 10.1007/s00394-015-1093-9

Zhang, Y., Zhang, H., Wang, M., Gao, S., Hong, L., Hou, T., et al. (2020). Shikonin ameliorates lipoteichoic acid-induced acute lung injury via promotion of neutrophil apoptosis. *Mol. Med. Rep.* 23:2. doi: 10.3892/mmr.2020.11772

Zhao, W., Yuan, X.-Y., Xiang, H., Ma, Z., Cui, H., Li, H., et al. (2023). Transcriptome-based analysis of key genes and pathways affecting the linoleic acid content in chickens. *J. Integr. Agric.* 22, 3744–3754. doi: 10.1016/j.jia.2023.02.019

Zhou, Y., Khan, H., Xiao, J., and Cheang, W. S. (2021). Effects of arachidonic acid metabolites on cardiovascular health and disease. *Int. J. Mol. Sci.* 22:12029. doi: 10.3390/ijms222112029

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 20 February 2024
DOI 10.3389/fmicb.2024.1352555

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Sidharth Prasad Mishra,
University of South Florida, United States
Yuyuan Li,
Dalian Medical University, China

*CORRESPONDENCE
Lichun Qian
✉ lcqian@zju.edu.cn

RECEIVED 08 December 2023
ACCEPTED 31 January 2024
PUBLISHED 20 February 2024

CITATION
Ye W, Fan J, Wu W, Chen Z, Huang Q and Qian L (2024) Effects of fecal microbiota transplantation on metabolic health of DBA mice.
Front. Microbiol. 15:1352555.
doi: 10.3389/fmicb.2024.1352555

COPYRIGHT
© 2024 Ye, Fan, Wu, Chen, Huang and Qian. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Effects of fecal microbiota transplantation on metabolic health of DBA mice

Wenxin Ye[1], Jinghui Fan[2], Wenzi Wu[3], Zhuo Chen[3], Qixin Huang[1] and Lichun Qian[1]*

[1]Key Laboratory of Animal Nutrition and Feed Science in East China, Ministry of Agriculture, College of Animal Sciences, Zhejiang University, Hangzhou, China, [2]Hangzhou Academy of Agricultural Sciences, Hangzhou, China, [3]Hainan Institute of Zhejiang University, Sanya, China

Introduction: Numerous studies have demonstrated that C57BL/6 mice exhibit superior growth rates and overall growth performance compared to DBA mice. To investigate whether this discrepancy in growth performance is linked to the composition of gut microorganisms, we conducted fecal microbiome transplantation (FMT) experiments.

Methods: Specifically, we transplanted fecal fluids from adult C57BL/6 mice, high-fat C57BL/6 mice, and Wistar rats into weaned DBA mice (0.2mL/d), and subsequently analyzed their gut contents and gene expression through 16S rRNA sequencing and transcriptome sequencing. During the test period, C57BL/6 mice and Wistar rats were provided with a normal diet, and high-fat C57BL/6 mice were provided with a high-fat diet.

Results: The results of our study revealed that mice receiving FMT from all three donor groups exhibited significantly higher daily weight gain and serum triglyceride (TG) levels compared to mice of CK group. 16S rRNA sequensing unveiled substantial differences in the abundance and function of the gut microbiota between the FMT groups and the CK group. Transcriptome analysis revealed a total of 988 differential genes, consisting of 759 up-regulated genes and 187 down-regulated genes, between the three experimental groups and the CK group. Functional Gene Ontology (GO) annotation suggested that these genes were primarily linked to lipid metabolism, coagulation, and immunity. Pearson correlation analysis was performed on the differential genes and clusters, and it revealed significant correlations, mainly related to processes such as fatty acid metabolism, fat digestion and absorption, and cholesterol metabolism.

Discussion: In summary, FMT from dominant strains improved the growth performance of DBA mice, including body weight gain, institutional growth, and immune performance. This change may be due to the increase of probiotic content in the intestinal tract by FMT and subsequent alteration of intestinal gene expression. However, the effects of cross-species fecal transplantation on the intestinal flora and gene expression of recipient mice were not significant.

KEYWORDS

DBA mice, C57BL/6 mice, fecal microbiota transplantation, transcriptome analysis, 16S RNA sequencing, Wistar rats

1 Introduction

In recent years, there has been a significant focus on understanding the impact of gut microbes on the performance of the host organism. In mammals, the gut microbiota plays a pivotal role in regulating lipid metabolism, primarily through the influence of compounds like short-chain fatty acids, secondary bile acids, trimethylamine, and proinflammatory agents derived from bacteria, such as lipopolysaccharides (Schoeler and Caesar, 2019). This intricate process predominantly centers around the bile acid signal transduction pathway, with a heavy reliance on key receptors like the farnesoid X receptor (FXR), the G-protein-coupled bile acid receptor (Gpbar/TGR5), the short-chain fatty acid signaling pathway, and the functioning of enteroendocrine cells (Yu et al., 2019). FMT is a method of transplanting bacteria from the feces of a pre-selected donor into the intestines of a recipient in order to restore the homeostasis of the recipient's intestinal flora, and thus restore its normal physiological function (Higgins et al., 2005). However, the development of FMT today is not only as a therapeutic method, but also has more applications, such as adjusting the intestinal flora of humans or animals through FMT to improve the growth rate or production performance, or altering the metabolism of obese populations as well as the establishment of disease models, and so on (Elangovan et al., 2022). Remarkably, this method traces its origins back 1,700 years to the fourth century when a Chinese scientist named Ge Hong administered oral suspensions of human feces to treat patients with food poisoning and severe diarrhea (McDonald et al., 2005). The earliest documented case of FMT being employed to treat *C. difficile* infection (CDI) dates back to 1983 (Higgins et al., 2005). FMT has proven therapeutic applications in a range of gastrointestinal disorders, including CDI, chronic fatigue syndrome, Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), and Ulcerative Colitis (UC) (Lewin, 2001; Cammarota et al., 2014; Rossen et al., 2015; Rodrigues et al., 2017). Moreover, research by Lin et al. (2018) has revealed that FMT can enhance metabolic processes in the gut. In the domain of aquaculture production, FMT has also demonstrated potential by improving the intestinal flora of livestock and poultry, consequently impacting their growth performance. A study conducted by Rosa et al. (2021) involved the transplantation of feces from adult dairy cows into newborn calves. The result showed that calves in the FMT group exhibited a gradual increase in body weight from birth to weaning, surpassing those in the CK group. This improvement was attributed to the alteration in the relative abundance of the *Firmicutes* and *Bacteroidetes*, suggesting that the interaction of transplanted intestinal flora played a role in enhancing the growth performance of the recipient calves. Similarly, Wang et al. (2019) conducted research by transplanting feces from healthy adult pigs into weaned piglets, with results that FMT significantly improved the growth performance of the pigs. The intestinal microbial composition and stage characteristics of the recipients did not change significantly, but the abundance of *Streptococcus* spp. and *Clostridium* spp. increased, which was hypothesized to be likely to promote the growth of the animals. The use of donor chickens with higher feed utilization for FMT to establish a more desirable microbiota in the intestinal tract of recipient chickens may serve as a new way to regulate microbial colonization in chickens at early stage (Siegerstetter et al., 2018).

Previous research has demonstrated that C57BL/6 mice tend to exhibit more favorable body weight characteristics when compared to DBA/2 mice (Xi et al., 2011). To investigate whether this variance might be linked to differences in their intestinal microbiota, we embarked on a series of experiments. For the first group (T1 group), we transplanted fecal bacteria from C57BL/6 mice into DBA mice. This was done to assess whether alterations in the intestinal flora could impact body weight in DBA mice. Building upon these findings, in the second group (T2 group), we transplanted fecal bacteria from high-fat diet-fed C57BL/6 mice of the same age into the intestines of DBA mice. Our goal was to explore how the high-fat diet affected the intestinal microbiota of C57BL/6 mice and the subsequent consequences on DBA mice's body weight. Furthermore, to investigate whether factors beyond genetic predisposition contribute to the differential growth rates observed in rats concerning their gut microbiota, we introduced an additional experimental group. In this third group (T3 group), we transplanted fecal bacteria from Wistar rats into the intestines of DBA mice. This was done to assess the potential role of gut microorganisms in the growth disparities between these species. During the test period, donors in the T1 and T3 groups were provided with a normal diet, and donors in the T2 group were provided with a high-fat diet.

2 Materials and methods

All procedures of animal experiments were carried out based on protocols approved by the Animal Care Advisory Committee of Zhejiang University, Hangzhou, China (No. ZJU20220438).

2.1 Animals

Use 10 SPF male adult C57BL/6 mice at 6 weeks old, 10 SPF male adult high-fat diet-fed C57BL/6 mice at 6 weeks old, and 6 SPF male adult Wistar rats at 10 weeks old (Hans Biotechnology Co., Ltd., Hangzhou, China) as donors, male DBA mice (bred in SPF-grade conditions at Hans Biotechnology Co., Ltd., Hangzhou, China) at 7 days old as accepters. All DBA mice were housed with their mothers before weaning, and after weaning, they were housed in separate cages, and after 1 week of acclimatization feeding, mice of similar body weight were selected for the experiment. All animals were housed under controlled conditions, including a constant temperature range of 21–24°C, humidity maintained at 50–60%, and exposure to natural lighting. Water was provided *ad libitum* to the animals throughout the study. Donors in the T1 and T3 groups were provided with a normal diet, and donors in the T2 group were provided with a high-fat diet.

All experimental procedures were conducted in strict adherence to the guidelines for animal care and use. The research protocols were approved by the Committee for Animal Research at Zhejiang University, Hangzhou, China (approval number ZJU20220438).

2.2 Experimental protocol

After a one-week acclimation period, the DBA mice were randomly divided into four groups, each consisting of 12 mice: Control group (CK); C57BL/6 mice fecal microbiota transplantation (FMT) group (T1); High-fat C57BL/6 FMT group (T2); Wistar rats FMT group (T3). Fresh fecal samples were collected daily from

C57BL/6 mice, high-fat C57BL/6 mice, and Wistar rats to serve as donors. The collected fecal material was placed in sterile tubes and homogenized using sterile normal saline (Sinopharm Chemical Reagent Co. Ltd., China). This mixture was then subjected to centrifugation at 6,000 × g for 10 min, followed by filtration through a 70-μm filter (Nest, China). The resultant material was quantified to achieve an optical density (OD) at 600 nm of 1.0, corresponding to an estimated bacterial concentration of 10^9 colony-forming units (CFU) per milliliter. Subsequently, the fecal bacterial slurry was diluted to a final concentration of 5.0×10^9 CFU/mL using a 30% sterile medical glycerin phosphate-buffered saline (PBS) (Sinopharm Chemical Reagent Co. Ltd., China). Any remaining fecal fluid was stored at −80°C for future use. The mice in the CK group were administered sterile normal saline via gavage once a day for a duration of 28 days using disposable sterile syringes (1 mL, DKBT, China) with sterile gavage needles (45 mm, Shenzhen Longreen Lighting Co., Ltd., China) (Zhang et al., 2020; Elangovan et al., 2022). In contrast, the mice in the T1 group received fecal fluid from normal C57BL/6 mice, the T2 group received fecal fluid from high-fat C57BL/6 mice, and the T3 group received fecal fluid from Wistar rats through gavage over the same 28-day period.

2.3 Sample collection

At the end of the experiment, the finishing mice were fasted for 24 h (6-h daytime and 16-h overnight) (Zhang et al., 2016) and at which time, blood samples were collected at baseline into a vacuum tube. Mice were anesthetized by intraperitoneal injection of 80 mg/kg sodium pentobarbital and were euthanized by intraperitoneal injection of 200 mg/kg sodium pentobarbital under deep anesthesia. The colon and liver tissues were collected immediately from all mice. After euthanasia of the mice, the same intestinal segments from the posterior half of the colon were taken and their contents squeezed into cryopreservation tubes, which were immediately stored at −80°C for subsequent sequencing. A part of the liver and colon was fixed using 4% paraformaldehyde solution, the rest of liver and colon tissue was saved at −80°C.

2.4 Growth performance indicators

The mice and food intakes were meticulously weighed at the end of each week, specifically on the 1st, 2nd, 3rd, 4th, and 5th weeks. This data was then used to calculate both the daily weight gain and the daily feed intake of the mice.

2.5 Serum biochemical indicators

The level of serum biochemical indicators were measured by Albumin (ALB) assay kit, Total Cholesterol (TC) assay kit, Triglyceride (TG) assay kit and Superoxide Dismutase (SOD) assay kit (Nanjing Jiancheng Institute of Bioengineering, China), as well as the inflammatory markers of the mice including Immunoglobulin A (IgA), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-22 (IL-22), Tumor Necrosis Factor-α (TNF-α), Chemokine (C-X-C motif) ligand 1 (CXCL1) and Chemokine (C-X-C motif) ligand 2 (CXCL2) (Jiangsu Meimian Industrial Co., Ltd., China) were measured with assay kits according to the manufacturer's instructions with UV–VIS Spectrophotometer (UV1100, MAPADA, Shanghai, China) following the manufacturer's instructions.

2.6 Microbial 16S rRNA gene sequencing library construction and sequencing

2.6.1 DNA extraction and PCR amplification

Total microbial genomic DNA was extracted from colon contents samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States) following the manufacturer's instructions. The quality and concentration of DNA was determined by 1.0% agarose gel electrophoresis and a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Inc., United States) and stored at −80°C before further use. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using primer pairs 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) on an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, United States) (Liu et al., 2016). The PCR reaction mixture contained 4 μL 5 × Fast Pfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL each primer (5 μM), 0.4 μL Fast Pfu polymerase, 10 ng template DNA and ddH2O to a final volume of 20 μL. PCR amplification cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and a single extension at 72°C for 10 min, terminating at 4°C. All samples were amplified in triplicate. The PCR product was extracted from a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and quantified using the Quantus™ Fluorometer (Promega, United States).

2.6.2 Illumina MiSeq sequencing

The main stages of library construction using the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, United States), including linkage of junctions, removal of junction self-associated fragments, PCR amplification for library template enrichment, and PCR product recovery. The sequencing process was performed using Illumina's Miseq PE300 (Illumina, United States), which involves complementing one end of the DNA fragment with the junction bases embedded in the chip to form a fixed bridge structure, followed by PCR amplification to produce DNA clusters, which are subsequently linearized into single strands. Sequence information of the template DNA fragments is obtained by scanning the surface of the reaction plate with a laser, synthesizing only one base per round, and by chemical cleavage and counting fluorescent signals (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China[1]).

2.6.3 Amplicon sequence processing and analysis

Following the demultiplexing process, the sequences produced underwent quality filtration with fastp (version 0.19.6) (Chen et al., 2018) and were merged with FLASH (version 1.2.11) (Magoč and

1 https://cloud.majorbio.com

Salzberg, 2011). Next, using DADA2 (Callahan et al., 2016) plugin within the Qiime2 (Bolyen et al., 2019) (version 2020.2) pipeline with recommended parameters, the high-quality sequences were de-noised. This process obtained single nucleotide resolution based on error profiles within samples, resulting in the creation of amplicon sequence variants (ASVs) using the DADA2 denoised sequences. Taxonomic assignments of ASVs were made using the Naive Bayes (or Vsearch or Blast) consensus taxonomy classifier implemented in Qiime2 along with the SILVA 16S rRNA database (v138).

2.7 Transcriptomics

Colon RNA samples from 20 different male mice were used for mRNA sequencing.

2.7.1 Extraction of total RNA

Total RNA was extracted from tissue samples, and the concentration and purity of the extracted RNA were examined using Nanodrop2000, RNA integrity was detected by agarose gel electrophoresis, and RNA integrity number (RIN) value was determined by Agilent2100. Total RNA amount ≥1ug, concentration ≥35 ng/μL, OD260/280 ≥ 1.8, OD260/230 ≥ 1.0 were required for single library construction.

2.7.2 Oligo dT mRNA enrichment

The 3′ end of eukaryotic mRNA has the structure of ployA tail, and the magnetic beads with Oligo (dT) are utilized to perform A-T base pairing with ployA. Using magnetic beads with Oligo (dT) to perform A-T base pairing with ployA, mRNA can be isolated from total RNA and used to analyze transcriptome information.

2.7.3 Fragmented mRNAs

The Illumina platform sequences short fragments, and the mRNAs obtained from enrichment are complete RNA sequences, averaging several kb in length, and therefore need to be randomly interrupted. By adding fragmentation buffer, mRNA can be randomly fragmented, and small fragments of about 300 bp can be separated by magnetic bead screening.

2.7.4 Reverse cDNA synthesis

Under the action of reverse transcriptase, six-base random hexamers are added, and mRNA is used as a template for reverse transcription. One-stranded cDNA is synthesized by adding six-base random hexamers under the action of reverse transcriptase, using mRNA as a template for reverse transcription, followed by two-stranded synthesis to form a stable double-stranded structure.

2.7.5 Linkage adaptor

The double-stranded cDNA structure has sticky ends, which are made flat by adding End Repair Mix, followed by the addition of an "A" base at the 3′ end. Then an "A" base was added to the 3′ end to connect the Y-shaped connector.

Finally, we performed up-sequencing on the illumination platform (Majorbiobio, Shanghai, China) (Schoeler and Caesar, 2019). Library enrichment, PCR amplification of 15 cycles; (Yu et al., 2019) 2% agarose gel recovery of the target bands; (Elangovan et al., 2022) TBS380 (Picogreen) quantification, mixing the data proportionally on the machine; (McDonald et al., 2005) Bridge PCR amplification on cBot to generate clusters; (Higgins et al., 2005) Sequencing on Illumina platform (PE library, read length 2 × 150 bp) (MiSeq Reagent Kit v3, Illumina, United States).

2.8 Statistical analysis

All data except for the 16S RNA results were analyzed by one-way analysis of variance (ANOVA) using SPSS statistical software (Ver. 20.0 for windows, SPSS, Inc., Chicago, IL, United States). Differences among treatments were examined using the Tukey–Kramer's multiple range tests, which were considered significant when the *p*-value was less than 0.05. Results are presented as means alongside their pooled standard errors of means (SEM).

For 16S RNA analysis, based on the ASVs information, α diversity indices (including the Sobs index, Chao richness estimator, Shannon diversity index, and Simpson index) between the mice of three groups were calculated to evaluate microbial species richness and evenness (Schloss et al., 2009). The similarity among the microbial communities in different samples was determined by principal coordinate analysis (PCoA) based on Bray–curtis dissimilarity using Vegan v2.5-3 package. Use Wilcox rank-sum test analysis to analyze which bacteria differ in the intestinal flora of different groups of mice at the phylum level and genus level, respectively. Using the two-sample *t*-test, we analyzed the community abundance data and rigorously determined the significance of differences in species abundance between the two groups of sampled microbial communities. The hypothesis of species between the groups was tested using R-3.3.1 (stat) software and enabled identification of the species that exhibited significant differences between the groups (Mojorbio, Shanghai, China). The raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1050037).

3 Results

3.1 Impact of FMT on the growth performance of DBA mice

Initial body weights of DBA mice were measured at 4 weeks of age, following 28 days of FMT gavage, the final body weight (FBW) and average daily gain (ADG) of mice that received FMT from C57BL/6 mice, high-fat C57BL/6 mice, and Wistar rats exhibited substantial increased compared to the CK group, and the overall growth curve was better than that of the mice in CK group (Figure 1). These results suggested that FMT indeed enhances the growth performance of DBA mice. Moreover, it is worth noting that the growth performance improvement observed in DBA mice that received FMT from high-fat C57BL/6 mice was significantly greater than that in DBA mice receiving FMT from normal C57BL/6 mice and Wistar rats. This observation implied that the high-fat model also induces notable alterations in the intestinal flora of mice.

3.2 Impact of FMT on immune function in DBA mice

In order to evaluate the effect of FMT on the immune function of DBA mice, we performed a series of serum ELISA assays, including

CXCL1, CXCL2, TNF-α, IL-1β, IL-6, IL-22, IL-10, and IgA (Supplementary Table S2). The results demonstrated a significant reduction in the levels of CXCL2 and IL-22 in the T1 group compared to the CK group, a significant decrease in TNF-α levels in the T2 group compared to the CK group, a significant decrease in the levels of IL-22 in the T3 group compared to the CK group, and a significant increase in IgA levels (Figure 2).

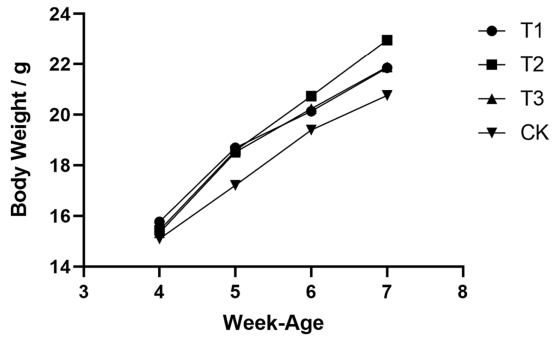


FIGURE 1
Comparison of growth curves of four groups of mice. CK: DBA mice accepting saline gavage; T1: DBA mice receiving FMT from C57BL/6 mice; T2: DBA mice receiving FMT from high-fat C57BL/6 mice; T3: DBA mice receiving FMT from Wistar rats.

3.3 Impact of FMT on lipid profiles, liver function, and antioxidant capacity in DBA mice

To investigate the effects of fecal microbiota transplantation on lipids, liver function and antioxidant capacity in DBA mice, serum TG, TC, TP, ALB, and SOD were measured in four groups of mice (Supplementary Table S3). Notably, serum triglyceride (TG) levels exhibited a significant increase in both the T1 and T2 groups compared to the CK group (Figure 3).

3.4 Microbial 16S rRNA gene sequencing library construction and sequencing

Information on the richness, diversity and coverage of species in the community was obtained through α diversity index analysis, and the test of difference between groups was applied to detect whether the values of A diversity index were significantly different between every two groups and above. The results showed a significant increase in α-diversity indices, including ace, chao and sobs indices of the gut microbiota of T1 group than T3 and CK group (Figures 4A–C), which suggested that FMT from normal C57BL/6 mice increased bacterial richness of DBA mice (as determined by rising ace, chao and sobs indices). Then, we analyzed the β-diversity with the Bray-Curtis

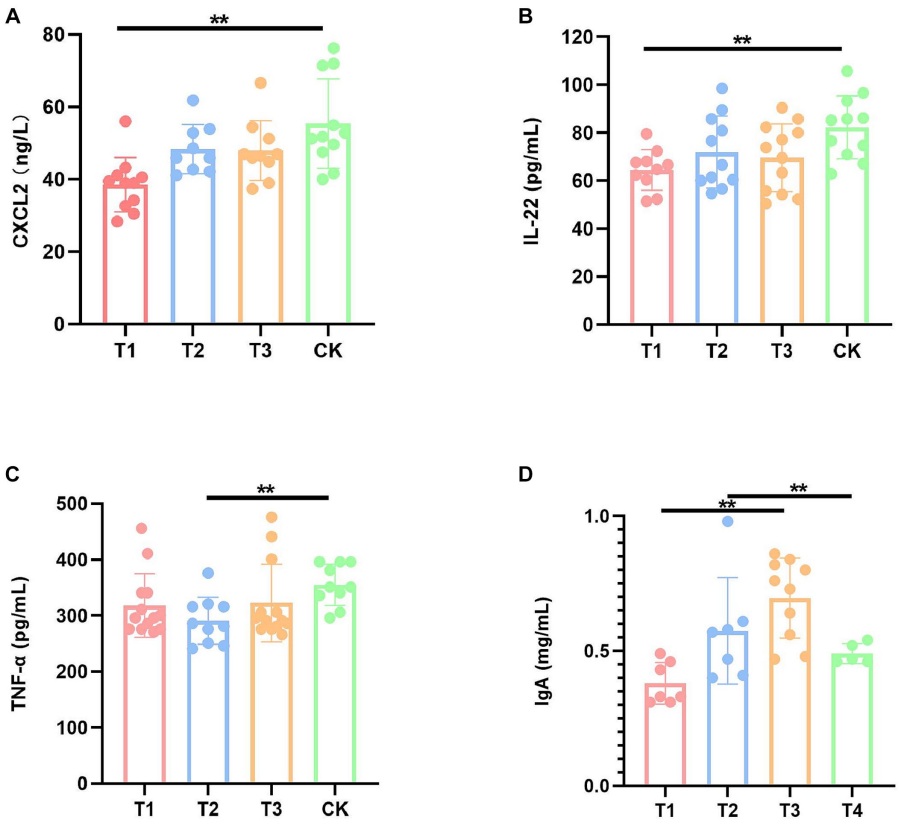


FIGURE 2
Immunization factors with significant differences of mice in four groups. **(A)** Serum CXCL2 levels. **(B)** Serum IL-22 levels. **(C)** Serum TNF-α levels. **(D)** Serum IgA level. CK: DBA mice accepting saline gavage; T1: DBA mice receiving FMT from C57BL/6 mice; T2: DBA mice receiving FMT from high-fat C57BL/6 mice; T3: DBA mice receiving FMT from Wistar rats. ***P*<0.01.

10.3389/fmicb.2024.1352555

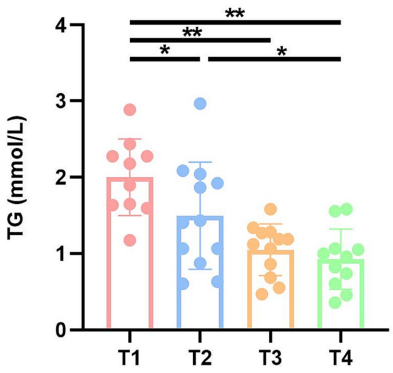


FIGURE 3
TG levels of mice in four groups. CK: DBA mice accepting saline gavage; T1: DBA mice receiving FMT from C57BL/6 mice; T2: DBA mice receiving FMT from high-fat C57BL/6 mice; T3: DBA mice receiving FMT from Wistar rats. **P<0.05, **P<0.01.

principal coordinate analysis (PCoA) on ASV level, to identify potential principal components that influence differences in the composition of the sample communities through dimensionality reduction (Figure 4D). PCoA exposed significant difference in gut microbiota between T1 group and CK group (R = 0.622, p = 0.003). Also, T2 group and CK group showed significant difference in gut microbiota (R = 0.744, p = 0.004), same was between T3 group and CK group (R = 0.507, p = 0.004). These results indicated that FMT modulates gut microbiota composition of DBA mice.

In order to identify high-dimensional biomarkers and reveal genomic features, we performed Linear discriminant analysis Effect Size (LFfSe) analysis of the flora in the mouse gut from the phylum level to the genus level. Figures 4E–F shows the results for all LDA values greater than 2. After FMT, the four groups of mice with important roles in the gut of microbial taxa differed significantly. Compared with the control group, mice in group T1 were significantly enriched in RF39 at the Order level (LDA = 3.014, p = 0.022), at the Family level Ruminococcaceae (LDA = 3.375, p = 0.047), Rikenellaceae (LDA = 3.980, p = 0.21) and norank_o_RF39 (LDA = 3.014, p = 0.022) were significantly enriched, at the Genus level *unclassified_f_Ruminococcaceae* (LDA = 2.943, p = 0.011), *Negativibacillus* (LDA = 2.865, p = 0.020), Blautia (LDA = 2.981, p = 0.036), *Alistipes* (LDA = 3.817, p = 0.047), *g_norank_f_norank_o_RF39* (LDA = 3.025, p = 0.022) and *g_Rikenellaceae_RC9_gut_group* (LDA = 3.345, p = 0.024) were significantly enriched. T2 group only enriched at Genus level, which were *Roseburia* (LDA = 3.364, p = 0.009), *norank_f_Ruminococcaceae* (LDA = 3.094, p = 0.026), *Eubacterium_xylanophilum_group* (LDA = 2.950, p = 0.017), *Eubacterium_oxidoreducens_group* (LDA = 3.045, p = 0.027), *NK4A214_group* (LDA = 2.936, p = 0.049) and *Anaerotruncus* (LDA = 2.942, p = 0.033). T3 group only enriched in Genus *A2* (LDA = 2.871, p = 0.044).

Thereafter, we assessed the relative abundance of bacteria on phylum and genus levels. Firmicutes and Bacteroidota were the most abundant, accounting for over 80% of all microorganisms in all groups at Phylum level (Figure 4G). At genus level, Lactobacillus and Bacillus were the most abundant, accounting for over 50% of all microorganisms in T1, T2, and CK groups, meanwhile Lactobacillus and Staphylococcus were the most abundant accounting for over 50% of all microorganisms in T3 group (Figure 4H).

In order to assess the level of significance of the differences in species abundance and to obtain information on the species that were significantly different between the two groups, we utilized the between-groups test of variance methodology, applying rigorous statistical methods based on the community abundance data obtained to test hypotheses about the species between the two groups of sampled microbial communities. The results showed that only T2 group and CK group showed significant difference at Phylum level, which was a significant rise in Verrucomicrobiota (p = 0.045, Figure 4I). At genus level, the abundance values of *Alistipes*, *Rikenellaceae_RC9_gut_group*, *unclassified_f_Ruminococcaceae*, *Roseburia*, and *Family_XIII_AD3011_group* in group T1 were all significantly increased relative to CK group (Figure 4J). The abundance of *Bacillus*, *unclassified_f_Lachnospiraceae*, *Lachnospiraceae_NK4A136_group*, *Rikenellaceae_RC9_gut_group*, *Roseburia*, *norank_f_Ruminococcaceae*, *Eubacterium_xylanophilum_group*, *Anaerotruncus*, *unclassified_f_Ruminococcaceae*, *norank_f_Erysipelotrichaceae*, *norank_f_Peptococcaceae*, *Negativibacillus* and *Akkermansia* of T2 group all showed a significant increase in abundance values relative to the CK group, while *Aerococcus* and *Staphylococcus* showed a decrease in abundance values relative to the CK group (Figure 4K). As is shown in Figure 4L, T3 group significantly decreased the abundance of *norank_f_norank_o_RF39* and increased the abundance of *Christensenellaceae_R-7_group*. Thus, FMT altered the gut flora composition of DBA mice.

3.5 Transcriptome construction and sequencing

Since this experiment involved FMT across strains and breeds, in order to investigate whether changes in intestinal flora would alter gene expression in the gut, we took colon tissues from four groups of mice for transcriptome sequencing. By quantitatively analyzing the genes sequenced in the four groups of mice and obtaining their reads, the samples were analyzed for differential expression of genes between groups, identifying differentially expressed genes between groups, and then studying the functions of the differential genes (Figures 5A,B). Mice in group T1 had a total of 356 differential genes compared to CK group, with 216 up-regulated and 140 down-regulated genes. Mice in group T2 had a total of 222 differential genes compared to CK group, with 176 up-regulated and 46 down-regulated genes. Mice in group T3 had a total of 416 differential genes compared to CK group, with 367 up-regulated and 43 down-regulated genes.

Based on the information about the expression number of genes in different samples, the distance between genes and samples is calculated, and then an iterative method was used to classify the genes and samples, and the differentially expressed genes between each experimental group and the CK group were plotted as a heatmaps (Figures 5C–E). Acceptance of FMT from normal C57BL/6 mice up-regulated cholesterol metabolism related genes (Apoh, Apob, Apoc3, Apoa1, Apoa2), PPAR signaling pathway related genes (Apoa2, Apoa5, Apoc3, Apoa1, Fabp1) and complement and coagulation cascades related genes (Fga, C8b, Fgb, Fgg, C8a, Plg) in DBA mice. Acceptance of FMT from high-fat C57BL/6 mice similarly upregulated genes that increase the cancer related genes (Mycn, C4bp) (Figure 5D). The Pla2g3b gene was down-regulated by fecal microbiota transplants from Wistar rats (Figure 5E).

10.3389/fmicb.2024.1352555

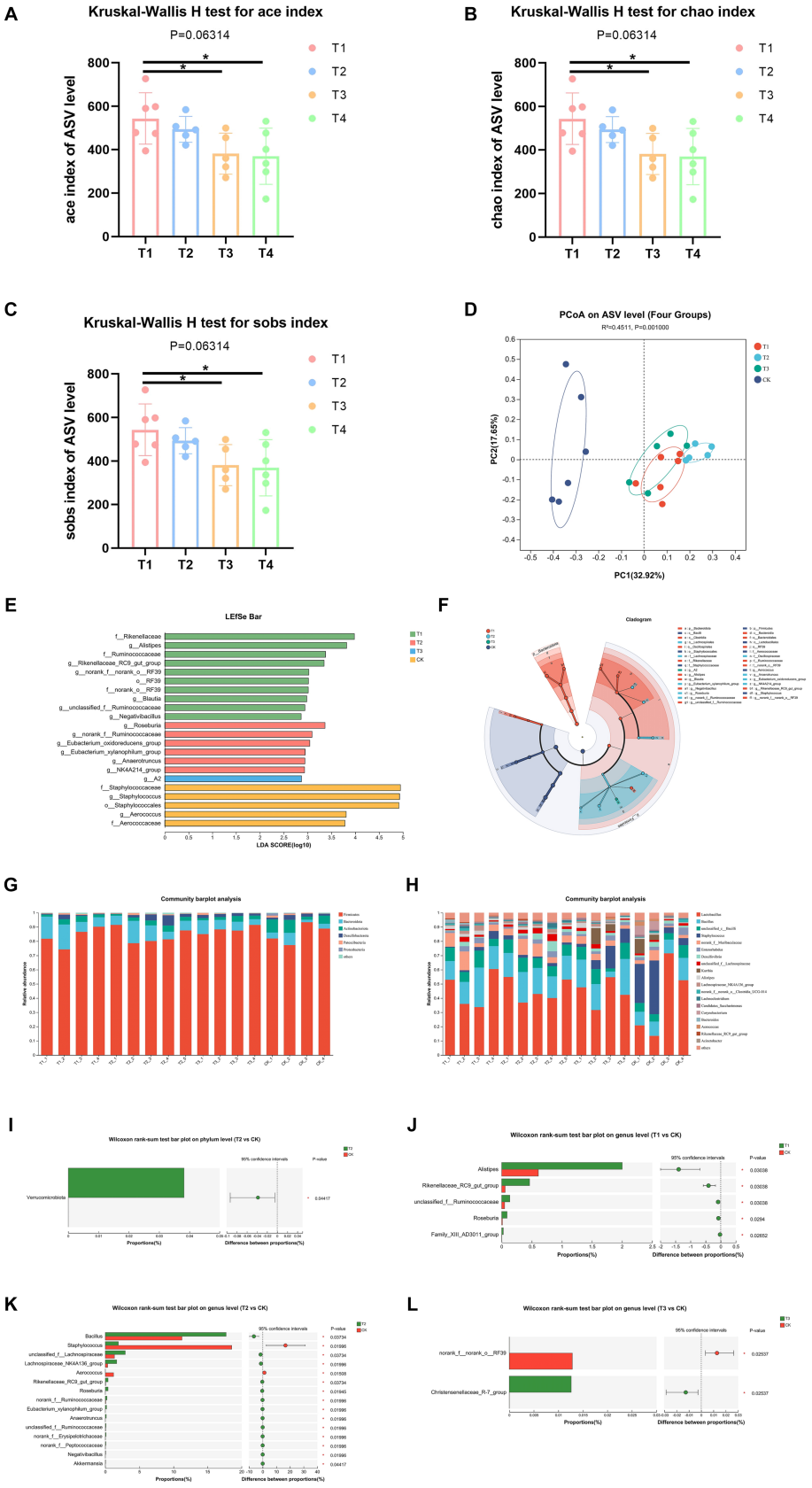


FIGURE 4
FMT modulated microbiota composition. **(A–C)** Ace, chao, and sobs index of ASV level. **(D)** β-diversity between experiment groups and CK group on ASV level. **(E,F)** LEfSe analysis of four groups. o: Order, f: Family, g: Genus. **(G,H)** The relative abundance of bacteria on phylum and genus. **(I)** Comparative analysis of the relative abundance of bacteria at phylum level between T2 group and CK group. **(J–L)** Comparative analysis of the relative abundance of bacteria at genus level between experimental groups and CK group; CK: DBA mice accepting saline gavage; T1: DBA mice receiving FMT from C57BL/6 mice; T2: DBA mice receiving FMT from high-fat C57BL/6 mice; T3: DBA mice receiving FMT from Wistar rats.

10.3389/fmicb.2024.1352555

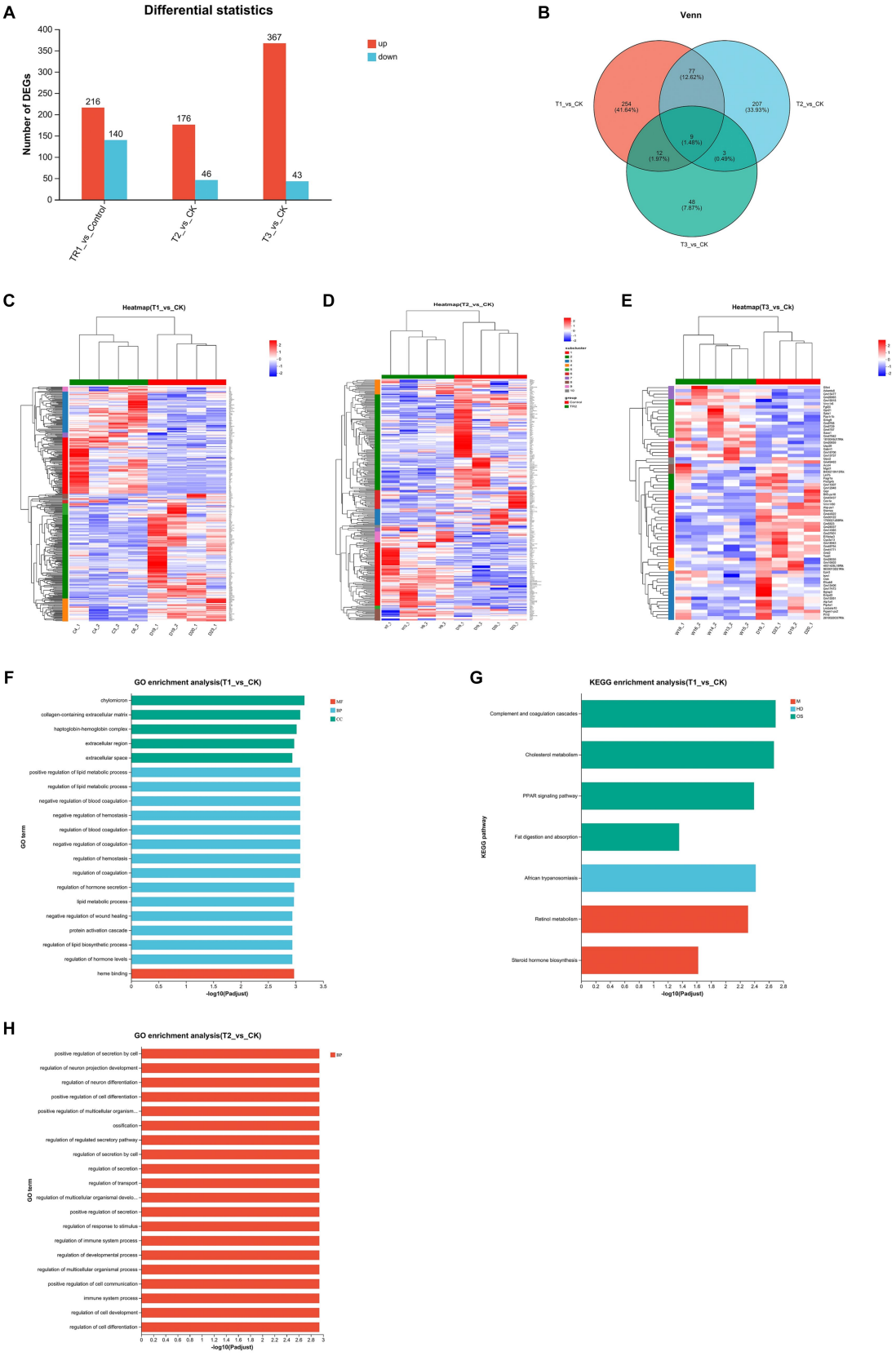


FIGURE 5
Analysis of DEGs in the colon among four groups. **(A,B)** Differential gene expression statistics. **(C–E)** Heatmap showing the differential expression of genes among the groups. **(F–H)** GO and KEGG pathway enrichment analysis of DEGs; CK: DBA mice accepting saline gavage; T1: DBA mice receiving FMT from C57BL/6 mice; T2: DBA mice receiving FMT from high-fat C57BL/6 mice; T3: DBA mice receiving FMT from Wistar rats.

GO functional and KEGG pathway enrichment analysis were performed with the DEGs among the four groups. Enrichment results of top20 were shown at padjust <0.05, where T1 group versus CK group had enrichment results in both GO and KEGG pathway, T2 group versus CK group had only GO enrichment results, and T3 group versus CK group did not produce enrichment results in both GO and KEGG pathway. The results showed that the DEGs between T1 group and CK group were categorized into three groups [molecular function (MF), biological process (BP), and cellular component (CC)] based on GO enrichment analysis, and many of them were associated with lipid metabolic process, such as positive regulation of lipid metabolic process, regulation of lipid metabolic process, lipid metabolic process, regulation of lipid biosynthetic process, regulation of lipid biosynthetic process in BP. There were also many related to hemostasis and coagulation, such as negative regulation of blood coagulation, negative regulation of hemostasis, regulation of blood coagulation, negative regulation of coagulation, regulation of hemostasis, regulation of coagulation in BP, and heme binding in CC. Other high enriched GOs were chylomicron, collagen-containing extracellular matrix, haptoglobin-hemoglobin complex, extracellular region and extracellular space in MF, regulation of hormone secretion, negative regulation of wound healing, protein activation cascade and regulation of hormone levels in BP (Figure 5F). KEGG pathway enrichment showed that the DEGs between T1 group and CK group were categorized into three groups [metabolism (M), human disease (HD), organism system (OS)]. And many of them were associated to fat and sterol such as Cholesterol metabolism, Fat digestion and absorption in OS, and Steroid hormone biosynthesis of HD. Other high enriched KEGG pathways were African trypanosomiasis of M and Complement and coagulation cascades, PPAR signaling pathway in OS, together with Retinol metabolism in HD (Figure 5G). The DEGs between T2 group and CK group were categorized into biological process (BP), and most of them are related to the regulation of cellular activity (positive regulation of secretion by cell, positive regulation of cell differentiation, positive regulation of multicellular organismal process, regulation of secretion by cell, regulation of multicellular organismal development, regulation of multicellular organismal process, positive regulation of cell communication, regulation of cell development, regulation of cell differentiation). Others were related to neuron-related regulation, secretory regulation and the immune system (Figure 5H).

3.6 Differentially expressed genes related to the composition of gut bacterial communities

Pearson correlation was used to infer the relationship between the composition of the microbial communities on the genus level and DEGs. The top candidate genes with genus-level relationships with the bacterial community are listed in Supplementary Tables S4, S5. Correlation analysis of the 357 differential genes in the T1 and CK groups with the five genus-level differential flora yielded 111 genes with significant correlations, most of which had KEGG functional annotations related to fatty acid metabolism (Apoa5, Angptl4), fat digestion and absorption (Dgat2), cholesterol metabolism (Apoa2, Kng1), inflammation regulation (Cyp2c29) and coagulation (C8b, Fgb, C8a, Plg) (Figure 6). Correlation analysis of 221 differential genes from the T2 and CK groups with 15 genus-level differential flora yielded 111 genes with significant correlations, most of which had KEGG functional annotations related to fatty acid metabolism (Cyp8b1), cholesterol metabolism (Angptl8), and coagulation (C4bp) (Figure 7). Correlation analysis of 416 differential genes from the T3 and CK groups with 2 genus-level differential flora yielded 7 genes with significant correlations, most of which had KEGG functional annotations related to cancer and cell apoptosis (Prima1, Pidd1, Pmel, Dner, Rnf208, Igfn1) (Sun et al., 2009; Izetti et al., 2014; Weiler et al., 2022) and Nervous-System Function (Susd4, Elfn2) (Figure 8).

4 Discussion

4.1 Relationship between the gut bacterial community structure and growth performance

Normal steady-state intestinal flora is closely related to host health and forms a reciprocal relationship with the host through complex interactions. Its unique community structure and metabolites are essential for the regulation of host metabolism, growth and development, pathogen resistance, immunomodulation, adaptation, and evolution (Bäckhed et al., 2005; Engel et al., 2012; Ezenwa et al., 2012; Zhang et al., 2016). To investigate whether the effect of fecal transplantation on the growth performance of DBA mice is related to the intestinal flora, mice from three experimental groups and CK group were cultured in similar environments during the reproductive period to explore the differences in the structure of their intestinal bacterial communities. The PCoA analysis of the bacterial flora revealed that the three experimental groups and CK group were clearly distinguishable. The results showed that the T1 group significantly enhanced five dominant bacteria genera including the genera *Alistipes*, *Rikenellaceae_RC9_gut_group*, *unclassified_f_Ruminococcaceae*, *Roseburia*, and *Family_XIII_AD3011_group*. *Alistipes* and *Rikenellaceae_RC9_gut_group* belong to Bacteroidetes. It has been shown that *Alistipes* may be protective against certain diseases, including liver fibrosis, cancer immunotherapy and cardiovascular disease. In addition to this, *Alistipes* spp. have been directly linked to cardiovascular disease (CVD) and may also be considered as a potential producer of short-chain fatty acids (SCFA) (Jie et al., 2017; Kim et al., 2018; Parker et al., 2020). Genus *Rikenellaceae_RC9_gut_group* was proved to promotes lipid metabolism (Zhou et al., 2018). *Unclassified_f_Ruminococcaceae* (*Ruminococcus* belongs to this family) and *Alistipes* are associated with immune regulation and healthy homeostasis and are regarded as potentially beneficial bacteria (Kong et al., 2016; Shang et al., 2016; Wang et al., 2018), Which might relate to the decrease in pro-inflammatory factors in T1 group. *Alistipes* have been shown to produce sulfolipids (SL) as a marker of a high-fat diet (Walker et al., 2017). This may explain why the growth performance of mice in the T2 group was better than that of mice in other three groups, and why the serum TG content of the mice in the T2 group was significantly higher than that of the mice in CK group. *Roseburia* belongs to the phylum Firmicutes, class Clostridia, order Clostridiales, and family Lachnospiraceae. The five well-characterized species of *Roseburia* (*Roseburia intestinalis*, *Roseburia hominis*, *Roseburia inulinivorans*, *Roseburia faecis*, and *Roseburia cecicola)* all produce SCFA, such as acetate, propionate, and butyrate (Nie et al., 2021). *Roseburia* has been

shown to prevent intestinal inflammation and maintain energy homeostasis through the production of metabolites (Kumari, 2013; Rajilić-Stojanović et al., 2013). Similarly, the *Family_XIII_AD3011_group* has probiotic properties and improves intestinal health in weaned piglets after inoculation (Yu et al., 2020). The main species that significantly increased at the genus level in the T2 group compared to the CK group were *Bacillus*, *unclassified_f_Lachnospiraceae* and *Lachnospiraceae_NK4A136_group*, and the main species that significantly decreased were *Staphylococcus* and *Aerococcus*. The genus *Bacillus* is a diverse group of gram-positive, spore-forming aerobic bacilli (Weber and Rutala, 2016). Scientists at the National Institutes of Health (NIH) found that probiotic *Bacillus* can comprehensively eradicate intestinal as well as nasal *S. aureus* colonization (Piewngam et al., 2018), this might be the reason why *Staphylococcus* significantly decreased in T2 group compared to CK group. *Lachnospiraceae_NK4A136_group* can produce SCFAs through

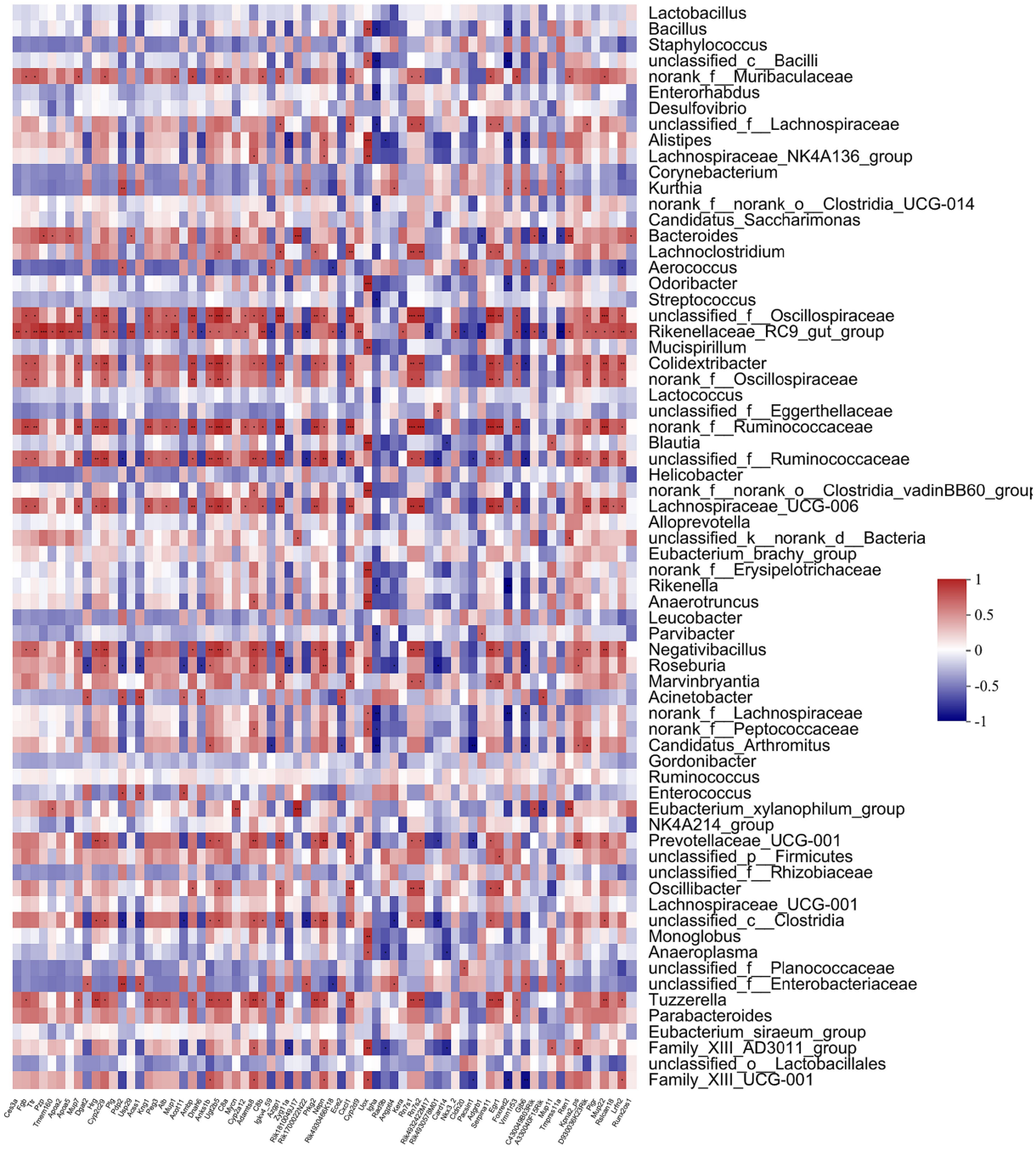


FIGURE 6
Pearson Correlation Heatmap of microbial communities on the genus level and DEGs between T1 and CK groups.

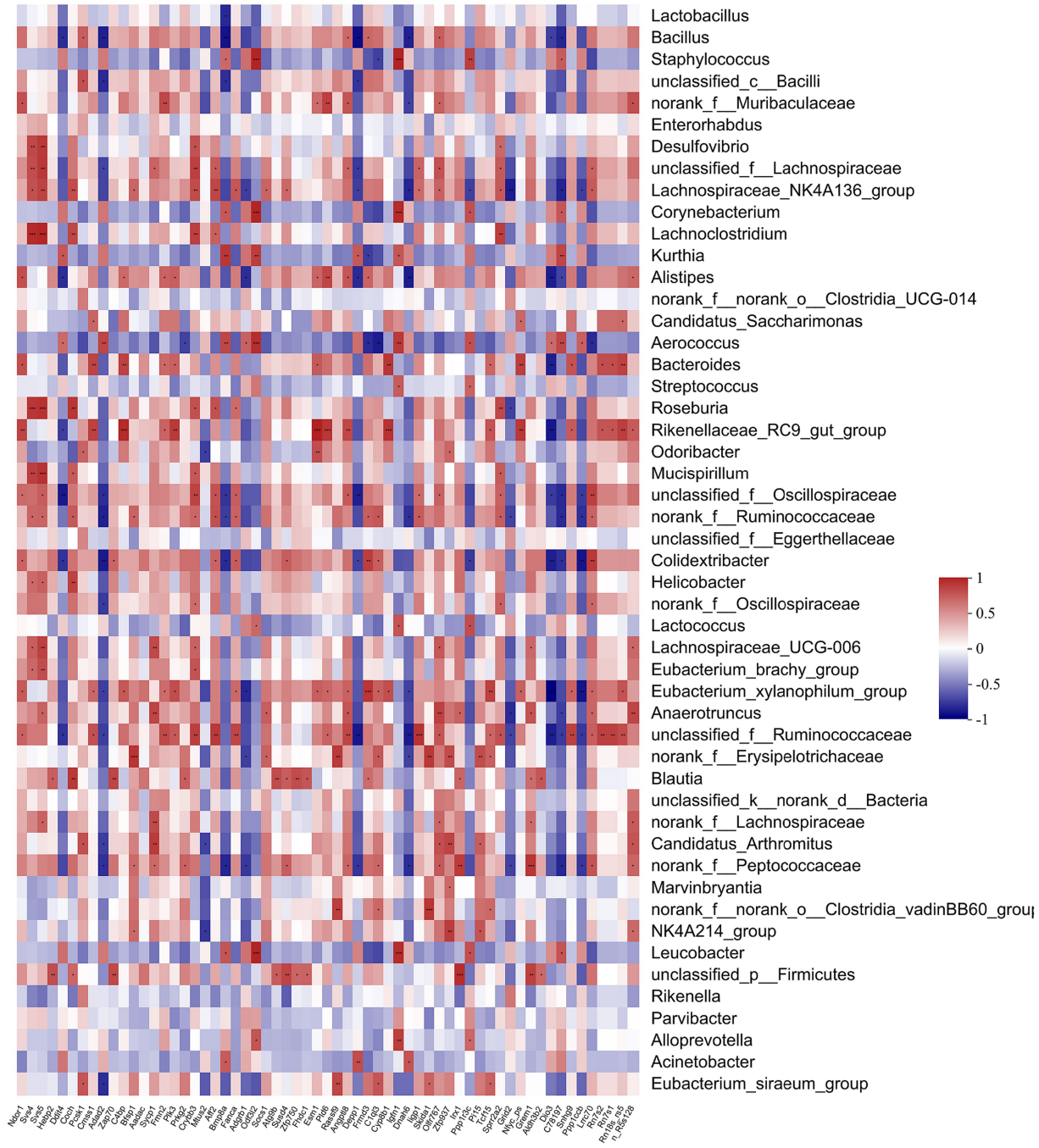


FIGURE 7
Pearson Correlation Heatmap of microbial communities on the genus level and DEGs between T2 and CK groups.

fermentation of dietary polysaccharides and has been negatively associated with several metabolic diseases and chronic inflammation (Truax et al., 2018; Hu et al., 2019; Ma et al., 2020). *Staphylococcus aureus* is a gram-positive bacterium with a diameter of 1 μm that can cause many forms of infection (Cheung et al., 2021). *Aerococcus* are granulocyte-positive bacteria that grow in clusters, and studies have shown that *Aerococcus* are human pathogens that can cause invasive human infections, such as urogenital focal sepsis and infective endocarditis (Williams et al., 1953; Senneby et al., 2012; Senneby et al., 2014; Senneby et al., 2015; Opota et al., 2016). The improved growth performance of mice in the T3 group may be associated with a significant increase in *Christensenellaceae_R-7_group* bacteria spp. *Christensenellaceae_R-7_group* is a member of the *Christensenellaceae* family. The *Christensenellaceae* family is a relatively new family of bacteria previously associated with host health (Waters and Ley, 2019). *Christensenellaceae* have been shown to be associated with the

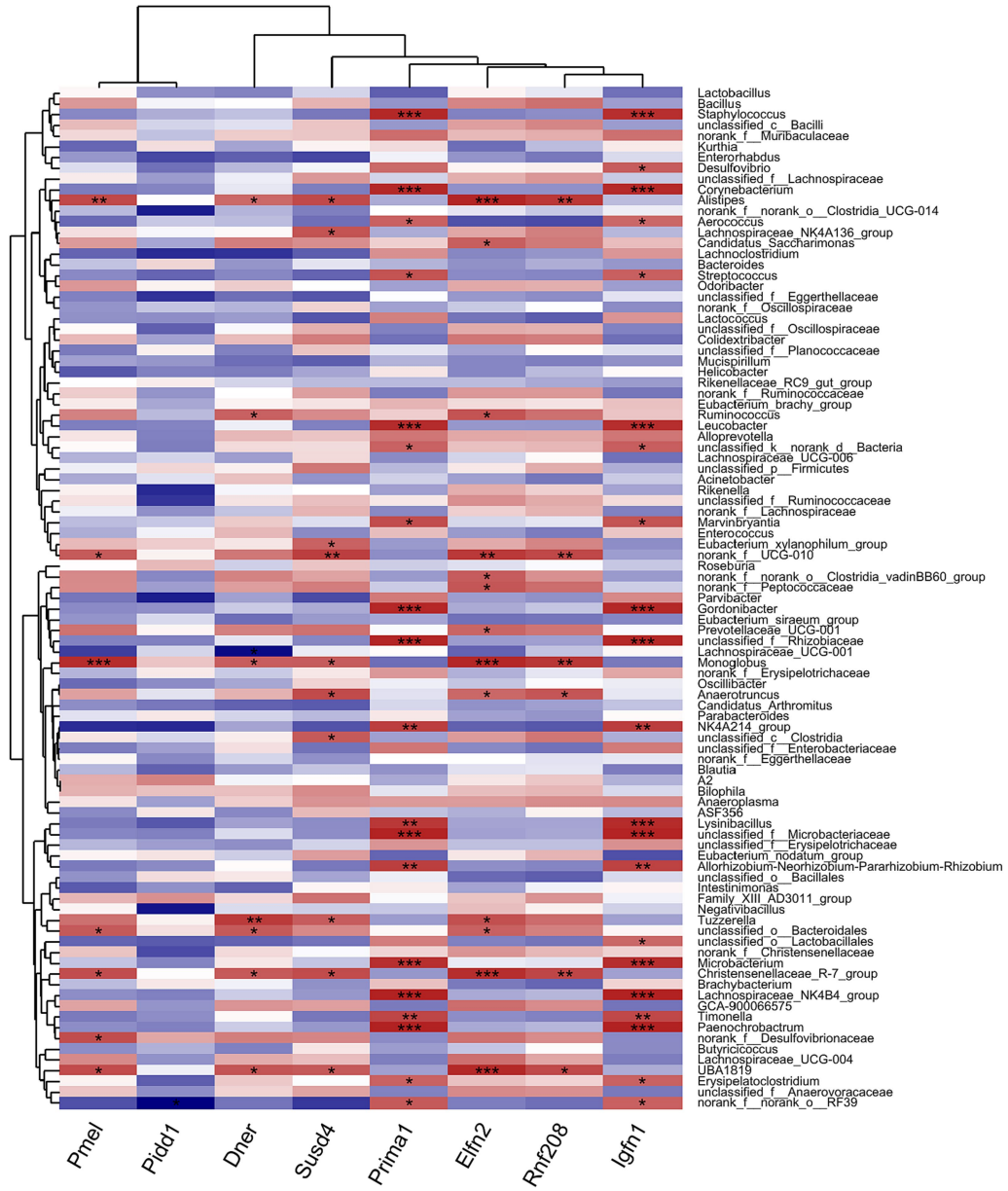


FIGURE 8
Pearson Correlation Heatmap of microbial communities on the genus level and DEGs between T3 and CK groups.

proteolytic metabolism of god is animal proteins and intestinal metabolites are positively correlated and are strongly associated with butyrate production (Beaumont et al., 2017; Haas and Blanchard, 2017; Manor et al., 2018).

4.2 Relationship between the gene expression in the gut and growth performance

The productive performance of the host can be modulated by regulating the structure of the gut microflora, which in turn is largely influenced by genetics and feed. Gut microbes are also able to influence host gene expression and methylation levels (Levy et al., 2015). Anhê et al. (2019) found that altering the intestinal flora of mice can change gene expression in the gut and can prevent obesity by increasing the energy trumpet in diet-induced obese mice. Similarly, in the current study, we found significant differences in intestinal gene expression in several experimental groups of mice compared to CK group. In the T1 group, the up-regulated genes mainly included lipid metabolism-related genes such as Apoh, Apoa2, Apob, Apoa5, Apoc3, Apoa1, and Fabp1. We found these genes in the National Center for Biotechnology Information (NCBI) database to be homologous to both human and mouse. The Apoh

gene was found to inhibit fatty acid oxidation and promote lipid synthesis (Pan et al., 2022). Zaki et al. (2013) found that Apoa2 is strongly associated with obesity and lipid metabolism, and that it has a potential role in regulating appetite in humans; the Apob gene is also involved in fat digestion and absorption (Honda et al., 2022). Apoa5 is a major gene involved in triglyceride metabolism (Tai and Ordovas, 2008). Apoc3 is expressed predominantly in the intestine, where it contributes to postprandial coeliac formation, and in the liver, where it regulates central lipid metabolism and turnover (Kohan, 2016). The *in vivo* association of Apoc3, Apoa1, and Apoa5 with hyperlipidemia, particularly TG levels, has been demonstrated in genome-wide association studies (GWAS) (Mar et al., 2004; Lai et al., 2005; Qi et al., 2007; Pouwer et al., 2020). This may account for the significant increase in serum TG levels in DBA mice that received fecal microbiome transplants from normal C57BL/6 mice. However, DBA mice that received fecal colony transplants from high-fat C57BL/6 mice upregulated cancer-related genes containing Mycn and C4bp (Kopylov et al., 2020; Massó-Vallés et al., 2020). This may be due to the fact that a high-fat diet enhances cancer incidence by modulating gut microbes and metabolites (Yang et al., 2022).

4.3 Relationship between the DEGs and bacterial community composition in the gut

Generally, intestinal bacteria interact with the expression of certain genes in intestinal epithelial cells (Larsson et al., 2012; Broderick et al., 2014; Richards et al., 2019). When human colonic epithelial cells (HCoEpiC) were treated with live gut microbiota extracted from five healthy humans, the most intense response (3,240 genes in any of the five microbiota samples) occurred at 2 h; 588 pairs of taxon-by-taxon unit transcripts corresponded to 121 host genes, and changes in expression correlated with the abundance of 46 taxa (Richards et al., 2019). Larsson et al. (2012) and Broderick et al. (2014) found that MyD88 is essential for microbiota-induced colonic expression of the antimicrobial genes Reg3β and Reg3γ in the epithelium. Lack of MyD88 resulted in altered bacterial diversity and a greater proportion of segmented filamentous bacteria in the small intestine. Su et al. found that the Aeromonas and Roseomonas percentage, as well as differential expression of IL12, were related to anti-disease ability (Su et al., 2021), and that the percentage of thick-walled bacteria was associated with growth performance of a new strain of Yellow River carp. Alenghant et al. found that a mouse model with tissue-specific deletion of histone deacetylase 3 (HDAC3) resulted in dysregulation of microbiome-dependent gene regulation in intestinal epithelial cells (Alenghat et al., 2013). Specifically, an intestinal epithelial cell-specific HDAC3 knockout mouse line (HDAC3 ΔIEC) was bred to study IBD progression. Conventionalized HDAC3 ΔIEC mice exhibit dysregulated host gene expression and disrupted homeostasis *in vivo*. In contrast, no such dysregulation was observed in the germ-free HDAC3ΔIEC mouse model, suggesting that these effects are mediated by the microbiome (Alenghat et al., 2013). From a molecular perspective, transcription factors are host proteins that bind to DNA and regulate gene transcription, and elements of the microbiome bind directly to transcription factors (Krautkramer et al., 2016; Davison et al., 2017), which may be the molecular mechanism that facilitates the linkage between microbiome and host gene expression. In this study, we analyzed the selection and CK groups in conjunction with the structure of gut bacterial communities and DEGs, and identified the Apoa2 and Apoa5 genes that were significantly associated with the *Rikenellaceae_RC9_gut_group* bacteria, which have been found to promote lipid digestion and absorption, and Apoa2 and Apoa5, on the other hand, are important genes in lipid metabolism. Thus, FMT from C57BL/6 mice might enhance the lipid metabolism of DBA mice by increasing the *Rikenellaceae_RC9_gut_group* and finally upregulated the expression of Apoa2 and Apoa5 genes in the colon.

These results suggest that gut bacteria can influence gene expression in the gut, where dominant species or bacterial structure may reflect the genetic characteristics of the host, C57BL/6 mice. Gene profiling of gut bacteria in the gut contributes to host health and performance. In this study, we used multisource FMT experimental design, in which fecal bacterial fluids derived from C57BL/6 mice, high-fat C57BL/6 mice, and Wistar rats were transplanted into DBA mice. This multisource FMT design facilitated a more comprehensive comparison of the effects of different microbial compositions on host growth and physiological functions. In addition, the effects of FMT on DBA mice were investigated by combining multilevel analyses such as 16 s rRNA sequencing and colonic gene expression sequencing. This combined multi-omics analysis helps to deeply explore the microbial regulatory mechanisms on the host, providing more precise targets for future intervention and treatment of related diseases or growth promotion. However, the mechanism of the interaction between gut flora and intestinal gene expression, as well as the mechanism of the differences between normal C57BL/6 mice and high-fat C57BL/6 mice produced as donors still need to be validated by further studies.

5 Conclusion

In the present study, we found that cross-strain FMT can improve the production performance of mice, which may be due to FMT altering the structure of the intestinal bacterial community in the recipient mice and subsequently enhancing lipid metabolism production, and lipid metabolites can alter intestinal gene expression in mice.

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/sra/PRJNA1050037, NCBI-PRJNA1050037.

Ethics statement

The animal study was approved by Animal Care Advisory Committee of Zhejiang University, Hangzhou, China. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WY: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. JF: Conceptualization, Visualization, Writing – review & editing. WW: Formal analysis, Writing – original draft. ZC: Investigation, Writing – original draft. QH: Methodology, Writing – original draft. LQ: Supervision, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Key R&D Program of China (Ministry of Science and Technology of China, no. 2021YFC2103005), Hangzhou Science and Technology Bureau Agricultural Society Key Projects (no. 202003A02), and Zhejiang Province Science and Technology Commissioner Team Project (2020-2024).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1352555/full#supplementary-material

References

Alenghat, T., Osborne, L. C., Saenz, S. A., Kobuley, D., Ziegler, C. G. K., Mullican, S. E., et al. (2013). Histone deacetylase 3 coordinates commensal-bacteria-dependent intestinal homeostasis. *Nature* 504, 153–157. doi: 10.1038/nature12687

Anhê, F. F., Nachbar, R. T., Varin, T. V., Trottier, J., Dudonné, S., Le Barz, M., et al. (2019). Treatment with camu camu (*Myrciaria dubia*) prevents obesity by altering the gut microbiota and increasing energy expenditure in diet-induced obese mice. *Gut* 68, 453–464. doi: 10.1136/gutjnl-2017-315565

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

Beaumont, M., Portune, K. J., Steuer, N., Lan, A., Cerrudo, V., Audebert, M., et al. (2017). Quantity and source of dietary protein influence metabolite production by gut microbiota and rectal mucosa gene expression: a randomized, parallel, double-blind trial in overweight humans. *Am. J. Clin. Nutr.* 106, 1005–1019. doi: 10.3945/ajcn.117.158816

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Broderick, N. A., Buchon, N., and Lemaitre, B. (2014). Microbiota-induced changes in *Drosophila melanogaster* host gene expression and gut morphology. McFall-Ngai MJ, editor. *MBio* 5, e01117–e01114. doi: 10.1128/mBio.01117-14

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cammarota, G., Ianiro, G., and Gasbarrini, A. (2014). Fecal microbiota transplantation for the treatment of *Clostridium difficile* infection a systematic review. *J. Clin. Gastroenterol.* 48, 693–702. doi: 10.1097/MCG.0000000000000046

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

Cheung, G. Y. C., Bae, J. S., and Otto, M. (2021). Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence* 12, 547–569. doi: 10.1080/21505594.2021.1878688

Davison, J. M., Lickwar, C. R., Song, L., Breton, G., Crawford, G. E., and Rawls, J. F. (2017). Microbiota regulate intestinal epithelial gene expression by suppressing the transcription factor hepatocyte nuclear factor 4 alpha. *Genome Res.* 27, 1195–1206. doi: 10.1101/gr.220111.116

Elangovan, S., Borody, T. J., and Holsinger, R. M. D. (2022). Fecal microbiota transplantation reduces pathology and improves cognition in a mouse model of Alzheimer's disease. *Cell* 12:119. doi: 10.3390/cells12010119

Engel, P., Martinson, V. G., and Moran, N. A. (2012). Functional diversity within the simple gut microbiota of the honey bee. *Proc. Natl. Acad. Sci. USA* 109, 11002–11007. doi: 10.1073/pnas.1202970109

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M., and Xavier, J. B. (2012). Animal behavior and the microbiome. *Science* 338, 198–199. doi: 10.1126/science.1227412

Haas, K. N., and Blanchard, J. L. (2017). Kineothrix alysoides, gen. Nov., sp. nov., a saccharolytic butyrate-producer within the family Lachnospiraceae. *Int. J. Syst. Evol. Microbiol.* 67, 402–410. doi: 10.1099/ijsem.0.001643

Higgins, P. D. R. Schwartz, M, and Mapili, J, (2005). Patient defined dichotomous end points for remission and clinical improvement in ulcerative colitis. *Gut* 54, 782–788. doi: 10.1136/gut.2004.056358

Honda, Y., Ogawa, N., Wong, M. K. S., Tokunaga, K., Kuraku, S., Hyodo, S., et al. (2022). Molecular mechanism of nutrient uptake in developing embryos of oviparous cloudy catshark (*Scyliorhinus torazame*). Angers a, editor. *PLoS One* 17:e0265428. doi: 10.1371/journal.pone.0265428

Hu, S., Wang, J., Xu, Y., Yang, H., Wang, J., Xue, C., et al. (2019). Anti-inflammation effects of fucosylated chondroitin sulphate from *Acaudina molpadioides* by altering gut microbiota in obese mice. *Food Funct.* 10, 1736–1746. doi: 10.1039/C8FO02364F

Izetti, P., Hautefeuille, A., Abujamra, A. L., De Farias, C. B., Giacomazzi, J., Alemar, B., et al. (2014). PRIMA-1, a mutant p53 reactivator, induces apoptosis and enhances chemotherapeutic cytotoxicity in pancreatic cancer cell lines. *Investig. New Drugs* 32, 783–794. doi: 10.1007/s10637-014-0090-9

Jie, Z., Xia, H., Zhong, S. L., Feng, Q., Li, S., Liang, S., et al. (2017). The gut microbiome in atherosclerotic cardiovascular disease. *Nat. Commun.* 8:845. doi: 10.1038/s41467-017-00900-1

Kim, S., Goel, R., Kumar, A., Qi, Y., Lobaton, G., Hosaka, K., et al. (2018). Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clin. Sci.* 132, 701–718. doi: 10.1042/CS20180087

Kohan, A. B. (2016). ApoC-III: A potent modulator of hypertriglyceridemia and cardiovascular disease. *Curr. Opin. Endocrinol. Diabetes Obes.* 22, 119–125. doi: 10.1097/MED.0000000000000136

Kong, F., Hua, Y., Zeng, B., Ning, R., Li, Y., and Zhao, J. (2016). Gut microbiota signatures of longevity. *Curr. Biol.* 26, R832–R833. doi: 10.1016/j.cub.2016.08.015

Kopylov, A. T., Stepanov, A. A., Malsagova, K. A., Soni, D., Kushlinsky, N. E., Enikeev, D. V., et al. (2020). Revelation of proteomic indicators for colorectal Cancer in initial stages of development. *Molecules* 25:619. doi: 10.3390/molecules25030619

Krautkramer, K. A., Kreznar, J. H., Romano, K. A., Vivas, E. I., Barrett-Wilt, G. A., Rabaglia, M. E., et al. (2016). Diet-microbiota interactions mediate global epigenetic programming in multiple host tissues. *Mol. Cell* 64, 982–992. doi: 10.1016/j.molcel.2016.10.025

Kumari, R. (2013). Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India. *WJG* 19, 3404–3414. doi: 10.3748/wjg.v19.i22.3404

Lai, C. Q., Parnell, L. D., and Ordovas, J. M. (2005). The APOA1/C3/A4/A5 gene cluster, lipid metabolism and cardiovascular disease risk. *Curr. Opin. Lipidol.* 16, 153–166. doi: 10.1097/01.mol.0000162320.54795.68

Larsson, E., Tremaroli, V., Lee, Y. S., Koren, O., Nookaew, I., Fricker, A., et al. (2012). Analysis of gut microbial regulation of host gene expression along the length of the gut and regulation of gut microbial ecology through MyD88. *Gut* 61, 1124–1131. doi: 10.1136/gutjnl-2011-301104

Levy, M., Thaiss, C. A., and Elinav, E. (2015). Metagenomic cross-talk: the regulatory interplay between immunogenomics and the microbiome. *Genome Med.* 7:120. doi: 10.1186/s13073-015-0249-9

Lewin, R. A. (2001). More on Merde. *Perspect. Biol. Med.* 44, 594–607. doi: 10.1353/pbm.2001.0067

Lin, C., Wan, J., Su, Y., and Zhu, W. (2018). Effects of early intervention with maternal fecal microbiota and antibiotics on the gut microbiota and metabolite profiles of piglets. *Meta* 8:89. doi: 10.3390/metabo8040089

Liu, C., Zhao, D., Ma, W., Guo, Y., Wang, A., Wang, Q., et al. (2016). Denitrifying sulfide removal process on high-salinity wastewaters in the presence of Halomonas sp. *Appl. Microbiol. Biotechnol.* 100, 1421–1426. doi: 10.1007/s00253-015-7039-6

Ma, L., Ni, Y., Wang, Z., Tu, W., Ni, L., Zhuge, F., et al. (2020). Spermidine improves gut barrier integrity and gut microbiota function in diet-induced obese mice. *Gut Microbes* 12, 1832857–1832819. doi: 10.1080/19490976.2020.1832857

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

Manor, O., Zubair, N., Conomos, M. P., Xu, X., Rohwer, J. E., Krafft, C. E., et al. (2018). A multi-omic association study of trimethylamine N-oxide. *Cell Rep.* 24, 935–946. doi: 10.1016/j.celrep.2018.06.096

Mar, R., Pajukanta, P., Allayee, H., Groenendijk, M., Dallinga-Thie, G., Krauss, R. M., et al. (2004). Association of the *APOLIPOPROTEIN A1/C3/A4/A5* gene cluster with triglyceride levels and LDL particle size in familial combined hyperlipidemia. *Circ. Res.* 94, 993–999. doi: 10.1161/01.RES.0000124922.61830.F0

Massó-Vallés, D., Beaulieu, M. E., and Soucek, L. (2020). MYC, MYCL, and MYCN as therapeutic targets in lung cancer. *Expert Opin. Ther. Targets* 24, 101–114. doi: 10.1080/14728222.2020.1723548

McDonald, L. C., Killgore, G. E., Thompson, A., Owens, R. C., Kazakova, S. V., Sambol, S. P., et al. (2005). An epidemic, toxin gene–variant strain of *Clostridium difficile*. *N. Engl. J. Med.* 353, 2433–2441. doi: 10.1056/NEJMoa051590

Nie, K., Ma, K., Luo, W., Shen, Z., Yang, Z., Xiao, M., et al. (2021). *Roseburia intestinalis*: a beneficial gut organism from the discoveries in genus and species. *Front. Cell. Infect. Microbiol.* 11:757718. doi: 10.3389/fcimb.2021.757718

Opota, O., Prod'hom, G., Andreutti-Zaugg, C., Dessauges, M., Merz, L., Greub, G., et al. (2016). Diagnosis of *Aerococcus urinae* infections: importance of matrix-assisted laser desorption ionization time-of-flight mass spectrometry and broad-range 16S rDNA PCR. *Clin. Microbiol. Infect.* 22, e1–e2. doi: 10.1016/j.cmi.2015.08.026

Pan, Z., Du, G., Li, G., Wu, D., Chen, X., and Geng, Z. (2022). Apolipoprotein H: a novel regulator of fat accumulation in duck myoblasts. *J. Anim. Sci. Technol.* 64, 1199–1214. doi: 10.5187/jast.2022.e60

Parker, B. J., Wearsch, P. A., Veloo, A. C. M., and Rodriguez-Palacios, A. (2020). The genus Alistipes: gut Bacteria with emerging implications to inflammation, Cancer, and mental health. *Front. Immunol.* 11:906. doi: 10.3389/fimmu.2020.00906

Piewngam, P., Zheng, Y., Nguyen, T. H., Dickey, S. W., Joo, H. S., Villaruz, A. E., et al. (2018). Pathogen elimination by probiotic Bacillus via signalling interference. *Nature* 562, 532–537. doi: 10.1038/s41586-018-0616-y

Pouwer, M. G., Pieterman, E. J., Worms, N., Keijzer, N., Jukema, J. W., Gromada, J., et al. (2020). Alirocumab, evinacumab, and atorvastatin triple therapy regresses plaque lesions and improves lesion composition in mice. *J. Lipid Res.* 61, 365–375. doi: 10.1194/jlr.RA119000419

Qi, L., Liu, S., Rifai, N., Hunter, D., and Hu, F. B. (2007). Associations of the apolipoprotein A1/C3/A4/A5 gene cluster with triglyceride and HDL cholesterol levels in women with type 2 diabetes. *Atherosclerosis* 192, 204–210. doi: 10.1016/j.atherosclerosis.2006.05.006

Rajilić-Stojanović, M., Shanahan, F., Guarner, F., and De Vos, W. M. (2013). Phylogenetic analysis of dysbiosis in ulcerative colitis during remission. *Inflamm. Bowel Dis.* 19, 481–488. doi: 10.1097/MIB.0b013e31827fec6d

Richards, A. L., Muehlbauer, A. L., Alazizi, A., Burns, M. B., Findley, A., Messina, F., et al. (2019). Gut microbiota has a widespread and modifiable effect on host gene regulation. Grice EA, editor. *mSystems* 4, e00323–e00318. doi: 10.1128/mSystems.00323-18

Rodrigues, R., Barber, G. E., and Ananthakrishnan, A. N. (2017). A comprehensive study of costs associated with recurrent *Clostridium difficile* infection. *Infect. Control Hosp. Epidemiol.* 38, 196–202. doi: 10.1017/ice.2016.246

Rosa, F., Michelotti, T. C., St-Pierre, B., Trevisi, E., and Osorio, J. S. (2021). Early life fecal microbiota transplantation in neonatal dairy calves promotes growth performance and alleviates inflammation and oxidative stress during weaning. *Animals* 11:2704. doi: 10.3390/ani11092704

Rossen, N. G., Fuentes, S., van der Spek, M. J., Tijssen, J. G., Hartman, J. H. A., Duflou, A., et al. (2015). Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis. *Gastroenterology* 149, 110–118.e4. doi: 10.1053/j.gastro.2015.03.045

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schoeler, M., and Caesar, R. (2019). Dietary lipids, gut microbiota and lipid metabolism. *Rev. Endocr. Metab. Disord.* 20, 461–472. doi: 10.1007/s11154-019-09512-0

Senneby, E., Eriksson, B., Fagerholm, E., and Rasmussen, M. (2014). Bacteremia with *Aerococcus sanguinicola*: case series with characterization of virulence properties. *Open Forum Infect. Dis.* 1:ofu025. doi: 10.1093/ofid/ofu025

Senneby, E., Petersson, A. C., and Rasmussen, M. (2012). Clinical and microbiological features of bacteraemia with *Aerococcus urinae*. *Clin. Microbiol. Infect.* 18, 546–550. doi: 10.1111/j.1469-0691.2011.03609.x

Senneby, E., Petersson, A. C., and Rasmussen, M. (2015). Epidemiology and antibiotic susceptibility of aerococci in urinary cultures. *Diagn. Microbiol. Infect. Dis.* 81, 149–151. doi: 10.1016/j.diagmicrobio.2014.11.009

Shang, Q., Shan, X., Cai, C., Hao, J., Li, G., and Yu, G. (2016). Dietary fucoidan modulates the gut microbiota in mice by increasing the abundance of *Lactobacillus* and *Ruminococcaceae*. *Food Funct.* 7, 3224–3232. doi: 10.1039/C6FO00309E

Siegerstetter, S. C., Petri, R. M., Magowan, E., Lawlor, P. G., Zebeli, Q., O'Connell, N. E., et al. (2018). Fecal microbiota transplant from highly feed-efficient donors shows little effect on age-related changes in feed-efficiency-associated fecal microbiota from chickens. Dudley EG, editor. *Appl. Environ. Microbiol.* 84, e02330–e02317. doi: 10.1128/AEM.02330-17

Su, S., Jing, X., Zhang, C., Hou, Y., Li, Z., Yang, X., et al. (2021). Interaction between the intestinal microbial community and transcriptome profile in common carp (*Cyprinus carpio* L.). *Front. Microbiol.* 12:659602. doi: 10.3389/fmicb.2021.659602

Sun, P., Xia, S., Lal, B., Eberhart, C. G., Quinones-Hinojosa, A., Maciaczyk, J., et al. (2009). *DNER*, an epigenetically modulated gene, regulates glioblastoma-derived Neurosphere cell differentiation and tumor propagation. *Stem Cells* 27, 1473–1486. doi: 10.1002/stem.89

Tai, E. S., and Ordovas, J. M. (2008). Clinical significance of apolipoprotein A5. *Curr. Opin. Lipidol.* 19, 349–354. doi: 10.1097/MOL.0b013e328304b681

Truax, A. D., Chen, L., Tam, J. W., Cheng, N., Guo, H., Koblansky, A. A., et al. (2018). The inhibitory innate immune sensor NLRP12 maintains a threshold against obesity by regulating gut microbiota homeostasis. *Cell Host Microbe* 24, 364–378.e6. doi: 10.1016/j.chom.2018.08.009

Walker, A., Pfitzner, B., Harir, M., Schaubeck, M., Calasan, J., Heinzmann, S. S., et al. (2017). Sulfonolipids as novel metabolite markers of Alistipes and Odoribacter affected by high-fat diets. *Sci. Rep.* 7:11047. doi: 10.1038/s41598-017-10369-z

Wang, X., Tsai, T., Deng, F., Wei, X., Chai, J., Knapp, J., et al. (2019). Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome* 7:109. doi: 10.1186/s40168-019-0721-7

Wang, Y., Zhang, H., Zhu, L., Xu, Y., Liu, N., Sun, X., et al. (2018). Dynamic distribution of gut microbiota in goats at different ages and health states. *Front. Microbiol.* 9:2509. doi: 10.3389/fmicb.2018.02509

Waters, J. L., and Ley, R. E. (2019). The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* 17:83. doi: 10.1186/s12915-019-0699-4

Weber, D. J., and Rutala, W. A. (2016). Bacillus species. *Infect. Control Hosp. Epidemiol.* 9, 368–373. doi: 10.2307/30145465

Weiler, E. S., Szabo, T. G., Garcia-Carpio, I., and Villunger, A. (2022). PIDD1 in cell cycle control, sterile inflammation and cell death. *Biochem. Soc. Trans.* 50, 813–824. doi: 10.1042/BST20211186

Williams, R. E. O., Hirch, A., and Cowan, S. T. (1953). Aerococcus, a new bacterial genus. *J. Gen. Microbiol.* 8, 475–480. doi: 10.1099/00221287-8-3-475

Xi, X., Cheng, J., Tian, Y., Zhang, J., Zhang, W., and Wu, R. (2011). Determination of growth curves and major organ weights in 3 SPF-grade mice. *Heilongjiang Anim. Husbandry Vet. Med.* 61, 136–138. doi: 10.13881/j.cnki.hljxmsy.2011.01.061

Yang, J., Wei, H., Zhou, Y., Szeto, C. H., Li, C., Lin, Y., et al. (2022). High-fat diet promotes colorectal tumorigenesis through modulating gut microbiota and metabolites. *Gastroenterology* 162, 135–149.e2. doi: 10.1053/j.gastro.2021.08.041

Yu, Y., Raka, F., and Adeli, K. (2019). The role of the gut microbiota in lipid and lipoprotein metabolism. *JCM* 8:2227. doi: 10.3390/jcm8122227

Yu, S., Zhang, G., Liu, Z., Wu, P., Yu, Z., and Wang, J. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. *BMC Microbiol.* 20:29. doi: 10.1186/s12866-020-1716-z

Zaki, M. E., Amr, K. S., and Abdel-Hamid, M. (2013). *APOA2* polymorphism in relation to obesity and lipid metabolism. *Cholesterol* 2013, 1–5. doi: 10.1155/2013/289481

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., et al. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Curr. Biol.* 26, 1873–1879. doi: 10.1016/j.cub.2016.05.012

Zhang, W., Zou, G., Li, B., Du, X., Sun, Z., Sun, Y., et al. (2020). Fecal microbiota transplantation (FMT) alleviates experimental colitis in mice by gut microbiota regulation. *J. Microbiol. Biotechnol.* 30, 1132–1141. doi: 10.4014/jmb.2002.02044

Zhou, L., Xiao, X., Zhang, Q., Zheng, J., Li, M., Yu, M., et al. (2018). Improved glucose and lipid metabolism in the early life of female offspring by maternal dietary Genistein is associated with alterations in the gut microbiota. *Front. Endocrinol.* 9:516. doi: 10.3389/fendo.2018.00516

frontiers | Frontiers in Microbiology

TYPE Systematic Review
PUBLISHED 09 May 2024
DOI 10.3389/fmicb.2024.1374774

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Ravina Kullar,
Expert Stewardship, Inc., United States
Changming Chen,
The Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, China

*CORRESPONDENCE
Najla A. Obaid
✉ naobaid@uqu.edu.sa

RECEIVED 22 January 2024
ACCEPTED 18 April 2024
PUBLISHED 09 May 2024

CITATION
Obaid NA (2024) Alternative treatment of recurrent *Clostridioides difficile* infection in adults by fecal transplantation: an overview of phase I–IV studies from Clinicaltrials.gov. *Front. Microbiol.* 15:1374774. doi: 10.3389/fmicb.2024.1374774

COPYRIGHT
© 2024 Obaid. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Alternative treatment of recurrent *Clostridioides difficile* infection in adults by fecal transplantation: an overview of phase I–IV studies from Clinicaltrials.gov

Najla A. Obaid*

Pharmaceutical Sciences Department, College of Pharmacy, Umm Al-Qura University, Makkah, Saudi Arabia

Background: Fecal microbiota transplantation (FMT) is an interventional approach to treat chronic and recurrent *Clostridioides difficile* infection (CDI). However, there is insufficient evidence regarding its effectiveness and safety. Clinical trials have been conducted to inspect the safety and effectiveness of FMT with and without comparison to pharmacological treatments.

Aim: This review explored the treatment of CDI in adults using FMT and evaluated the safety of this intervention based on phase I–IV studies registered on Clinicaltrials.gov.

Method: A comprehensive search of Clinicaltrials.gov was conducted to identify relevant studies that investigated CDI in adults. Data on study type, study design, sample size, intervention details, and outcomes related to FMT were examined and evaluated.

Results: In total, 13 clinical trials on FMT for CDI published through 17 November 2023 were identified, all of which were interventional studies. The investigation focused on both terminated and completed studies. Basic and advanced outcome measures were examined.

Conclusion: Some studies were terminated during phase II, and FMT was less effective than antibiotics such as vancomycin and fidaxomicin. However, colonoscopy and oral FMT were explored in several completed studies with promising results, but the evidence remains limited and inconclusive.

KEYWORDS

***Clostridioides difficile* infection, fecal microbiota transplant, full-spectrum microbiota, clinical trials, recurrent CDI (rCDI)**

1 Introduction

Clostridioides difficile infection (CDI) is a concerning global health issue, and the incidence of hospital-acquired CDI has substantially increased in recent years globally (Wiegand et al., 2012). CDI remains a major contributor to diarrhea in healthcare settings, even with established prevention and treatment protocols (Hensgens et al., 2014). CDI places a major economic burden on the healthcare system due to prolonged hospitalization and on patients due to comorbidities, repeated infections, extended length of stay, increased treatment costs, and indirect societal costs (Gupta and Ananthakrishnan, 2021).

In total, 30% of patients with CDI develop recurrence and are associated with 33% higher hazards of death (Olsen et al., 2015). The difficulty of CDI treatment illustrates the need to prevent recurrence and develop effective therapies.

Updated recommendations on CDI treatment and prevention have been issued by the Infectious Diseases Society of America and Society for Healthcare Epidemiology of America (IDSA), the American College of Gastroenterology, and the European Society of Clinical Microbiology and Infectious Diseases (Bainum et al., 2023). CDI treatment has progressed, with vancomycin and fidaxomicin being the primary treatments, whereas metronidazole is only recommended for non-severe cases when patients are unable to obtain or to be treated with oral vancomycin or fidaxomicin (Jarmo et al., 2020). Some studies concluded that fidaxomicin has advantages over vancomycin in terms of reducing recurrence and that fidaxomicin is cost-effective as a first-line therapy (Louie et al., 2011; Cornely et al., 2012; Beinortas et al., 2018; Mikamo et al., 2018). The University of Pittsburgh Medical Center released guidelines (2018) to use fidaxomicin for the first recurrence of CDI or subsequent recurrences (Bariola, 2019). However, fecal microbiota transplantation (FMT) has been recommended by many studies for treating CDI recurrence (Bariola, 2019; Jarmo et al., 2020). Recently, some FMT products were approved by the Food and Drug Administration (FDA) for treating recurrent CDI (Aschenbrenner, 2023; Jain et al., 2023). Strict clinical studies, good manufacturing processes, and donor and pathogen screening are important ways that this approach differs from live biotherapeutic products (LBPs) and traditional FMT (Monday et al., 2024). Donor banks are not established to guarantee that the donors' health is in good condition. To prevent CDI recurrence, the FDA approved a live fecal microbiota product called "Rebyota" (Aschenbrenner, 2023). This product was specifically approved after the failure of antibiotic treatment and the recurrence of CDI in adults. This product provides a potential solution to recurrent CDI following the failure of antibiotic treatment (Cornely et al., 2012). A novel FMT product named Vowst was also approved by the FDA as a prophylactic therapy to prevent the recurrence of CDI. Vowst consists of live fecal microbiota spores and works by re-establishing the gut microbiota and providing a better microbiome for the patient (Jain et al., 2023). Although an endoscopic technique is the recommended mode of delivery for FMT, limited research has examined the potential of frozen oral tablets for FMT. FMT formulated as frozen tablets or capsules is not inferior to FMT performed via colonoscopy (Jain et al., 2023). The approval of Vowst represented a major advancement in the prevention of recurrent CDI (Jain et al., 2023). There are uncertainties regarding the effectiveness of this product for CDI patients other than the participants in its clinical trials, and a prior study recommended that the donor screening process should be improved (Jain et al., 2023).

Approved FMT products are the recommended options for recurrent CDI. Current treatment options for CDI include vancomycin and fidaxomicin as the primary treatments (Jarmo et al., 2020). Metronidazole is reserved for mild cases, and bezlotoxumab, a monoclonal antibody against *C. difficile* toxin B, is used as an additional treatment for recurrent CDI (Jarmo et al., 2020). Fidaxomicin and FMT are more expensive but more effective in preventing recurrences, and thus, they are becoming the standard of care for CDI (Gupta and Ananthakrishnan, 2021). However, there are limited data on the preferred administration route of FMT, the cost-effectiveness of preventing recurrent FMT, and the use of unconventional therapies such as bezlotoxumab. This review explored the latest update on the prevention of CDI recurrence and the effectiveness of FMT treatment by analyzing the Clinicaltrials.gov dataset.

2 Methods

2.1 Search strategy

On 17 November 2023, a search was conducted on ClinicalTrials.gov to identify relevant studies using the single search term "*Clostridioides difficile* infection recurrence" for the disease or condition together with "fecal microbiota transplant."

2.2 Search results for the review

Clinical trials of any phase that used FMT as an observational measure were eligible for inclusion, and other trials were excluded (Figure 1).

2.3 Extracted data from the screened database

All study information, such as study title, study status, study type, intervention details, characteristics of the studies, and outcomes, was extracted from the database.

3 Results

3.1 The number of studies returned by the search

In total, 21 clinical trials were identified in the search. Of these, only 13 clinical trials of any phase that performed "fecal microbiota transplant" in adults for recurrent CDI were included in this review.

3.2 The characteristics of the clinical trials

These 13 studies mainly assessed recurrent CDI in patients aged 18 years and older, including elderly patients. Only six trials in the registry were completed studies with results. These completed studies included 1,164 participants in total. The remaining trials were terminated for several reasons, such as administrative reasons, a lack of available funds for follow-up, ineffectiveness, and the investigators no longer considering FMT products for CDI. Completed details are presented in Table 1.

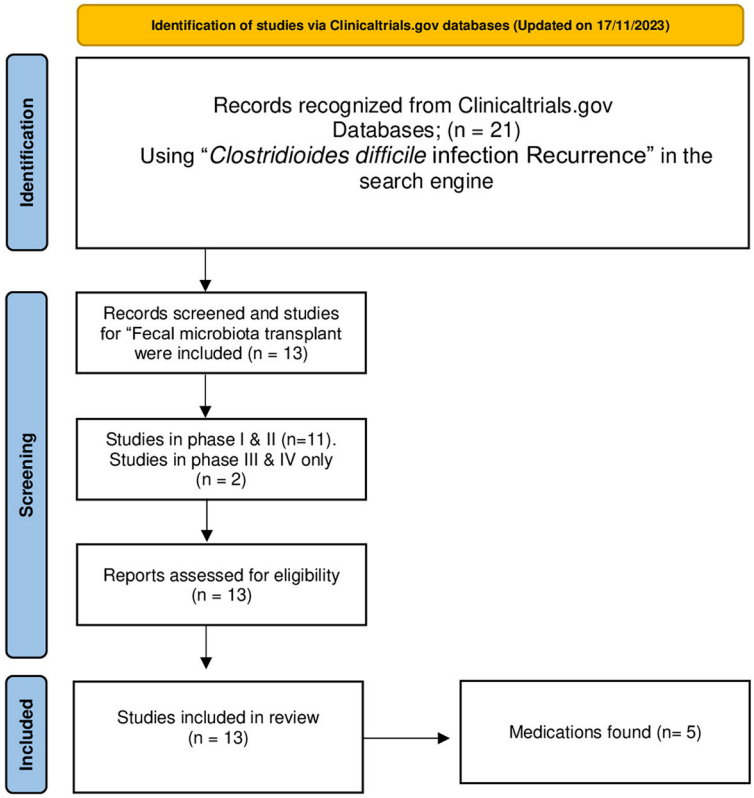


FIGURE 1
PRISMA flow chart of the identification, screening, and inclusion of studies in this review. Adopted from Page et al. (2021).

3.3 Outcome measures

Most of the clinical trials focused on the clinical resolution of symptoms. The primary outcomes included diarrhea, abdominal pain, length of hospital stay (90 days), mortality within 90 days, ≤4 stools per day for 2 days with no stool categorized as type 7 on the Bristol Stool Scale, no positive result on enzyme immunoassay for *C. difficile* toxin after treatment, no recurrence of the infection within 8 weeks after the transplant, and no additional antibiotic prescription. The secondary outcomes included the evaluation of the safety of FMT and the absence of adverse effects; measurement of serious adverse events (SAEs), including death or life-threatening events; prolonged hospitalization; and significant incapacity of normal life function. Only two studies included a placebo group for comparison, and one study compared FMT to antibiotic treatment. A complete list is provided in Table 2.

3.4 Success rate and the safety of FMT

FMT was performed using different formulations, as presented in Table 3, together with a description of the success rate and safety (mortality and SAEs). The success rate varied between the primary outcomes measured in these studies. Some studies focused on the absence of CDI recurrence within 8 or 24 weeks. Other studies examined the occurrence of symptoms such as nausea, vomiting, and constipation. Many studies recorded a mortality rate of 0%. All studies reported SAEs, excluding the one that did not measure SAEs.

4 Discussion

Disruption of the intestinal microbiome contributes to many human conditions and symptoms (Weiss and Hennet, 2017). Recurrence of CDI is a serious condition caused by disruption of the intestinal microbiome because of the use of antibiotics (Seekatz et al., 2022). Hospitalized patients who have been prescribed antibiotics for a long period have a high risk of *C. difficile* infection, and *C. difficile* toxins cause abdominal pain and diarrhea (Goldberg et al., 2015). Antibiotics prescribed as standard treatments for both acute and recurring illnesses do not treat abdominal syndromes such as dysbiosis; in fact, they frequently make them worse (Seekatz et al., 2022). Monoclonal antibodies, such as bezlotoxumab, can reduce the risk of recurrences, but they do not treat the underlying dysbiosis (Wilcox et al., 2017; Seekatz et al., 2022). They are recommended to be used in conjunction with standard-of-care antibiotics for preventing recurrent CDI in patients at high risk of recurrence. FMT is a developing therapeutic approach in which the gut microbiome of a recipient is restored by introducing bacteria from a healthy donor's stool into the recipient's gut. There are several FMT preparation methods and routes of administration. The effectiveness of FMT is considered to vary between different

TABLE 1 The characteristics of the clinical trials (from ClinicalTrials.gov 17th November 2023).

	Title	Status	Conditions	Intervention	Phase	*n*
1	Penn Microbiome Therapy for Recurrent Clostridium Difficile Infection	Terminated	Recurrent *Clostridium difficile* Infection	• Penn Microbiome Therapy – 001 • Penn Microbiome Therapy – 002 • Penn Microbiome Therapy - 003	2	9
2	FMT Versus Antimicrobials for Initial Treatment of Recurrent CDI	Terminated	*Clostridium difficile* Infection	Biological FMT and antimicrobial	2	6
3	A Prospective Trial of Frozen-and-Thawed Fecal Microbiota Transplantation for Recurrent Clostridium Difficile Infection	Terminated	*Clostridium difficile*	Frozen fecal microbiota	2	140
4	Dose Ranging Study of the Safety and Efficacy of Orally Administered Lyophilized Fecal Microbiota Product (PRIM-DJ2727) for the Treatment of Recurrent Clostridium Difficile Infection (CDI)	Terminated	Recurrent C. *difficile* Infection	Low (collected from 50-g of stool for 2 consecutive days), mild (collected from 100-g of stool on the 1st day and then from 50 g of stool on the consecutive day) and high (collected from 100-g of stool for 2 consecutive days) fecal microbiota dose	2	17
5	Safety of FMT: OpenBiome Outcomes and Longitudinal Follow-up (STOOL) for Recurrent Clostridium Difficile Infection	Terminated	*Clostridium difficile*	Fecal microbiota preparation (frozen processed fecal material)	2	17
6	Microbiota Restoration Therapy for Recurrent Clostridium Difficile Infection (PUNCHCD2)	Completed	Enterocolitis *Clostridium difficile* Recurrent	RBX2660 (microbiota suspension)	2	150
7	Efficacy, Safety, and Tolerability Study of Oral Full-Spectrum MicrobiotaTM (CP101) in Subjects With Recurrent C. Diff (PRISM3)	Completed	*Clostridium difficile* Infection recurrent	Full-spectrum microbiota capsule	2	206
8	Microbiota Restoration Therapy for Recurrent *Clostridium difficile* Infection	Completed	*Clostridium difficile* Infection	Rebiotix RBX2660 (microbiota suspension)	2	272
9	The ICON Study: Outcomes After FMT for Patients With IBD and CDI	Completed	Inflammatory bowel diseases, *Clostridium difficile* Infection	Fecal microbiota transplantation	1 and 2	50
10	Open-Label Extension of CP101 Trials Evaluating Oral Full-Spectrum Microbiota^{TM} (CP101) in Subjects With Recurrence of Clostridium Difficile Infection (PRISM-EXT)	Completed	*Clostridium difficile* Infection Recurrent *Clostridium difficile* Infection	Full-spectrum microbiota	2	132
11	Microbiota Restoration Therapy for Recurrent Clostridium Difficile-associated Diarrhea (PUNCH CD)	Completed	Recurrent *Clostridium difficile* Infection	RBX2660 (microbiota suspension)	2	34
12	A Trial of CP101 for the Prevention of Recurrent CDI (PRISM4) (PRISM4)	Terminated	Recurrent *Clostridium difficile* Infection	CP101 (an investigational microbiome therapeutic designed to deliver a complete and functional microbiome)	3	19
13	Microbiota Restoration Therapy for Recurrent Clostridium Difficile Infection (PUNCHCD3)	Completed	*Clostridium difficile* Infection (CDI)	RBX2660 microbiota suspension	3	320

n, number of participants.

routes (Gough et al., 2011). The oldest technique followed for FMT is the direct infusion of donor stool through colonoscopy, and it is considered to be simple, safe, and 92% effective in the treatment of recurrent CDI (Kelly et al., 2012). Other FMT administration methods include nasogastric or nasoenteric tube and enema (Brandt and Aroniadis, 2013). Several barriers to the use of FMT have emerged, such as determining the features of a healthy microbiome, assuring the receiver's safety in terms of long-term consequences, sufficiently monitoring the recipient of the fecal material, and attaining high-quality control (Kim and Gluck, 2019). Therefore, the preferred administration method and the route of FMT depend on overcoming these barriers to ensure treatment efficacy.

FMT delivered via intestinal suspensions is reported to provide a high rate of symptom resolution, reaching 92% in some studies (Gough et al., 2011). Most of the clinical trials included in this review were performed using fecal suspensions delivered via enema and colonoscopy. RBX2660 is one of the FMT preparations used in the clinical trials described in this review (Table 2), and it was approved by the FDA under the name Rebyota (Kim et al., 2023).

TABLE 2 Outcome measures with the prevention of recurrent CDI with FMT (from ClinicalTrials.gov 17th November 2023).

Product used	Outcome measures	The presence of the placebo group
FMT G3 capsules	- A comparison between the stool microbiome with and without FMT administration - The number of FMT pills administered after the completion of a course of oral vancomycin. - The emergence of adverse events - The incidence of gastrointestinal symptoms - CDI recurrence within 60 days - The number of hospital readmissions	
50 g of fecal material suspended in bacteriostatic normal saline and glycerol	- 90 days of clinical resolution of symptoms (diarrhea and abdominal pain) - Patients' hospital length of stay after 90 days of transplantation. - Re-admission of patients to the hospital for recurrent CDI - Mortality rate	
1 enema RBX2660 (microbiota suspension) A suspension of intestinal microbes	- The absence of the syptoms at 56 days after FMT - Successful treatment between the groups (I enema of RBX2660 and 1 enema of placebo) - Validated SF-36 scale was used by the study to identify the changes to quality of life. - Time to CDI recurrence between groups using Kaplan–Meier analysis	Yes
Frozen fecal microbiota kept at−20°C and thawed prior treatment	- No CDI recurrence within 13 weeks - The evaluation of the safety of FMT for any serious adverse event up to 13 weeks	
Single and two doses of Penn Microbiome Therapy (PMT) PMT-001 PMT-002 PMT-003	- The number of patients with clinical resolution of diarrhea within 8 weeks - Colectomy within 30 days after FMT - Hospital length stay within 30 days of FMT - The number of readmissions to hospital within 60 days - Mortality rate (following 30 days then 90 days of FMT)	
Frozen processed human fecal material	- Measuring for absence or presence of serious adverse events post 6 weeks of FMT - Measuring for absence or presence of serious adverse events post 6 weeks to 1 year of FMT	
Low, mid, and high fecal microbiota dose	- Safety assessed by the number of participants with nausea, vomiting, diarrhea, bloating, and constipation post FMT - The number of participants with recurrent CDI	
CP101 Full spectrum microbiota capsule	- The number of participants with absence of recurrence more than 8 weeks - The number of participants with occurrence of treatment adverse event	Yes
Fecal microbiota transplantation	- Recurrence of CDI within 8 weeks - Positive stool testing for *C. difficile* via polymerase chain reaction	

TABLE 3 Successful rate and indication for safety (from ClinicalTrials.gov 17th November 2023).

Product used	Percentage of successful for FMT	Indications for safety
1 enema RBX2660 (microbiota suspension)	55.6% with overall efficacy (88.8%) (Dubberke et al., 2018)	Mortality 16.67%
		SAE 42.86%
50 g of the fecal material suspended in bacteriostatic normal saline and glycerol	50%	Mortality 0%
Frozen fecal microbiota kept at −20°C and thawed prior to treatment	89.47%	Mortality 8.3%
		SAE 12.03%
Single and two doses of Penn Microbiome Therapy (PMT) PMT-001 PMT-002 PMT-003	20% for a single PMT 50% for two PMT	Mortality 0% SAE for single PMT 60% SAE for two PMT 75%
Fecal microbiota preparation (frozen processed fecal material)	Not specified	Mortality 0%
		SAE 26.67%
Low, mid, and high fecal microbiota dose	Safety assessed by the percentage of participants with nausea (66.7% mid FMT and 12.5% high FMT), vomiting (33.3% mid FMT), diarrhea (25% low FMT and 25% high FMT), bloating (25% high FMT), and constipation (12.5% FMT).	Mortality 0%
	Recurrent CDI (50% low FMT and 25% high FMT)	SAE low FMT 25%
CP101 Full-spectrum microbiota capsule	The absence of recurrent CDI in week 8: 74.5%	Mortality 0.96%
	The absence of recurrent CDI in week 24: 73.5%	SAE 15.38%
Full spectrum microbiota	The absence of recurrent CDI in week 8: 80%	Mortality 0.76%
	The absence of recurrent CDI in week 24: 78.8%	SAE 12.88%
Fecal microbiota transplantation	FMT failure within 8 weeks 8.2%	Mortality 0%
	Colonized with *C.difficile* 10.2%	SAE 6%

SAE, serious adverse event.

The potential of this preparation to restore the microbiome can alleviate CDI recurrence, and it was more effective than the placebo in terms of restoring the microbiome (Blount et al., 2019). The standardization of the material for FMT preparation from the donor can significantly simplify the clinical use of FMT for recurrent CDI (Hamilton et al., 2012). The transplant material is prepared from a donor stool from two sources: current patients

and universal donors from a stool bank (Edelstein et al., 2016). The major drawback in the use of FMT for recurrent CDI is the lack of standardization regarding the preparation process and administration techniques (Berry and Khanna, 2023). In addition, patients who have completed antibiotic therapy for recurrent CDI and who are 18 years and older can receive Rebyota as a prophylactic biotherapeutic treatment (Berry and Khanna, 2023). It should be taken rectally and needs to be administered only once (Kim et al., 2023).

FMT can also be performed using prepared capsules. FMT capsule formulations combine the ease of administration of an antibiotic with the efficacy of FMT for treating recurrent CDI (Varga et al., 2021). One study in this review (Table 2) examined FMT performed using prepared capsules, and two studies delivered a full-spectrum microbiota using an oral formulation (CP101). One study was terminated in phase III, and the primary outcomes were not analyzed because no patients completed treatment (Table 1). Another study using oral CP101 was completed, with the primary outcome being achieved in 80.3% of patients (Table 3). The third study using CP101 presented some results assessing its efficacy (primary outcome) and safety (secondary outcome) in subjects with recurrent CDI (Table 2). Based on the percentage of participants with adverse events after oral FMT, safety was not achieved in comparison with the placebo, and many adverse events from recurrent CDI were observed. The Penn microbiome therapy for recurrent CDI was examined in a terminated clinical trial that administered three preparations (the Penn microbiome therapy 001 [enema product], the Penn microbiome therapy 002 [suspension product], and the Penn microbiome therapy 003 [capsule product]), as detailed in Tables 1, 2. This study observed a low rate of clinical resolution with one or two doses. However, the mortality rate was 0% (Table 3). From these results, the achievement of the primary and secondary outcomes was inconsistent for oral FMT preparations, and further clinical trials are needed.

Regarding the cost-effectiveness of preventing CDI recurrence, FMT performed via colonoscopy was identified as the most cost-effective approach in one clinical trial included in this review (Konijeti et al., 2014). The study recorded cure rates exceeding 88.4% and CDI recurrence rates lower than 14.9% with colonoscopy-based FMT compared with antibiotics (Konijeti et al., 2014). This finding was also suggested in a study published nearly 10 years before this review. The recently approved orally administered FMT named Vowst was examined in a clinical trial (SER-109) and then received FDA approval on 26 April 2023 (Jain et al., 2023). This product is non-invasive and more patient-friendly, and it minimizes the risk of iatrogenic consequences (Jain et al., 2023). Regardless of the number of past CDIs by age or antibiotic type (vancomycin or fidaxomicin), a remarkable clinical response was observed at week 8 in 91.3% of patients receiving Vowst, and the rate increased to 94.6% at week 24 (Khanna et al., 2022). Moreover, 87.6% of patients in the Vowst group were free from recurring CDI at the end of 8 weeks in comparison to 60.2% of participants in the placebo group, indicating that Vowst decreased CDI recurrence. Along with having a better safety profile, Vowst also caused comparatively mild-to-moderate transient side effects. The rigorous inclusion criteria used in the clinical trials (phase 1) raise some uncertainties about the specific efficacy of Vowst in patients with CDI outside those who participated in the trials (McGovern et al., 2021). For instance, patients with cancer, those requiring additional antibacterial medication (surgical prophylaxis and urinary tract infections), and those with a history of inflammatory bowel disease were excluded, as were female participants who were pregnant, nursing, or lactating (Jain et al., 2023). Regarding the safety profile, mild-to-moderate adverse effects were noted in a phase III trial (McGovern et al., 2021). We can conclude that the better safety profile along with a low number of FMT courses (either coloscopy or oral) can be considered more cost-effective than expensive antibiotics. However, economic analyses need to be conducted to explore the benefit and effectiveness of oral capsule formulations for FMT (Jain et al., 2023), as the cost-effectiveness of Rebyota and Vowst was not examined in their clinical trials.

This review had several limitations. Because the variables linked to the FMT technique were inconsistently classified across trials, operational definitions were established beforehand to facilitate data abstraction. Limited clinical trials progressed to completion, and from these publications, data on the techniques of FMT and cost-effectiveness were not sufficiently recorded. In addition, three trials were terminated without generating data because no patients could be analyzed.

5 Conclusion

Based on the records of FMT treatment for recurrent CDI from ClinicalTrials.gov, the completed clinical trials recorded high clinical resolution rates of CDI symptoms with mild-to-moderate SAEs but extremely low mortality rates. However, resolution rates can be affected by variations in the FMT process. Colonoscopy in the earliest studies achieved a high success rate, and even higher success rates were achieved in later trials using oral formulations for full-spectrum FMT. Furthermore, the data indicate that, in cases in which conventional therapies have failed, FMT using FDA-approved products could be an extremely safe and effective treatment for recurrent CDI. These methods proved to be differentially effective, suggesting that personalized approaches to FMT may enhance its success rate. Moreover, several studies highlighted the potential cost-effectiveness of FMT, which is a significant consideration given the economic burden of CDI. The analysis encompassed a diverse range of interventional studies, which demonstrated a notable variance in success rates, reflecting the heterogeneity of FMT applications and patient demographics. Despite these variations, the collective data highlight a trend toward positive outcomes with the use of FMT in the management of CDI.

In conclusion, the analyzed database substantiates that FMT is a safe and effective treatment for recurrent CDI. It highlights the potential for FMT to be incorporated more prominently into clinical practice as a therapeutic strategy against CDI. Future research should focus on standardizing FMT procedures, optimizing delivery methods, and monitoring for long term to fully exploit its therapeutic benefits and ensure the consistent safety and effectiveness of FMT.

Data availability statement

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

Author contributions

NO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Aschenbrenner, D. S. (2023). New treatment to prevent recurring *Clostridioides difficile* infections. *AJN* 123, 16–17. doi: 10.1097/01.NAJ.0000944904.20242.ae

Bainum, T. B., Reveles, K. R., Hall, R. G., and Cornell, K. (2023). Controversies in the prevention and treatment of clostridioides difficile infection in adults: a narrative review. *Microorganisms* 11:387. doi: 10.3390/microorganisms11020387

Bariola, J. R. (2019). "1987. Impact of updated IDSA *Clostridium difficile* Guidelines on the use of Fidaxomicin in a Large Health System," in *Open Forum Infectious Diseases* (US: Oxford University Press), S666. doi: 10.1093/ofid/ofz360.1667

Beinortas, T., Burr, N. E., and Wilcox, M. H. (2018). Comparative efficacy of treatments for *Clostridium difficile* infection: a systematic review and network meta-analysis. *Lancet Infect. Dis.* 18, 1035–1044. doi: 10.1016/S1473-3099(18)30285-8

Berry, P., and Khanna, S. (2023). Recurrent *Clostridioides difficile* infection: current clinical management and microbiome-based therapies. *BioDrugs* 37, 757–773. doi: 10.1007/s40259-023-00617-2

Blount, K. F., Shannon, W. D., and Deych, E. (2019). "Restoration of bacterial microbiome composition and diversity among treatment responders in a phase 2 trial of RBX2660: an investigational microbiome restoration therapeutic," in *Open Forum Infectious Diseases* (US: Oxford University Press), ofz095. doi: 10.1093/ofid/ofz095

Brandt, L. J., and Aroniadis, O. C. (2013). An overview of fecal microbiota transplantation: techniques, indications, and outcomes. *Gastrointest. Endosc.* 78, 240–249. doi: 10.1016/j.gie.2013.03.1329

Cornely, O. A., Crook, D. W., Esposito, R., Poirier, A., Somero, M. S., Weiss, K., et al. (2012). Fidaxomicin versus vancomycin for infection with *Clostridium difficile* in Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial. *Lancet Infect. Dis.* 12, 281–289. doi: 10.1016/S1473-3099(11)70374-7

Dubberke, E. R., Lee, C. H., Orenstein, R., Khanna, S., Hecht, G., Gerding, D. N., et al. (2018). Results from a randomized, placebo-controlled clinical trial of a RBX2660-A microbiota-based drug for the prevention of recurrent *Clostridium difficile* infection. *Clin. Infect. Dis.* 67, 1198–1204. doi: 10.1093/cid/ciy259

Edelstein, C., Daw, J. R., and Kassam, Z. (2016). Seeking safe stool: Canada needs a universal donor model. *CMAJ* 188, E431–E432. doi: 10.1503/cmaj.150672

Goldberg, E. J., Bhalodia, S., Jacob, S., Patel, H., Trinh, K. V., Varghese, B., et al. (2015). *Clostridium difficile* infection: a brief update on emerging therapies. *Am. J. Health-Syst. Phar.* 72, 1007–1012. doi: 10.2146/ajhp140645

Gough, E., Shaikh, H., and Manges, A. R. (2011). Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Clin. Infect. Dis.* 53, 994–1002. doi: 10.1093/cid/cir632

Gupta, A., and Ananthakrishnan, A. N. (2021). Economic burden and cost-effectiveness of therapies for *Clostridiodes difficile* infection: a narrative review. *Therap. Adv. Gastroenterol.* 14:17562848211018654. doi: 10.1177/17562848211018654

Hamilton, M. J., Weingarden, A. R., and Sadowsky, M. J. (2012). Standardized frozen preparation for transplantation of fecal microbiota for *Recurrentclostridium difficileinfection*. *ACG* 107, 761–767. doi: 10.1038/ajg.2011.482

Hensgens, M. P. M., Dekkers, O. M., Demeulemeester, A., Buiting, A. G. M., Bloembergen, P., van Benthem, B. H. B., et al. (2014). Diarrhoea in general practice: when should a *Clostridium difficile* infection be considered? Results of a nested case-control study. *Clin. Microbiol. Infect.* 20, O1067–O1074. doi: 10.1111/1469-0691.12758

Jain, N., Umar, T. P., Fahner, A. F., and Gibietis, V. (2023). Advancing therapeutics for recurrent clostridioides difficile infections: an overview of vowst's FDA approval and implications. *Gut Microb.* 15:2232137. doi: 10.1080/19490976.2023.2232137

Jarmo, O., Veli-Jukka, A., and Eero, M. (2020). Treatment of *Clostridioides (Clostridium) difficile* infection. *Ann. Med.* 52, 12–20. doi: 10.1080/07853890.2019.1701703

Kelly, C. R., Leon, d. e., Jasutkar, L., and Fecal, N. (2012). microbiota transplantation for relapsing *Clostridium difficile* infection in 26 patients: methodology and results. *J. Clin. Gastroenterol.* 46, 145–149. doi: 10.1097/MCG.0b013e318234570b

Khanna, S., Sims, M., Louie, T. J., Fischer, M., LaPlante, K., Allegretti, J., et al. (2022). SER-109: An oral investigational microbiome therapeutic for patients with recurrent *Clostridioides difficile* infection (rCDI). *Antibiotics* 11:1234. doi: 10.3390/antibiotics11091234

Kim, K., Kang, M., and Cho, B. K. (2023). Systems and synthetic biology-driven engineering of live bacterial therapeutics. *Front. Bioeng. Biotechnol.* 11:1267378. doi: 10.3389/fbioe.2023.1267378

Kim, K. O., and Gluck, M. (2019). Fecal microbiota transplantation: an update on clinical practice. *Clin. Endosc.* 52, 137–143. doi: 10.5946/ce.2019.009

Konijeti, G. G., Sauk, J., Shrime, M. G., and Gupta, M. (2014). Cost-effectiveness of competing strategies for management of recurrent *Clostridium difficile* infection: a decision analysis. *Clin. Infect. Dis.* 58, 1507–1514. doi: 10.1093/cid/ciu128

Louie, T. J., Miller, M. A., Mullane, K. M., Weiss, K., Lentnek, A., Golan, Y., et al. (2011). Fidaxomicin versus vancomycin for *Clostridium difficile* infection. *Engl. J. Med.* 364, 422–431. doi: 10.1056/NEJMoa0910812

McGovern, B. H., Ford, C. B., Henn, M. R., Pardi, D. S., Khanna, S., Hohmann, E. L., et al. (2021). SER-109, an investigational microbiome drug to reduce recurrence after *Clostridioides difficile* infection: lessons learned from a phase 2 trial. *Clin. Infect. Dis.* 72, 2132–2140. doi: 10.1093/cid/ciaa387

Mikamo, H., Tateda, K., Yanagihara, K., Kusachi, S., Takesue, Y., Miki, T., et al. (2018). Efficacy and safety of fidaxomicin for the treatment of *Clostridioides (Clostridium) difficile* infection in a randomized, double-blind, comparative phase III study in Japan. *J. Infect. Chemother.* 24, 744–752. doi: 10.1016/j.jiac.2018.05.010

Monday, L., Tillotson, G., and Chopra, T. (2024). Microbiota-based live biotherapeutic products for *Clostridioides difficile* infection- the devil is in the details. *Infect. Drug Resist.* 17, 623–639. doi: 10.2147/IDR.S419243

Olsen, M. A., Yan, Y., Reske, K. A., and Zilberberg, M. D. (2015). Recurrent *Clostridium difficile* infection is associated with increased mortality. *Clin. Microbiol. Infect.* 21, 164–170. doi: 10.1016/j.cmi.2014.08.017

Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., et al. (2021). The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ* 372:n71. doi: 10.1136/bmj.n71

Seekatz, A. M., Safdar, N., and Khanna, S. (2022). The role of the gut microbiome in colonization resistance and recurrent *Clostridioides difficile* infection. *Therap. Adv. Gastroenterol.* 15:17562848221134396. doi: 10.1177/17562848221134396

Varga, A., Kocsis, B., Sipos, D., Kása, P., Vigvári, S., Pál, S., et al. (2021). How to apply FMT more effectively, conveniently and flexible–a comparison of FMT methods. *Front. Cell. Infect. Microbiol.* 11:657320. doi: 10.3389/fcimb.2021.657320

Weiss, G. A., and Hennet, T. (2017). Mechanisms and consequences of intestinal dysbiosis. *Cell. Molec. Life Sci.* 74, 2959–2977. doi: 10.1007/s00018-017-2509-x

Wiegand, P. N., Nathwani, D., Wilcox, M. H., Stephens, J., Shelbaya, A., Haider, S., et al. (2012). Clinical and economic burden of *Clostridium difficile* infection in Europe: a systematic review of healthcare-facility-acquired infection. *J. Hospital Infect.* 81, 1–14. doi: 10.1016/j.jhin.2012.02.004

Wilcox, M. H., Gerding, D. N., Poxton, I. R., Kelly, C., Nathan, R., Birch, T., et al. (2017). Bezlotoxumab for prevention of recurrent *Clostridium difficile* infection. *New Engl. J. Med.* 376, 305–317. doi: 10.1056/NEJMoa1602615

frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 10 June 2024
DOI 10.3389/fmicb.2024.1390046

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Mahmoud Al-Khrasani,
Semmelweis University, Hungary
Pingchuan Ma,
Washington University in St. Louis, United States

*CORRESPONDENCE
Bo Hua
✉ ysb75hb@163.com
Zhiheng Liu
✉ zhiheng_liu_tongji@163.com

RECEIVED 22 February 2024
ACCEPTED 24 May 2024
PUBLISHED 10 June 2024

CITATION
Gong Z, Xue Q, Luo Y, Yu B, Hua B and Liu Z (2024) The interplay between the microbiota and opioid in the treatment of neuropathic pain.
Front. Microbiol. 15:1390046.
doi: 10.3389/fmicb.2024.1390046

COPYRIGHT
© 2024 Gong, Xue, Luo, Yu, Hua and Liu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The interplay between the microbiota and opioid in the treatment of neuropathic pain

Zexiong Gong[1], Qingsheng Xue[2], Yan Luo[2], Buwei Yu[2], Bo Hua[1]* and Zhiheng Liu[1]*

[1]Department of Anesthesiology, Health Science Center, Shenzhen Second People's Hospital/The First Affiliated Hospital of Shenzhen University, Shenzhen, China, [2]Department of Anesthesiology, School of Medicine, Ruijin Hospital, Shanghai Jiaotong University, Shanghai, China

Neuropathic pain (NP) is characterized by its complex and multifactorial nature and limited responses to opioid therapy; NP is associated with risks of drug resistance, addiction, difficulty in treatment cessation, and psychological disorders. Emerging research on gut microbiota and their metabolites has demonstrated their effectiveness in alleviating NP and augmenting opioid-based pain management, concurrently mitigating the adverse effects of opioids. This review addresses the following key points: (1) the current advances in gut microbiota research and the challenges in using opioids to treat NP, (2) the reciprocal effects and benefits of gut microbiota on NP, and (3) the interaction between opioids with gut microbiota, as well as the benefits of gut microbiota in opioid-based treatment of NP. Through various intricate mechanisms, gut microbiota influences the onset and progression of NP, ultimately enhancing the efficacy of opioids in the management of NP. These insights pave the way for further pragmatic clinical research, ultimately enhancing the efficacy of opioid-based pain management.

KEYWORDS

gut microbiota, gut-brain axis, neuropathic pain, opioids, pain

1 Introduction

The pathological pain resulting from damage or diseases that affects the somatosensory nervous system is called neuropathic pain (NP) (Jensen et al., 2011). NP is often accompanied by neuronal damage, and accumulating experimental evidence has highlighted the involvement of various non-neuronal cells, including glial cells, regulatory T cells, and tumor cells, as pivotal contributors to the onset of pain (Fiore et al., 2023). In this article, we describe the close interplay between gut microbiota and NP and consider the therapeutic potential of utilizing gut microbiota to augment the management of NP, particularly within the context of opioid therapy.

Recent findings in epidemiological and genomic research, coupled with cellular and animal experiments, underscore the profound influence of the microbiome on human health, acting as a mediator or modifier of environmental variables. Most of these microorganisms reside in the gastrointestinal tract, where they synthesize and modify an array of metabolites and chemical compounds. Predominantly classified into the phyla Firmicutes and Bacteroidetes, these microorganisms constitute the principal gut microbiota populations (Zádori et al., 2023). They intricately participate in modulating various physiological functions within the human body, including neuroinflammatory responses and other vital physiological

processes (Belkaid and Harrison, 2017; Fan and Pedersen, 2021; Fiore et al., 2023). The development of the gut microbiota system is a gradual and intricate process beginning in infancy (Schmidt et al., 2018; Cassidy-Bushrow et al., 2023; Ruan et al., 2023). Its makeup can change over time, influenced by factors such as age, dietary habits, and prolonged medication use (Minerbi et al., 2019; Fan and Pedersen, 2021). Due to the heterogeneity of external perturbations, the rate of change in the gut microbiota varies among individuals (Flores et al., 2014). Empirical evidence consistently demonstrates that the composition of gut microbiota exhibits notable stability, with the ability to return to its baseline profile after disturbances (Dogra et al., 2020). The advent of genome sequencing technologies has significantly expedited the progress of gut microbiota research (Jovel et al., 2016). In recent years, whole-genome sequencing has unveiled the association between gut microbiota and a diverse spectrum of diseases. Initially, distinct microbial profiles were identified in the progression of gastrointestinal disorders (Barko et al., 2018). Subsequently, deviations in gut microbiota composition have been identified as playing a role inconditions spanning metabolism-related disorders, tumors, cardiovascular diseases, and pain-related disorders (Minerbi and Shen, 2022). In various forms of pain, including NP, distinctive gut microbiota signatures have been observed (Guo et al., 2019; Pane et al., 2022).

For a long time, predominant approaches to treating NP have targeted the central nervous system (CNS). Opioid medications, including morphine, tramadol, and hydrocodone have emerged as the cornerstone of clinical intervention (Balanaser et al., 2023). The escalating use of opioid medications is attributable to their excellent pain-relieving effects (Xu et al., 2022). Despite their effectiveness in helping manage moderate to severe pain, opioids often exhibit suboptimal analgesic efficacy in the treatment of NP (Martínez-Navarro et al., 2019). Classic opioids such as morphine and hydrocodone have shown limited effectiveness in this context, with clinical trials reporting unsatisfactory levels of efficacy and safety (Martínez-Navarro et al., 2019). Prolonged opioid therapy frequently leads to tolerance, dependence, and gastrointestinal adverse effects, leaving a growing number of patients unable to attain satisfactory pain relief within tolerable dose ranges. Encouragingly, a growing body of literature suggests that gut microbiota can effectively mitigate the adverse effects of opioids and contribute to the treatment of NP (Fiore et al., 2023). It is critical to note the bidirectional relationship between gut microbiota and opioids in NP treatment. Gut microbiota influence the analgesic effects of opioids, while opioids, in turn, induce alterations in the gut microenvironment (Wang et al., 2018; Zádori et al., 2023). Therefore, the gut microbiota emerges as a critical target for optimizing opioid therapy in the treatment of NP.

2 Gut microbiota in neuropathic pain

2.1 The gut-brain axis

The gut microbiota has long been considered for its beneficial impact on intestinal motility (Dey et al., 2015), local immune function (Cai et al., 2022), and visceral pain perception (Luczynski et al., 2017; Aguilera et al., 2021). Recent evidence has further elucidated its regulatory influence on organs beyond the gastrointestinal tract, most notably its association with the nervous system (Thaiss, 2023). The concept of the gut-brain axis was first proposed in the 1880s, subsequently undergoing continuous refinements (Russo et al., 2018). The gut-brain axis constitutes a complex bidirectional communication network (Cussotto et al., 2018; Russo et al., 2018). The gut microbiota and its metabolites, such as γ- aminobutyric acid (GABA), serotonin, bile acids, and amino acids, directly influence the nervous system, and indirectly mediate cell interactions through the regulation of the vagus nerve (Figure 1). The gut-brain axis is believed to be closely linked to a range of disorders, including depression (Foster, 2022), Alzheimer's disease (Nguyen and Endres, 2022), and autism spectrum disorders (Coccurello et al., 2022). NP, the focus of this article, has also been extensively reported to be robustly connected with the gut-brain axis. Notably, mediators derived from gut microbiota exert their effects by influencing the dorsal root ganglion (DRG) and specific T cells, thereby modulating neuronal excitability and nociception (Guo et al., 2019). Furthermore, the enteric nervous system (ENS), often referred to as the "second brain," comprises the submucosal plexus, myenteric plexus, and interstitial cells of Cajal. It facilitates bidirectional communication with the CNS, transmitting impulses on both directions (Morreale et al., 2022). While typically under the regulatory control of the CNS, the ENS can also function independently, a process involving interactions between enteric glial cells and immune regulation (Dora et al., 2021).

At the dawn of the 21st century, propelled by technological advancements in computational power and data processing capabilities, research in the field of metabolomics has accelerated substantially. These advancements in metabolomics have significantly enhanced our understanding of the gut microbiota, thereby enriching the landscape of gastrointestinal microbiome studies. It has been discovered that the activation of Toll-like receptors by lipopolysaccharides (LPS) plays an important role in the nervous system (Araldi et al., 2023), involving neuroimmune responses and the genesis of pain. Derived from gut Gram-negative bacteria, LPS gains access to the spinal cord through the bloodstream (Shen et al., 2017), subsequently activating Toll-like receptors (TLR) to partake in NP processing (Lin et al., 2022). Additionally, short-chain fatty acids (SCFAs), metabolic byproducts of the gut microbiota, play a critical role in microglial development (Erny et al., 2015). Multiple studies have demonstrated the key contributions of sphingolipids and polysaccharides produced by fragile rod-shaped bacteria in processes such as myelin formation, neuroinflammation, and the development of chronic pain (Mao et al., 2013; An et al., 2014; Joscelyn and Kasper, 2014; Blaho et al., 2015). These findings affirm the close connection between the CNS, microglia, and the gut microbiota.

At the same time, regarding tryptophan metabolism direction. Research evidence indicates that serotonin and quinolinic acid affect mood via the tryptophan metabolic pathway. In instances of intestinal inflammation, increased levels of quinolinic acid may contribute to anxiety (Kelly et al., 2016). Another crucial compound produced by a healthy gut microbiota is glutamate, which constitutes a vital link in the gut-brain axis (Zhang et al., 2019a). Mice subjected to acute stress-induced dysbiosis of the gut microbiota (due to antibiotic (ABX) treatment), exhibit a notable reduction in hippocampal N-Methyl-D-Aspartate (NMDA) and Brain-Derived neurotrophic factor (BDNF) levels, which can be restored by gut microbiota supplementation (Sudo et al., 2004; Gronier et al., 2018; Jiang et al., 2019). Furthermore, the gut microbiota directly engages in the regulation of certain neurotransmitters, such as GABA (Strandwitz et al., 2019) and

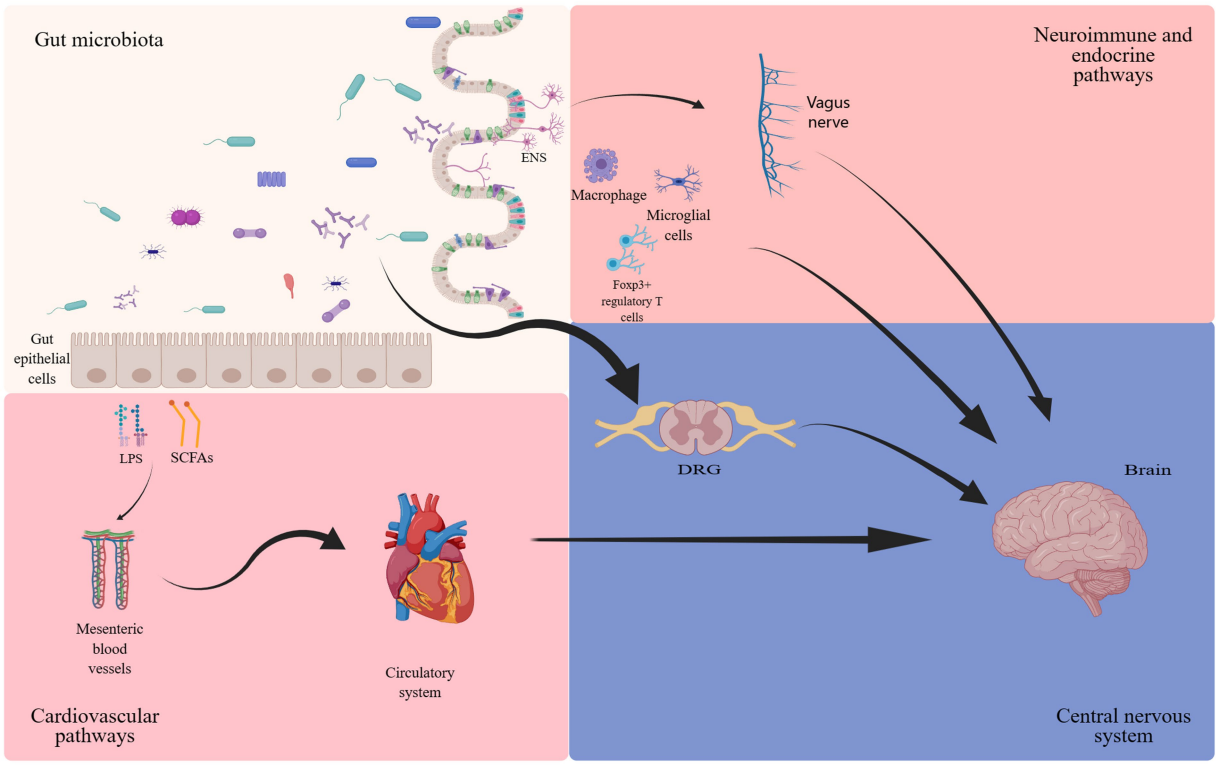


FIGURE 1
The involvement of gut microbiota in the gut-brain axis. The bidirectional communication between the gut microbiota and the brain occurs through endocrine (cortisol), immune (cytokines) and neural (vagus nerve and enteric nervous system) mechanisms (Josefowicz et al., 2012; Zhang et al., 2016; Cussotto et al., 2018; Russo et al., 2018; Guo et al., 2019; Ding et al., 2021; Dora et al., 2021; Ma et al., 2022; Araldi et al., 2023).

5-hydroxytryptamine (5-HT) (Yano et al., 2015), thereby influencing neuronal function. These findings greatly expand our understanding of the gut-brain axis.

2.2 The gut microbiota as a therapeutic target in neuropathic pain

Changes in gut microbiota were discernible in experiments employing various NP animal models. In an experimental model of NP induced by peripheral nerve injury, rats exhibiting dysbiosis in their gut microbiota were observed to be more susceptible to the development of NP (Yang et al., 2019). In the chronic sciatic nerve compression injury model, the abundances of Helicobacter, Phascolarctobacterium, Christensenella, Blautia, Streptococcus, Rothia and Lactobacillus were significantly increased, whereas the abundances of Ignatzschineria, Butyricimonas, Escherichia and Corynebacterium were significantly decreased (Chen et al., 2021). Additionally, experimental data indicate alterations in gut microbiota in C57BL/6 mice which are sensitive to Chemotherapy-induced peripheral neuropathy (CIPN). The abundance of bacteria *Akkermansia muciniphila* is significantly decreased, which may damage the integrity of the intestinal barrier, exposing the gut to bacterial metabolites, ultimately leading to brain dysfunction through the gut-brain axis (Ramakrishna et al., 2019). These significant changes in the gut microbiota imply a robust correlation between the gut microbiota and NP.

In fact, the critical role of gut microbiota in NP has been extensively reported. In animal experiments, researchers have demonstrated that the gut microbiota can to mitigate NP induced by chronic-constriction injury of the sciatic nerve (Ding et al., 2021). Furthermore, in the oxaliplatin (OXA) mice model, gut microbiota depletion effectively inhibits glial cell proliferation and cytokine production in the DRG induced by chronic constriction injury (CCI) or streptozotocin (STZ). The opposite is the partial recovery of the gut microbiota, especially Akkermansia and Bifidobacterium, which can either worsen or alleviate NP (Ma et al., 2022). CIPN is a prevalent adverse effect of cancer treatment. A sensitive neuronal *in vitro* model experiment demonstrated that gut probiotics hold promise for treating paclitaxel-induced NP, offering a long-term safe and effective treatment option (Castelli et al., 2018). Intriguingly, gut microbiota may not only contribute to the management of cancer-related NP but also potentially influence the effects of chemotherapy on tumors (Castelli et al., 2018; Vázquez-Baeza et al., 2018). These foundational experimental findings suggest that gut microbiota could represent a potential therapeutic target in CIPN.

Regarding the therapeutic role of the gut microbiome in NP, its underlying mechanisms have also garnered considerable attention and discussion. This intricate mechanism involves factors such as the metabolic products of the intestinal flora, inflammatory responses, and the recently focused neuroimmune responses. Apart from the involvement of gut microbiota, SCFAs, among the main metabolites produced by gut microbiota, have been implicated in NP. Depletion of SCFAs has been observed to alleviate NP induced by CCI (Zhou

et al., 2021). Similarly, in the CCI model, following correlation analysis, it was revealed that the decreased abundance of gut microbiota producing butyrate is closely associated with levels of β-hydroxybutyric acid in both serum and spinal cord (Chen et al., 2021). This suggests that therapeutically targeting gut microbiota responsible for SCFA/β-hydroxybutyric acid production may holdpromise in ameliorating NP. Moreover, in examining the mechanism underlying inflammatory response, our investigation reveals that in the CIPN model, the therapeutic effect of probiotics on NP may be attributed to inflammation-related responses associated with the Interleukin (IL)-8 signaling pathway (Castelli et al., 2018). Conversely, modifying the gut microbiota composition through ABX treatment suppresses the TLR-4 signaling pathway mediated by macrophages, leading to improvements in CIPN and reduced secretion of inflammatory factors in the DRG (Lian et al., 2021; Ma et al., 2022). Additionally, a study revealed a significant proliferation of spinal cord microgliosis in mice receiving paclitaxel treatment, indicating a causal relationship. Gut microbiota plays a role in the development of CIPN by modulating microgliosis (Ramakrishna et al., 2019). This offers a promising avenue for the precise manipulation of gut microbiota in future NP management.

Recent studies on NP found that the gut microbiota actively participates in the onset and progression of pain by interacting with the neuroimmune system. In a mouse model of CCI, the alteration of gut microbiota via ABX led to an attenuation of NP induced by CCI. This phenomenon may be attributed to the modulation of the balance between pro-inflammatory and anti-inflammatory T cells (Ding et al., 2021). In terms of utilizing the gut microbiota for treatment (Figure 2; Table 1): (a) It has been confirmed that judicious supplementation of probiotics holds promise for pain management. In a pain model induced by lumbar disk herniation, supplementation with *Lactobacillus paracasei* significantly alleviated pain and mitigated inflammatory responses (Wang et al., 2021). (b). Fecal microbiota transplantation (FMT) is a common strategy in gut microbiota research. In a mouse model of NP triggered by obesity, FMT from lean mice effectively alleviated pain. One potential mechanism underlying this effect could be the reduction of RYR2-dependent calcium release in DRG neurons of obese mice following FMT (Bonomo et al., 2020). Additionally, M2-like macrophages play a pivotal role in FMT treatment for obesity-induced peripheral NP, actively participating in the pain relief process and displaying a high association with SCFAs (Bonomo et al., 2020). In mouse model of spinal cord injury, significant enrichment of the Firmicutes phylum was observed following FMT, ultimately promoting functional recovery (Jing et al., 2021). In a clinical study, alterations in the composition of gut microbiota were confirmed in patients with complex regional pain syndrome, characterized by a decrease in diversity and similar shifts in the Firmicutes and Bacteroidetes phyla as observed in animal models (Reichenberger et al., 2013). (c). ABX treatment presents another common approach for investigating gut microbiota-directed therapies. It has been demonstrated that the elimination of gut microbiota through ABX can inhibit the production of IL-6 in the DRG and improve NP (Ma et al., 2022). Additionally, ABX treatment can also alleviate CIPN through the macrophage pathway (Lian et al., 2021). In addition, clinical evidence suggests that chronic NP is often associated with cognitive impairments (Fonseca-Rodrigues et al., 2021). Supplementation with probiotics or FMT has shown potential in mitigating the harm caused by chronic NP and its associated cognitive impairment (Van Laar et al., 2019; Bonomo et al., 2020; Cuozzo et al., 2021). Unfortunately, due to ethical considerations, there is currently limited research on the direct therapeutic effects of gut microbiota in the treatment of NP.

In summary, accumulating preclinical evidence suggests an important role of the gut microbiota in NP, involving complex mechanisms such as gut microbiota composition and metabolites, related immune responses, and activation of the gut-brain axis. We look forward to obtaining more relevant findings in clinical studies.

3 Gut microbiome and opioids in neuropathic pain

3.1 Opioid-induced gut microbiota dysbiosis

Opioids primarily exert their effects through G protein-coupled receptors, inhibiting the transmission of nociceptive signals from the periphery to the CNS (Zádori et al., 2023). Research indicates a close correlation between the use of opioids and the composition and function of the gut microbiota (Acharya et al., 2017; Guo et al., 2019). Prolonged opioid therapy can lead to a systemic inflammatory environment, evidenced in the gut microbiota by compromised barrier function, bacterial translocation, release of gut inflammatory factors, and disrupted gastrointestinal motility (Figure 3) (Zhang et al., 2019a). Recent studies report that prolonged morphine therapy can lead to the onset of intestinal inflammation, with its mechanism closely related to the bacterial product LPS (Bhave et al., 2017). Morphine treatment directly leads to increased activity of P2X receptors in enteric glia, ultimately triggering enhanced inflammatory responses (Bhave et al., 2017). An earlier report mentioned that Systemic administration of morphine can activate a glial -mediated inflammatory cascade in the spinal dorsal horn (Raghavendra et al., 2004). Additionally, morphine sulfate attenuate the reward behavior associated with dopamine due to the activation of the BDNF signaling pathway (Taylor et al., 2016). This leads to the activation of glial cells and impaired reward mechanisms, ultimately reshaping the composition of the gut microbiota through the gut-brain axis (Zhang et al., 2019a). Additionally, opioid medications (morphine) can induce gastrointestinal motility disorders such as constipation, primarily attributed to alterations in the gut microbiota (Touw et al., 2017). In summary, opioids affect the gut microbiome through intricate mechanisms, predominantly inducing the change that typically manifests unfavorably.

3.1.1 The composition of gut microbiota

Intestinal motility and transit time are the primary determinants of gut microbiota composition (Zádori et al., 2023). Constipation induced by opioid therapy and compromised intestinal motility exerts a profound influence on the composition of the gut microbiome. In a mouse model of long-term morphine treatment, dysbiosis of the gut microbiota was observed (Table 2). This dysbiosis comprised an increase in the abundance of Staphylococcus, Enterococcus, and Proteus, alongside a decrease in the abundance of Bacteroidales, Clostridiales, and Lactobacillales (Akbarali and Dewey, 2017; Kim et al., 2021). Furthermore, alterations in gut microbiota composition also extend to other physiological functions, including the inhibition

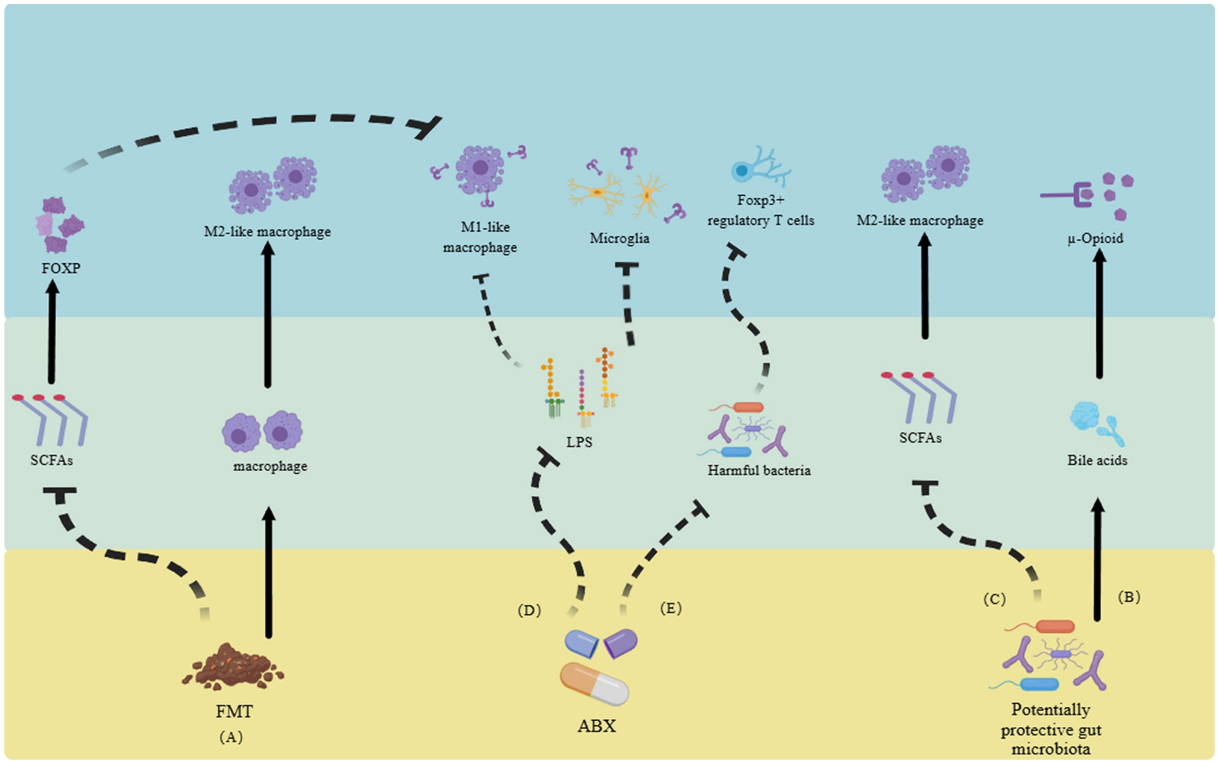


FIGURE 2
Utilizing gut microbiota and their metabolites to alleviate neuropathic pain through the neuroimmune system. **(A)** FMT reduces SCFAs, inhibiting the transformation of M1-like macrophages and promoting the increase of M2-like macrophages, ultimately relieving neuropathic pain. **(B)** Gut microbiota itself can promote the generation of bile acids, activate opioid receptors, and alleviate pain. **(C)** Some gut microbiota can increase the number of M2-like macrophages by inhibiting the production of SCFAs. **(D)**. ABX alleviate pain by reducing the activation of Toll-like receptors in LPS-mediated M1-like macrophages and microglia. **(E)** ABX can also improve pain by altering gut microbiota and regulating regulatory T cells. FMT, fecal microbiota transplantation. SCFAs, short-chain fatty acids. ABX, antibiotics. LPS, lipopolysaccharides (Reichenberger et al., 2013; Van Laar et al., 2019; Bonomo et al., 2020; Cuozzo et al., 2021; Fonseca-Rodrigues et al., 2021; Jing et al., 2021; Lian et al., 2021; Wang et al., 2021; Ma et al., 2022).

TABLE 1 Probiotics treatment.

Probiotics	Function
Probiotics VSL#3 (*Lactobacillus acidophilus*, *Bacillus subtilis*, plant-derived Lactobacillus, *Escherichia coli*)	Effective relief of morphine-induced opioid tolerance in mice (Zhang et al., 2019a)
Bifidobacterium bifidum	Maintain intestinal homeostasis (Wang et al., 2014)
Lactobacillus reuteri NK33 and *Bifidobacterium adolescentis* NK98, *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175	It alleviates the depressive state during pain treatment through neuroimmune mechanisms (Messaoudi et al., 2011; Jang et al., 2019; Yang et al., 2024)
Lactobacillus paracasei S16	It noticeably relieves pain and inflammatory responses in patients with lumbar disk herniation-related neuralgia (Wang et al., 2021)
Probiotic DSF (*Lactobacillus plantarum*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Bifidobacterium longum*)	*In vitro* experiments have demonstrated that probiotics rich in these bacterial communities can treat neuropathic pain caused by chemotherapy drugs and alleviate inflammatory responses (Castelli et al., 2018)

of bile acid secretion (Hu et al., 2022), suppression of bicarbonate secretion (Xu et al., 2022) and the inhibition of immune surveillance (Brosnahan et al., 2014). Similar studies have also highlighted that morphine enhances the toxicity of *Pseudomonas aeruginosa* (Babrowski et al., 2012). Representative Gram-negative bacteria in the gut microbiota, such as those belonging to the genera Bacteroides, play an important role in ameliorating gut inflammation and maintaining the homeostasis of intestinal epithelial cells through the production of SCFAs (Smith et al., 2013). Prolonged treatment with morphine can lead to a reduction in Bacteroidetes, consequently giving rise to intestinal inflammation and compromising the integrity of the intestinal epithelial barrier (Kang et al., 2017). Another illustrative example lies in the Gram-positive Firmicutes phylum (Lactobacillales), which mitigates intestinal inflammation through butyrate salts and regulatory T cells (Furusawa et al., 2013). Prolonged morphine treatment also leads to a significant reduction in the abundance of the Firmicutes phylum (Kang et al., 2017). Interestingly, naloxone (a classic antagonist of opioid stimulants) can effectively alleviate the reduction of thick-walled microbial organisms caused by prolonged morphine treatment (Meng et al., 2015). In another study, naloxone was also shown to normalize morphine treatment-induced gut dysbiosis (Wang et al., 2018; Essmat et al., 2023). This could mean that the dysregulation of gut microbiota caused by opioid drugs can potentially be alleviated through the judicious use of peripheral

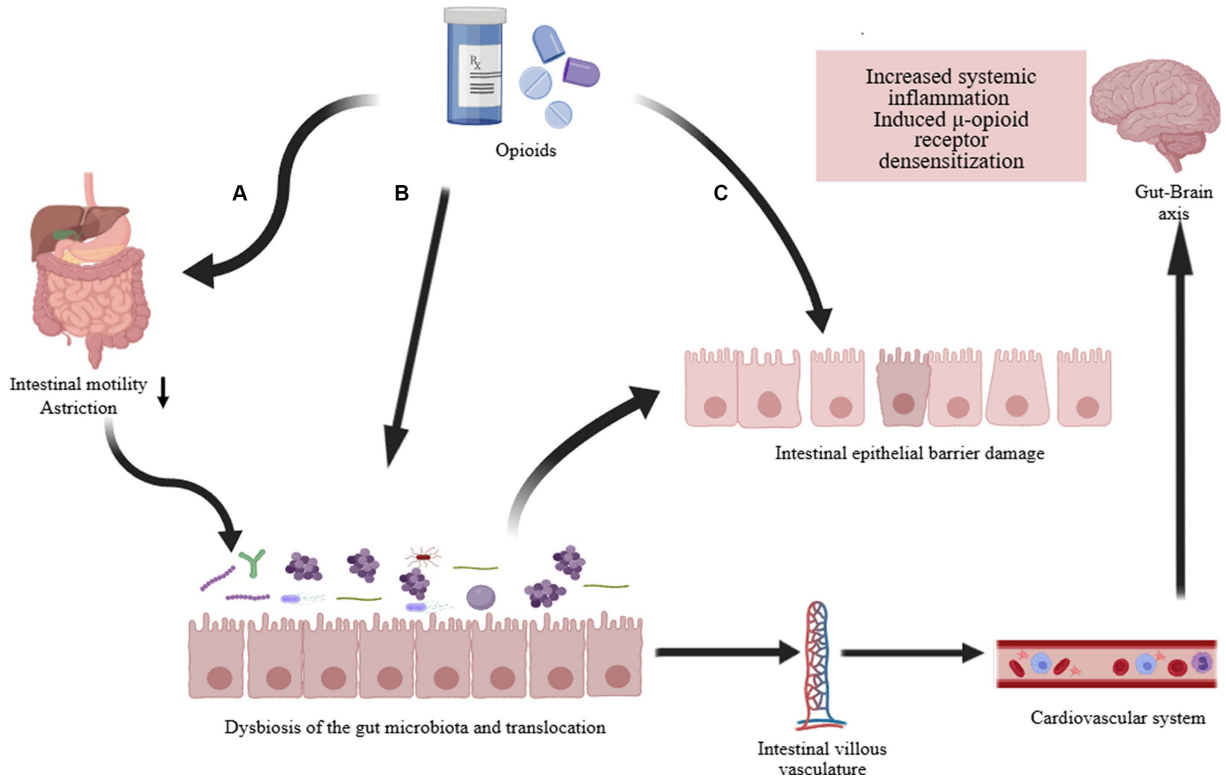


FIGURE 3
Opioid-induced gut microbiota dysbiosis. The long-term effects of opioid treatment on the intestines are: **(A)** Directly weakening gastrointestinal motility, commonly resulting in complications such as constipation. Changes in intestinal motility and transit time further alter the composition of the intestinal microbiota and cause translocation of gut microbiota. **(B)** Directly altering the composition and dysbiosis of the intestinal microbiota, such as a decrease in Lactobacillus and Bifidobacterium, an increase in Staphylococcus and Enterobacter, and potentially increasing other pathogenic bacteria. **(C)** Damage to the intestinal epithelial barrier (increased permeability, disruption of tight junction proteins, decreased immune function). The dysregulation of intestinal microbiota and function ultimately affects systemic inflammation and contributes to the development of opioid resistance via the gut-brain axis (Acharya et al., 2017; Bhave et al., 2017; Guo et al., 2019; Zhang et al., 2019a).

μ-opioid receptor antagonists, thereby reducing the side effects associated with opioid medications.

Recent research has revealed that morphine treatment induces an enrichment of bacteria producing β-glucuronidase in the mouse gut. This leads to the accumulation of chemotherapy drugs in the intestine, culminating in the onset of gut dysbiosis (Meng et al., 2023). For cancer patients undergoing long-term opioid treatment for pain management, the development of interventional drugs targeting the gut microbiota associated with this treatment could potentially mitigate the adverse effects of chemotherapy. In addition, an elevated abundance of Enterobacteriaceae is frequently considered as an indicator of gut dysbiosis (Rizzatti et al., 2017). Recent studies have confirmed that Enterobacteriaceae are markedly enriched in histological samples of inflammatory bowel disease (Gevers et al., 2014). Prolonged exposure to morphine results in a significant increase in the abundance of Enterobacteriaceae (Kang et al., 2017). Furthermore, disparities may exist in secondary intestinal microbiota, such as filamentous bacteria (Earley et al., 2023). Additionally, there have been reports suggesting that the presence of certain less abundant genera, such as Verrucomicrobia and the genus Akkermansia, may also be augmented under the influence of opioid analgesics (Kim et al., 2021; Abu et al., 2022).

The results of animal experiments regarding changes in gut microbiota species due to opioid use are robust and compelling. However, two remaining questions remain concerning the translatability of these findings to humans. First, the use of opioids in animal studies involves high doses and short treatment durations. Second, there is a notable discrepancy in the presence of 85% of secondary phyla between the murine gastrointestinal tract used for preclinical experiments and the human body (Hu et al., 2024). Whether these subtle differences will influence the outcomes of clinical experiments remains to be investigated. Encouragingly, although the data are limited, there have been reports in human studies that parallel those from animal research. Notably, these include a decrease in Ruminococcaceae (Acharya et al., 2017) and an increase in Enterobacteriaceae (Gicquelais et al., 2020).

3.1.2 Translocation and immunomodulation

In recent years, there has been growing interest concerning the mechanism by which opioid drugs influence the gut microbiota. The latest data has forged a connection between opioid treatment and gut microbiota translocation. Several preclinical studies have corroborated that classical opioid drugs, including morphine (Meng et al., 2015; Wang et al., 2018; Zhang et al., 2019a) and hydromorphone (Sharma et al., 2020), can disrupt intestinal epithelial integrity and barrier function in mice. This disruption ultimately leads to bacterial translocation and gut inflammation. On one hand, opioids impede gastrointestinal motility and substance transport by binding to the

TABLE 2 Changes in gut microbiota diversity with opioid treatment regimen (animal studies).

Opioid	Dose, dosing regimen	Finding	References
Morphine	25 mg pellet (4 days)	↑Staphylococcus, Enterococcus	Meng et al. (2015)
	25 mg pellet (5–7 days)	↑Enterococcaceae, Staphylococcaceae, Bacillaceae, Streptococcaceae, Erysipelotrichaceae ↓Bacteroidetes, Proteobacteria, Actinobacteria, Acidobacteria, Actinobacteria, Acidobacte	Banerjee et al. (2016)
	10 mg/kg i.p. (4 days)	↑Ruminococcus ↓Lactobacillus	Lee et al. (2018)
	25 mg pellet (3 days)	↑Flavobacterium, Enterococcus, Fusobacterium	Wang et al. (2018)
	75 mg pellet (5 days)	↑Enterobacteriales ↓Bacteroidales, Clostridiales,	Kang et al. (2017)
	5–40 mg pellet (8 days)	↑Allobaculum, Peptostreptococcaceae ↓Lactobacillus, Bifidobacterium	Zhang et al. (2019a)
	10 mg/kg i.p. (12 days)	↑Verrucomicrobia ↓Bacteroides	Zhang et al. (2021)
Oxycodone	2 mg s.c (5 days)	↑Firmicutes ↓Bacteroidetes	Simpson et al. (2020)
Hydromorphone	7.5 mg pellet (8 days)	↑Bacteroides, Enterococcus ↓Lactobacillus, Lachnospiraceae	Sharma et al. (2020)

opioid mu-receptors in the myenteric plexus of the gut (Goudsward et al., 2024), This impairment in bowel motility can lead to the overgrowth of harmful gut bacteria, potentially resulting in gram-negative sepsis (Meng et al., 2013). On the other hand, in a mouse model of sepsis, it has been shown that morphine treatment induces the dissemination of Gram-positive bacteria (Meng et al., 2015). The translocation of Gram-positive bacteria subsequently triggers an excessive expression of IL-17A and IL-6, ultimately compromis of intestinal epithelial barrier function and further exacerbating systemic inflammation (Meng et al., 2015; Kang et al., 2017). Similar conclusions have also been drawn from clinical data (Meng et al., 2015).

The displacement of gut microbiota induced by opioid medications can lead to disruptions in intestinal epithelial integrity (Banerjee et al., 2016) and trigger inflammatory immune responses (Thomas et al., 2022). We posit that future research might delve into the intricate interplay between gut microbiota and immune responses within the intestinal epithelial barrier. In animal models, it has been found that gut microbiota serves as a key determinant in initiating autoimmune T-cell responses (Berer et al., 2017). The gut microbiota is likely to influence the function of many innate immune cells, thereby actively participating in the regulation of immune responses (Santa-Cecília et al., 2019). Notably, research has demonstrated that morphine exhibits no discernible impact on the gut microbiota of mice with TLR2 knockout or immunodeficiency (Banerjee et al., 2016). This observation underscores the critical role of immune function in maintaining intestinal microbial homeostasis and preventing dysbiosis.

Opioids often exert a dual effect on immune regulation. They typically suppress the immune response of T cells and B cells (Roy et al., 2011). However, in the gastrointestinal tract, they can paradoxically activate the immune system, giving rise to inflammatory responses (Meng et al., 2013; Kang et al., 2017). Research has shed light on the key role of neuroimmune effects induced by opioids in the gastrointestinal tract. Morphine directly induce changes in intestinal epithelial function and compromise the integrity of tight junction proteins through TLR regulation (Banerjee et al., 2016). Evidence suggests that these neuroimmune effects actively contribute to maintaining intestinal epithelial barrier function (Talbot et al., 2020). However, prolonged opioid therapy inhibits immune responses (Banerjee et al., 2016), ultimately leading to dysbiosis and an altered composition of the gut microbiota. This, in turn, leads to the disruption of the intestinal epithelial immune barrier and the development of analgesic tolerance, presenting a significant challenge in opioid management.

3.2 The benefits of gut microbiota in opioid treatment

3.2.1 Analgesic tolerance

Opioids represent the most commonly prescribed medications for pain management. However, their prolonged usage carries inherent risks including the development of analgesic tolerance, potential overdose, and even mortality. In cases of NP, the downregulation or decreased sensitivity of opioid receptors frequently leads to diminished effectiveness of opioids, resulting in opioid tolerance (Balogh et al., 2019; Li et al., 2023). The prevailing view posits that alterations in opioid receptors are the primary drivers of opioid analgesic tolerance. These changes encompass a variety of mechanisms, such as the downregulation of spinal opioid receptors (Balogh et al., 2019), desensitization stemming from adaptive modifications within opioid receptor cells (Bai et al., 2020), and the functional modulation of cell membranes resulting from the repeated use of opioid analgesics (Kliewer et al., 2019). In addition, the mechanism of opioid-induced tolerance to pain relief is a topic of ongoing debate and investigation. Several potential mechanisms have emerged from relevant studies, including immune activation and neuroinflammation (Deleo et al.,

2004; Hutchinson et al., 2011; Wang et al., 2012), receptor desensitization and downregulation (Balogh et al., 2019), upregulation of protein kinase A, and the release of norepinephrine and acetylcholine (Ma et al., 2001). Recent research has highlighted the significant role played by gut microbiota dysbiosis in pain modulation and tolerance through the activation of the gut-brain axis. Changes in the composition of gut microbiota have been shown to directly impact opioid tolerance in mouse experiments (Kang et al., 2017). Notably, the use of vancomycin alone, resulting in a reduction of gram-positive bacteria, prevented the development of opioid tolerance (Kang et al., 2017). While prior research on opioid-induced analgesic tolerance primarily focused on spinal mechanisms (Porreca et al., 1998), recent years have witnessed a growing emphasis on peripheral dysregulation mechanisms (Rivat, 2016; Zhang et al., 2016). Studies have revealed the pivotal role of the dorsal DRG in downregulating mu-opioid receptor (MOR) gene expression in the spinal dorsal horn, consequently diminishing the analgesic efficacy of opioids such as morphine (Zhang et al., 2016). The gut microbiota primarily contributes to the onset and progression of pain through its interaction with DRG and nerve endings within the intestine (Xu et al., 2022). Furthermore, recent research underscores the importance of the DRG in the development of pain tolerance (Jin et al., 2023). Another study has revealed that the gut microbiota is involved in the development of morphine tolerance in primary afferent neurons through its interaction with tetrodotoxin-resistant (TTX-R) sodium channels (Mischel et al., 2018). These research findings confirm the important role of gut microbiota in the peripheral analgesic mechanism of opioids.

Furthermore, a substantial body of evidence supports the key role of neuroinflammation in the development of morphine tolerance (Trang et al., 2015; Gei et al., 2024; Wang et al., 2024). Within neuroinflammatory responses, microglia have long been recognized as important markers for observation. Laboratory findings indicate that the elimination of microglia can effectively prevent opioid-induced pain hypersensitivity (Burma et al., 2017). In addition to its role in the analgesic tolerance process, glial activation actively participates in the development of NP (Gei et al., 2024) and is a key element in the mechanisms related to opioid treatment (Martínez-Navarro et al., 2019). MOR stimulation can lead to the activation of microglia (Ferrini et al., 2013). Interestingly, this opioid-induced glial response may counteract some of the analgesic effects (Martínez-Navarro et al., 2019). Furthermore, specific metabolic products of gut microbiota, such as lipoteichoic acid and LPS, can also induce the activation of glial cells within the nervous system (Obata and Pachnis, 2016; Bhave et al., 2017). Once activated, glial cells release an array of cytokines into the internal environment (Leduc-Pessah et al., 2017), consequently facilitating the development of analgesic tolerance (Drossman and Szigethy, 2014). These reports suggest that reducing harmful gut bacteria, which are associated with increased pain tolerance, can effectively alleviate the development of pain tolerance.

Recent studies have indicated that a portion of morphine analgesic tolerance arises from a decrease in the levels of intestinal β-glucuronidase, resulting in reduced hydrolysis of morphine metabolites back into morphine (Wang and Roy, 2016). Compelling evidence indicates that specific bacteria in the gut, such as Bifidobacteria and Lactobacilli, produce β-glucuronidase (Zhang et al., 2019a). Moreover, further research has confirmed the association of Bifidobacterium and Lactobacillus with opioid tolerance. Probiotics enriched with these microbial strains have demonstrated their effectiveness in alleviating analgesic tolerance in morphine-treated mice (Zhang et al., 2019a). The observed changes in Bifidobacterium and Lactobacillus are particularly intriguing, as these strains themselves contribute to maintaining intestinal homeostasis (Wang et al., 2014). Furthermore, recent investigations into opioids have revealed that FMT significantly reduces opioid withdrawal responses (Zhang et al., 2021). In summary, these studies underscore the substantial role of gut microbiota in opioid treatment, suggesting that supplementation with associated gut microbial strains may potentially prolong the analgesic efficacy of opioids.

3.2.2 The addictive properties of opioids

Opioid addiction poses a significant challenge in the context of long-term opioid use for NP, often leading to varying degrees of withdrawal symptoms upon discontinuation of the medication (Simpson et al., 2020). Preclinical evidence strongly suggests that the gut microbiota actively influences behaviors associated with opioid addiction (Han et al., 2018; Lee et al., 2018; Hofford et al., 2021; Thomaz et al., 2021). Recent research has been exploring the impact of gut microbiota on opioid withdrawal. A recent preclinical study yielded results supporting the effectiveness of FMT and ABX treatment in reducing naloxone-induced withdrawal responses (Thomaz et al., 2021). Furthermore, a study identified significant gender differences in the relationship between gut microbiota and opioid addiction (Ren and Lotfipour, 2023). Among the abundant evidence, a key produced by the gut microbiota, SCFAs, plays a crucial role in cocaine reward behavior (Kiraly et al., 2016). In instances where gut microbiota diversity is inhibited by ABX, there is an increase in cocaine-conditioned place preference (CPP), a phenomenon that can be reversed through SCFA supplementation (Kiraly et al., 2016; Hofford et al., 2021). This suggests that diminished gut microbiota diversity, likely closely linked to SCFAs, instigates changes in opioid reward behavior. Research on SCFAs holds promise for the development of safer and more effective opioid treatment options.

Chronic morphine treatment activate the BDNF pathway through microglia, resulting in a reduction of dopamine-dependent reward behavior (Taylor et al., 2016). This underscores microglia as a key factor in the development of reward dysfunction induced by opioid drugs. Excessive activation of microglia in the ventral tegmental area leads to impaired brain reward behavior and diminished pleasure (Taylor et al., 2016), necessitating higher opioid doses for achieving reward and ultimately contributing to opioid addiction. Importantly, the gut microbiota can influence the morphology and activity of microglia (Gicquelais et al., 2020), consequently leading to reduced dopamine activity and disruptions in reward mechanisms (Taylor et al., 2016). Recent animal experiments have demonstrated that animals treated with FMT exhibited a significant restoration of normal reward behavior, accompanied by an observed increase in resting-state microglia (Lee et al., 2018). These research findings suggest that modulating the gut microbiota can exert a positive influence on the restoration of reward behavior and microglial function. Advancements in gut-brain axis research have suggested intriguing connections between gut microbiota, neuroimmune responses, and opioid withdrawal,

offering fresh insights into opioid pain management. The development of gut microbiota-based therapies has introduced a fresh perspective to opioid withdrawal treatment and neuroimmune mechanisms. However, extensive clinical research is required to conclusively validate these possibilities and to further investigate the interactions between gut microbiota, opioid management, and neuroimmunity.

3.2.3 Mental Health

NP is often accompanied by a spectrum of depressive symptoms (Janakiram et al., 2023). Notably, the severity of depression tends to worsen in tandem with the intensity of pain (Agüera-Ortiz et al., 2011). Clinical psychologists often use sedative medication as an adjunct treatment during the process of treating depression. When considering the combination of opioid drugs and sedative drugs, particularly benzodiazepines, it is imperative to exercise a high degree of vigilance due to the potential risks associated with these medications (Liu et al., 2021). Previous studies have confirmed that when compared to using opioids in isolation for managing NP, the combination of opioids and sedative drugs may lead to a notable increase in the risk of drug overdose (Meyer et al., 2023). Furthermore, it can escalate to a critical level, potentially culminating in fatal respiratory depression (Liu et al., 2021). Research has shown that the activation of opioid receptors in the context of pain treatment can lead to or exacerbate depressive symptoms (Dagher et al., 2022). This pertains not only to patients already affected by mental health issues, but even to those deemed mentally healthy, long-term use of opioids significantly increases the risk of developing depression (Scherrer et al., 2016).

The gut microbiota plays a pivotal role in the development of depression by secreting neurotransmitters, including serotonin (Yano et al., 2015). The utilization of opioid medications can disrupt the gut microbiota, resulting in a depletion of serotonin levels and an augmented risk of developing depression (Parker et al., 2020). Notably, Lactobacillus and Bifidobacterium have been implicated in associations with depression and anxiety (Han and Kim, 2019). Their depletion can occur as a consequence of morphine treatment (Zhang et al., 2019a), potentially increasing the susceptibility to experiencing anxiety and depression during medication therapy. Encouragingly, specific strains of probiotics, such as Bifidobacterium, have demonstrated the capacity to mitigate depression by increasing BDNF levels and reducing oxidative stress (Messaoudi et al., 2011). Research has reported a decrease in BDNF levels in instances of depression, and clinical studies have shown that supplementation with probiotics can lower depressive symptoms (Jang et al., 2019). Another study on Bifidobacterium revealed that it, along with Lactobacillus, contributes to the reduction of depression levels by activating the vagus nerve and eliciting an immunostress response (Yang et al., 2024). Furthermore, research findings also suggest that increasing the abundance of the Ruminococcaceae family could also be a potential avenue for reducing depression levels (Zhu et al., 2019). A recent study suggests that Lactobacillus may help alleviate depression and anxiety induced by chronic stress by sustaining levels of interferon gamma (IFN-γ). Therefore, maintaining healthy levels of Lactobacillus or IFN-γ could potentially prevent or treat symptoms of depression (Clark et al., 2023). Although further exploration is warranted to fully understand the connection between depression and the gut microbiota, it is worth considering supplementation with specific gut microbiota, especially in cases necessitating long-term opioid medication use for NP treatment.

A clinical study has revealed that patients with higher depression scores often exhibit elevated levels of kynurenine, serotonin, and histamine (Han et al., 2022). These compounds are primarily produced by enterochromaffin cells under the influence of gut microbiota, ultimately contributing to the onset of depression. This also underscores the indirect influence of gut microbiota on the development of depression. In addition, another article highlights the serotonin-generating capability of *Clostridium nexile*, which demonstrates significant elevation in patients with severe depression (Williams et al., 2014). These clinical findings suggest a potential avenue for alleviating the depressive effects induced by opioid treatment, by targeting specific gut microbiota, such as *Clostridium nexile*, to modulate neurotransmitter production.

4 Conclusions and treatment perspectives

With the rapid development of genomic sequencing technology, research on gut microbiota has entered a transformative phase (Jovel et al., 2016). These microorganisms, cohabiting with humans, have demonstrated significant influence over various aspects of our physiological and pathological behaviors (Barko et al., 2018; Pane et al., 2022). It is imperative that we focus on and deepen our understanding of gut microbiota and their metabolites within the human body, to gain insights into human health and address unresolved diseases. Research on the gut-brain axis has received considerable attention, with emerging evidence indicating that gut microbiota and their metabolites (SCFAs, LPS, butyrate, and glutamate) play pivotal roles in many diseases (Furusawa et al., 2013; Bhave et al., 2017; Zhang et al., 2019a), including NP. The mechanisms underlying these effects involve neuroimmune interactions. Extensive experimental data have demonstrated the intricate and complex connections between gut microbiota and the development of pain, including NP (Vázquez-Baeza et al., 2018; Ramakrishna et al., 2019; Lian et al., 2021; Ma et al., 2022). Probiotic supplementation or FMT has demonstrated significant reductions in NP (Vázquez-Baeza et al., 2018; Bonomo et al., 2020; Wang et al., 2021). Exploring the involvement of gut microbiota in the pathogenesis of NP holds promise for the development of novel therapeutic approaches in pain management for the future.

In traditional approaches to treating NP, opioids have held a pivotal role (Balanaser et al., 2023). However, their effectiveness is often limited due to shared neurophysiological mechanisms with NP (Martínez-Navarro et al., 2019), along with associated risks of tolerance, addiction, and potential overuse crises. This article elucidates the intricate interplay between gut microbiota and opioid drugs in the context of NP. Opioid therapy alters gut microbiota diversity (Kim et al., 2021) and physiological metabolic processes (Sharma et al., 2020; Kim et al., 2021) by affecting gastrointestinal motility and neuroimmune function. Simultaneously, interventions such as probiotics, ABX, and FMT significantly reduce opioid tolerance and addiction (Wang et al., 2014; Wang and Roy, 2016; Kim et al., 2021; Thomaz et al., 2021) while also positively affecting the psychological well-being of the host (Table 3) (Jang et al., 2019). These findings hold relevance in clinical practice, including observational

TABLE 3 The benefits of gut microbiota in the management of opioids (animal studies).

Side effects of opioids	Involvement of gut microbiota
Analgesic tolerance	A reduction in Gram-positive bacteria has led to a decrease in the activity of microglial cells (Obata and Pachnis, 2016; Bhave et al., 2017), which has alleviated opioid tolerance (Kang et al., 2017)
	The gut microbiota can actively participate in the development of opioid tolerance through the dorsal root ganglia (Corder et al., 2017)
	Bifidobacteria and Lactobacilli effectively increase the hydrolysis of opioid metabolites back to morphine through β-glucuronidase (Wang and Roy, 2016; Zhang et al., 2019b)
Addictive	FMT and ABX can reduce the withdrawal reactions induced by naloxone (Thomaz et al., 2021)
	In a FMT animal model, increased activity of microglia and restoration of normal opioid reward behavior were observed (Lee et al., 2018)
Mental health	Lactobacillus and Bifidobacterium alleviate depressive and anxiety behaviors through activation of the neuroimmune system (Clapp et al., 2019), but they can be depleted by opioid treatment (Zhang et al., 2019a)
	Lactobacillus relieves depression and anxiety by maintaining the level of compound interferon gamma (Clark et al., 2023)
	Opioid treatment leads to a decrease in serotonin levels, while certain gut microbiota can improve depressive behavior by secreting serotonin (Agüera-Ortiz et al., 2011; Janakiram et al., 2023)
	Clostridium nexile exacerbate the depressive state induced by opioids through the production of serotonin (Williams et al., 2014)

studies, suggesting that gut microbiota can play a valuable role in the management of opioid-induced NP, thereby mitigating the adverse effects of opioids and ultimately enhancing the overall quality of treatment.

The nexus between gut microbiota, opioids, and NP presents both intriguing and challenging dimensions. Presently, most research is centered around animal models, underscoring the need for further clinical studies and long-term dynamic data to support the underlying mechanisms involving gut microbiota. Therefore, future clinical research must focus on generating a robust body of evidence and thoroughly investigating the relevant mechanisms to elucidate the complex relationship between gut microbiota and various types of NP. Furthermore, the influence of numerous confounding factors, including patients lifestyles and dietary habits, complex psychological factors, and diverse treatment regimens, must be carefully considered in studies exploring the interactions between advanced gut microbiota-dietary interventions and gut microbiota-based therapies. Additionally, advancements in detection methods are expected to unveil previously overlooked microorganisms, thereby prompting scientists to deepen their understanding of the interconnections among these variables. We look forward to the widespread integration of gut microbiota-based approaches in opioid management for NP in the future.

Author contributions

ZG: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. QX: Conceptualization, Data curation, Investigation, Writing – review & editing. YL: Investigation, Supervision, Validation, Writing – review & editing. BY: Methodology, Validation, Writing – review & editing. BH: Writing – review & editing, Supervision. ZL: Funding acquisition, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by Guangdong Basic and Applied Basic Research Nature Science Foundation (2023A1515011649), Sanming Project of Medicine in Shenzhen (No. SZSM202211007).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Abu, Y., Tao, J. Y., Dutta, R., Yan, Y., Vitari, N., Kolli, U., et al. (2022). Brief hydromorphone exposure during pregnancy sufficient to induce maternal and neonatal mcrobial dysbiosis. *J. Neuroimmune Pharmacol.* 17, 367–375. doi: 10.1007/s11481-021-10019-2

Acharya, C., Betrapally, N. S., Gillevet, P. M., Sterling, R. K., Akbarali, H., White, M. B., et al. (2017). Chronic opioid use is associated with altered gut microbiota and predicts readmissions in patients with cirrhosis. *Aliment. Pharmacol. Ther.* 45, 319–331. doi: 10.1111/apt.13858

Agüera-Ortiz, L., Failde, I., Mico, J. A., Cervilla, J., and López-Ibor, J. J. (2011). Pain as a symptom of depression: Prevalence and clinical correlates in patients attending psychiatric clinics. *J. Affect. Disord.* 130, 106–112. doi: 10.1016/j.jad.2010.10.022

Aguilera, M., Rossini, V., Hickey, A., Simnica, D., Grady, F., Felice, V. D., et al. (2021). Inflammasome signaling regulates the microbial–neuroimmune axis and visceral pain in mice. *Int. J. Mol. Sci.* 22:8336. doi: 10.3390/ijms22158336

Akbarali, H. I., and Dewey, W. L. (2017). The gut–brain interaction in opioid tolerance. *Curr. Opin. Pharmacol.* 37, 126–130. doi: 10.1016/j.coph.2017.10.012

An, D., Oh, S. F., Olszak, T., Neves, J. F., Avci, F. Y., Erturk-Hasdemir, D., et al. (2014). Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* 156, 123–133. doi: 10.1016/j.cell.2013.11.042

Araldi, D., Khomula, E. V., Bonet, I. J. M., Bogen, O., Green, P. G., and Levine, J. D. (2023). Role of pattern recognition receptors in chemotherapy-induced neuropathic pain. *Brain* 147, 1025–1042. doi: 10.1093/brain/awad339

Babrowski, T., Holbrook, C., Moss, J., Gottlieb, L., Valuckaite, V., Zaborin, A., et al. (2012). *Pseudomonas aeruginosa* virulence expression is directly activated by morphine and is capable of causing lethal gut derived sepsis in mice during chronic morphine administration. *Ann. Surg.* 255, 386–393. doi: 10.1097/SLA.0b013e3182331870

Bai, X., Wang, C., Zhang, X., Feng, Y., and Zhang, X. (2020). The role of testosterone in mu-opioid receptor expression in the trigeminal ganglia of opioid-tolerant rats. *Neurosci. Lett.* 723:134868. doi: 10.1016/j.neulet.2020.134868

Balanaser, M., Carley, M., Baron, R., Finnerup, N. B., Moore, R. A., Rowbotham, M. C., et al. (2023). Combination pharmacotherapy for the treatment of neuropathic pain in adults: Systematic review and meta-analysis. *Pain* 164, 230–251. doi: 10.1097/j.pain.0000000000002688

Balogh, M., Zádor, F., Zádori, Z. S., Shaqura, M., Király, K., Mohammadzadeh, A., et al. (2019). Efficacy-Based Perspective to Overcome Reduced Opioid Analgesia of Advanced Painful Diabetic Neuropathy in Rats. *Front. Pharmacol.* 10:347. doi: 10.3389/fphar.2019.00347

Banerjee, S., Sindberg, G., Wang, F., Meng, J., Sharma, U., Zhang, L., et al. (2016). Opioid-induced gut microbial disruption and bile dysregulation leads to gut barrier compromise and sustained systemic inflammation. *Mucosal Immunol.* 9, 1418–1428. doi: 10.1038/mi.2016.9

Barko, P. C., McMichael, M. A., Swanson, K. S., and Williams, D. A. (2018). The gastrointestinal microbiome: A review. *J. Vet. Intern. Med.* 32, 9–25. doi: 10.1111/jvim.14875

Belkaid, Y., and Harrison, O. J. (2017). Homeostatic immunity and the microbiota. *Immunity* 46, 562–576. doi: 10.1016/j.immuni.2017.04.008

Berer, K., Gerdes, L. A., Cekanaviciute, E., Jia, X., Xiao, L., Xia, Z., et al. (2017). Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc. Natl. Acad. Sci. USA* 114, 10719–10724. doi: 10.1073/pnas.1711233114

Bhave, S., Gade, A., Kang, M., Hauser, K. F., Dewey, W. L., and Akbarali, H. I. (2017). Connexin–purinergic signaling in enteric glia mediates the prolonged effect of morphine on constipation. *FASEB J.* 31, 2649–2660. doi: 10.1096/fj.201601068R

Blaho, V. A., Galvani, S., Engelbrecht, E., Liu, C., Swendeman, S. L., Kono, M., et al. (2015). HDL-bound sphingosine-1-phosphate restrains lymphopoiesis and neuroinflammation. *Nature* 523, 342–346. doi: 10.1038/nature14462

Bonomo, R. R., Cook, T. M., Gavini, C. K., White, C. R., Jones, J. R., Bovo, E., et al. (2020). Fecal transplantation and butyrate improve neuropathic pain, modify immune cell profile, and gene expression in the PNS of obese mice. *Proc. Natl. Acad. Sci. USA* 117, 26482–26493. doi: 10.1073/pnas.2006065117

Brosnahan, A. J., Jones, B. J., Dvorak, C. M., and Brown, D. R. (2014). Morphine attenuates apically-directed cytokine secretion from intestinal epithelial cells in response to enteric pathogens. *Pathogens* 3, 249–257. doi: 10.3390/pathogens3020249

Burma, N. E., Bonin, R. P., Leduc-Pessah, H., Baimel, C., Cairncross, Z. F., Mousseau, M., et al. (2017). Blocking microglial pannexin-1 channels alleviates morphine withdrawal in rodents. *Nat. Med.* 23, 355–360. doi: 10.1038/nm.4281

Cai, J., Sun, L. L., and Gonzalez, F. J. (2022). Gut microbiota-derived bile acids in intestinal immunity, inflammation, and tumorigenesis. *Cell Host Microbe* 30, 289–300. doi: 10.1016/j.chom.2022.02.004

Cassidy-Bushrow, A. E., Sitarik, A. R., Johnson, C. C., Johnson-Hooper, T. M., Kassem, Z., Levin, A. M., et al. (2023). Early-life gut microbiota and attention deficit hyperactivity disorder in preadolescents. *Pediatr. Res.* 93, 2051–2060. doi: 10.1038/s41390-022-02051-6

Castelli, V., Palumbo, P., d'Angelo, M., Moorthy, N. K., Antonosante, A., Catanesi, M., et al. (2018). Probiotic DSF counteracts chemotherapy induced neuropathic pain. *Oncotarget* 9, 27998–28008. doi: 10.18632/oncotarget.25524

Chen, P., Wang, C., Ren, Y. N., Ye, Z. J., Jiang, C., and Wu, Z. B. (2021). Alterations in the gut microbiota and metabolite profiles in the context of neuropathic pain. *Mol. Brain* 14:50. doi: 10.1186/s13041-021-00765-y

Clark, D. N., O'Neil, S. M., Xu, L., Steppe, J. T., Savage, J. T., Raghunathan, K., et al. (2023). Prolonged STAT1 activation in neurons drives a pathological transcriptional response. *J. Neuroimmunol.* 382:578168. doi: 10.1016/j.jneuroim.2023.578168

Coccurello, R., Marrone, M. C., and Maccarrone, M. (2022). The endocannabinoids-microbiota partnership in gut-brain axis homeostasis: Implications for autism spectrum disorders. *Front. Pharmacol.* 13:869606. doi: 10.3389/fphar.2022.869606

Corder, G., Tawfik, V. L., Wang, D., Sypek, E. I., Low, S. A., Dickinson, J. R., et al. (2017). Loss of μ opioid receptor signaling in nociceptors, but not microglia, abrogates morphine tolerance without disrupting analgesia. *Nat. Med.* 23, 164–173. doi: 10.1038/nm.4262

Cuozzo, M., Castelli, V., Avagliano, C., Cimini, A., d'Angelo, M., Cristiano, C., et al. (2021). Effects of chronic oral probiotic treatment in paclitaxel-induced neuropathic pain. *Biomedicines* 9:346. doi: 10.3390/biomedicines9040346

Cussotto, S., Sandhu, K. V., Dinan, T. G., and Cryan, J. F. (2018). The neuroendocrinology of the microbiota-gut-brain axis: A behavioural perspective. *Front. Neuroendocrinol.* 51, 80–101. doi: 10.1016/j.yfrne.2018.04.002

Dagher, M., Cahill, C. M., and Evans, C. J. (2022). Opioid systems and depression: The relationship is strengthening. *Biol. Psychiatry* 92, 920–922. doi: 10.1016/j.biopsych.2022.09.020

Deleo, J. A., Tanga, F. Y., and Tawfik, V. L. (2004). Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. *Neuroscientist* 10, 40–52. doi: 10.1177/1073858403259950

Dey, N., Wagner, V. E., Blanton, L. V., Cheng, J., Fontana, L., Haque, R., et al. (2015). Regulators of gut motility revealed by a gnotobiotic model of diet-microbiome interactions related to travel. *Cell* 163, 95–107. doi: 10.1016/j.cell.2015.08.059

Ding, W., You, Z., Chen, Q., Yang, L., Doheny, J., Zhou, X., et al. (2021). Gut microbiota influences neuropathic pain through modulating proinflammatory and anti-inflammatory T cells. *Anesth. Analg.* 132, 1146–1155. doi: 10.1213/ANE.0000000000005155

Dogra, S. K., Doré, J., and Damak, S. (2020). Gut microbiota resilience: Definition, link to health and strategies for intervention. *Front. Microbiol.* 11:572921. doi: 10.3389/fmicb.2020.572921

Dora, D., Ferenczi, S., Stavely, R., Toth, V. E., Varga, Z. V., Kovacs, T., et al. (2021). Evidence of a myenteric plexus barrier and its macrophage-dependent degradation during murine colitis: Implications in enteric neuroinflammation. *Cell. Mol. Gastroenterol. Hepatol.* 12, 1617–1641. doi: 10.1016/j.jcmgh.2021.07.003

Drossman, D., and Szigethy, E. (2014). The narcotic bowel syndrome: A recent update. *Am. J. Gastroenterol. Suppl.* 2, 22–30. doi: 10.1038/ajgsup.2014.6

Earley, Z. M., Lisicka, W., Sifakis, J. J., Aguirre-Gamboa, R., Kowalczyk, A., Barlow, J. T., et al. (2023). GATA4 controls regionalization of tissue immunity and commensal-driven immunopathology. *Immunity* 56, 43–57.e10. doi: 10.1016/j.immuni.2022.12.009

Erny, D., Hrabě de Angelis, A. L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., et al. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* 18, 965–977. doi: 10.1038/nn.4030

Essmat, N., Karádi, D. Á., Zádor, F., Király, K., Fürst, S., and Al-Khrasani, M. (2023). Insights into the Current and Possible Future Use of Opioid Antagonists in Relation to Opioid-Induced Constipation and Dysbiosis. *Molecules* 28:7766. doi: 10.3390/molecules28237766

Fan, Y., and Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 19, 55–71. doi: 10.1038/s41579-020-0433-9

Ferrini, F., Trang, T., Mattioli, T. A. M., Laffray, S., Del'Guidice, T., Lorenzo, L. E., et al. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl− homeostasis. *Nat. Neurosci.* 16, 183–192. doi: 10.1038/nn.3295

Fiore, N. T., Debs, S. R., Hayes, J. P., Duffy, S. S., and Moalem-Taylor, G. (2023). Pain-resolving immune mechanisms in neuropathic pain. *Nat. Rev. Neurol.* 19, 199–220. doi: 10.1038/s41582-023-00777-3

Flores, G. E., Caporaso, J. G., Henley, J. B., Rideout, J. R., Domogala, D., Chase, J., et al. (2014). Temporal variability is a personalized feature of the human microbiome. *Genome Biol.* 15:531. doi: 10.1186/s13059-014-0531-y

Fonseca-Rodrigues, D., Amorim, D., Almeida, A., and Pinto-Ribeiro, F. (2021). Emotional and cognitive impairments in the peripheral nerve chronic constriction injury model (CCI) of neuropathic pain: A systematic review. *Behav. Brain Res.* 399:113008. doi: 10.1016/j.bbr.2020.113008

Foster, J. A. (2022). Modulating brain function with microbiota. *Science* 376, 936–937. doi: 10.1126/science.abo4220

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504, 446–450. doi: 10.1038/nature12721

Gei, L., Yan, Y., Xing, W., Li, Q., Chen, X., Yan, F., et al. (2024). Amiloride alleviates morphine tolerance by suppressing ASIC3-dependent neuroinflammation in the spinal cord. *Eur. J. Pharmacol.* 963:176173. doi: 10.1016/j.ejphar.2023.176173

Gevers, D., Kugathasan, S., Denson, L. A., Vázquez-Baeza, Y., Van Treuren, W., Ren, B., et al. (2014). The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe* 15, 382–392. doi: 10.1016/j.chom.2014.02.005

Gicquelais, R. E., Bohnert, A. S. B., Thomas, L., and Foxman, B. (2020). Opioid agonist and antagonist use and the gut microbiota: associations among people in addiction treatment. *Sci. Rep.* 10:19471. doi: 10.1038/s41598-020-76570-9

Goudsward, H. J., Ruiz-Velasco, V., Stella, S. L., Willing, L. B., and Holmes, G. M. (2024). Coexpressed δ-, μ-, and κ-Opioid Receptors Modulate Voltage-Gated Ca2+ Channels in Gastric-Projecting Vagal Afferent Neurons. *Mol. Pharmacol.* 105, 250–259. doi: 10.1124/molpharm.123.000774

Gronier, B., Savignac, H. M., Di Miceli, M., Idriss, S. M., Tzortzis, G., Anthony, D., et al. (2018). Increased cortical neuronal responses to NMDA and improved attentional set-shifting performance in rats following prebiotic (B-GOS®) ingestion. *Eur. Neuropsychopharmacol.* 28, 211–224. doi: 10.1016/j.euroneuro.2017.11.001

Guo, R., Chen, L. H., Xing, C. G., and Liu, T. (2019). Pain regulation by gut microbiota: Molecular mechanisms and therapeutic potential. *Br. J. Anaesth.* 123, 637–654. doi: 10.1016/j.bja.2019.07.026

Han, S. K., and Kim, D. H. (2019). Lactobacillus mucosae and *Bifidobacterium longum* synergistically alleviate immobilization stress-induced anxiety/depression in mice by suppressing gut dysbiosis. *J. Microbiol. Biotechnol.* 29, 1369–1374. doi: 10.4014/jmb.1907.07044

Han, W. F., Tellez, L. A., Perkins, M. H., Perez, I. O., Qu, T., Ferreira, J., et al. (2018). A neural circuit for gut-induced reward. *Cell* 175, 665–78.e23. doi: 10.1016/j.cell.2018.08.049

Han, L. J., Zhao, L., Zhou, Y., Yang, C., Xiong, T., Lu, L., et al. (2022). Altered metabolome and microbiome features provide clues in understanding irritable bowel syndrome and depression comorbidity. *ISME J.* 16, 983–996. doi: 10.1038/s41396-021-01123-5

Hofford, R. S., Mervosh, N. L., Euston, T. J., Meckel, K. R., Orr, A. T., and Kiraly, D. D. (2021). Alterations in microbiome composition and metabolic byproducts drive behavioral and transcriptional responses to morphine. *Neuropsychopharmacology* 46, 2062–2072. doi: 10.1038/s41386-021-01043-0

Hu, H., Shao, W., Liu, Q., Liu, N., Wang, Q., Xu, J., et al. (2022). Gut microbiota promotes cholesterol gallstone formation by modulating bile acid composition and biliary cholesterol secretion. *Nat. Commun.* 13:252. doi: 10.1038/s41467-021-27758-8

Hu, X., Yu, C., He, Y., Zhu, S., Wang, S., Xu, Z., et al. (2024). Integrative metagenomic analysis reveals distinct gut microbial signatures related to obesity. *BMC Microbiol.* 24:119. doi: 10.1186/s12866-024-03278-5

Hutchinson, M. R., Shavit, Y., Grace, P. M., Rice, K. C., Maier, S. F., and Watkins, L. R. (2011). Exploring the neuroimmunopharmacology of opioids: An integrative review of mechanisms of central immune signaling and their implications for opioid analgesia. *Pharmacol. Rev.* 63, 772–810. doi: 10.1124/pr.110.004135

Janakiram, C., Okunev, I., Tranby, E. P., Fontelo, P., Iafolla, T. J., and Dye, B. A. (2023). Opioids for acute and chronic pain when receiving psychiatric medications. *PLoS One* 18:e0286179. doi: 10.1371/journal.pone.0286179

Jang, H. M., Lee, K. E., and Kim, D. H. (2019). The preventive and curative effects of *Lactobacillus reuteri* NK33 and *Bifidobacterium adolescentis* NK98 on immobilization stress-induced anxiety/depression and colitis in mice. *Nutrients* 11:819. doi: 10.3390/nu11040819

Jensen, T. S., Baron, R., Haanpää, M., Kalso, E., Loeser, J. D., Rice, A. S. C., et al. (2011). A new definition of neuropathic pain. *Pain* 152, 2204–2205. doi: 10.1016/j.pain.2011.06.017

Jiang, C., Lin, W. J., and Salton, S. R. (2019). Role of a VGF/BDNF/TrkB autoregulatory feedback loop in rapid-acting antidepressant efficacy. *J. Mol. Neurosci.* 68, 504–509. doi: 10.1007/s12031-018-1124-0

Jin, D., Chen, H., Zhou, M. H., Chen, S. R., and Pan, H. L. (2023). mGluR5 from Primary Sensory Neurons Promotes Opioid-Induced Hyperalgesia and Tolerance by Interacting with and Potentiating Synaptic NMDA Receptors. *J. Neurosci.* 43, 5593–5607. doi: 10.1523/JNEUROSCI.0601-23.2023

Jing, Y. L., Yu, Y., Bai, F., Wang, L. M., Yang, D. G., Zhang, C., et al. (2021). Effect of fecal microbiota transplantation on neurological restoration in a spinal cord injury mouse model: Involvement of brain-gut axis. *Microbiome* 9:59. doi: 10.1186/s40168-021-01007-y

Joscelyn, J., and Kasper, L. H. (2014). Digesting the emerging role for the gut microbiome in central nervous system demyelination. *Mult. Scler. J.* 20, 1553–1559. doi: 10.1177/1352458514541579

Josefowicz, S. Z., Niec, R. E., Kim, H. Y., Treuting, P., Chinen, T., Zheng, Y., et al. (2012). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* 482, 395–399. doi: 10.1038/nature10772

Jovel, J., Patterson, J., Wang, W. W., Hotte, N., O'Keefe, S., Mitchel, T., et al. (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. *Front. Microbiol.* 7:459. doi: 10.3389/fmicb.2016.00459

Kang, M., Mischel, R. A., Bhave, S., Komla, E., Cho, A., Huang, C., et al. (2017). The effect of gut microbiome on tolerance to morphine mediated antinociception in mice. *Sci. Rep.* 7:42658. doi: 10.1038/srep42658

Kelly, J. R., Borre, Y., O' Brien, C., Patterson, E., el Aidy, S., Deane, J., et al. (2016). Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat. *J. Psychiatr. Res.* 82, 109–118. doi: 10.1016/j.jpsychires.2016.07.019

Kim, M. G., Jo, K., Cho, K., Park, S. S., Suh, H. J., and Hong, K. B. (2021). Prebiotics/probiotics mixture induced changes in cecal microbiome and intestinal morphology alleviated the loperamide-induced constipation in rat. *Food Sci. Anim. Resour.* 41, 527–541. doi: 10.5851/kosfa.2021.e17

Kiraly, D. D., Walker, D. M., Calipari, E. S., Labonte, B., Issler, O., Pena, C. J., et al. (2016). Alterations of the host microbiome affect behavioral responses to cocaine. *Sci. Rep.* 6:35455. doi: 10.1038/srep35455

Kliewer, A., Schmiedel, F., Sianati, S., Bailey, A., Bateman, J. T., Levitt, E. S., et al. (2019). Phosphorylation-deficient G-protein-biased μ-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat. Commun.* 10:367. doi: 10.1038/s41467-018-08162-1

Leduc-Pessah, H., Weilinger, N., Fan, C. Y., Burma, N. E., Thompson, R. J., and Trang, T. (2017). Site-specific regulation of P2X7 receptor function in microglia gates morphine analgesic tolerance. *J. Neurosci.* 37, 10154–10172. doi: 10.1523/JNEUROSCI.0852-17.2017

Lee, K., Vuong, H. E., Nusbaum, D. J., Hsiao, E. Y., Evans, C. J., and Taylor, A. M. W. (2018). The gut microbiota mediates reward and sensory responses associated with regimen-selective morphine dependence. *Neuropsychopharmacology* 43, 2606–2614. doi: 10.1038/s41386-018-0211-9

Li, L., Chen, J., and Li, Y. Q. (2023). The downregulation of opioid receptors and neuropathic pain. *Int. J. Mol. Sci.* 24:5981. doi: 10.3390/ijms24065981

Lian, N. Q., Shen, M. X., Zhang, K., Pan, J., Jiang, Y., Yu, Y., et al. (2021). Drinking hydrogen-rich water alleviates chemotherapy-induced neuropathic pain through the regulation of gut microbiota. *J. Pain Res.* 14, 681–691. doi: 10.2147/JPR.S288289

Lin, L., Lin, J., Qiu, J., Wei, F., Bai, X., Ma, W., et al. (2022). Gut microbiota alterations may increase the risk of prescription opioid use, but not vice versa: A two-sample bi-directional Mendelian randomization study. *Front. Microbiol.* 13:994170. doi: 10.3389/fmicb.2022.994170

Liu, E. Y., Tamblyn, R., Filion, K. B., and Buckeridge, D. L. (2021). Concurrent prescriptions for opioids and benzodiazepines and risk of opioid overdose: Protocol for a retrospective cohort study using linked administrative data. *BMJ Open* 11:e042299. doi: 10.1136/bmjopen-2020-042299

Luczynski, P., Tramullas, M., Viola, M., Shanahan, F., Clarke, G., O'Mahony, S., et al. (2017). Microbiota regulates visceral pain in the mouse. *eLife* 6:e25887. doi: 10.7554/eLife.25887

Ma, P., Mo, R., Liao, H., Qiu, C., Wu, G., Yang, C., et al. (2022). Gut microbiota depletion by antibiotics ameliorates somatic neuropathic pain induced by nerve injury, chemotherapy, and diabetes in mice. *J. Neuroinflammation* 19:169. doi: 10.1186/s12974-022-02523-w

Ma, W., Zheng, W. H., Powell, K., Jhamandas, K., and Quirion, R. (2001). Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an *in vitro* and *in vivo* study. *Eur. J. Neurosci.* 14, 1091–1104. doi: 10.1046/j.0953-816x.2001.01731.x

Mao, Y. K., Kasper, D. L., Wang, B. X., Forsythe, P., Bienenstock, J., and Kunze, W. A. (2013). *Bacteroides fragilis* polysaccharide A is necessary and sufficient for acute activation of intestinal sensory neurons. *Nat. Commun.* 4:1465. doi: 10.1038/ncomms2478

Martínez-Navarro, M., Maldonado, R., and Baños, J. E. (2019). Why mu-opioid agonists have less analgesic efficacy in neuropathic pain? *Eur. J. Pain* 23, 435–454. doi: 10.1002/ejp.1328

Meng, J. J., Abu, Y. F., Zhang, Y., Zhou, Y., Xie, Y., Yan, Y., et al. (2023). Opioid-induced microbial dysbiosis disrupts irinotecan (CPT-11) metabolism and increases gastrointestinal toxicity in a murine model. *Br. J. Pharmacol.* 180, 1362–1378. doi: 10.1111/bph.16020

Meng, J. J., Banerjee, S., Li, D., Sindberg, G. M., Wang, F., Ma, J., et al. (2015). Opioid exacerbation of Gram-positive sepsis, induced by gut microbial modulation, is rescued by IL-17A neutralization. *Sci. Rep.* 5:10918. doi: 10.1038/srep10918

Meng, J., Yu, H., Ma, J., Wang, J., Banerjee, S., Charboneau, R., et al. (2013). Morphine induces bacterial translocation in mice by compromising intestinal barrier function in a TLR-dependent manner. *PLoS One* 8:e54040. doi: 10.1371/journal.pone.0054040

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., et al. (2011). Assessment of psychotropic-like properties of a probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) in rats and human subjects. *Br. J. Nutr.* 105, 755–764. doi: 10.1017/S0007114510004319

Meyer, M., Gygli, F., Westenberg, J. N., Schmid, O., Strasser, J., Lang, U. E., et al. (2023). Benzodiazepine use, quality of life and psychiatric symptom burden in oral and injectable opioid agonist treatment: a cross-sectional study. *Addict. Sci. Clin. Pract.* 18:43. doi: 10.1186/s13722-023-00397-8

Minerbi, A., Gonzalez, E., Brereton, N. J. B., Anjarkouchian, A., Dewar, K., Fitzcharles, M. A., et al. (2019). Altered microbiome composition in individuals with fibromyalgia. *Pain* 160, 2589–2602. doi: 10.1097/j.pain.0000000000001640

Minerbi, A., and Shen, S. Q. (2022). Gut microbiome in anesthesiology and pain medicine. *Anesthesiology* 137, 93–108. doi: 10.1097/ALN.0000000000004204

Mischel, R. A., Dewey, W. L., and Akbarali, H. I. (2018). Tolerance to morphine-induced inhibition of TTX-R sodium channels in dorsal root ganglia neurons is modulated by gut-derived mediators. *iScience* 2, 193–209. doi: 10.1016/j.isci.2018.03.003

Morreale, C., Bresesti, I., Bosi, A., Baj, A., Giaroni, C., Agosti, M., et al. (2022). Microbiota and pain: Save your gut feeling. *Cells* 11:971. doi: 10.3390/cells1106097

Nguyen, V. T. T., and Endres, K. (2022). Targeting gut microbiota to alleviate neuroinflammation in Alzheimer's disease. *Adv. Drug Deliv. Rev.* 188:114418. doi: 10.1016/j.addr.2022.114418

Obata, Y., and Pachnis, V. (2016). The effect of microbiota and the immune system on the development and organization of the enteric nervous system. *Gastroenterology* 151, 836–844. doi: 10.1053/j.gastro.2016.07.044

Pane, K., Boccella, S., Guida, F., Franzese, M., Maione, S., and Salvatore, M. (2022). Role of gut microbiota in neuropathy and neuropathic pain states: A systematic preclinical review. *Neurobiol. Dis.* 170:105773. doi: 10.1016/j.nbd.2022.105773

Parker, B. J., Wearsch, P. A., Veloo, A. C. M., and Rodriguez-Palacios, A. (2020). The genus *Alistipes*: Gut bacteria with emerging implications to inflammation, cancer, and mental health. *Front. Immunol.* 11:906. doi: 10.3389/fimmu.2020.00906

Porreca, F., Tang, Q. B., Bian, D., Riedl, M., Robert, E., and Lai, J. (1998). Spinal opioid mu receptor expression in lumbar spinal cord of rats following nerve injury. *Brain Res.* 795, 197–203. doi: 10.1016/s0006-8993(98)00292-3

Raghavendra, V., Tanga, F. Y., and DeLeo, J. A. (2004). Attenuation of morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammatory immune responses by propentofylline in rats. *Neuropsychopharmacology* 29, 327–334. doi: 10.1038/sj.npp.1300315

Ramakrishna, C., Corleto, J., Ruegger, P. M., Logan, G. D., Peacock, B. B., Mendonca, S., et al. (2019). Dominant role of the gut microbiota in chemotherapy induced neuropathic pain. *Sci. Rep.* 9:20324. doi: 10.1038/s41598-019-56832-x

Reichenberger, E. R., Alexander, G. M., Perreault, M. J., Russell, J. A., Schwartzman, R. J., Hershberg, U., et al. (2013). Establishing a relationship between bacteria in the human gut and complex regional pain syndrome. *Brain Behav. Immun.* 29, 62–69. doi: 10.1016/j.bbi.2012.12.005

Ren, M., and Lotfipour, S. (2023). Antibiotic knockdown of gut bacteria sex-dependently enhances intravenous fentanyl self-administration in adult sprague dawley rats. *Int. J. Mol. Sci.* 24:409. doi: 10.3390/ijms24010409

Rivat, C. (2016). Neuropathic pain: When nerve lesion turns off peripheral analgesia. *Anesthesiology* 125, 627–629. doi: 10.1097/ALN.0000000000001262

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., and Gasbarrini, A. (2017). Proteobacteria: A common factor in human diseases. *Biomed. Res. Int.* 2017:9351507. doi: 10.1155/2017/9351507

Roy, S., Ninkovic, J., Banerjee, S., Charboneau, R. G., Das, S., Dutta, R., et al. (2011). Opioid drug abuse and modulation of immune function: Consequences in the susceptibility to opportunistic infections. *J. Neuroimmune Pharmacol.* 6, 442–465. doi: 10.1007/s11481-011-9292-5

Ruan, J. W., Liao, Y. C., Chen, P. C., Chen, Y. J., Tsai, Y. H., Tsai, P. J., et al. (2023). The composition of the maternal breastmilk microbiota influences the microbiota network structure during early infancy. *J. Microbiol. Immunol. Infect.* 56, 1084–1097. doi: 10.1016/j.jmii.2023.07.005

Russo, R., Cristiano, C., Avagliano, C., De Caro, C., La Rana, G., Raso, G. M., et al. (2018). Gut-brain axis: Role of lipids in the regulation of inflammation, pain and CNS diseases. *Curr. Med. Chem.* 25, 3930–3952. doi: 10.2174/0929867324666170216113756

Santa-Cecília, F. V., Ferreira, D. W., Guimaraes, R. M., Cecilio, N. T., Fonseca, M. M., Lopes, A. H., et al. (2019). The NOD2 signaling in peripheral macrophages contributes to neuropathic pain development. *Pain* 160, 102–116. doi: 10.1097/j.pain.0000000000001383

Scherrer, J. F., Salas, J., Copeland, L. A., Stock, E. M., Ahmedani, B. K., Sullivan, M. D., et al. (2016). Prescription opioid duration, dose, and increased risk of depression in 3 large patient populations. *Ann. Fam. Med.* 14, 54–62. doi: 10.1370/afm.1885

Schmidt, T. S. B., Raes, J., and Bork, P. (2018). The human gut microbiome: From association to modulation. *Cell* 172, 1198–1215. doi: 10.1016/j.cell.2018.02.044

Sharma, U., Olson, R. K., Erhart, F. N., Zhang, L., Meng, J., Segura, B., et al. (2020). Prescription opioids induce gut dysbiosis and exacerbate colitis in a murine model of inflammatory bowel disease. *J. Crohns Colitis* 14, 801–817. doi: 10.1093/ecco-jcc/jjz188

Shen, S., Lim, G., You, Z., Ding, W., Huang, P., Ran, C., et al. (2017). Gut microbiota is critical for the induction of chemotherapy-induced pain. *Nat. Neurosci.* 20, 1213–1216. doi: 10.1038/nn.4606

Simpson, S., Kimbrough, A., Boomhower, B., McLellan, R., Hughes, M., Shankar, K., et al. (2020). Depletion of the microbiome alters the recruitment of neuronal ensembles of oxycodone intoxication and withdrawal. *eNeuro* 7, 7:ENEURO.0312-19.2020. doi: 10.1523/ENEURO.0312-19.2020

Smith, P. M., Howitt, M. R., Panikov, N., Michaud, M., Gallini, C. A., Bohlooly-Y, M., et al. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. *Science* 341, 569–573. doi: 10.1126/science.1241165

Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., et al. (2019). GABA-modulating bacteria of the human gut microbiota. *Nat. Microbiol.* 4, 396–403. doi: 10.1038/s41564-018-0307-3

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X. N., et al. (2004). Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J. Physiol.* 558, 263–275. doi: 10.1113/jphysiol.2004.063388

Talbot, J., Hahn, P., Kroehling, L., Nguyen, H., Li, D., and Littman, D. R. (2020). Feeding-dependent VIP neuron-ILC3 circuit regulates the intestinal barrier. *Nature* 579, 575–580. doi: 10.1038/s41586-020-2039-9

Taylor, A. M. W., Castonguay, A., Ghogha, A., Vayssiere, P., Pradhan, A. A., Xue, L., et al. (2016). Neuroimmune regulation of GABAergic neurons within the ventral tegmental area during withdrawal from chronic morphine. *Neuropsychopharmacology* 41, 949–959. doi: 10.1038/npp.2015.221

Thaiss, C. A. (2023). A microbiome exercise. *Science* 381:38. doi: 10.1126/science.adi6329

Thomas, K. R., Watt, J., Wu, C. M. J., Akinrinoye, A., Amjad, S., Colvin, L., et al. (2022). Pain and opioid-induced gut microbial dysbiosis. *Biomedicines* 10:1815. doi: 10.3390/biomedicines10081815

Thomaz, A. C., Iyer, V., Woodward, T. J., and Hohmann, A. G. (2021). Fecal microbiota transplantation and antibiotic treatment attenuate naloxone-precipitated opioid withdrawal in morphine-dependent mice. *Exp. Neurol.* 343:113787. doi: 10.1016/j.expneurol.2021.113787

Touw, K., Ringus, D. L., Hubert, N., Wang, Y., Leone, V. A., Nadimpalli, A., et al. (2017). Mutual reinforcement of pathophysiological host-microbe interactions in intestinal stasis models. *Physiol. Rep.* 5:e13182. doi: 10.14814/phy2.13182

Trang, T., Al-Hasani, R., Salvemini, D., Salter, M. W., Gutstein, H., and Cahill, C. M. (2015). Pain and poppies: The good, the bad, and the ugly of opioid analgesics. *J. Neurosci.* 35, 13879–13888. doi: 10.1523/JNEUROSCI.2711-15.2015

Van Laar, T., Boertien, J. M., and Herranz, A. H. (2019). Faecal transplantation, pro- and prebiotics in Parkinson's disease; hope or hype? *J. Parkinsons Dis.* 9, S371–S379. doi: 10.3233/JPD-191802

Vázquez-Baeza, Y., Callewaert, C., Debelius, J., Hyde, E., Marotz, C., Morton, J. T., et al. (2018). Impacts of the human gut microbiome on therapeutics. *Annu. Rev. Pharmacol. Toxicol.* 58, 253–270. doi: 10.1146/annurev-pharmtox-042017-031849

Wang, X., Loram, L. C., Ramos, K., de Jesus, A. J., Thomas, J., Cheng, K., et al. (2012). Morphine activates neuroinflammation in a manner parallel to endotoxin. *Proc. Natl. Acad. Sci. USA* 109, 6325–6330. doi: 10.1073/pnas.1200130109

Wang, F. Y., Meng, J. J., Zhang, L., Johnson, T., Chen, C., and Roy, S. (2018). Morphine induces changes in the gut microbiome and metabolome in a morphine dependence model. *Sci. Rep.* 8:3596. doi: 10.1038/s41598-018-21915-8

Wang, F. Y., and Roy, S. (2016). Gut homeostasis, microbial dysbiosis, and opioids. *Toxicol. Pathol.* 45, 150–156. doi: 10.1177/0192623316679898

Wang, B., Wang, L. N., Wu, B., Guo, R., Zhang, L., Zhang, J. T., et al. (2024). Astrocyte PERK and IRE1 Signaling Contributes to Morphine Tolerance and Hyperalgesia through Upregulation of Lipocalin-2 and NLRP3 Inflammasome in the Rodent Spinal Cord. *Anesthesiology* 140, 558–577. doi: 10.1097/ALN.0000000000004858

Wang, Z. C., Wu, H. Q., Chen, Y., Chen, H. J., Wang, X. W., and Yuan, W. (2021). *Lactobacillus paracasei S16* alleviates lumbar disc herniation by modulating inflammation response and gut microbiota. *Front. Nutr.* 8:701644. doi: 10.3389/fnut.2021.701644

Wang, B. G., Xu, H. B., Wei, H., Zeng, Z. L., and Xu, F. (2014). Oral administration of Bifidobacterim bifidum for modulating microflora, acid and bile resistance, and physiological indices in mice. *Can. J. Microbiol.* 61, 155–163. doi: 10.1139/cjm-2014-0694

Williams, B. B., Van Benschoten, A. H., Cimermancic, P., Donia, M. S., Zimmermann, M., Taketani, M., et al. (2014). Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. *Cell Host Microbe* 16, 495–503. doi: 10.1016/j.chom.2014.09.001

Xu, R., Miao, L., Yang, C., and Zhu, B. (2022). Gut microbiota plays a pivotal role in opioid-induced adverse effects in gastrointestinal system. *Crit. Care* 26:5. doi: 10.1186/s13054-021-03867-0

Yang, C., Fang, X., Zhan, G. F., Huang, N., Li, S., Bi, J., et al. (2019). Key role of gut microbiota in anhedonia-like phenotype in rodents with neuropathic pain. *Transl. Psychiatry* 9:57. doi: 10.1038/s41398-019-0379-8

Yang, Y., Yang, L., Wan, M., Pan, D., Sun, G., and Yang, C. (2024). Assessment of optimal combinations of therapeutic probiotics for depression, anxiety, and stress. *Psychol. Med.* 1–15, 1–15. doi: 10.1017/S0033291724000679

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., et al. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* 161, 264–276. doi: 10.1016/j.cell.2015.02.047

Zádori, Z. S., Király, K., Al-Khrasani, M., and Gyires, K. (2023). Interactions between NSAIDs, opioids and the gut microbiota - Future perspectives in the management of inflammation and pain. *Pharmacol. Ther.* 241:108327. doi: 10.1016/j.pharmthera.2022.108327

Zhang, J. B., Deji, C., Fan, J. N., Chang, L., Miao, X., Xiao, Y., et al. (2021). Differential alteration in gut microbiome profiles during acquisition, extinction and reinstatement of morphine-induced CPP. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 104:110058. doi: 10.1016/j.pnpbp.2020.110058

Zhang, J., Liang, L. L., Miao, X. R., Wu, S., Cao, J., Tao, B., et al. (2016). Contribution of the suppressor of variegation 3-9 homolog 1 in dorsal root ganglia and spinal cord dorsal horn to nerve injury–induced nociceptive hypersensitivity. *Anesthesiology* 125, 765–778. doi: 10.1097/ALN.0000000000001261

Zhang, L., Meng, J. J., Ban, Y. G., Jalodia, R., Chupikova, I., Fernandez, I., et al. (2019a). Morphine tolerance is attenuated in germfree mice and reversed by probiotics, implicating the role of gut microbiome. *Proc. Natl. Acad. Sci. USA* 116, 13523–13532. doi: 10.1073/pnas.1901182116

Zhang, L., Wang, R. Y., Bai, T., Xiang, X., Qian, W., Song, J., et al. (2019b). EphrinB2/ephB2-mediated myenteric synaptic plasticity: Mechanisms underlying the persistent muscle hypercontractility and pain in postinfectious IBS. *FASEB J.* 33, 13644–13659. doi: 10.1096/fj.201901192R

Zhou, F., Wang, X., Han, B. Y., Tang, X., Liu, R., Ji, Q., et al. (2021). Short-chain fatty acids contribute to neuropathic pain via regulating microglia activation and polarization. *Mol. Pain* 17:1744806921996520. doi: 10.1177/17448069219 96520

Zhu, H. Z., Liang, Y. D., Ma, Q. Y., Hao, W. Z., Li, X. J., Wu, M. S., et al. (2019). Xiaoyaosan improves depressive-like behavior in rats with chronic immobilization stress through modulation of the gut microbiota. *Biomed. Pharmacother.* 112:108621. doi: 10.1016/j.biopha.2019.108621

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 26 June 2024
DOI 10.3389/fcimb.2024.1424376

Check for updates

OPEN ACCESS

EDITED BY
Ludovico Abenavoli,
Magna Græcia University, Italy

REVIEWED BY
Peng An Khun,
University of Western Australia, Australia
Anna Boroń-Kaczmarska,
Silesian Academy, Poland

*CORRESPONDENCE
Adorján Varga
adorjanvarga@ymail.com

†These authors have contributed equally to this work and share last authorship

RECEIVED 27 April 2024
ACCEPTED 10 June 2024
PUBLISHED 26 June 2024

CITATION
Sipos D, Varga A, Kappéter Á, Halda-Kiss B, Kása P, Pál S, Kocsis B and Péterfi Z (2024) Encapsulation protocol for fecal microbiota transplantation.
Front. Cell. Infect. Microbiol. 14:1424376.
doi: 10.3389/fcimb.2024.1424376

COPYRIGHT
© 2024 Sipos, Varga, Kappéter, Halda-Kiss, Kása, Pál, Kocsis and Péterfi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Encapsulation protocol for fecal microbiota transplantation

Dávid Sipos[1], Adorján Varga[2*], Ágnes Kappéter[1], Bernadett Halda-Kiss[1], Péter Kása[3], Szilárd Pál[3], Béla Kocsis[2†] and Zoltán Péterfi[1†]

[1]1st Department of Internal Medicine – Department of Infectology, University of Pécs Clinical Centre, Pécs, Hungary, [2]Department of Medical Microbiology and Immunology, University of Pécs Medical School, Clinical Centre, Pécs, Hungary, [3]Institute of Pharmaceutical Technology and Biopharmacy, University of Pécs Faculty of Pharmacy, Pécs, Hungary

Introduction: *Clostridioides difficile* infections (CDI) continue to pose a challenge for clinicians. Fecal microbiota transplantation (FMT) is an effective treatment option in CDI. Furthermore, recent and ongoing studies suggest potential benefits of FMT in other diseases as well.

Methods: We would like to present a novel protocol for encapsulation of lyophilized fecal material. Our method provides with better compliance as well as improved flexibility, storage and safety.

Results: FMT was conducted in 28 patients with an overall success rate of 82,14% using apsules containing lyophilized stool. 16 of patients were given capsules with lessened bacteria counts. The success rate in this group was 93,75%.

Discussion: The results highlight the still unanswered questions about the mechanism of action and contribute to a wider use of FMT in the clinical praxis and in research.

KEYWORDS

***Clostridioides difficile*, *Clostridium difficile* infection, FMT, protocol, capsule, lyophilizate**

1 Introduction

Healthcare-associated infections are diseases that occur despite the work of health care workers or as a side effect of health care interventions. They impose a significant burden on patients and the healthcare system. *Clostridioides difficile* infection (CDI) is a typical example of this group of diseases.

Most often, *C. difficile* is unlikely to enter the body during hospital care. The bacteria are often carried by clinically symptomatic patients even before admission (Péterfi, 2015). It

has also been shown to be an essential element of the intestinal flora of infants (Jangi and Lamont, 2010) and is not uncommon in the (even healthy) population without symptoms of CDI (Nagy et al., 2013).

Restoring colonization resistance with bactericidal or bacteriostatic drugs is a significant challenge. It can be restored by introducing healthy gut flora or even bacterial strains specific to healthy gut flora into the intestinal tract. It is recorded to have been a common procedure in ancient China (Zhang et al., 2012) and in 18th century Europe for the treatment of various gut-related pathological conditions (DePeters and George, 2014).

The whole gut flora is in fact a "finished product", but this can also allow the transmission of pathogens (DeFilipp et al., 2019; Zellmer et al., 2021). It is also possible to introduce a known composition of bacterial strains, but the complexity of the gut flora makes it very difficult to determine the correct composition.

The main aim of this research was to improve the already proven effectiveness of fecal transplantation and, as part of this, to make it a more acceptable procedure for patients and easier to perform in clinical practice. For this purpose, we considered capsules filled with lyophilized stool to be the most suitable due to their ease of storage, transport and use. Sample preparation inevitably became more complex; therefore, the workflow was designed to fit in with the daily tasks of a normal working day.

1.1 Pathophysiology of CDI

Clostridioides difficile is resistant to commonly used surface disinfectants due to its spore-forming ability and is also tolerant to desiccation, allowing it to survive for extended periods on various surfaces (Kramer et al., 2006). Removal requires a sporicidal disinfectant and hand washing with soap (Cooper et al., 2016). It is also commonly found in the gut flora of asymptomatic individuals. Illness is typically caused when the colonization resistance of the intestinal flora is weakened. Elderly, immunosuppressed state, prolonged antibiotic use predicts more severe clinical symptoms.

Its spread between patients in a healthcare or nursing facility is of particular importance in the case of toxin-producing (toxin A, toxin B and binary toxin) strains, which cause a more severe course (Chandrasekaran and Lacy, 2017). Ribotype 027 produces 16 times more toxin A and 23 times more toxin B (Warny et al., 2005).

Toxin A is an enterotoxin that destroys cell-to-cell contacts, while toxin B and the binary toxin are cytotoxins, with toxin B targeting the actin filament that builds the cytoskeleton. Together, the toxins therefore induce symptoms by disrupting the integrity of the intestinal mucosa. The disease may be associated with mild intestinal symptoms, but also with painful, mucopurulent (rarely bloody) diarrhea, for example in pseudomembranous enterocolitis.

Persistent, asymptomatic *C. difficile* carriage is a protective factor for CDI. This is likely to be due to elevated serum levels of antibodies against toxins A and B. A similar protective effect has been observed in non-toxin-producing strains in both human and animal studies (Johnson et al., 2021).

Recurrence of the disease is also an unfavorable sign both for the remission of the current disease and for new recurrences. The frequency of recurrence of CDI and the time and cost of hospitalization places a heavy burden on the healthcare system (Ghantoji et al., 2010; Dubberke and Olsen, 2012).

1.2 Treatment of CDI

The conventional first-line treatment is metronidazole, with vancomycin used for more severe cases or relapses. Multiple relapses or a very severe course justifies the use of fidaxomicin (van Prehn et al., 2021). Other drug therapy options include nitazoxanide, rifaximin, tigecycline and teicoplanin and certain monoclonal antibodies (Wilcox et al., 2017).

Non-drug treatment is fecal transplantation, which rivals the efficacy of the most effective anti-CDI drugs (Vigvári et al., 2015). Recent publications have shown similarly reliable results with a concentrate of *Firmicutes* strain spores (SER-109).

1.2.1 Antimicrobials

Metronidazole has bactericidal activity against anaerobic bacteria. It can be administered orally and parenterally. Due to its efficacy in mild to moderate CDI and their relatively low cost (Venugopal and Johnson, 2012) it is still in widespread use. According to the latest guidelines, they should only be used under specific circumstances.

Vancomycin inhibits bacterial cell wall synthesis. It is normally used as an agent in intravenous formulations. In CDI, it is dissolved in water and administered orally. An argument against its use in CDI is that it may cause the proliferation of resistant strains of other pathogens in the intestinal tract (e.g. *Enterococcus* and *Staphylococcus* strains) (Mathias et al., 2019).

Fidaxomicin has a lower MIC against *C. difficile* strains (including ribotype NAP1/B1/027) than metronidazole or vancomycin under *in vitro* conditions. Due to its narrow antibiotic spectrum, it is less disruptive to the diversity of the microbiome and thus allows rapid recovery of colonization resistance. It has been observed to reduce sporulation and toxin production. Its effect in reducing the incidence of recurrent CDI makes it cost-effective in the group of patients at highest risk of relapse (Mullane, 2014).

Tigecycline, an intravenously administered tetracycline derivative, is effective against a number of multi-drug resistant pathogens (Mathur et al., 2014).

The following antibiotics are not part of the ESCMID recommendation but have also been shown to be effective in the treatment of CDI.

Nitazoxanide is comparable to vancomycin in terms of efficacy against *C. difficile*. It is considered a possible choice in refractory or recurrent cases (Mathur et al., 2014).

Rifaximin is a gastrointestinal-selective antibiotic with broad-spectrum bactericidal activity, but only negligible effect on normal gut flora (Mathur et al., 2014).

Teicoplanin also shows good results, but its availability and high cost limit its use (Mathur et al., 2014).

Ridinilazole is a benzimidazole derivative. According to ongoing trials, its efficacy in CDI exceeds that of vancomycin (Vickers et al., 2017).

1.2.2 Fecal microbiota transplant

Its use in the therapy of pseudomembranous colitis was first reported in 1958 (Eisenman et al., 1958) and has since been shown to be an effective and safe procedure in numerous studies (Kelly et al., 2014). It is currently part of the therapeutic protocol for recurrent CDI. The exact criteria for ideal donors are not clear. In principle, healthy individuals with no infectious disease based on their medical history and screening results are requested for donation. A general bacteriology, parasitology and *C. difficile* toxin testing of stool samples and screening of serum samples for HIV-1, HIV-2, EBV, CMV, hepatitis A, B, C, *Treponema pallidum subspecies pallidum* are routinely performed (Vigvári et al., 2015). SARS-Cov-2 virus RNA can be detected in stool long after recovery and protocols have been developed for screening donors (Ianiro et al., 2020; Nagy et al., 2020; Segal et al., 2020).

There are currently four different options for fecal transplantation. It can be performed rectally, through a nasogastric (NG) or a nasojejunal (NJ) tube. Samples prepared for this purpose can be stored frozen (up to -20°C) for later use (Youngster et al., 2014; Vigvári et al., 2015). Encapsulation is the fourth option. Among these, the use of frozen stool-filled capsules (Vigvári et al., 2019) and lyophilized inoculum-filled capsules (Varga et al., 2021; Varga et al., 2023) is possible. Case series studies have shown the efficacy of stool transplants in recurrent CDI, including the prevention of relapse after multiple relapses (Vigvári et al., 2015).

The microbiome is likely to play an important role in irritable bowel disease (Ford et al., 2020), obesity, metabolic syndrome and diabetes (Aron-Wisnewsky et al., 2021). The protocol of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID, Table 1) recommends it for severe complicated and refractory infections (van Prehn et al., 2021).

The most important potential side effect is the risk of pathogen transmission and the possibility of developing contaminated small bowel syndrome in case of oral administration. Other possible complications include, depending on the method used, intestinal perforation caused by colonoscopy (the risk of which is increased by local inflammation) and, when using NG or NJ tubes, vomiting and subsequent aspiration, which may lead to pneumonia.

1.2.3 Further treatment options

There are other ways to restore normal gut flora besides antibiotics or FMT. Toxins of *C. difficile* can be neutralized by monoclonal antibodies (bezlotoxumab, actoxumab (Džunková et al., 2016; Wilcox et al., 2017; Gerding et al., 2018; Prabhu et al., 2018; Salavert et al., 2018; Chen et al., 2021)), while re-establishment of a healthy microenvironment can be attempted through inoculation of certain bacterial strains (SER-109) in a similar way to FMT (McGovern et al., 2021; Feuerstadt et al., 2022). Bacteriophage therapy can also be used to target the bacterium directly (Mondal et al., 2020; Selle et al., 2020; Whittle et al., 2022). The first FDA approved fecal microbiota product (RBX2660, "Rebyota") has shown success rates above 70% (Orenstein et al., 2016; Khanna et al., 2022; Orenstein et al., 2022). This product is made of human stool and administered as an enema. It is stored in form of a suspension in a single-dose ready-to-use enema bag at -80°C. The product can be stored at room temperature for up to 2 days prior to administration (Orenstein et al., 2016).

2 Materials and methods

2.1 Study design

The main aim of this research was to develop an effective encapsulation protocol to improve the practical application of fecal transplantation. We investigated whether the administration of a

TABLE 1 The protocol of the European Society of Clinical Microbiology and Infectious Diseases in CDI.

		Initial CDI	First recurrence	Further recurrences
Standard of care (SoC)	1st	Fidaxomicin 200 mg bid 10 days		FMT
	2nd	Vancomycin 125 mg qid 10 days		SoC + Bezlotoxumab
High risk of recurrence	1st	Fidaxomicin 200 mg bid 10 days		
	2nd	SoC + Bezlotoxumab		
Preferred option not available		Metronidazole 500 mg tid 10 days	Vancomycin taper and pulse	Vancomycin taper and pulse
Severe CDI		Vancomycin or Fidaxomicin Oral administration not possible: local delivery +/- adjunctive therapy with i.v. metronidazole or i.v. tigecycline		
Severe-complicated CDI and refractory severe complicated CDI		Vancomycin or Fidaxomicin Multidisciplinary approach with surgical consultation Consider i.v. tigecycline and FMT when refractory		

10.3389/fcimb.2024.1424376

TABLE 2 The baseline characteristics of group A and B.

	Female/ male/Sum	Mean age	Previous recurrences (median)
Group A	10/6/16	64,79 years	2
Group B	7/5/12	66,47 years	2

bacteria-free (or significantly reduced bacterial count) formulation is sufficient in CDI. Lyophilized fecal preparation was filled into capsules and stored at -20°C until administration.

The cure rate was recorded after capsule administration and the recurrence rate at one-year follow-up.

In parallel with the clinical trial, we assessed bacterial survival rates in the lyophilizates by periodic inoculation.

2.2 Population

Patients treated for CDI at the Department of Infectious Diseases, 1st Department of Internal Medicine, University of Pécs Clinical Centre between January 2018 and December 2019 were included in the study. During this period, a total of 28 patients received FMT with capsules. The intervention was offered for recurrent CDI when at least two recurrences occurred within 1 year.

After signing the informed consent form, patients were randomly assigned to receive either the supernatant (group A: 16 patients) or the sediment (group B: 12 patients).

The mean age of group A was 64.79 years, 10 women and 6 men. Group B had an average age of 66.47 years, 7 women and 5 men. The median number of previous relapses was 2 in both groups. Table 2 summarizes the baseline characteristics of the two groups.

2.3 Donor criteria

Stool samples were provided by one donor throughout the clinical trial. Donor selection was based on criteria in line with international recommendations and was done by direct recruitment. The selection criteria were as follows:

Age should be between 18 and 65 years, BMI below 30 kg/m^2, there should be no moderate or severe malnutrition. No chronic internal medicine, immunologic, neuropsychiatric or oncologic disease. No transmissible infectious diseases and no therapy influencing the immune system and the gut microbiome.

The donor was a tall, thin, Caucasian man in his twenties with a normal BMI, on a normal diet, not a healthcare worker. After evaluation of a risk assessment questionnaire and protocol-based screening, we provided him with the necessary information and tools for appropriate sample collection. The clinical trial was conducted before the first confirmed European appearance of the SARS-Cov-2 virus and therefore no screening was performed in this direction. Laboratory screening tests are summarized in Table 3.

TABLE 3 Laboratory screening tests for donors.

	From blood	From stool	From perianal swab
Bacteria	- *Treponema pallidum subspecies pallidum* - enzyme immunoassay	- Enteral pathogen culture: *Salmonella, Shigella, Campylobacter* spp., *Yersinia enterocolitica* - *Helicobacter pylori* - enzyme immunoassay	- *Vancomycin resistant Enterococcus* - antibiotic susceptibility test to detect poly-resistant strains - ESBL producing *Enterobacteriales* strains
	From blood	**From stool**	
Viruses	- Hepatitis A virus IgM – enzyme immunoassay - Hepatitis B virus surface antigen - enzyme immunoassay - Hepatitis C and E virus surface antibody - enzyme immunoassay - HIV 1 and 2 - enzyme immunoassay	- Norovirus - enzyme immunoassay or PCR - Rotavirus - enzyme immunoassay - ESBL and VRE – anuclease	
Parasites	- Entamoeba histolytica - enzyme immunoassay - Strongyloides stercoralis - enzyme immunoassay	- Parasites or their eggs - microscopic examination - Microsporidia - microscopic examination - Giardia antigen - enzyme immunoassay - Cryptosporidium - enzyme immunoassay - Isospora and Cyclospora - acid-fast staining	
Others	- Complete blood count - Liver function test - Sedimentation, C-reactive protein - HbA1c	- *C. difficile* toxin and glutamate dehydrogenase antigen detection	

2.4 Sample collection

The stool was collected at the donor's home in a non-sterile, airtight specimen collection container and the specimen was transported at room temperature to the sample processing laboratory within 1 hour.

2.5 Sample preparation

Sample processing was supposed to start within 6 hours of defecation according to our protocol. A dedicated laminar suction

box was used, which was disinfected with sporicidal disinfectant before each sample processing.

2.5.1 Homogenization, separation

During this phase, 60 g of feces were homogenized in a household mixer in a container with a sealable lid in 200 ml of physiological (0.9%) saline solution at room temperature. The resulting suspension was filtered twice to remove the larger solids. Solids that were difficult to remove by mechanical filtration were removed by a short-time, low g centrifugation (MPW-380R, Poland) (10 min, 827 g, room temperature) using 50 ml centrifuge tubes (Sarstedt Inc. Nümbrecht, Germany). With this method, approximately 120 ml of macroscopically homogeneous suspension can be produced from a 60 g dose of feces. Subsequently, the sample was treated in 100 ml portions. According to our previous protocol, this is the volume that the patient would receive if injected by nasogastric tube; therefore, this was chosen as the initial volume. Samples prepared for capsule preparation were then subjected to further centrifugation, this time for 15 min at 3309 g in a centrifuge precooled to 4°C. This step resulted in a sediment of around 30 ml, to which 10 ml of physiological (0.9%) saline was added to facilitate further treatment. The supernatant (around 70 ml volume) was divided into two parts and the sediment (around 40 ml volume) was stored whole in 5 cm diameter glass containers at -20°C for 12 hours. Samples at -20°C were then lyophilized (Freeze Dryer Heto Drywinner model DW1.0) at -40°C under vacuum 4*10–4 mbar for 36 hours, and the lyophilized samples were homogenized in a porcelain mortar on room temperature.

2.5.2 Lyophilization, encapsulation

The homogeneous lyophilizates were loaded into hard gelatin capsules of size "00" corresponding to the given volume using a capsulation device (Capsule Machine, Capsule Connection, LLC, Prescott AR, USA). From the initial volume of 100 ml, 4–6 capsules containing lyophilized sediment and 4–6 capsules containing lyophilized supernatant were prepared, depending on the dry matter content of the sample and the degree of compressibility during encapsulation. The capsules were then stored at -20°C until administration. The appearance of the lyophilized supernatant and sediment-filled capsules differed only in color shade. These capsules are water soluble and not resistant to gastric acid.

We also planned to test enterosolvent capsules (Vcaps® Enteric Capsules, Capsugel, Cambridge, MA, USA) to protect the lyophilizates against gastric acid. These capsules were one size smaller, size "0", resulting in a 45% increase in the number of capsules required to encapsulate a single dose of lyophilizate. Given the success of the normal capsules, enterosolvent capsules were ultimately not used in the clinical trial.

We also planned to encapsulate an unlyophilized fecal suspension. This required the creation of a water-insoluble coating on the inner wall of the capsules. We tried two formulations:

- Eudragit® L 30 D-55 (Ph. Eur. Methacrylic Acid - Ethyl Acrylate Copolymer 187 (1:1) 30% dispersion).

- Eudragit® RS 30 D (Ph. Eur. 0.3% Sodium Lauryl Sulphate, 1.2% 188 Polysorbate 80 - aqueous dispersion with 30% solids content).

Both dispersions deformed the capsules, which remained properly sealable (if not perfectly) after coating. The degree of deformation was found to be reduced by the use of a hair dryer. After contact with water, the pre-treated capsules retained their stiffness for 60 minutes for Eudragit® L 30 D-55 and 5 minutes for Eudragit® RS 30 D.

2.6 Administration

In patients scheduled for FMT, we stopped metronidazole or vancomycin treatment the day before the procedure and started proton pump inhibitor therapy (if not already ongoing) to raise pH of the gastric fluid and hence improve bacterial survival. We tried to achieve a more rapid intestinal transit of the drug and a reduction in the likelihood of vomiting by administering metoclopramide. Patients were not allowed to eat before taking the capsules on the day of transplantation. Patients were required to take 4–6 capsules, one at a time, in about 5 minutes. They were then not allowed to eat for 2 hours, during which time they had to remain in at least a semi-recumbent position.

2.7 Evaluation, follow up

Cure was defined as the resolution of clinical symptoms, relapse as the recurrence of symptoms within 6 months, and follow-up of patients was defined as one year.

2.8 *In vitro* survival of bacteria

Survival studies were performed on donor samples before and after lyophilization to determine how long samples could be stored using the presented method without loss of efficiency.

At the time of sample processing, a dilution series was prepared from 1–1 ml of the samples to be lyophilized (supernatant, sediment) and 1 ml of the fecal suspension before separation of the two fractions. Inoculation of these samples was performed immediately. At the end of lyophilization, volumes of around 0.01 g were measured from the lyophilization samples for immediate inoculation and for inoculation at 2 days, 1 week, 1 month, 3 months and 6 months. The measured samples were stored at 4 different temperatures: -80°C, -20°C, +4°C and +20°C (room temperature), i.e. 21–21 samples were taken from the two different lyophilizates. The samples were taken from 9 processed fecal samples (378 samples in total.) The samples to be inoculated were first diluted to the original concentration based on the masses measured before and after lyophilization, and then ten-fold serial dilutions were made. From the dilution series, a single loopful of inoculum was spread over the surface of the agar media and the media were incubated aerobically and anaerobically at 30°C.

One inoculation per dilution was prepared for each of the following media: blood agar (aerobic and anaerobic incubation), chocolate agar, chocolate agar with vancomycin, eosin-methylene blue agar, Sabouraud agar.

The census was carried out after 48 hours of incubation. Clearly infected media were excluded from the counts. All media where, despite higher dilution, multiple counts or colonies not complying with the dilution were detected were marked as clearly infected. Germ counts were expressed in CFU/ml (Colony Forming Unit).

3 Results

3.1 Capsule making workflow

The fundamental objective was to create an efficient encapsulation workflow. Using our protocol, an assistant can prepare a total of 4–6 doses of capsules within 72 hours, with a total of about 8 hours of work.

The most complex was to organize the ideal time to bring the donor stool to the lab, due to the time-consuming and time-critical nature of the initial steps. After 2–3 hours of preparation, the stool sample could be placed in the freezer overnight after the necessary centrifugation steps. The next morning, the lyophilization process could begin, lasting 36–48 hours. After lyophilization, approximately 2 hours were required to pulverize and encapsulate the lyophilizates.

3.2 Clinical results (sediment, supernatant)

The reference point for this clinical trial was the effectiveness of previous modalities of FMT. As expected, patients showed less aversion to the concept of taking capsules as opposed to taking them through different tubes. It should be noted, however, that in recurrent CDI in general, no significant persuasion was needed for the other modalities either.

In total, 28 cases of FMT with capsules were performed in the clinical trial. No anomalies were observed during their implementation; the most serious complication was mild abdominal discomfort.

In group A (where patients were given capsules containing lyophilized supernatant), 15 out of 16 patients recovered after a single dose of capsules (93.75%). Two of the recovered patients relapsed shortly after transplantation (within 3 weeks), but they were again asymptomatic after administration of fidaxomicin. (In the group of cured patients, one patient underwent colectomy for IBD.) During follow-up, one patient passed away due to a comorbidity unrelated to CDI. The history of the patient who did not respond to treatment prior to FMT included a total of 22 recurrences.

In group B (where patients were given capsules containing lyophilized sediment), 8 out of 12 patients recovered after the first dose (66.67%). None of them experienced a relapse, but one of them remained positive for *C. difficile* A toxin in stool without clinical symptoms. Two of the patients who did not recover did not respond to the rest of the therapeutic options, one of them recovered after another FMT and the other one finally recovered after treatment with fidaxomicin. One patient passed away during follow-up due to another disease.

The overall cure rate was 23/28 (82.14%). The results are summarized in Table 4.

TABLE 4 The clinical results with FMT capsules.

	Successful	Unsuccessful	Sum
Group A	15	1	16
Group B	8	4	12

3.3 Laboratory results (CFU)

The results of the inoculation experiments have already been reported in a previous publication (Varga et al., 2023).

Using concurrent aerobic and anaerobic cultures, we found a difference of approximately 600-fold between supernatant and sediment CFU counts. We did not investigate the possible contribution of spores to the residual bacterial counts in the supernatant. The results showed that the lyophilized supernatants did not contain any viable bacteria or bacterial spores after 6 months of storage at -20°C.

The experiment showed that survival was inversely related to storage temperature and storage time. Thus, survival was most favorable at -80°C, but the greatest difference was seen between samples kept at -20°C and +4°C. This suggests that storage at -20°C for 6 months seems to be adequate for survival.

4 Discussion

The presented clinical trial was conducted to improve the methodology of fecal transplantation. The described encapsulation workflow makes fecal transplantation significantly simpler and more flexible, providing a new tool for the investigation of other uses. The use of lyophilized, encapsulated stools avoids the use of nasogastric or nasojejunal tubes, thus reducing the risk of future clinical trial planning and making the intake of the capsules less burdensome for the patients. The planning of interventions is improved, and their constraints are reduced. These factors will hopefully lead to easier obtaining of the necessary licenses for trials. The capsulation protocol results in a longer sample preparation time than is required for the preparation of stool suspensions used in NG, NJ, or colonoscopic administration. However, the prepared capsules can be easily and reliably stored in a -20°C freezer, even for long periods of time. Due to the small volume, FMT can be performed immediately after taking the capsules out of the freezer.

As the inoculations during sample processing showed, the lyophilized supernatant-filled capsules were not nearly bacteria-free. However, they had significantly lower bacterial counts than those containing lyophilized sediment or the whole feces administered according to the previous procedure. A report has also shown that bacteria-free stool preparations produced by mechanical filtration can be used effectively (Ott et al., 2017), suggesting that it is not the bacteria or their spores that are administered, but rather the smaller size components of the stool that may be responsible for the effect. The inoculation results also showed that after 6 months, although the lyophilized supernatants did not contain viable bacteria or bacterial spores, they were still effective in CDI. These bacterial survival results provide a clue as to

how much less viable bacteria may have been present in capsules used 1–2, or even 6 months later (stored at -20°C until then) than in freshly used capsules. In contrast, results with bacterial concentrates of known composition suggest that the administration of some strains is effective (McGovern et al., 2021; Feuerstadt et al., 2022). It should be noted that these strains were not obtained by culture but by purification of donor stool, for example with ethanol.

For the bacteria and fungi that can grow on the media we used, we obtained information on some of the microorganisms introduced during the FMT, but we did not investigate the quantity and diversity of viruses at all. Several new viruses have been discovered in fecal samples using metagenomic studies (Xie et al., 2013), but even before this, a bewilderingly high diversity was to be taken into account.

To date, none of the current therapeutic recommendations mention stool transplantation as a first-line treatment for CDI or any other disease. Although it is already routinely used in the treatment of recurrent CDI in several centers, it is unlikely to be the first-line treatment of choice in this disease in the future.

The complexity of the sample to be administered always carries the risk of iatrogenic contamination, as has unfortunately been the case in recent years (DeFilipp et al., 2019; Zellmer et al., 2021). Normally, such a risk does not apply to antibiotics, as the manufacturer can control exactly what is included in the product. Bacterial concentrates of known composition are halfway between the two approaches (Orenstein et al., 2016; McGovern et al., 2021; Feuerstadt et al., 2022; Khanna et al., 2022; Orenstein et al., 2022).

The efficacy of bacteria-free formulations highlights what is probably the most important grey area at present. If a proportion of CDI infections can be cured with a bacteria-free stool preparation, the reason for the failure of bacterial concentrates may be the lack of, or insufficient quantity of a component that has been killed, denatured or diluted in the preparation of the concentrates. This component is naturally present in all feces, so it is likely that fecal transfusions will continue to be part of the protocol. However, in some cases, neither antibiotics nor the use of whole feces has led to cure. The existence of this group may be the result of inappropriate donor selection but is certainly a result of the lack of information available on the microbiome.

Our research suggests that fecal transplantation should be performed as shown in Table 5.

C. difficile infection is the only disease for which fecal transplantation is currently routinely used, so most of the information comes from this field. Clinical trials are also being carried out in other intestinal diseases, such as ulcerative colitis (CU) and Crohn's disease. A significant number of cases have been reported in several places, but the evidence is still awaited. Based on individual case reports (Blanchaert et al., 2019; Costello et al., 2019; Crothers et al., 2021), as we have also seen in one of the patients, FMT in CU may improve the patient's condition. However, it should be kept in mind that these are all individual observations. Patients received transplantation for essentially different indications, so the results should be interpreted with caution.

The lack of evidence combined with the cumbersome nature of the commonly used FMT methodology hampers further research in this field. It is anticipated that the use of capsules will remove a number of current barriers, both in terms of patients (burdensome intervention, aversions), approval (risks of intervention) and infrastructure (equipment, time and space requirements).

TABLE 5 Suggested faecal transplantation protocol.

Preparations	Regularly request stool samples from eligible donors who have undergone the necessary screening tests, depending on the expected use.
	Stool sample processing should begin within 6 hours of defecation.
	Process the stool sample according to the methodology described in this publication.
	Use '00' size hard gelatin capsules without protective coating can be used appropriately.
	Store lyophilized samples at -20°C until use (samples stored at -20°C can be used with adequate efficacy within 6 months.)
Administration	Discontinue metronidazole or vancomycin the day before the procedure.
	Start proton pump inhibitor therapy the day before the procedure.
	Administer metoclopramide to increase intestinal motility and reduce the risk of vomiting.
	Patients should not eat before taking capsules on the day of transplantation.
	Patients should not eat until at least 2 hours after taking the capsules, during which time they should remain in at least a semi-recumbent position.
Follow up	6-month follow-up after a successful procedure.

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 human participants were reviewed and approved by Hungarian Medical Research Council (ETT-TUKEB), Budapest, Hungary. Approval number: IF-7762-4/2015. Local ethical approvement number: 7083 – PTE 2018. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained

from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

DS: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. AV: Methodology, Project administration, Writing – review & editing. ÁK: Investigation, Writing – review & editing. BH-K: Investigation, Writing – review & editing. PK: Conceptualization, Writing – review & editing. SP: Conceptualization, Writing – review & editing. BK: Conceptualization, Methodology, Writing – review & editing. ZP: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The publication was funded by the University of Pécs, Medical School, Pécs, Hungary.

Acknowledgments

We would like to thank Dr. Gábor Reuter (head of the Department of Medical Microbiology and Immunology, University of Pécs Medical School) for supporting this study.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Aron-Wisnewsky, J., Warmbrunn, M. V., Nieuwdorp, M., and Clément, K. (2021). Metabolism and metabolic disorders and the microbiome: the intestinal microbiota associated with obesity, lipid metabolism, and metabolic health-pathophysiology and therapeutic strategies. *Gastroenterology* 160, 573–599. doi: 10.1053/j.gastro.2020.10.057

Blanchaert, C., Strubbe, B., and Peeters, H. (2019). Fecal microbiota transplantation in ulcerative colitis. *Acta Gastroenterol. Belg.* 82, 519–528.

Chandrasekaran, R., and Lacy, D. B. (2017). The role of toxins in *Clostridium difficile* infection. *FEMS Microbiol. Rev.* 41, 723–750. doi: 10.1093/femsre/fux048

Chen, J., Gong, C. L., Hitchcock, M. M., Holubar, M., Deresinski, S., and Hay, J. W. (2021). Cost-effectiveness of bezlotoxumab and fidaxomicin for initial *Clostridioides difficile* infection. *Clin. Microbiol. Infect.* 27, 1448–1454. doi: 10.1016/j.cmi.2021.04.004

Cooper, C. C., Jump, R. L. P., and Chopra, T. (2016). Prevention of infection due to Clostridium difficile. *Infect. Dis. Clin. North Am.* 30, 999–1012. doi: 10.1016/j.idc.2016.07.005

Costello, S. P., Hughes, P. A., Waters, O., Bryant, R. V., Vincent, A. D., Blatchford, P., et al. (2019). Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: a randomized clinical trial. *JAMA* 321, 156–164. doi: 10.1001/jama.2018.20046

Crothers, J. W., Chu, N. D., Nguyen, L. T. T., Phillips, M., Collins, C., Fortner, K., et al. (2021). Daily, oral FMT for long-term maintenance therapy in ulcerative colitis: results of a single-center, prospective, randomized pilot study. *BMC Gastroenterol.* 21, 281. doi: 10.1186/s12876-021-01856-9

DeFilipp, Z., Bloom, P. P., Torres Soto, M., Mansour, M. K., Sater, M. R. A., Huntley, M. H., et al. (2019). Drug-resistant E. coli bacteremia transmitted by fecal microbiota transplant. *N Engl. J. Med.* 381, 2043–2050. doi: 10.1056/NEJMoa1910437

DePeters, E. J., and George, L. W. (2014). Rumen transfaunation. *Immunol. Lett.* 162, 69–76. doi: 10.1016/j.imlet.2014.05.009

Dubberke, E. R., and Olsen, M. A. (2012). Burden of *Clostridium difficile* on the healthcare system. *Clin. Infect. Dis.* 55 Suppl 2, S88–S92. doi: 10.1093/cid/cis335

Džunková, M., D'Auria, G., Xu, H., Huang, J., Duan, Y., Moya, A., et al. (2016). The monoclonal antitoxin antibodies (Actoxumab-Bezlotoxumab) treatment facilitates normalization of the gut microbiota of mice with *Clostridium difficile* infection. *Front. Cell Infect. Microbiol.* 6, 119.

Eisenman, B., Silen, W., Bascom, G. S., and Kauvar, A. J. (1958). Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery* 44, 854–859.

Feuerstadt, P., Louie, T. J., Lashner, B., Wang, E. E. L., Diao, L., Bryant, J. A., et al. (2022). SER-109, an oral microbiome therapy for recurrent *Clostridioides difficile* infection. *N Engl. J. Med.* 386, 220–229. doi: 10.1056/NEJMoa2106516

Ford, A. C., Sperber, A. D., Corsetti, M., and Camilleri, M. (2020). Irritable bowel syndrome. *Lancet* 396, 1675–1688. doi: 10.1016/S0140-6736(20)31548-8

Gerding, D. N., Kelly, C. P., Rahav, G., Lee, C., Dubberke, E. R., Kumar, P. N., et al. (2018). Bezlotoxumab for prevention of recurrent *Clostridium difficile* infection in patients at increased risk for recurrence. *Clin. Infect. Dis.* 67, 649–656. doi: 10.1093/cid/ciy171

Ghantoji, S. S., Sail, K., Lairson, D. R., DuPont, H. L., and Garey, K. W. (2010). Economic healthcare costs of *Clostridium difficile* infection: a systematic review. *J. Hosp. Infect.* 74, 309–318. doi: 10.1016/j.jhin.2009.10.016

Ianiro, G., Mullish, B. H., Kelly, C. R., Kassam, Z., Kuijper, E. J., Ng, S. C., et al. (2020). Reorganisation of faecal microbiota transplant services during the COVID-19 pandemic. *Gut* 69, 1555–1563. doi: 10.1136/gutjnl-2020-321829

Jangi, S., and Lamont, J. T. (2010). Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in later life. *J. Pediatr. Gastroenterol. Nutr.* 51, 2–7. doi: 10.1097/MPG.0b013e3181d29767

Johnson, S., Lavergne, V., Skinner, A. M., Gonzales-Luna, A. J., Garey, K. W., Kelly, C. P., et al. (2021). Clinical practice guideline by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA): 2021 focused update guidelines on management of *Clostridioides difficile* infection in adults. *Clin. Infect. Dis.* 73, e1029–e1044. doi: 10.1093/cid/ciab718

Kelly, C. R., Ihunnah, C., Fischer, M., Khoruts, A., Surawicz, C., Afzali, A., et al. (2014). Fecal microbiota transplant for treatment of *Clostridium difficile* infection in immunocompromised patients. *Am. J. Gastroenterol.* 109, 1065–1071. doi: 10.1038/ajg.2014.133

Khanna, S., Assi, M., Lee, C., Yoho, D., Louie, T., Knapple, W., et al. (2022). Efficacy and safety of RBX2660 in PUNCH CD3, a phase III, randomized, double-blind, placebo-controlled trial with a bayesian primary analysis for the prevention of recurrent *Clostridioides difficile* infection. *Drugs* 82, 1527–1538. doi: 10.1007/s40265-022-01797-x

Kramer, A., Schwebke, I., and Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Diseases* 6, 130. doi: 10.1186/1471-2334-6-130

Mathias, F., Curti, C., Montana, M., Bornet, C., and Vanelle, P. (2019). Management of adult *Clostridium difficile* digestive contaminations: a literature review. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 209–231. doi: 10.1007/s10096-018-3419-z

Mathur, H., Rea, M. C., Cotter, P. D., Ross, R. P., and Hill, C. (2014). The potential for emerging therapeutic options for *Clostridium difficile* infection. *Gut. Microbes* 5, 696–710. doi: 10.4161/19490976.2014.983768

McGovern, B. H., Ford, C. B., Henn, M. R., Pardi, D. S., Khanna, S., Hohmann, E. L., et al. (2021). SER-109, an investigational microbiome drug to reduce recurrence after *Clostridioides difficile* infection: lessons learned from a phase 2 trial. *Clin. Infect. Dis.* 72, 2132–2140. doi: 10.1093/cid/ciaa387

Mondal, S. I., Draper, L. A., Ross, R. P., and Hill, C. (2020). Bacteriophage endolysins as a potential weapon to combat *Clostridioides difficile* infection. *Gut. Microbes* 12, 1813533. doi: 10.1080/19490976.2020.1813533

Mullane, K. (2014). Fidaxomicin in *Clostridium difficile*infection: latest evidence and clinical guidance. *Ther. Adv. Chronic. Dis.* 5, 69–84. doi: 10.1177/2040622313511285

Nagy, G. G., Tudlik, Z., Gergely, L., Kónya, J., Orosi, P., Rákóczi, É., et al. (2020). Reconsidering the technical aspects and quality management background of faecal microbiota transplantation due to the novel coronavirus pandemic. *Orv. Hetil.* 161, 1858–1871. doi: 10.1556/650.2020.32023

Nagy, G. G., Várvölgyi, C., Balogh, Z., Orosi, P., and Paragh, G. (2013). Detailed methodological recommendations for the treatment of *Clostridium difficile*-associated diarrhea with faecal transplantation. *Orv. Hetil.* 154, 10–19. doi: 10.1556/OH.2013.29514

Orenstein, R., Dubberke, E., Hardi, R., Ray, A., Mullane, K., Pardi, D. S., et al. (2016). Safety and durability of RBX2660 (microbiota suspension) for recurrent *Clostridium difficile* infection: results of the PUNCH CD study. *Clin. Infect. Dis.* 62, 596–602. doi: 10.1093/cid/civ938

Orenstein, R., Dubberke, E. R., Khanna, S., Lee, C. H., Yoho, D., Johnson, S., et al. (2022). Durable reduction of *Clostridioides difficile* infection recurrence and microbiome restoration after treatment with RBX2660: results from an open-label phase 2 clinical trial. *BMC Infect. Dis.* 22, 245. doi: 10.1186/s12879-022-07256-y

Ott, S. J., Waetzig, G. H., Rehman, A., Moltzau-Anderson, J., Bharti, R., Grasis, J. A., et al. (2017). Efficacy of sterile fecal filtrate transfer for treating patients with *Clostridium difficile* infection. *Gastroenterology* 152, 799–811. doi: 10.1053/j.gastro.2016.11.010

Péterfi, Z. A. (2015). *Clostridium difficile* fertőzések korszerű terápiája. *Med. Tribune* 12, 14–14.

Prabhu, V. S., Dubberke, E. R., Dorr, M. B., Elbasha, E., Cossrow, N., Jiang, Y., et al. (2018). Cost-effectiveness of bezlotoxumab compared with placebo for the prevention of recurrent *Clostridium difficile* infection. *Clin. Infect. Dis.* 66, 355–362. doi: 10.1093/cid/cix809

Salavert, M., Cobo, J., Pascual, Á., Aragón, B., Maratia, S., Jiang, Y., et al. (2018). Cost-effectiveness analysis of bezlotoxumab added to standard of care versus standard of care alone for the prevention of recurrent *Clostridium difficile* infection in high-risk patients in Spain. *Adv. Ther.* 35, 1920–1934. doi: 10.1007/s12325-018-0813-y

Segal, J. P., Mullish, B. H., Quraishi, M. N., Iqbal, T., Marchesi, J. R., and Sokol, H. (2020). Mechanisms underpinning the efficacy of faecal microbiota transplantation in treating gastrointestinal disease. *Therap. Adv. Gastroenterol.* 13, 1756284820946904. doi: 10.1177/1756284820946904

Selle, K., Fletcher, J. R., Tuson, H., Schmitt, D. S., McMillan, L., Vridhambal, G. S., et al. (2020). *In vivo* targeting of *Clostridioides difficile* using phage-delivered CRISPR-Cas3 antimicrobials. *mBio* 11 (2), e00019-20. doi: 10.1128/mBio.00019-20

van Prehn, J., Reigadas, E., Vogelzang, E. H., Bouza, E., Hristea, A., Guery, B., et al. (2021). European Society of Clinical Microbiology and Infectious Diseases: 2021 update on the treatment guidance document for *Clostridioides difficile* infection in adults. *Clin. Microbiol. Infect.* 27 Suppl 2, S1–21. doi: 10.1016/j.cmi.2021.09.038

Varga, A., Kocsis, B., Sipos, D., Kása, P., Vigvári, S., Pál, S., et al. (2021). How to apply FMT more effectively, conveniently and flexible - a comparison of FMT methods. *Front. Cell Infect. Microbiol.* 11, 657320. doi: 10.3389/fcimb.2021.657320

Varga, A., Makszin, L., Bufa, A., Sipos, D., Kása, P., Pál, S., et al. (2023). Efficacy of lyophilised bacteria-rich faecal sediment and supernatant with reduced bacterial count for treating patients with *Clostridioides difficile* Infection - a novel method for capsule faecal microbiota transfer. *Front. Cell Infect. Microbiol.* 13, 1041384. doi: 10.3389/fcimb.2023.1041384

Venugopal, A. A., and Johnson, S. (2012). Current state of *Clostridium difficile* treatment options. *Clin. Infect. Dis.* 55 Suppl 2, S71–S76. doi: 10.1093/cid/cis355

Vickers, R. J., Tillotson, G. S., Nathan, R., Hazan, S., Pullman, J., Lucasti, C., et al. (2017). Efficacy and safety of ridinilazole compared with vancomycin for the treatment of *Clostridium difficile* infection: a phase 2, randomised, double-blind, active-controlled, non-inferiority study. *Lancet Infect. Dis.* 17, 735–744. doi: 10.1016/S1473-3099(17)30235-9

Vigvári, S., Nemes, Z., Vincze, Á., Solt, J., Sipos, D., Feiszt, Z., et al. (2015). Faecal microbiota transplantation in *Clostridium difficile* infections. *Infect. Dis. (Lond)* 47, 114–116. doi: 10.3109/00365548.2014.969305

Vigvári, S., Sipos, D., Solt, J., Vincze, Á., Kocsis, B., Nemes, Z., et al. (2019). Faecal microbiota transplantation for *Clostridium difficile* infection using a lyophilized inoculum from non-related donors: A case series involving 19 patients. *Acta Microbiol. Immunol. Hung.* 66, 69–78. doi: 10.1556/030.64.2017.042

Warny, M., Pepin, J., Fang, A., Killgore, G., Thompson, A., Brazier, J., et al. (2005). Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* 366, 1079–1084. doi: 10.1016/S0140-6736(05)67420-X

Whittle, M. J., Bilverstone, T. W., van Esveld, R. J., Lücke, A. C., Lister, M. M., Kuehne, S. A., et al. (2022). A novel bacteriophage with broad host range against *Clostridioides difficile* ribotype 078 supports SlpA as the likely phage receptor. *Microbiol. Spectr.* 10, e0229521. doi: 10.1128/spectrum.02295-21

Wilcox, M. H., Gerding, D. N., Poxton, I. R., Kelly, C., Nathan, R., Birch, T., et al. (2017). Bezlotoxumab for prevention of recurrent *Clostridium difficile* infection. *N Engl. J. Med.* 376, 305–317. doi: 10.1056/NEJMoa1602615

Xie, G., Yu, J., and Duan, Z. (2013). New strategy for virus discovery: viruses identified in human feces in the last decade. *Sci. China Life Sci.* 56, 688–696. doi: 10.1007/s11427-013-4516-y

Youngster, I., Sauk, J., Pindar, C., Wilson, R. G., Kaplan, J. L., Smith, M. B., et al. (2014). Fecal microbiota transplant for relapsing *Clostridium difficile* infection using a frozen inoculum from unrelated donors: a randomized, open-label, controlled pilot study. *Clin. Infect. Dis.* 58, 1515–1522. doi: 10.1093/cid/ciu135

Zellmer, C., Sater, M. R. A., Huntley, M. H., Osman, M., Olesen, S. W., and Ramakrishna, B. (2021). Shiga toxin-producing *Escherichia coli* transmission *via* fecal microbiota transplant. *Clin. Infect. Dis.* 72, e876–e880. doi: 10.1093/cid/ciaa1486

Zhang, F., Luo, W., Shi, Y., Fan, Z., and Ji, G. (2012). Should we standardize the 1,700-year-old fecal microbiota transplantation? *Am. J. Gastroenterol.* 107, 1755–1756. doi: 10.1038/ajg.2012.251

frontiers | Frontiers in Microbiomes

TYPE Original Research
PUBLISHED 04 July 2024
DOI 10.3389/frmbi.2024.1404353

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Promi Das,
University College Cork, Ireland
Shaghayegh Baradaran Ghavami,
Shahid Beheshti University of Medical Sciences, Iran

*CORRESPONDENCE
Qingsheng Li
qli@unl.edu

†PRESENT ADDRESS
Lance Daharsh,
Genvax Technologies, Ames, IA, United States

‡These authors have contributed equally to this work

RECEIVED 20 March 2024
ACCEPTED 21 June 2024
PUBLISHED 04 July 2024

CITATION
Daharsh L, Lohani SC, Ramer-Tait AE and Li Q (2024) Characterization of double humanized BLT-mice with stable engraftment of a human gut bacterial microbiome. *Front. Microbiomes* 3:1404353. doi: 10.3389/frmbi.2024.1404353

COPYRIGHT
© 2024 Daharsh, Lohani, Ramer-Tait and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Characterization of double humanized BLT-mice with stable engraftment of a human gut bacterial microbiome

Lance Daharsh[1,2†‡], Saroj Chandra Lohani[1,2‡], Amanda E. Ramer-Tait[3,4] and Qingsheng Li[1,2*]

[1]School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE, United States, [2]Nebraska Center for Virology, University of Nebraska-Lincoln, Lincoln, NE, United States, [3]Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, United States, [4]Nebraska Food for Health Center, University of Nebraska-Lincoln, Lincoln, NE, United States

Humanized mice with human-like immune systems are commonly used to study immune responses to human-specific pathogens. However, one limitation of using humanized mice is their native murine gut microbiota, which significantly differs from that in humans. Given the importance of the gut microbiome to human health, these differences may profoundly impact the ability to translate results from humanized mouse studies to humans. Further, there is a critical need for improved pre-clinical models to study the complex *in vivo* relationships of the gut microbiome, immune system, and human disease. We previously created double humanized mice with a functional human immune system and a stable, human-like gut microbiome. Here, we characterized the engrafted human gut bacterial microbiome in our double humanized mouse model generated by transplanting fecal material from healthy human donors into the gut of humanized mice. Analysis of bacterial microbiomes in fecal samples from double humanized mice revealed they had unique 16S rRNA gene profiles consistent with those of the individual human donor samples. Importantly, transplanted human-like gut microbiomes were stable in mice for the duration of the study, extending up to 14.5 weeks post-transplant. Microbiomes of double humanized mice also harbored predicted functional capacities that more closely resembled those of the human donors than humanized mice. In conclusion, our study highlights the successful engraftment of human fecal microbiota in BLT humanized mice and underscores the stability of this model, offering a valuable platform for investigating the intricate interplay among the human gut microbiome, immune system, and various diseases *in vivo*.

KEYWORDS

double humanized mice, stable engraftment, human gut microbiota, mouse model, BLT mice

1 Introduction

The complex ecosystem of the gut microbiome plays a critical role in human health and disease (Turnbaugh et al., 2006; Qin et al., 2010; Clemente et al., 2018; Gopalakrishnan et al., 2018; Routy et al., 2018). Specifically, the gut microbiome has a highly reciprocal and dynamic relationship with the immune system. Antigens derived from the gut microbiome influence host immune responses, and the immune system, in turn, contributes to shaping the spatial distribution and composition of the gut microbiota (Kau et al., 2011; Hooper et al., 2012; Maynard et al., 2012). Humanized mice (hu-mice) with an engrafted human immune system have facilitated important advancements in the study of human cancer, autoimmune diseases, hematopoiesis, and infectious diseases (Sun et al., 2007; Simpson-Abelson et al., 2008; Bankert et al., 2011; Whitfield-Larry et al., 2011; Vudattu et al., 2014; Wang et al., 2014; Stary et al., 2015; Ernst, 2016; Yi et al., 2017). However, gut microbiomes of hu-mice are murine in origin and are often not well-characterized in translational studies. The murine gut microbiome differs substantially in composition and function from that of humans (Xiao et al., 2015), primarily due to anatomical or evolutionary differences as well as other factors such as diet (Nguyen et al., 2015). Considering the importance of the gut microbiota in proper immunological development and influencing immune responses, the murine origin of the microbiome harbored by hu-mice could affect translational study outcomes. Consequently, a need exists to not only characterize the gut microbiomes of hu-mice but also impart these mice with a more human-like gut microbiome to improve the translational aspects of the model for human medicine.

The creation of a new, pre-clinical hu-mice model to study the human immune system in the context of a human microbiome offers numerous benefits over existing options. Many aspects of human disease are difficult or impossible to study directly in humans due to practical or ethical concerns. Non-human primate models are informative; however, they are often resource and cost prohibitive. Many important discoveries in the microbiome field have been made using mouse models; however, translating results from mouse studies to humans has often proven difficult. The use of germ-free mice reconstituted with human-like gut microbiomes has been the gold standard in studying the relationship of the gut microbiome to human health and disease (Turnbaugh et al., 2009; Kennedy et al., 2018). However, working with or deriving germ-free animals requires expertise and facilities that are not always available. Further, many immunodeficient mouse strains commonly used to reconstitute a human immune system, such as NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) are currently not commercially available as germ-free. We previously created a double hu-mice model featuring a functional human immune system and a human-like gut microbiome under specific pathogen free (SPF) conditions (Daharsh L et al., 2019). Here, we characterized the gut microbiome of the double hu-mice, demonstrating their unique 16S rRNA gene profiles based on the individual human donor sample with which they were colonized. Importantly, the transplanted human-like microbiome was stable in the mice for the duration of the study, up to 14.5 weeks post-transplant. Double hu-mice also harbored gut microbiomes with a more human-like predicted functional capacity compared to their hu-mice counterparts.

2 Methods

2.1 Generation of hu-mice

All methods described here were conducted as we previously reported (Li et al., 2015; Destache et al., 2016; Yuan et al., 2016; Daharsh L et al., 2019) in accordance with Institutional Animal Care and Research Committee (IACUC)-approved protocols at the University of Nebraska-Lincoln (UNL). Briefly, 6- to 8-week-old female NSG mice (NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ, Cat# 005557; Jackson Laboratory, Bar Harbor, ME) were housed and maintained in individual microisolator cages in a rack system designed to regulate air circulation through pre-filters and HEPA filters and fed with irradiated Teklad global 14% protein rodent chow (Teklad 2914; Inotiv, Madison, WI) along with autoclaved acidified drinking water. The second cohort of mice (Table 1) was supplemented with a high-calorie gel (DietGel Boost). Prior to surgery to humanize the mouse immune system, mice were subjected to total-body irradiation with a dose of 12 cGy per gram of body weight using the RS200 X-ray irradiator (RAD Source Technologies, Inc., GA). Following irradiation, mice were implanted with a single human fetal thymic tissue fragment sandwiched between two human fetal liver tissue fragments within the left renal capsule. Human CD34+ hematopoietic stem cells were isolated from human fetal liver and injected into mice via tail vein at a range of 1.5-5 $\times$ 10^5 within a 6-hour window following surgery. The human fetal liver tissues and thymus used in this study were obtained from Advanced Bioscience Resources (Alameda, CA). After 9 to 12 weeks, human immune cell reconstitution in peripheral blood was measured by a fluorescence-activated cell sorter (FACS) Aria II flow cytometer (BD Biosciences, San Jose, CA) using antibodies against mCD45-APC, hCD45-FITC, hCD3-PE, hCD19-PE/Cy5, hCD4-Alexa 700, and hCD8-APC-Cy7 (Cat# 103111, 304006, 300408, 302209, 300526, and 301016, respectively; BioLegend, San Diego, CA). Raw data were analyzed with FlowJo (version 10.0; FlowJo LLC, Ashland, OR). All hu-mice used in this study had human immune cell reconstitution with an average of 85% hCD45+ cells in peripheral blood 12 weeks post-surgery. The assignment of mice into the experimental groups was done randomly, ensuring comparable levels of human immune cell reconstitution across the groups.

2.2 Generation of double hu-mice

The hu-mice generated described above in section 2.1 by the bone-marrow, liver, thymus (BLT) method of humanization was then used to create double hu-mice with both the human immune system and human microbiota based on antibiotic-induced microbiota depletion procedure followed by oral gavage of human fecal microbiota.

TABLE 1 Experimental summary of double hu-mice cohorts.

Cohort	Antibiotic start	Antibiotic duration	Number of fecal transplants	Human fecal donor	Maximum weeks post-transplant	Double hu-mice	Double hu-mice fecal samples	Hu-mice	Hu-mice fecal samples
Antibiotic Pilot	After immune reconstitution	9 days	0	NA	0	0	0	6	9
Donor 65 Cohort 1	After immune reconstitution	14 days	2 (24 & 48 hr post antibiotics)	65	13.5	3	35#	3	23
Donor 65 Cohort 2	After immune reconstitution	14 days	2 (24 & 48 hr post antibiotics)	65	14.5	2	16#	8	8
Donor 74	After immune reconstitution	7 days	2 (24 & 48 hr post antibiotics)	74	6	3	17#	3*	3*
Donor 82	After immune reconstitution	7 days	2 (24 & 48 hr post antibiotics)	82	6	4	27#	3*	3*
Donor Mix	3 days after hu-mice surgery	14 days	2 (24 & 48 hr post antibiotics)	Equal mixture of 65, 74, & 82	9	4	21#	9	10

#Includes 1 pre-fecal transplant sample for each of the double hu-mice.
*Control hu-mice were the same for Donor 74 and Donor 82 groups.

2.2.1 Antibiotic treatment

A cocktail of broad-spectrum antibiotics consisting of Ampicillin (1 g/L), Metronidazole (1 g/L), Neomycin (1 g/L), and Vancomycin (0.5 g/L) was prepared fresh daily and given to the mice ad libitum in the drinking water along with grape-flavored Kool-Aid to improve palatability. Mice in the control group were provided solely with grape flavored Kool-Aid in the drinking water prepared fresh daily. During antibiotic treatment, cages were replaced daily to limit re-inoculation of pre-existing bacteria in the mice due to their coprophagic behavior. Antibiotics were given for 14 days for double hu-mice engrafted with Donor 65 and Donor Mix and for seven days for double hu-mice engrafted with Donor 74 and Donor 82. Mice in the pilot study were given antibiotics via oral gavage as follows: For three days, mice received anti-fungal Amphotericin B treatment (1 mg/kg) twice daily via oral gavage and were then given the antibiotic cocktail along with Amphotericin B twice daily via oral gavage. After four days of treatment, the Amphotericin B was stopped due to toxicity concerns, and after 10 days of treatment, oral gavage was reduced to once daily. Post-antibiotic treatment, all mice were given autoclaved non-acidified deionized drinking water.

During the first few days of antibiotic treatment, mice lost a considerable amount of body weight (10–20%). The weight loss plateaued after three to four days and remained steady for the remainder of antibiotic treatment. Throughout antibiotic treatment, we closely monitored the body weight of the mice, and when necessary, administered intraperitoneal (IP) injection of Ringer's solution to alleviate any effects of dehydration. After fecal transplant, the mice regained weight and returned to their pre-existing weight within two weeks post-transplant. During antibiotic treatment, there was a large reduction in spleen size and a large increase in cecum size compared to controls. This is similar to the morphology observed in germ-free mice, providing further evidence for the efficacy of the antibiotic regimen (Smith et al., 2007).

2.2.2 Donor samples and fecal microbiota transplant

Three unique microbiota preparations (Donor 65, Donor 74, and Donor 82) were procured from OpenBiome (Massachusetts, USA). An unbiased human donor sample (Donor mix) was prepared within an anaerobic chamber by mixing an equal portion of the three different donor microbiota preparations. Hu-mice were grouped into multiple cohorts and 200 µL of human fecal material from one of three unique healthy human donors or an equal mixture of all three (Donor mix) were given via oral gavage at 24 and 48 hours after the completion of antibiotic pre-treatment. 16S rRNA sequencing data on the three donors was also supplied by OpenBiome.

2.3 Mouse fecal collection and DNA extraction

Fresh fecal samples from the mice were collected within a biosafety hood by placing individual mice into autoclaved paper bags. Fecal samples were stored at −80°C in 1.5 mL Eppendorf tubes until DNA extraction. DNA was extracted from the fecal samples using the method described previously (Martinez et al., 2009). Briefly, fecal samples were washed three times with 1 mL PBS buffer (pH 7), followed by incubation at 60° C for 30 minutes along

with 750 µL of lysis buffer, 300 mg of autoclaved 0.1 mm zirconia/silica beads (Biospec), 85 µL of 10% SDS solution, and 40 µL of Proteinase K (15mg/mL, Promega, Cat# MC500B). After incubation, 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and cells were physically lysed using a bead beater (BioSpec Mini-Beadbeater-16) for 2 minutes. The upper phase of the sample was collected and additional 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added and spun down to separate upper phase containing the DNA, which was further purified twice with 500 µL of chloroform:isoamyl alcohol (24:1). DNA was then precipitated overnight at −20°C with 100% Ethanol (2.5 x volume of sample) and 3M sodium acetate (0.1 x volume of sample) and dried at room temperature. Dried DNA was resuspended in 100 µL of Tris-Buffer (10mM, pH8) and stored at −20°C for future use.

2.4 16S rRNA bacterial gene sequencing

16S rRNA bacterial gene sequencing was performed at the University of Nebraska Medical Center Genomics Core Facility using Illumina MiSeq. DNA normalization and library prep were performed, followed by V3-V4 16S rRNA amplicon gene sequencing using a MiSeqV2 (Illumina). The following primer sequences were used: (Primer sequences: Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Illumina overhangs: Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locusspecific sequence] Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locusspecific sequence]).

2.5 Generation of the amplicon sequence variant table and data analysis

Illumina-sequenced paired-end fastq files were demultiplexed by sample and barcodes were removed by the sequencing facility. Amplicon sequence variant (ASV) table was generated following the DADA2 v1.8 R package pipeline using the University of Nebraska Holland Computer Center Crane cluster (Callahan et al., 2016). Taxonomy was assigned using the Greengenes 13.8 database and RDP Classifier with a minimal confidence score of 0.80 (Wang et al., 2007; Caporaso et al., 2010). Analysis was performed using R package mctoolsr (https://github.com/leffj/mctoolsr/), and samples were rarified to 13,000 ASVs for downstream analysis. Additional testing of differences between groups was performed using LEfSe (Segata et al., 2011). SourceTracker was used to evaluate the stability of the transferred donor microbiome in the double hu-mice (Knights et al., 2011). GraphPad Prism 5 was used to create some figures. DADA2 generated ASVs were used to predict the functional metagenome capacity using PICRUSt (Langille et al., 2013) via the following pipeline (https://github.com/vmaffei/dada2_to_picrust).

3 Results

3.1 Gut microbiomes of double hu-mice were distinct and more human-like compared to hu-mice

Multiple cohorts of double hu-mice were created using fecal material from one of three unique healthy human donors or an equal mixture of all three (Table 1). We used 16S rRNA gene sequencing to characterize the gut bacterial microbiome of 100 fecal samples from 16 double hu-mice and compare them with 67 fecal samples representing the pre-existing murine gut microbiomes of hu-mice and the profiles of the four human fecal donor samples. To visualize beta-diversity relationships between hu-mice, double hu-mice, and the human donor samples, non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) plots were created. In the NMDS plot, the gut microbiome profiles of the three groups separated into distinct clusters (Figure 1A). In the PCoA plot, the human donor samples fell within the double hu-mice hull, which was distinct from the hu-mice profiles (Figure 1B). Both dimensionality reduction methods revealed that following human gut microbiome engraftment, double hu-mice exhibited a distinct gut microbial profile that clustered closer to the human donor samples when compared to hu-mice harboring murine gut microbiomes. Importantly, there was no reversion to hu-mice profiles post-transplant. Displaying the relatedness of the samples through a hierarchical dendrogram based on Bray-Curtis distances further confirmed the similarity of double hu-mice microbiomes to the human donor samples and differentiated them from those of hu-mice (Figure 2).

We also observed that double hu-mice maintained the pre-existing relationships among the microbiome profiles of the human fecal donors (Figures 1C, D). Importantly, pre-treatment samples from corresponding double hu-mice in each cohort had similar gut microbiome profiles as untreated control hu-mice. After engraftment, double hu-mice resembled the individual human donor that was transplanted as demonstrated by the relationships between the human donor microbiome profiles. Human donors 74 and 82 had more similar profiles to one another than to human donor 65. This relationship was maintained in the double hu-mice after engraftment. We also prepared an "unbiased" human sample by mixing equal parts of the three human donor fecal samples, designated hereafter as donor mix. The microbiome profile of this mixed sample resembled a mixture of the three individual human donor profiles. Specifically, the mixed donor sample more closely resembled individual donors 74 and 82, and this observation was mirrored in the double hu-mice engrafted with the donor mix sample.

We also investigated the impact of antibiotic treatment duration on the engraftment of the human gut microbiome. The double hu-mice engrafted with human donors 74 and 82 were generated after only seven days of antibiotic pre-treatment. Their microbiome profiles were less similar to the human donor profiles than to those from cohorts pre-treated with antibiotics for 14 days prior to fecal transplant (Figures 1C, D). We found the two weeks of antibiotic treatment was optimal for the creation of double hu-mice. Together, these results demonstrate that our approach of generating double hu-

10.3389/frmbi.2024.1404353

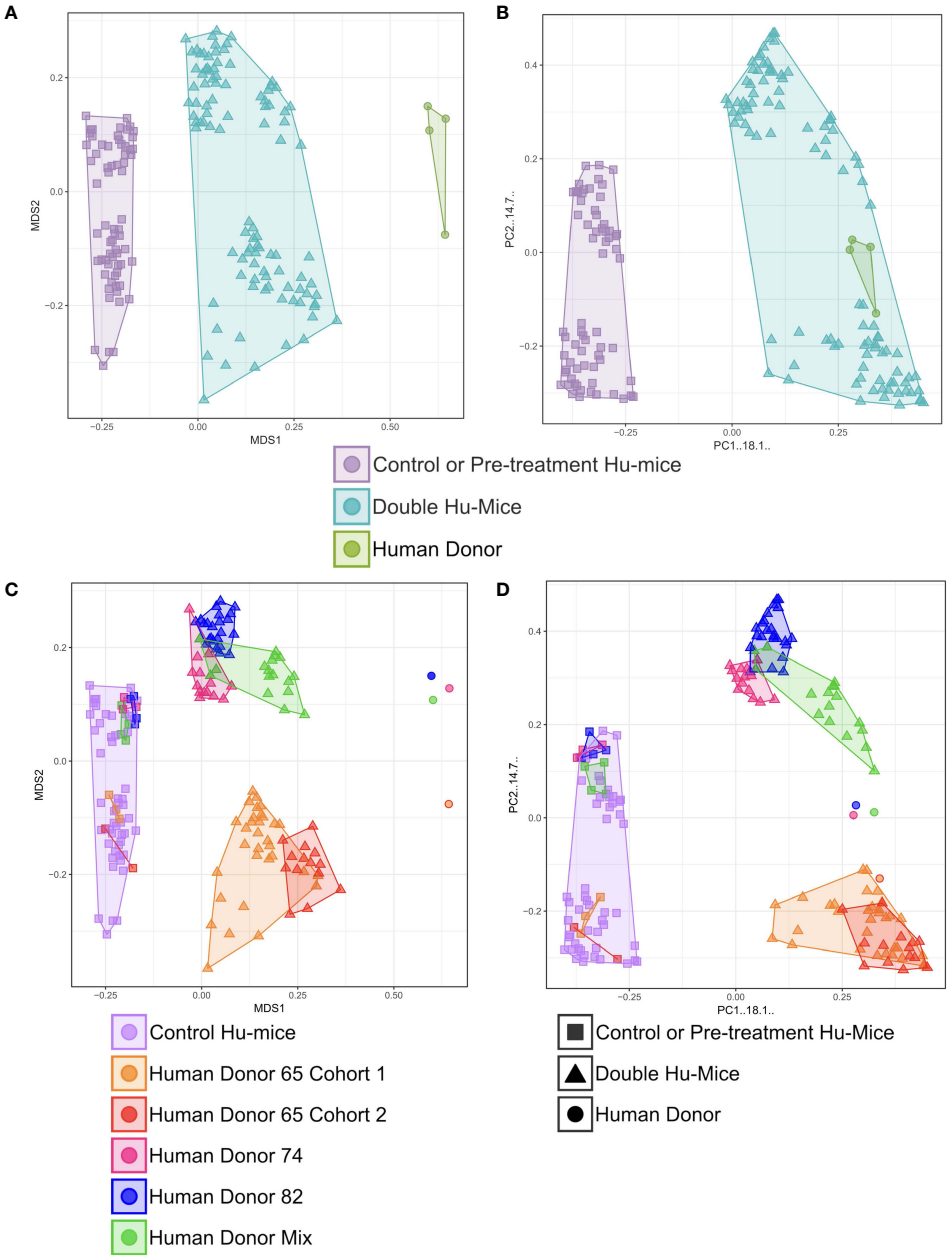


FIGURE 1
Gut microbiomes of double hu-mice were distinct and more human-like compared to hu-mice and feature donor specific profiles. Double hu-mice clustered distinctly than the human donor samples and pre- treatment or untreated control hu-mice in Non-metric multidimensional scaling (NMDS) plot **(A)** and in Principal coordinate analysis (PCoA) plot **(B)**. Plot displaying human donor specific profiles of the double hu-mice in NMDS plot **(C)** and in PCoA plot **(D)**.

mice is reproducible and able to create hu-mice with unique 16S rRNA gene profiles based on the individual human fecal donor.

3.2 Gut microbiomes of double hu-mice had increased levels of alpha diversity compared to hu-mice

Since the highly immunodeficient NSG mice used for generating hu-mice were raised under SPF conditions with limited exposure to outside sources of microbes, we hypothesized that successful engraftment of donor microbiota would significantly increase the gut microbial diversity of double hu-mice compared to the hu-mice. We tested this hypothesis and found that the gut microbial diversity of double hu-mice with a functional immune system significantly increased to similar levels observed in our human donor samples versus the hu-mice. Several alpha diversity measurements confirmed that hu-mice had very low measures of alpha diversity compared to our human donor samples (Figure 3). However, after engraftment, double hu-mice had increased species richness compared to hu-mice ($P < 0.001$) and did not differ significantly from the human donor samples (Figure 3A). Further, the Shannon index values of the human

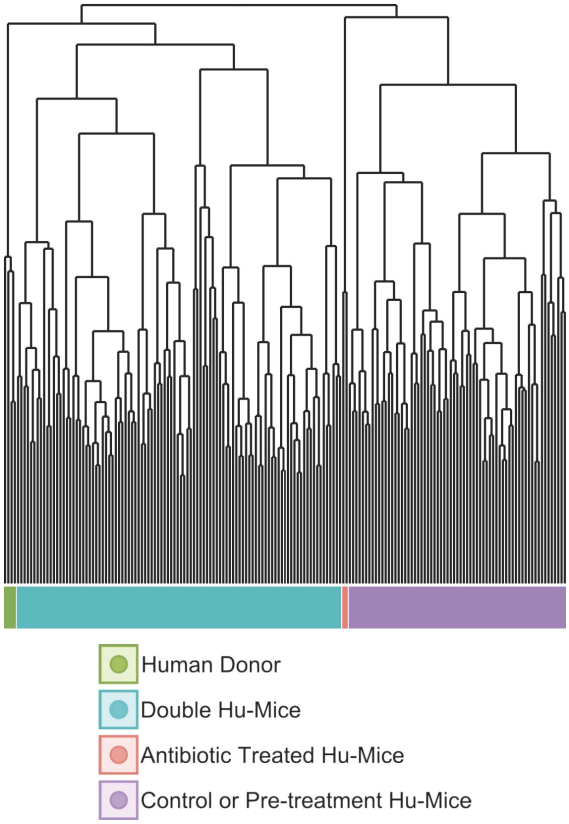


FIGURE 2
The gut microbiomes of double hu-mice clustered with those of human donor fecal samples. Dendrogram based on Bray-Curtis distances for the gut microbiome profiles of donor fecal samples (Human Donor), double hu-mice (Double Hu-Mice), antibiotic treated hu-mice (Antibiotic Treated Hu-Mice), and untreated control or pre-treatment hu-mice (Control or Pre-treatment Hu-Mice).

donor samples were significantly higher than hu-mice ($P < 0.05$) but were not significantly different from the double hu-mice samples. Double hu-mice had increased Simpson index values compared to hu-mice, but the human donor samples were significantly higher than both the double hu-mice ($P < 0.05$) and hu-mice ($P < 0.05$). As expected, samples taken during antibiotic treatment had the lowest measures of all three diversity metrics evaluated. Alpha diversity metrics were also measured based on the human donor sample engrafted (Supplementary Figure S1). Double hu-mice engrafted after 14 days of antibiotics (donor 65 cohorts 1 & 2, donor mix) had higher levels of alpha diversity compared to double hu-mice engrafted after only seven days of antibiotics (donors 74 & 82). Overall, double hu-mice had increased alpha diversity and were more similar to that observed in the human donor samples compared to hu-mice. The shorter antibiotic treatment duration (seven days versus 14 days) was associated with lower alpha diversity measurements in double hu-mice engrafted with human donors 74 or 82.

3.3 The relative abundance of gut bacteria in double hu-mice was similar to that found in human donor samples

We next hypothesized that successful engraftment of human gut microbiota would result in more taxonomic similarities between the human donor gut microbiome profiles and double hu-mice versus hu-mice. Several differences were observed in the relative abundances of double hu-mice based on the length of antibiotic treatment, the human fecal donor sample engrafted, and mouse cohort (Supplementary Figure S2). At the phylum level, both double hu-mice and hu-mice samples were largely represented by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Verrucomicrobia*. Notably, we found that hu-mice had high proportions of *Verrucomicrobia* that remained high even after antibiotic treatment and engraftment with human donor samples that had low abundances of *Verrucomicrobia*. At the family level, hu-mice samples had a higher relative abundance of *S24-7* than double hu-mice and human donor samples (Figure 4). Both hu-mice and double hu-mice samples had higher relative abundances of *Lactobacillaceae* and *Verrucomicrobiaceae* compared to human donor samples. The human donor and double hu-mice samples had higher relative abundances of *Bacteroidaceae* than hu-mice samples while *Lachnospiraceae* and *Ruminococcaceae* were relatively high in abundance in donor versus hu-mice and double hu-mice.

We next compared the taxonomic differences among microbiomes using Kruskal-Wallis testing with FDR adjusted P values below 0.05 (Supplementary Table S1). We found 195 significant differences between the hu-mice and human donor samples and 170 significant differences between hu-mice and

10.3389/frmbi.2024.1404353

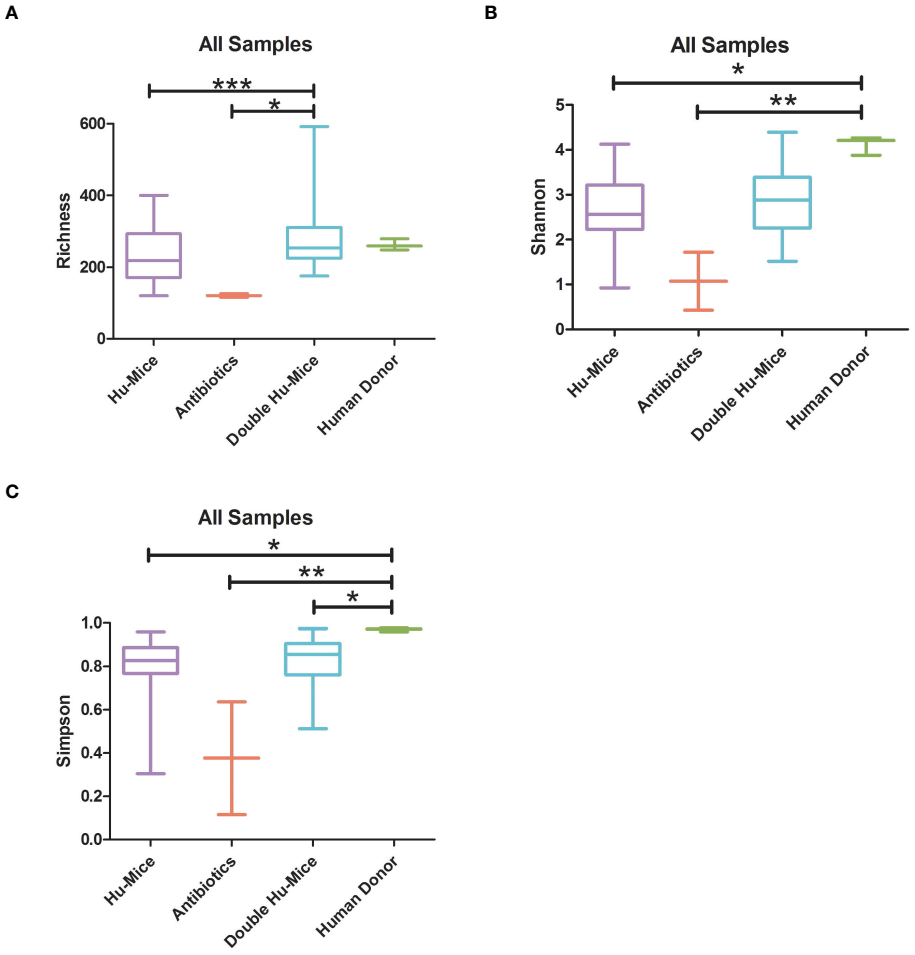


FIGURE 3
The gut microbiomes of double hu-mice had increased alpha diversity measures compared to that of pre-treatment or untreated control hu-mice. Alpha diversity indices Species richness **(A)**, Shannon index **(B)**, and Simpson index **(C)** between untreated control hu-mice, antibiotic-treated, double hu-mice, and human donor. Data are shown for pre-treatment or untreated control hu-mice (Hu-Mice), antibiotic treated mice (Antibiotics), double hu-mice (Double Hu-Mice), and human donor fecal samples (Human Donor). *, **, and *** indicate significant differences with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

double hu-mice. However, only 108 significant differences were observed between double hu-mice and human donor samples. Both the human donor and double hu-mice samples had significantly higher relative abundances of the family *Bacteroidaceae*, while hu-mice had a significantly higher relative abundance of the family *S24-7*. Human donor samples had a higher relative abundance of the phylum *Firmicutes* compared to both hu-mice and double hu-mice. Human donor samples had lower relative abundances of the class *Bacilli* and family *Lactobacillaceae* compared to both hu-mice and double hu-mice. Human donor samples also had a higher relative abundance of the class *Clostridia* than both hu-mice and double hu-mice samples (Supplementary Figure S2). Significant differences found within this class included the family *Lachnospiraceae*, genus *Blautia*, genus *Roseburia*, family *Ruminococcaceae*, genus *Ruminococcus*, and species *Faecalibacterium prausnitzii*. Some of these taxa did increase in abundance in double hu-mice as compared to hu-mice, as observed with significant differences in the genus *Blautia* and genus *Ruminococcaceae*. Levels of *Akkermansia muciniphila* were significantly higher in hu-mice and double hu-mice samples compared to human donor samples. However, double hu-mice had significantly less relative abundance of this species than hu-mice samples (Supplementary Table S1).

The increased similarity observed in double hu-mice and human donor gut microbiome profiles was driven by changes in the relative abundance of many taxa (Supplementary Figure S3). We categorized 70 bacterial taxa that had significantly higher relative abundance (Kruskal-Wallis testing with FDR adjusted P values below 0.05) in both human donor and double hu-mice samples compared to hu-mice. Further, 59 of the 70 higher abundance taxa were not significantly different between the human donor and double hu-mice samples. The largest changes were observed in members of *Bacteroidaceae* and *Blautia*. Additionally, increased abundance was observed in members of *Coriobacteriaceae*, [*Barnesiellaceae*], [*Odoribacteraceae*], *Porphyromonadaceae*, *Christensenellaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, and *Erysipelotrichaceae*. *Proteobacteria* was also increased in human donor and double hu-mice compared to hu-mice, including

10.3389/frmbi.2024.1404353

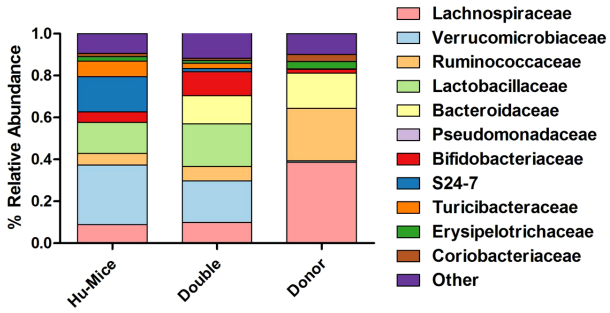


FIGURE 4
Comparison of relative abundance of taxa grouped by family. The 11 most abundant taxa by relative percent abundance grouped by family are shown for pre-treatment and untreated control hu-mice (Hu-Mice), double hu-mice (Double), and human fecal donor samples (Donor).

members of *Burkholderiales, Desulfovibrionales*, and *Enterobacteriales*. We also identified seven bacterial taxa that had significantly lower relative abundance in both human donor and double hu-mice samples compared to hu-mice. All seven lower abundance taxa were not significantly different between the human donor and double hu-mice samples and belonged to *Coriobacteriaceae, S24-7*, and *Erysipelotrichaceae*. Altogether, changes in the relative abundance of many taxa drove the increased similarity between double hu-mice and human donor gut microbiome profiles.

As shown previously, double hu-mice had increased species richness compared to hu-mice. Part of this increased diversity may be derived from the engraftment of donor specific bacteria, or bacteria that were not found to be present in any pre-treatment or control hu-mice but were present in at least one human donor. We found 28 examples of donor specific bacteria that were transferred into double hu-mice across different taxa levels (Supplementary Figure S4). The engrafted bacteria included members of *Bifidobacterium, Collinsella, Butyricimonas, Bacteroides, Streptococcus, Christensenellaceae, Eubacteriaceae, Anaerofustis, Pseudoramibacter Eubacterium, [Ruminococcus], Blautia, Coprococcus, Peptococcaceae*, [*Eubacterium*], and *cc-115*. The engraftment of these bacteria matched closely with the profiles of the human donor sample for each double hu-mice cohort. These results demonstrate that some of the increased diversity observed in double hu-mice is due to the engraftment of donor specific bacteria.

To further determine differences in relative abundances among microbiomes from the various treatments, we used Linear discriminant analysis Effect Size (LEfSe) with a P value < 0.05 and LDA score > 2 (Supplementary Table S2) (Segata et al., 2011). Taxa with LDA scores higher than four were plotted to show significant differences between double hu-mice and human donor samples and double hu-mice and hu-mice samples. Human donor samples were associated with higher relative abundances of several types of *Clostridia*, including *Lachnospiraceae*, *Blautia*, *Coprococcus*, *Roseburia*, *Facalibacterium*, and *Ruminococcus* compared to double hu-mice, while double hu-mice samples were associated with *Lactobacillus* and *Akkermansia muciniphila* (Figure 5A). Double hu-mice were associated with higher relative abundances of *Bacteroides* and several types of *Clostridia*, including *Blautia*, *Coprococcus*, and *Ruminococcaceae*, while hu-mice samples were associated with *S24-7* and *Mogibacteriaceae* (Figure 5B, Supplementary Table S2). These findings demonstrate that certain taxa, like members of *Bacteroides*, readily engrafted double hu-mice while others such as *Clostridia* were more difficult to transplant. Furthermore, several species such as those found in the phylum *Verrucomicrobia* were highly prevalent in hu-mice, and antibiotic treatment followed by fecal transplant did not fully diminish or replace this population based on relative abundances. Altogether, these results demonstrate that engraftment of human donor samples significantly changed the taxonomic profile of double hu-mice and that their human-like gut microbiomes were statistically more similar to human donor profiles compared to hu-mice.

3.4 Stable engraftment of a human-like gut microbiome in double hu-mice

To evaluate the stability of the engrafted human-like gut microbiome in double hu-mice after fecal transplant, the proportion of shared amplicon sequence variants (ASVs) with the human donor sample was calculated. After engraftment, double hu-mice had increased proportions of shared ASVs with their respective human donor samples, and those proportions remained higher than pre-treatment and control levels for the duration of the study. The first cohort of double hu-mice engrafted with human donor 65 had an average shared ASV proportion of 13.70% after transplant, while the pre-treatment samples had 1.06% and control samples levels had 1.42% (Figure 6A). This increased proportion of shared ASVs was maintained for the duration of the study, up to 14 weeks post-transplant (WPT). A second cohort of double hu-mice engrafted with human donor 65 was created, and a similar increase in the proportion of shared ASVs was observed (Figure 6B). The average proportion of shared ASVs was 17.65% post-transplant, while the pre-treatment samples had 2.60% and the control samples had 0.96%. This increased proportion of shared ASVs was maintained for the duration of the study, up to 14.5 WPT. In the case of the cohort treated with seven days of antibiotics prior to fecal transplant, the average shared ASVs proportions were 11.85% and

10.3389/frmbi.2024.1404353

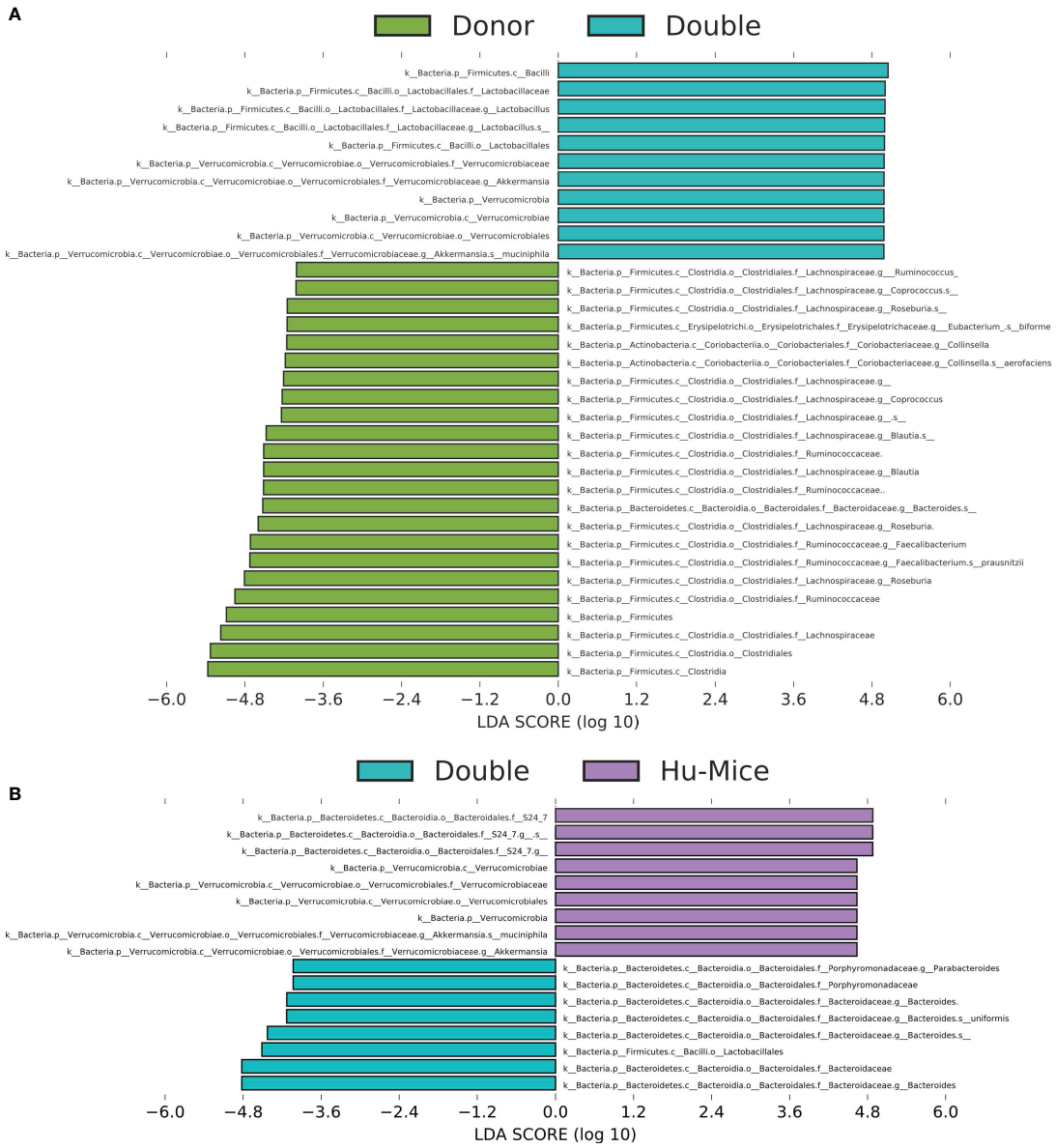


FIGURE 5
Engraftment of human fecal donor bacteria in double hu-mice as shown by LEfSe. All significant features with a linear discriminant analysis (LDA) score > 4.0 between human fecal donor samples (Donor) and double hu-mice (Double) **(A)** and between double hu-mice (Double) and pre-treatment or untreated control hu-mice (Hu-Mice) **(B)**.

10.56% for double hu-mice mice engrafted with human donors 74 and 85, respectively, while the percentages of shared ASVs in pre-treatment and controls were found to be 1.02% and 0.86%, respectively, in cohort 74 and 1.33% and 0.95%, respectively, in cohort 82 (Figure 6C). For the double hu-mice transplanted with the mixture of all three human donors, the average shared ASV proportion was 10.91% after transplant, while the pre-treatment samples had 0.31% and the control samples had 0.61% (Figure 6D). Altogether, these results suggest a portion of donor fecal microbiota successfully engrafted in our double hu-mice and remained stable for the duration of the study.

To further evaluate the stability of the engrafted human-like microbiome after transplant into double hu-mice, the contributions of the human donor and pre-treatment sample to the post-transplant samples were determined using SourceTracker (Knights et al., 2011). The first cohort of mice transplanted with human donor 65 had an average donor contribution percentage of 18.76% after transplant, while the pre-treatment samples had 0.00% and the control samples had 0.05% (Figure 7A). At the final time point collected at 14 WPT, the donor contribution was consistent at 18.10%. The second cohort of mice transplanted with human donor 65 had an average donor contribution percentage of 29.01% after transplant, while the pre-treatment and controls samples had no donor contribution (Figure 7B). The donor contribution percentage was 34.10% at 14.5 WPT, thus demonstrating the stable engraftment of donor bacteria. Double hu-mice engrafted with donor 74 had an average donor contribution percentage of 12.71% after transplant, while the pre-treatment samples had 0.00% and the control samples had 0.00%

10.3389/frmbi.2024.1404353

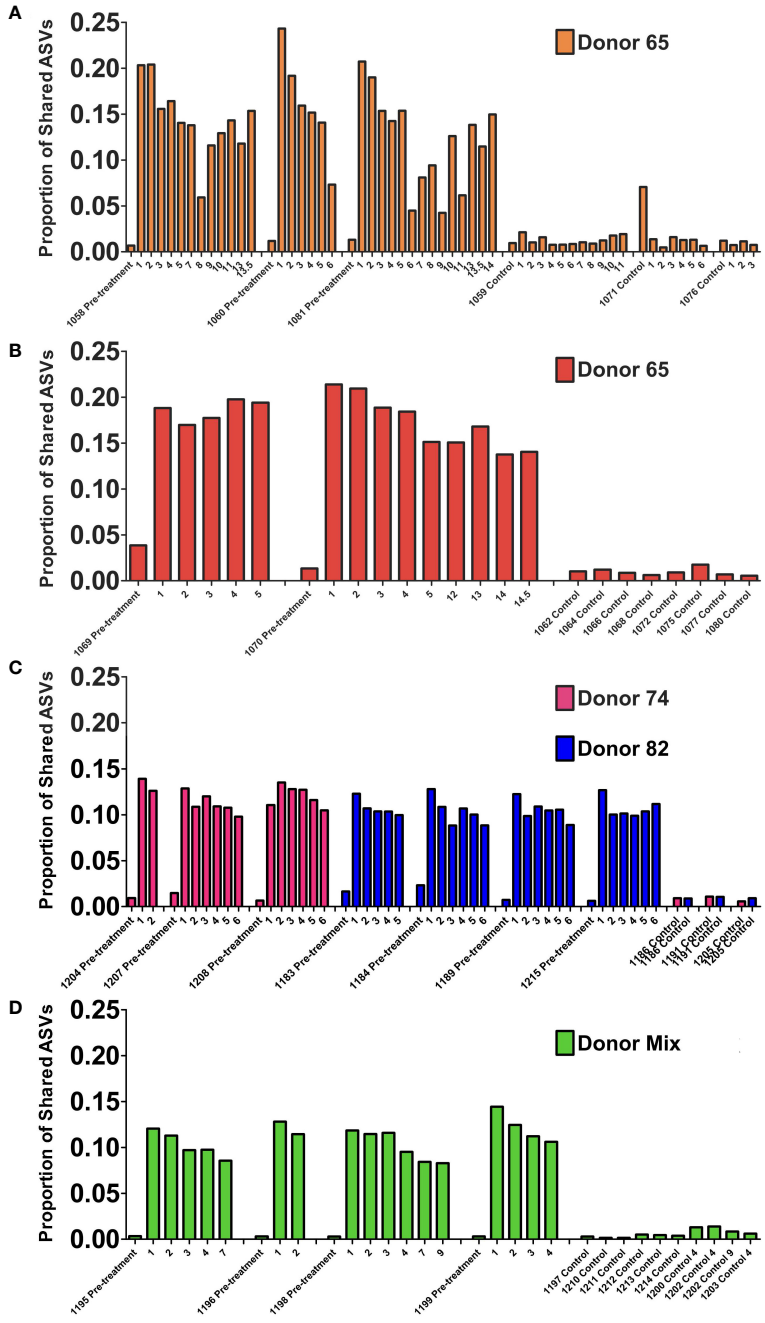


FIGURE 6
Engraftment and stability of a human-like gut microbiome in double hu-mice as determined by shared amplicon sequence variants (ASVs). Proportion of shared ASVs with the human donor from double hu-mice created using fecal material from human Donor 65, first cohort **(A)**; Donor 65, second cohort **(B)**; Donor 74 or Donor 82 **(C)**; and a mixture of all three donors **(D)**. On the x-axis, individual mouse IDs precede the Pre-treatment or Control designations followed by the number of weeks post fecal transplant.

(Figure 7C). At the final time point collected at 6 WPT, the average donor contribution was 18.75%.

The SourceTracker algorithm was unable to clearly distinguish donor 82 contributions as evidenced by both pre-treatment and control samples being assigned high human donor contribution percentages. Mice transplanted with human donor 82 had an average donor contribution percentage of 10.18% after transplant, while the pre-treatment samples had 11.95% and the control samples had 20.62% (Figure 7C). SourceTracker also assigned very high donor contribution percentages to the pre-treatment and control samples in the study of double hu-mice transplanted with a mixture of all three human donors. Mice transplanted with the mixture of all three human donors had an average donor contribution percentage of 14.88% after transplant, while the pre-treatment samples had 21.04% and the control samples had 15.89% (Figure 7D).

The high donor contribution percentages of donor 82 to the pre-treatment and control samples originated from an ASV with taxonomic assignment to *Akkermansia muciniphila*. This ASV was

10.3389/frmbi.2024.1404353

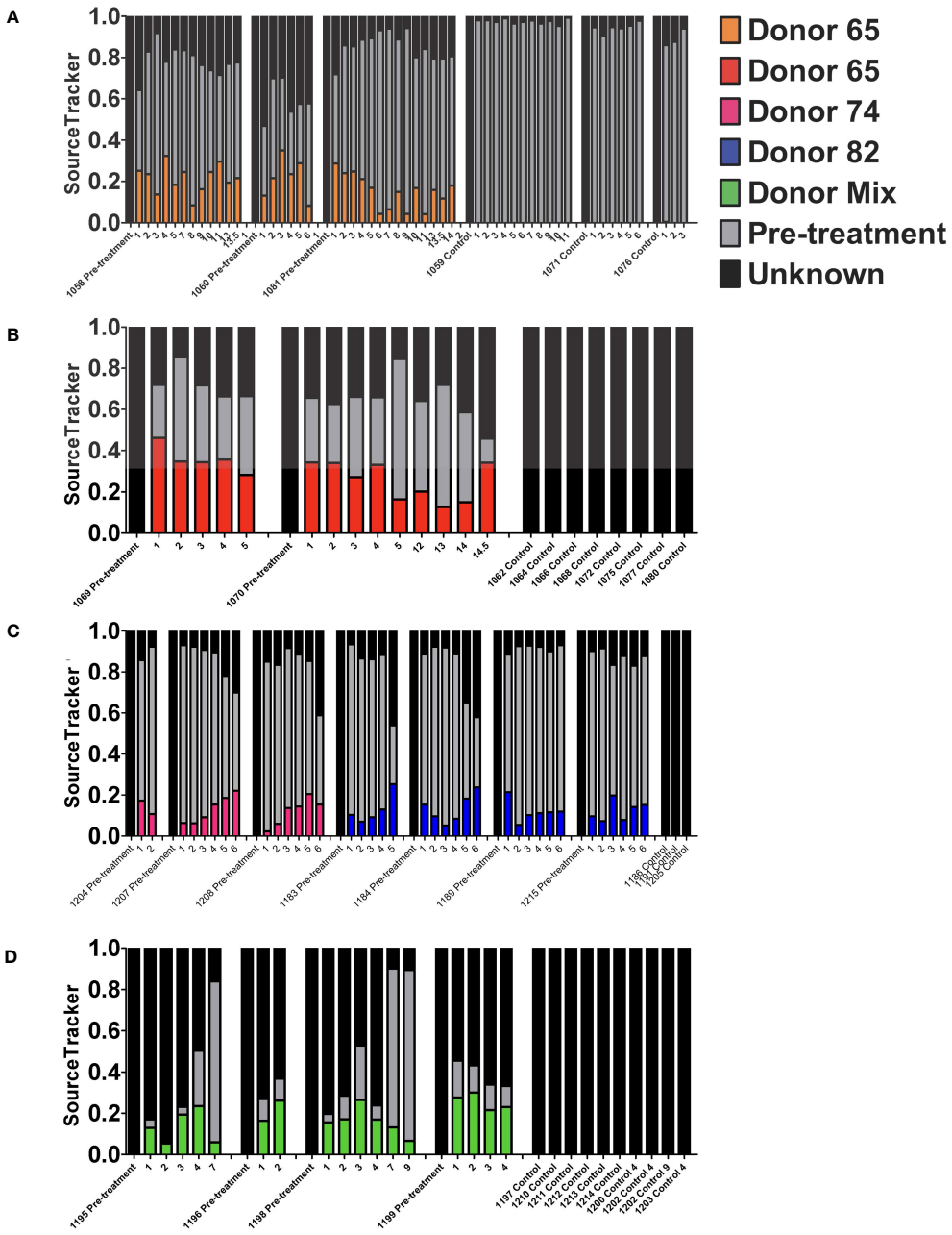


FIGURE 7
Stability of the engrafted human-like gut microbiome in double hu-mice as determined by SourceTracker. Contributions of the human fecal donor sample and pre-treatment sample in double hu-mice created using fecal material from Donor 65, first cohort **(A)**; Donor 65, second cohort **(B)**; Donor 74 or 82 **(C)**; and a mixture of all three donors **(D)**. On the x-axis, individual mouse IDs precede the Pre-treatment or Control designations followed by the number of weeks post fecal transplant.

highly abundant in both hu-mice and double hu-mice and was much more prevalent in human donor 82 and the mixed donor sample compared to donors 65 or 74. To get a more accurate account of the stability of the human-like gut microbiome in post-transplant samples, we removed this ASV that was resulting in false positive donor contributions and once again used SourceTracker (Supplementary Figure S5). After the removal of the *A. muciniphila* ASV, the double hu-mice engrafted with human donor 82 had an average donor contribution percentage of 12.64% after transplant and double hu-mice engrafted with the mixture of all three human donors had an average donor contribution percentage of 18.11% after transplant, while all pre-treatment control samples were at 0.00%. Taken together, the results from shared ASVs and SourceTracker analyses, demonstrate that double hu-mice had a stable human-like gut microbiome for the duration of the study, extending up to 14.5 weeks post-transplant.

3.5 Increased human-like predicted metagenome functional content in double hu-mice

In addition to evaluating microbiome classification, we also sought to assess the functional capacity of the microbiomes in double hu-mice. PICRUSt was used to predict the metagenome functional content from the 16S rRNA data after ASV inference (Langille et al., 2013), and the predicted KO features were graphed using both NMDS and PCoA plots. Many of the double hu-mice samples clustered closer to the human donor samples than the hu-mice samples (Figures 8A, B). When color-coded by donor and cohort, the microbiomes that clustered closest to the human donor samples belonged to mice from the second cohort of double hu-mice generated by engrafting bacteria from human donor 65 (Figures 8C, D). Similarly, several samples from the other double hu-mice cohorts also separated themselves from the hu-mice cluster and were closer to the human donor samples. (Figures 8C, D).

We next tested for differences of each predicted KO feature among the three different groups of mice (Supplementary Table S3).

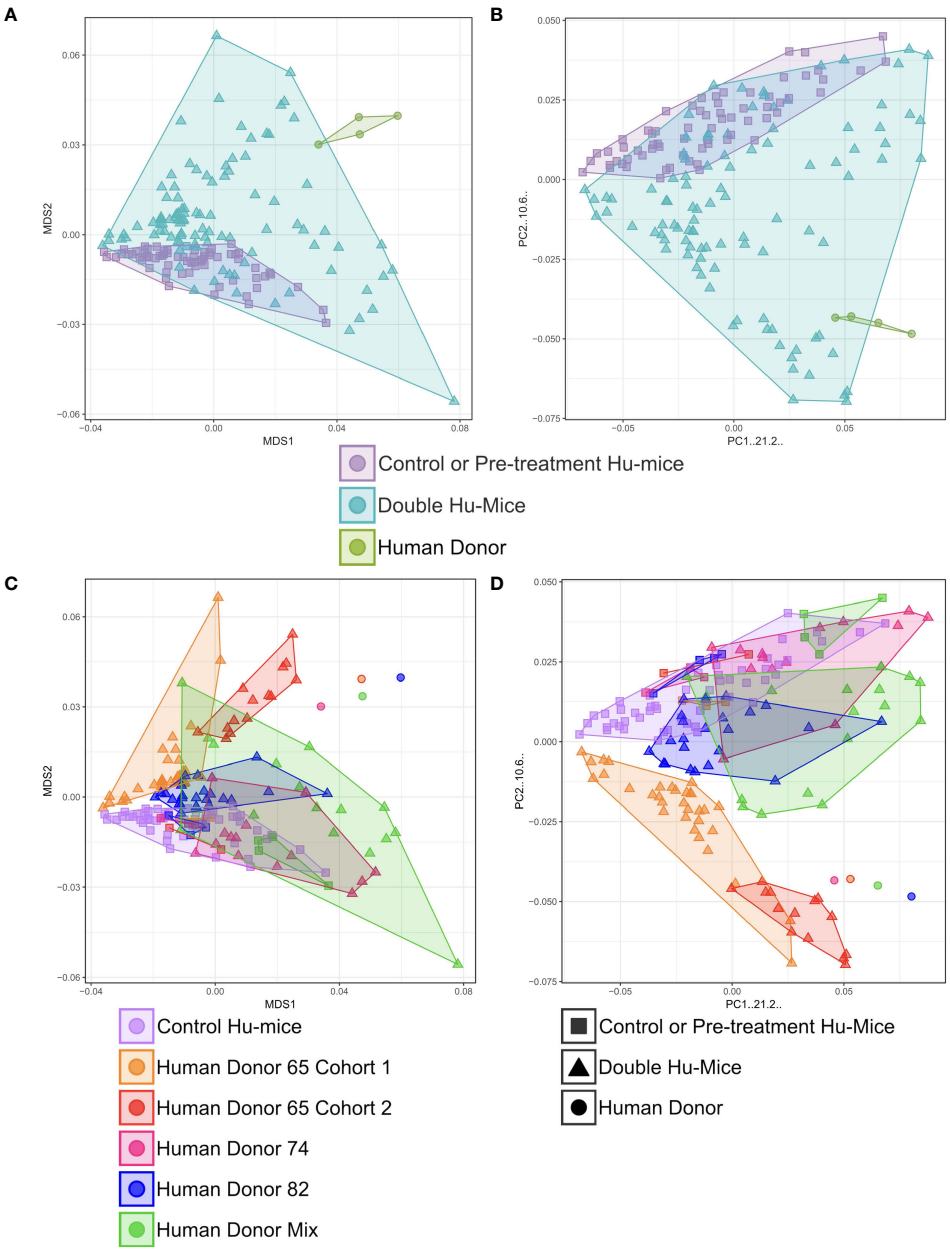


FIGURE 8
Increased human-like predicted metagenome functional content in double hu-mice. Rarified KEGG Orthology (KO) molecular functions were used to calculate Bray-Curtis dissimilarity after performing square-root transformations on the data. Non-metric multidimensional scaling (NMDS) or principal coordinates analysis (PCoA) ordination was used to create the coordinates for plotting the data. Double hu-mice clustered distinctly from the human fecal donor samples and pre-treatment or untreated control hu-mice in NMDS plot **(A)** and in PCoA plot **(B)**. Human donor specific functional profiles of double hu-mice in NMDS plot **(C)** and in PCoA plot **(D)**.

In total, there were 4,513 non-zero predicted KO features. Using Kruskal-Wallis testing and an FDR adjusted p-value of < 0.05, we found 35.54% (1,604/4,513) significantly different predicted KO features between hu-mice and human donor samples. There were 39.09% (1,764/4,513) significantly different predicted KO features between double hu-mice and hu-mice samples. However, when we compared double hu-mice and the human donor samples, there were only 1.35% (61/4,513) significantly different predicted KO features. To clarify what functional aspects were missing from the double hu-mice gut microbiomes, we determined the predicted ASV contribution for each of the 61 significantly different KO features between the double hu-mice and human donor samples (Supplementary Table S4). This analysis provided insight into which functionally significant bacteria were not successfully engrafted from the human donor samples to the double hu-mice. A total of 95 ASVs with 73 unique taxonomic assignments were found to contribute to the 61 significantly different KO features (Supplementary Figure S6). Family level taxa with the highest levels of contribution included *Actinomycetaceae* (10.46%), *Bifidobacteriaceae* (8.76%), *Streptococcaceae* (20.74%), *Lachnospiraceae* (23.95%), and *Peptococcaceae* (8.66%). *Bifidobacterium adolescentis* was the highest contributing species (7.85%), and the highest contributing ASV (13.01%) had the taxonomic assignment of an unclassified *Streptococcus* species. Collectively, these analyses demonstrate that the predicted functional capacity of double hu-mice is more similar to human donor samples compared to hu-mice.

6 Discussion

Currently, humanized mice with human-like immune systems, yet devoid of human gut microbiota, are extensively employed to investigate the immune responses to human-specific pathogens. However, the absence of human gut microbiota may significantly compromise the translational relevance of these mouse model studies to humans, given the critical role gut microbiota play in immune response. Therefore, this study aimed to characterize the engrafted human fecal microbiota in our double humanized mice, which possess both a functional human immune system and a human microbiome, by comparing them to the currently used humanized mouse as control that has solely a human immune system. Furthermore, we compared engrafted human microbiota with donor samples and evaluated its stability.

We found that double hu-mice have a gut microbiome profile similar to that of human donor samples. Our approach created reproducible and donor-specific human-like gut microbiomes across multiple cohorts of double hu-mice. Further, we showed that double hu-mice had increased measures of diversity and increased functional capacity compared to hu-mice. The engrafted human-like gut microbiomes were also stable for the duration of the study, extending up to 14.5 weeks post-transplant. Importantly, we also demonstrated that the predicted functional capacity of double hu-mice is more similar to the human donor samples than hu-mice.

One of the most significant findings of this study was that the double hu-mice had gut microbiome profiles that were unique to the human donor engrafted. Each of the four different donor samples resulted in a distinct population of double hu-mice resembling the human donor. This observation has several practical implications, particularly within personalized medicine and translational research domains. Patient-derived microbiotas can be used as donor microbiotas, and this mouse model can be utilized to study personalized responses of patient microbiomes to drug regimes, therapies, or dietary interventions. Additionally, this double hu-mice model could present a viable avenue to explore the mechanistic underpinnings of patients' pathological states, while concurrently offering a platform to understand the interplay between the patient's gut microbiome and the etiology of disease.

We found that double hu-mice had increased measures of alpha diversity compared to hu-mice. Several studies have highlighted the importance of microbiome diversity within the gut and have linked low gut microbiome diversity with several disease conditions (Chang et al., 2008; Willing et al., 2010). While not all low diversity conditions are detrimental, specifically when there is enrichment of potentially beneficial bacteria through prebiotic or probiotic treatments, the low pre-existing diversity found within the hu-mice was far below the levels observed in our human donor samples. After engraftment, the double hu-mice had increased levels of alpha diversity and maybe more importantly, had increased predicted functional capacities. This increased diversity may also offer a more realistic gut environment as it allows for diverse reciprocal interactions with the engrafted human immune system.

We also found that the engrafted human-like microbiome was very stable in our model for the length of the study, up to 14.5 weeks after transplant. We used several methods to determine the engraftment level and stability of the gut microbiome after transplant and found no reversion to the pre-existing murine profile. This stability allows for the longitudinal study of the role of the gut microbiome in many human diseases, such as HIV-1 and cancer. One outstanding question is whether the unique presence of human immune cells plays a role in stabilizing or enhancing engraftment of the human-like gut microbiome in our model compared to other non-humanized mouse models. Our data showed no reversion to the pre-existing murine gut microbiome profile, perhaps due to some enhanced stability or selection by the reconstituted human immune system. Further studies are needed to determine the relationship between the engrafted gut microbiome and the human immune system.

Many different methods and antibiotic regimens have been used for preconditioning of mice prior to fecal transplantation (Reikvam et al., 2011; Ericsson et al., 2017; Staley et al., 2017). Different combinations and durations of antibiotic treatments may increase the efficiency of the fecal transplant into the host (Staley et al., 2017). While the combination of metronidazole, ampicillin, neomycin, and vancomycin is widely used due to its broad spectrum of bacterial targets, the best methods are still being investigated. We found that the very rigorous method of gavaging antibiotics twice daily for 14 days used by Hintze et al. was too invasive for our NSG hu-mice and resulted in increased mortality (Hintze et al., 2014). Our study suggests that administrating broad-spectrum antibiotics in drinking water for a duration of 14 days

represents an effective approach for preconditioning NSG hu-mice prior to fecal transplantation, thus ensuring improved health and survival outcomes compared to more invasive regimens for generating double hu-mice.

Other important practical implications of our mouse model are the logistics and technical requirements. Although the germ-free mice model is considered a gold standard method for FMT studies and may exhibit better engraftment of most donor microbiota, germ-free animals require gnotobiotic facilities and expensive, specialized equipment (Kennedy et al., 2018; Le Roy et al., 2018). However, our double hu-mice have the advantage of requiring only SPF housing conditions, which are widely available and less costly compared to germ-free facilities. Our method also does not perturb the complex surgical procedures in generating BLT hu-mice, as it does not mandate the creation of a completely germ-free environment. Furthermore, our method of generating a double hu-mice model can be adapted to the neonatal intrahepatic hCD34 + hematopoietic stem progenitor cell (HSPC) method of humanization, which is a comparatively simpler process compared to the surgical approach integral to the BLT humanization procedure.

Our double hu-mice do not exhibit complete engraftment of donor fecal microbiota, which can be attributed to several hypothesized factors. There are major differences between the human and mouse digestive tract, including structure, function, and pH (Nguyen et al., 2015). Our mice are not germ-free to begin with, and the broad-spectrum antibiotic treatment can only reduce the prevalence of pre-existing murine gut bacteria. There were several key differences in the reconstituted mice compared to the human donors. Double hu-mice had significantly lower levels of several types of *Clostridia* including *Lachnospiraceae*, *Blautia*, *Coprococcus*, *Roseburia*, *Faecalibacterium*, and *Ruminococcus* compared to human donor samples. Many of these bacteria are well documented to be difficult to reconstitute within germ-free and SPF mouse models (Turnbaugh et al., 2009; Wos-Oxley et al., 2012; Hintze et al., 2014). Similar to fecal transplants in humans designed to treat *C. difficile* infections, the engrafted human-like gut microbiome in our double hu-mice is the result of a combination of host, donor, and environmental bacteria (Smillie et al., 2011). Despite these previously known limitations, our double hu-mice model reproducibly results in a donor-specific, stable, human-like gut microbiome in the presence of a human immune system.

7 Conclusion

Here, we describe the successful and stable engraftment of human fecal microbiomes into immunodeficient NSG mice surgically implanted with a functional human immune system to create double hu-mice with human donor-specific human gut microbiomes. This mouse model is a useful platform to study the impact of pathogen and dietary intervention on human health in the presence of both a functional human immune system and a human-like gut microbiota with minimal logistics and technical requirements.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI SRA, accession PRJNA507247.

Ethics statement

The studies involving humans were approved by the Scientific Research Oversight Committee (SROC) at the University of Nebraska-Lincoln (UNL) (SROC# 2016-1-002). The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from Advanced Bioscience Resources (Alameda, CA). 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. The animal study was conducted in accordance with Institutional Animal Care and Research Committee (IACUC)-approved protocols at the University of Nebraska-Lincoln (UNL). The IACUC at UNL has approved two protocols related to generating and using humanized BLT (hu-BLT) mice, including double hu-mice.

Author contributions

LD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SL: Writing – review & editing. AR-T: Supervision, Writing – review & editing. QL: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study is supported in part by the National Institutes of Health (NIH) Grants 1R01AI111862 and R21 AI143405 to QL. The funders had no role in study design, data collection and analysis, preparation of the manuscript, or decision for publication.

Acknowledgments

We thank Yanmin Wan, Guobin Kang, and Pallabi Kundu for their assistance in generating BLT hu-mice. We would like to

acknowledge the UNMC Genomics Core Facility who receives partial support from the Nebraska Research Network In Functional Genomics NE-INBRE P20GM103427-14, The Molecular Biology of Neurosensory Systems CoBRE P30GM110768, The Fred & Pamela Buffett Cancer Center – P30CA036727, The Center for Root and Rhizobiome Innovation (CRRI) 36-5150-2085-20, and the Nebraska Research Initiative. We want to thank the Institutional Animal Care Program staff at the University of Nebraska for their assistance.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frmbi.2024.1404353/full#supplementary-material

References

Bankert, R. B., Balu-Iyer, S. V., Odunsi, K., Shultz, L. D., Kelleher, R. J., Barnas, J. L., et al. (2011). Humanized mouse model of ovarian cancer recapitulates patient solid tumor progression, ascites formation, and metastasis. *PloS One* 6, e24420. doi: 10.1371/journal.pone.0024420

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M., et al. (2008). Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea. *J. Infect. Dis.* 197, 435–438. doi: 10.1086/525047

Clemente, J. C., Manasson, J., and Scher, J. U. (2018). The role of the gut microbiome in systemic inflammatory disease. *Bmj-Brit Med. J.* 360. doi: 10.1136/bmj.j5145

Daharsh L, Z. J., Ramer-Tait, A., and Li, Q. (2019). A double humanized BLT-mice model featuring a stable human-like gut microbiome and human immune system. *Jove-Journal Visualized Experiments*. doi: 10.3791/59773-v

Destache, C. J., Mandal, S., Yuan, Z., Kang, G., Date, A. A., Lu, W., et al. (2016). Topical tenofovir disoproxil fumarate nanoparticles prevent HIV-1 vaginal transmission in a humanized mouse model. *Antimicrob. Agents Chemother.* 60, 3633–3639. doi: 10.1128/AAC.00450-16

Ericsson, A. C., Personett, A. R., Turner, G., Dorfmeyer, R. A., and Franklin, C. L. (2017). Variable colonization after reciprocal fecal microbiota transfer between mice with low and high richness microbiota. *Front. Microbiol.* 8, 1–13. doi: 10.3389/fmicb.2017.00196

Ernst, W. (2016). Humanized mice in infectious diseases. *Comp. Immunol. Microb.* 49, 29–38. doi: 10.1016/j.cimid.2016.08.006

Gopalakrishnan, V., Spencer, C. N., Nezi, L., Reuben, A., Andrews, M. C., Karpinets, T. V., et al. (2018). Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science* 359, 97–103. doi: 10.1126/science.aan4236

Hintze, K. J., Cox, J. E., Rompato, G., Benninghoff, A. D., Ward, R. E., Broadbent, J., et al. (2014). Broad scope method for creating humanized animal models for animal health and disease research through antibiotic treatment and human fecal transfer. *Gut Microbes* 5, 183–191. doi: 10.4161/gmic.28403

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336, 1268–1273. doi: 10.1126/science.1223490

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., and Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature* 474, 327–336. doi: 10.1038/nature10213

Kennedy, E. A., King, K. Y., and Baldridge, M. T. (2018). Mouse microbiota models: comparing germ-free mice and antibiotics treatment as tools for modifying gut bacteria. *Front. Physiol.* 9, 1534. doi: 10.3389/fphys.2018.01534

Knights, D., Kuczynski, J., Charlson, E. S., Zaneveld, J., Mozer, M. C., Collman, R. G., et al. (2011). Bayesian community-wide culture-independent microbial source tracking. *Nat. Methods* 8, 761–763. doi: 10.1038/nmeth.1650

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Le Roy, T., Debédat, J., Marquet, F., Da-Cunha, C., Ichou, F., Guerre-Millo, M., et al. (2018). Comparative evaluation of microbiota engraftment following fecal microbiota transfer in mice models: age, kinetic and microbial status matter. *Front. Microbiol.* 9, 3289. doi: 10.3389/fmicb.2018.03289

Li, Q. S., Tso, F. Y., Kang, G. B., Lu, W. X., Li, Y., Fan, W. J., et al. (2015). Early initiation of antiretroviral therapy can functionally control productive HIV-1 infection in humanized-BLT mice. *Jaids-J Acq Imm Def.* 69, 519–527. doi: 10.1097/QAI.0000000000000687

Martinez, I., Wallace, G., Zhang, C., Legge, R., Benson, A. K., Carr, T. P., et al. (2009). Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. *Appl. Environ. Microbiol.* 75, 4175–4184. doi: 10.1128/AEM.00380-09

Maynard, C. L., Elson, C. O., Hatton, R. D., and Weaver, C. T. (2012). Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 489, 231–241. doi: 10.1038/nature11551

Nguyen, T. L. A., Vieira-Silva, S., Liston, A., and Raes, J. (2015). How informative is the mouse for human gut microbiota research? *Dis. Model. Mech.* 8, 1–16. doi: 10.1242/dmm.017400

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Reikvam, D. H., Erofeev, A., Sandvik, A., Grcic, V., Jahnsen, F. L., Gaustad, P., et al. (2011). Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PloS One* 6, e17996. doi: 10.1371/journal.pone.0017996

Routy, B., Le Chatelier, E., Derosa, L., Duong, C. P. M., Alou, M. T., Daillere, R., et al. (2018). Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science* 359, 91. doi: 10.1126/science.aan3706

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

Simpson-Abelson, M. R., Sonnenberg, G. F., Takita, H., Yokota, S. J., Conway, T. F., Kelleher, R. J., et al. (2008). Long-term engraftment and expansion of tumor-derived memory T cells following the implantation of non-disrupted pieces of human lung tumor into NOD-scid IL2R gamma(null) mice. *J. Immunol.* 180, 7009–7018. doi: 10.4049/jimmunol.180.10.7009

Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A., and Alm, E. J. (2011). Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* 480, 241–244. doi: 10.1038/nature10571

Smith, K., McCoy, K. D., and Macpherson, A. J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin. Immunol.* 19, 59–69. doi: 10.1016/j.smim.2006.10.002

Staley, C., Kaiser, T., Beura, L. K., Hamilton, M. J., Weingarden, A. R., Bobr, A., et al. (2017). Stable engraftment of human microbiota into mice with a single oral gavage following antibiotic conditioning. *Microbiome* 5. doi: 10.1186/s40168-017-0306-2

Stary, G., Olive, A., Radovic-Moreno, A. F., Gondek, D., Alvarez, D., Basto, P. A., et al. (2015). A mucosal vaccine against Chlamydia trachomatis generates two waves of protective memory T cells. *Science* 348. doi: 10.1126/science.aaa8205

Sun, Z. F., Denton, P. W., Estes, J. D., Othieno, F. A., Wei, B. L., Wege, A. K., et al. (2007). Intrarectal transmission, systemic infection, and CD4(+) T cell depletion in

humanized mice infected with HIV-1. *J. Exp. Med.* 204, 705–714. doi: 10.1084/jem.20062411

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., and Gordon, J. I. (2009). The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* 1, 6ra14. doi: 10.1126/scitranslmed.3000322

Vudattu, N. K., Waldron-Lynch, F., Truman, L. A., Deng, S. Y., Preston-Hurlburt, P., Torres, R., et al. (2014). Humanized mice as a model for aberrant responses in human T cell immunotherapy. *J. Immunol.* 193, 587–596. doi: 10.4049/jimmunol.1302455

Wang, L. X., Kang, G. B., Kumar, P., Lu, W. X., Li, Y., Zhou, Y., et al. (2014). Humanized-BLT mouse model of Kaposi's sarcoma-associated herpesvirus infection. *P Natl. Acad. Sci. U.S.A.* 111, 3146–3151. doi: 10.1073/pnas.1318175111

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

Whitfield-Larry, F., Young, E. F., Talmage, G., Fudge, E., Azam, A., Patel, S., et al. (2011). HLA-A2 Matched Peripheral Blood Mononuclear Cells From Type 1 Diabetic Patients, but Not Nondiabetic Donors, Transfer Insulitis to NOD-scid/gamma c(null)/HLA-A2 Transgenic Mice Concurrent With the Expansion of Islet-Specific CD8(+) T cells. *Diabetes* 60, 1726–1733. doi: 10.2337/db10-1287

Willing, B. P., Dicksved, J., Halfvarson, J., Andersson, A. F., Lucio, M., Zheng, Z., et al. (2010). A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology* 139, 1844–U105. doi: 10.1053/j.gastro.2010.08.049

Wos-Oxley, M., Bleich, A., Oxley, A. P., Kahl, S., Janus, L. M., Smoczek, A., et al. (2012). Comparative evaluation of establishing a human gut microbial community within rodent models. *Gut Microbes* 3, 234–249. doi: 10.4161/gmic.19934

Xiao, L., Feng, Q., Liang, S. S., Sonne, S. B., Xia, Z. K., Qiu, X. M., et al. (2015). A catalog of the mouse gut metagenome. *Nat. Biotechnol.* 33, 1103. doi: 10.1038/nbt.3353

Yi, G. H., Xu, X. Q., Abraham, S., Petersen, S., Guo, H., Ortega, N., et al. (2017). A DNA vaccine protects human immune cells against zika virus infection in humanized mice. *Ebiomedicine* 25, 87–94. doi: 10.1016/j.ebiom.2017.10.006

Yuan, Z., Kang, G., Ma, F., Lu, W., Fan, W., Fennessey, C. M., et al. (2016). Recapitulating cross-species transmission of simian immunodeficiency virus SIVcpz to humans by using humanized BLT mice. *J. Virol.* 90, 7728–7739. doi: 10.1128/JVI.00860-16

frontiers | Frontiers in Microbiology

TYPE Perspective
PUBLISHED 09 September 2024
DOI 10.3389/fmicb.2024.1449133

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Yiming Meng,
China Medical University, China

*CORRESPONDENCE
Olaf F. A. Larsen
✉ o.f.a.larsen@vu.nl

RECEIVED 14 June 2024
ACCEPTED 26 August 2024
PUBLISHED 09 September 2024

CITATION
Larsen OFA and Brummer RJM (2024) Perspective: on the future of fecal microbiota transplantation.
Front. Microbiol. 15:1449133.
doi: 10.3389/fmicb.2024.1449133

COPYRIGHT
© 2024 Larsen and Brummer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Perspective: on the future of fecal microbiota transplantation

Olaf F. A. Larsen[1]* and Robert J. M. Brummer[2]

[1]Athena Institute, Vrije Universiteit Amsterdam, Amsterdam, Netherlands, [2]Faculty of Medicine and Health, School of Medical Sciences, Nutrition-Gut-Brain Interactions Research Centre, Örebro University, Örebro, Sweden

Fecal Microbiota Transplantation (FMT) has shown to possess impressive potential benefit for a wide range of clinical indications. Due to its inherent safety issues and efficacy constraints, the use of personalized FMT analogs could be a promising avenue. The development of such analogs will require a detailed understanding of their functionality, encompassing not only microbe-host interactions of the microbial taxa that are involved, but also of the ecological dimensions of the analogs and an overview of the gastrointestinal sites where these relevant microbial interactions take place. Moreover, characterization of taxa that have been lost due to diminished exposure to beneficial microbes, as a consequence of Western lifestyle, may lead to creation of future FMT analogs with the capacity to restore functionalities that we have lost.

KEYWORDS

fecal microbiota transplantation, gut microbiota, probiotics, personalized, analogs, One Health

1 Introduction

Fecal microbiota transplantation (FMT) encompasses the transfer of fecal matter from a healthy donor to a recipient in order to restore its gut microbial ecosystem with the aim to prevent, treat or alleviate a clinical disorder. The technique has already been described in the fourth century in China, in which a human fecal suspension was orally administered to patients suffering from food poisoning or severe diarrhea (Zhang et al., 2012). In modern times, the first published FMT treatment originated from 1958, when it was successfully applied to four patients suffering from pseudomembranous colitis (Halkjær et al., 2023). It is known that pseudomembranous colitis is caused by infection with *Clostridioides difficile* (*C. diff.*) bacteria. After the landmark paper by Van Nood et al. (2013), in which FMT was shown to be highly efficacious for the treatment of recurrent *C. diff.* infections, the technique has drawn huge attention. In general, heterologous transplantation is commonly utilized, however autologous transplantations have also been reported, for example to boost microbial diversity after antibiotic treatment (Taur et al., 2018). A plethora of studies investigating the potential of FMT for various disorders have been published, ranging from Irritable Bowel Syndrome (IBS) (Wang et al., 2023), to Inflammatory Bowel Disease (Imdad et al., 2023) and autism spectrum disorder (Zhang et al., 2023). Currently, the potential of FTM to improve tumor immunotherapy by remodeling the gut microbiota toward a composition resulting in a more favorable efficacy of immune checkpoint inhibitors, has been recognized as well (Yang et al., 2024). Despite its promising potential, FMT suffers from an inherent safety risk, as transfer of non-pathogenic microorganisms only cannot be warranted. Furthermore, FMT is not as a standardized treatment compared to conventional pharmaceutical therapy, as the composition of the stool is inherently dependent on the donor. This has initiated an intense debate whether FMT should be regarded as tissue transplantation

or as pharmacological treatment which has substantial consequences for its research. Classifying conventional FMT as a pharmacologic treatment would infer severe limitations to its research. Consequently, the development of defined consortia is an area of intense research, with recently two products being approved by the FDA for the eradication of *C. diff*. (FDA website). However, treatment of other disorders than specifically related to *C. diff*. Probably need intervention with more complex microbiological ecosystems to be successful. As the gut microbiota is known to be unique for every individual, it is to be expected that the number needed to treat for future successful FTM applications will be (much) higher than that for the eradication of a single pathogen. A study by Kootte et al. (2017), in which FMT was being investigated to improve insulin sensitivity among patients suffering from metabolic syndrome, hinted already in this direction (Kootte et al., 2017). In this study, it was shown that efficacy was correlated to the baseline fecal microbiota composition of the patient. A recent study also reports on the drivers determining the efficacy of FMT colonization (Schmidt et al., 2022). Hence, to improve efficacy of future FMT products, a more personalized approach seems to be necessary. In this perspective, we will touch upon factors that should be taken into account for the development of such future rationalized products.

2 On the ecological dimensions of future FMT analogs

FMT represents a "bulk approach," in which the total gastrointestinal microbiota of a donor is being transferred to a patient. As the aim of such a transfer is the clearance or alleviation of a clinical disorder, it seems plausible, instead, only to transfer the microbes needed to treat that specific pathologic condition. As such, one would like to know which specific functionality (or functionalities) is (are) lost or malfunctioning. This can be due to a lack or low abundance of keystone taxa. Keystone taxa act as "interactional hubs" within a microbial network, and are known to be crucial for the structure of such networks (Banerjee et al., 2018). Absence of keystone taxa will result in a structural collapse of the microbial network it makes part of and consequently its associated functionality. Alternatively, a lack or low abundance of microbial guilds can be the causing of the pathophysiologic condition. A microbial guild is a small ecosystem, comprising a limited number of taxa that work together, and dedicated to a single specific functionality (Wu et al., 2021). It has been shown that such microbial guilds indeed exists in the human gastrointestinal tract and are, among others, involved in the etiology of type 2 diabetes mellitus (Zhang et al., 2015; Zhao et al., 2018). Hence, a future FTM analog may comprise such keystone taxa and/or microbial guilds. Earlier mathematical modeling on such small ecosystems demonstrated that a higher richness of taxa will contribute to both stability and efficiency of the process the guild is dedicated to Larsen and Claassen (2018). In order to produce a stable FTM analog, such preparations should consist of networks comprising at least five to six different taxa (Larsen et al., 2019).

3 On the microbial composition of future FMT analogs

Currently, most research on the composition of the gut microbiota is still focusing on the bacterial composition, the so-called bacteriome. This is not surprising, knowing that the number of gut-bacteria ($\approx 4 \times 10^{13}$) roughly equals the total number of somatic cells (Sender et al., 2016). However, other domains present in the intraluminal intestinal ecosystem also play an essential role in maintaining homeostasis or causing pathophysiological aberrations in the body. For example, the archea (collectively designated as the archeome) have been mapped (Chibani et al., 2022) and recent evidence suggests a synergistic metabolic relationship between the bacterium *Bacteroides thetaiotaomicron* and the archeon *Methanobrevibacter smithii* (Catlett et al., 2022). Both microorganisms are known to play important roles in health, suggesting that the crosstalk between different domains has to be understood to adequately develop FTM analogs. *Methanobrevibacter smithii* levels are shown to be increased in constipation-predominant IBS (Chong et al., 2019). Hence, one needs knowledge on these types of interdomain crosstalk to effectively treat IBS using FMT analogs, instead of solely focusing on the bacteriome. Similar arguments can be made for the phageome, which can be considered as a subset of the virome, of which its interactions with the bacteriome alter the functional activities of the bacteria (Cao et al., 2022; Kirk et al., 2024). On top of this, the collection of parasites, the parasitome (Ianiro et al., 2022), will undoubtedly also play an important role, as it is known to influence the immune system (Ianiro et al., 2022). In addition to these different microbial domains, microbial components and the produced microbial metabolites may also play a role in the overall efficacy of FMT, which was suggested by a study in which a sterile FMT showed to be sufficient to effectively treat patients suffering from *C. diff*. Infection (Ott et al., 2017). A study by Paramsothy et al. (2019) indeed showed that short-chain fatty acids and secondary bile acids were instrumental for the efficacy of FMT for the treatment of ulcerative colitis (Paramsothy et al., 2019). Hence, future FMT analogs will most likely not only consist of bacteria, but of taxa from different domains, possibly enriched with postbiotics.

4 On the mode of action of future FTM analogs

The intraluminal ecosystem is generally used as a proxy for the microbial composition and associated diversity (Tang et al., 2020). As such, fecal samples can be used. However, it is well known that human colonic microbiota interact with the intestinal mucosa both in a healthy as well as in pathological conditions (ulcerative colitis, post infectious IBS, rheumatoid arthritis, among others) and triggers immune-responses (Bachmann et al., 2022; Holster et al., 2020; Holster et al., 2019). As it is known that the microbial composition of the mucosa-associated microbiota significantly differs from its luminal counterpart (Holster et al., 2019; Rangel et al., 2015), it is of importance that the composition of a possible FMT analog also adequately matches with this mucosa-associated ecosystem and the intestinal immune system in a functional perspective. In case of an inadequately overstimulated mucosal immune response, such as could be hypothesized in post-infectious IBS, the aim of FMT would be normalizing this immune response rather than changing microbe-microbe interactions or metabolic effects. In ulcerative colitis or microscopic colitis, however, increased production and mucosal utilization of the microbial metabolite butyrate could also be the therapeutic target. The route of administration of FMT analogs should also be considered in relation to its composition and potential

mode-of-action. Proximal small bowel administration will trigger a substantially stronger and qualitative different immune response compared to applying the traditional colonic administration. Moreover, the gut microbial composition gradually changes (among others, from a more aerobic to a more anaerobic nature) along the gastrointestinal tract (Hillman et al., 2017). Hence, there are various reasons why an FMT analog should be tailored to the specific location its anticipated action should take place.

5 Discussion

In general, the development of future FTM analogs requires a highly detailed functional understanding of how homeostasis and dysbiosis maintain health or cause disease. Depending on the complexity of these (patho)physiological processes one aims to intervene, this understanding may be on the level of generic features at a population level for relatively "simple" disorders, like recurrent *C. diff*. Infections. Alternatively, this knowledge should be at a personalized level like for neurological indications. As such, the comparison can be made with probiotics, for which its therapeutic target ranges from the prevention of antibiotic-associated diarrhea, which is applicable to many probiotics, up to very specific effects that are strain specific (Hill et al., 2014). This detailed understanding will not only encompass all microbial domains involved, but also all sites where possible microbe-microbe and microbe-host interactions may take place, which is clearly not limited to the lumen only. Furthermore, we argue that future FTM analogs ultimately require a One Health approach. Our Western lifestyle with, among others, high antibiotics usage, less direct contact to nature and a low fiber diet, has brough us close to the point of a "catastrophic collapse" in which we are losing functionalities due to a strongly diminished microbial repertoire (Larsen and van de Burgwal, 2021). A detailed understanding of the interactions between the human species and its adjacent ecosystems like soil, plants, and animals, each harboring its own unique microbiota, may lead to the identification of key taxa and/or guilds that may be used to restore functionalities in our body that were lost already, or for reinforcement of such functionalities. To conclude, future FTM analogs may not only be used to alleviate or even cure indications on a personalized level, but also could be applied to (partly) restore the taxa and associated functionalities we have lost as a consequence of our Westernized lifestyle.

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.

Author contributions

OL: Conceptualization, Writing – original draft, Writing – review & editing. RB: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

OL is Senior Manager Science at Yakult Nederland B. V. RB is also a member of the Scientific Advisory Board of The Akkermansia Company.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Bachmann, R., van Hul, M., Baldin, P., Léonard, D., Delzenne, N. M., Belzer, C., et al. (2022). *Akkermansia muciniphila* reduces peritonitis and improves intestinal tissue wound healing after a colonic transmural defect by a MyD88-dependent mechanism. *Cells* 11:2666. doi: 10.3390/cells11172666

Banerjee, S., Schlaeppi, K., and van der Heijden, M. G. A. (2018). Keystone taxa as drivers of microbiome structure and functioning. *Nat. Rev. Microbiol.* 16, 567–576. doi: 10.1038/s41579-018-0024-1

Cao, Z., Sugimura, N., Burgermeister, E., Ebert, M. P., Zuo, T., and Lan, P. (2022). The gut virome: a new microbiome component in health and disease. *EBioMedicine* 81:104113. doi: 10.1016/j.ebiom.2022.104113

Catlett, J. L., Carr, S., Cashman, M., Smith, M. D., Walter, M., Sakkaff, Z., et al. (2022). Metabolic synergy between human symbionts Bacteroides and Methanobrevibacter. *Microbiol. Spectr.* 10, e01067–e01022. doi: 10.1128/spectrum.01067-22

Chibani, C. M., Mahnert, A., Borrel, G., Almeida, A., Werner, A., Brugère, J.-F., et al. (2022). A catalogue of 1,167 genomes from the human gut archaeome. *Nat. Microbiol.* 7, 48–61. doi: 10.1038/s41564-021-01020-9

Chong, P. P., Chin, V. K., Looi, C. Y., Wong, W. F., Madhavan, P., and Yong, V. C. (2019). The microbiome and irritable bowel syndrome–a review on the pathophysiology, current research and future therapy. *Front. Microbiol.* 10:1136. doi: 10.3389/fmicb.2019.01136

Halkjær, S. I., Lo, B., Cold, F., Højer Christensen, A., Holster, S., König, J., et al. (2023). Fecal microbiota transplantation for the treatment of irritable bowel syndrome: a systematic review and meta-analysis. *World J. Gastroenterol.* 29, 3185–3202. doi: 10.3748/wjg.v29.i20.3185

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hillman, E. T., Lu, H., Yao, T., and Nakatsu, C. H. (2017). Microbial ecology along the gastrointestinal tract. *Microbes Environ.* 32, 300–313. doi: 10.1264/jsme2.ME17017

Holster, S., Hooiveld, G. J., Repsilber, D., de Vos, W. M., Brummer, R. J., and König, J. (2019). Allogenic faecal microbiota transfer induces immune-related gene sets in the colon mucosa of patients with irritable bowel syndrome. *Biomol. Ther.* 9:586. doi: 10.3390/biom9100586

Holster, S., Rode, J., Bohr, J., Kumawat, A. K., Veress, G., Hultgren Hörnquist, E., et al. (2020). Faecal microbiota transfer in patients with microscopic colitis–a pilot study in collagenous colitis. *Scand. J. Gastroenterol.* 55, 1454–1466. doi: 10.1080/00365521.2020.1839544

Ianiro, G., Iorio, A., Porcari, S., Masucci, L., Sanguinetti, M., Perno, C. F., et al. (2022). How the gut parasitome affects human health. *Ther. Adv. Gastroenterol.* 15:17562848221091524. doi: 10.1177/17562848221091524

Imdad, A., Pandit, N. G., Zaman, M., Minkoff, N. Z., Tanner-Smith, E. E., and Gomez-Duarte, O. G. (2023). Fecal transplantation for treatment of inflammatory bowel disease. *Cochrane Database Syst. Rev.* 2023, 1–14. doi: 10.1002/14651858.CD012774.pub3

Kirk, D., Costeira, R., Visconti, A., Khan Mirzaei, M., Deng, L., Valdes, A. M., et al. (2024). Bacteriophages, gut bacteria, and microbial pathways interplay in cardiometabolic health. *Cell Rep.* 43:113728. doi: 10.1016/j.celrep.2024.113728

Kootte, R. S., Levin, E., Salojärvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., et al. (2017). Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. *Cell Metab.* 26, 611–619. doi: 10.1016/j.cmet.2017.09.008

Larsen, O. F. A., and Claassen, E. (2018). The mechanistic link between health and gut microbiota diversity. *Sci. Rep.* 8:2183. doi: 10.1038/s41598-018-20141-6

Larsen, O. F. A., Koning, A. H. J., van der Spek, P. J., and Claassen, E. (2019). Towards a rational design of faecal transplant analogues. *Sci. Rep.* 9:5558. doi: 10.1038/s41598-019-42043-x

Larsen, O. F. A., and van de Burgwal, L. H. M. (2021). On the verge of a catastrophic collapse? The need for a multi-ecosystem approach to microbiome studies. *Front. Microbiol.* 12:784797. doi: 10.3389/fmicb.2021.784797

Ott, S. J., Waetzig, G. H., Rehman, A., Moltzau-Anderson, J., Bharti, R., Grasis, J. A., et al. (2017). Efficacy of sterile fecal filtrate transfer for treating patients with *Clostridium difficile* infection. *Gastroenterology* 152, 799–811. doi: 10.1053/j.gastro.2016.11.010

Paramsothy, S., Nielsen, S., Kamm, M. A., Deshpande, N. P., Faith, J. J., Clemente, J. C., et al. (2019). Specific bacteria and metabolites associated with response to fecal microbiota transplantation in patients with ulcerative colitis. *Gastroenterology* 156, 1440–1454. doi: 10.1053/j.gastro.2018.12.001

Rangel, I., Sundin, J., Fuentes, S., Repsilber, D., de Vos, W. M., and Brummer, R. J. (2015). The relationship between faecal-associated and mucosal-associated microbiota in irritable bowel syndrome patients and healthy subjects. *Aliment. Pharmacol. Ther.* 42, 1211–1221. doi: 10.1111/apt.13399

Schmidt, T. S. B., Li, S. S., Maistrenko, O. M., Akanni, W., Coelho, L. P., Dolai, S., et al. (2022). Drivers and determinants of strain dynamics following fecal microbiota transplantation. *Nat. Med.* 28, 1902–1912. doi: 10.1038/s41591-022-01913-0

Sender, R., Fuchs, S., and Milo, R. (2016). Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. *Cell* 164, 337–340. doi: 10.1016/j.cell.2016.01.013

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., Wang, B., et al. (2020). Current sampling methods for gut microbiota: a call for more precise devices. *Front. Cell. Infect. Microbiol.* 10:151. doi: 10.3389/fcimb.2020.00151

Taur, Y., Coyte, K., Schluter, J., Robilotti, E., Figueroa, C., Gjonbalaj, M., et al. (2018). Reconstitution of the gut microbiota of antibiotic-treated patients by autologous fecal microbiota transplant. *Sci. Transl. Med.* 10:eaap9489. doi: 10.1126/scitranslmed.aap9489

Van Nood, E., Vrieze, A., Nieuwdorp, M., Fuentes, S., Zoetendal, E. G., de Vos, W. M., et al. (2013). Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *N. Engl. J. Med.* 368, 407–415. doi: 10.1056/NEJMoa1205037

Wang, M., Xie, X., Zhao, S., Ma, X., Wang, Z., and Zhang, Y. (2023). Fecal microbiota transplantation for irritable bowel syndrome: a systematic review and meta-analysis of randomized controlled trials. *Front. Immunol.* 14:1136343. doi: 10.3389/fimmu.2023.1136343

Wu, G., Zhao, N., Zhang, C., Lam, Y. Y., Zhao, L., et al. (2021). Guild-based analysis for understanding gut microbiome in human health and diseases. *Genome Med.* 13, 1–12. doi: 10.1186/s13073-021-00840-y

Yang, Y., An, Y., Dong, Y., Chu, Q., Wei, J., Wang, B., et al. (2024). Fecal microbiota transplantation: no longer cinderella in tumour immunotherapy. *EBioMedicine* 100:104967. doi: 10.1016/j.ebiom.2024.104967

Zhang, F., Luo, W., Shi, Y., Fan, Z., and Ji, G. (2012). Should we standardize the 1,700-year-old fecal microbiota transplantation? *Am. J. Gastroenterol.* 107:1755. doi: 10.1038/ajg.2012.251

Zhang, C., Yin, A., Li, H., Wang, R., Wu, G., Shen, J., et al. (2015). Dietary modulation of gut microbiota contributes to alleviation of both genetic and simple obesity in children. *EBioMedicine* 2, 968–984. doi: 10.1016/j.ebiom.2015.07.007

Zhang, J., Zhu, G., Wan, L., Liang, Y., Liu, X., Yan, H., et al. (2023). Effect of fecal microbiota transplantation in children with autism spectrum disorder: a systematic review. *Front. Psychiatr.* 14:1123658. doi: 10.3389/fpsyt.2023.1123658

Zhao, L., Zhang, F., Ding, X., Wu, G., Lam, Y. Y., Wang, X., et al. (2018). Gut bacteria selectively promoted by dietary fibers alleviate type 2 diabetes. *Science* 359, 1151–1156. doi: 10.1126/science.aao5774

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 11 September 2024
DOI 10.3389/fmicb.2024.1458754

Check for updates

OPEN ACCESS

EDITED BY
Ludovico Abenavoli,
Magna Græcia University, Italy

REVIEWED BY
Bon-Sang Koo,
Korea Research Institute of Bioscience and Biotechnology, Republic of Korea
Jianquan He,
Xiamen University, China

*CORRESPONDENCE
Zhongsi Hong
✉ hongzhs@mail.sysu.edu.cn
Mengdang Ou
✉ oumd@mail.sysu.edu.cn

†These authors have contributed equally to this work

RECEIVED 11 July 2024
ACCEPTED 20 August 2024
PUBLISHED 11 September 2024

CITATION
Deng L, Guo X, Chen J, Li B, Liu N, Xia J, Ou M and Hong Z (2024) Effect of intestinal microbiota transplantation on chronic hepatitis B virus infection associated liver disease.
Front. Microbiol. 15:1458754.
doi: 10.3389/fmicb.2024.1458754

COPYRIGHT
© 2024 Deng, Guo, Chen, Li, Liu, Xia, Ou and Hong. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Effect of intestinal microbiota transplantation on chronic hepatitis B virus infection associated liver disease

Lisi Deng†, Xiaozhen Guo†, Jiehua Chen, Baoyi Li, Na Liu, Jinyu Xia, Mengdang Ou* and Zhongsi Hong*

Department of Infectious Diseases, the Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, China

Background: Research on the effects of intestinal microbiota transplantation (IMT) on chronic HBV infection (CHB) progression associated liver disease (HBV-CLD) and alterations in the microbiota post-IMT are quite limited for the moment.

Methods: By integrating microbiome with metabolome analyses, we aimed to the function of IMT and the alterations of gut microbiota in patients with HBV-CLD. First, this study included 20 patients with HBV-CLD and ten healthy controls. Then, 16 patients with CHB were given IMT with donor feces (heterologous) via oral capsule. Fecal samples from CHB patients were obtained before and after IMT, as well as healthy controls, for 16S rDNA sequencing and untargeted metabolomics analysis.

Results: The proalbuminemia were significantly increased after IMT, and the HBsAg and TBA showed a significant decrease after IMT in the HBV-CLD patients. There was statistical difference in the Chaol indexes between between CHB patients and healthy controls, suggesting a lower abundance of the gut microbiota in HBV-CLD patients. In addition, there was statistical difference in the Shannon and Simpson indexes between prior to IMT and post-IMT, indicating that the impaired abundance of the gut microbiota had been improved after IMT. The host-microbiota-metabolite interplay, amino acid metabolism, nicotinate and nicotinamide metabolism, starch and sucrose metabolism, steroid biosynthesis, and vitamins metabolism, were significantly lower in HBV-CLD patients than healthy controls.

Conclusion: IMT may improve the therapeutic effects on patients HBV-CLD. Furthermore, IMT appears to improve amino acid metabolism by impaired abundance of the gut microbiota and therefore improve liver prealbumin synthesis.

KEYWORDS

chronic hepatitis B virus infection, microbiome, metabolome, intestinal microbiota transplantation, alterations

Introduction

Hepatitis B virus infection-associated liver disease (HBV-CLD) places a significant economic burden on the state and society, despite ongoing good interventions in many countries and areas. In the normal course of HBV infection, an estimated 15–40% of individuals with chronic hepatitis B (CHB) infection develop to cirrhosis, liver failure, or hepatocellular carcinoma (Arora et al., 2018).

The ideal endpoint for patients with HBV (both HBeAg positive and HBeAg negative) infection is hepatitis B surface antigen (HBsAg) loss, with or without seroconversion to anti-HBs. This is associated with complete and definitive remission of the activity of HBV (Chotiyaputta and Lok, 2010; Chen et al., 2002). During the natural history of chronic HBV infection, the loss of serum HBeAg and the development of anti-HBe antibodies (HBeAg seroconversion) mark a transition from the immune-active phase of the disease to the inactive carrier state (Chen and Yang, 2011).

The liver secretes bile into the bile duct system, while a variety of gut-derived signals, such as bacterial products, environmental toxins, and dietary antigens, enter the gut and travel through the biliary tract to the liver (Albillos et al., 2020). Based on the "gut-liver axis, "an increasing number of research have discovered a link between hepatic damage and disturbance of gut microbial homeostasis. Growing data suggests that gut microbe-derived metabolites such as trimethylamine, bile acids, and short-chain fatty acids play a substantial role in the beginning and progression of liver diseases (Zhao et al., 2020; Jia et al., 2018). Furthermore, a large signaling network regulated by microbiota-derived metabolism continues to be studied, providing gut-liver targets for disease therapy (Li et al., 2023).

Intestinal microbiota transplantation (IMT) is a novel approach to restoring and reconstructing the intestinal microecological balance and diversity. IMT has been shown to be effective in the treatment of a variety of disorders, including *Clostridium difficile* infection, metabolic diseases, malignancies, autoimmune diseases, nonalcoholic fatty liver disease (NAFLD), and hepatic encephalopathy (Surawicz et al., 2013; de Groot et al., 2017; Vaughn et al., 2019; Meighani et al., 2020). Besides, IMT appears to be safe and potentially effective in terms of viral suppression and HBeAg clearance in patients with HBeAg-positive CHB (Chauhan et al., 2021).

Although some studies have been conducted on the diversity of gut microbiota in chronic hepatitis B, as well as the clinical effects of IMT on E antigen and liver cirrhosis, research on the effects of IMT on early stage cirrhosis or pre-cirrhosis patients and changes in the microbiota post-IMT is quite limited. Therefore, in the present study, we performed a clinical study to characterize gut microbiota in CHB progression, followed by an investigation into the role of IMT in HBeAg-negative CHB patients, and finally, we examined the impact of IMT on gut microbiota composition and metabolomics signature.

Methods

Subjects and study design

There were three study groups, a cross-sectional study was conducted to see if there was any alternation in gut microbiota and metabonomics in HBV-CLD patients with healthy controls. Afterward, a perspective study was performed in the cohort of patients before IMT and after IMT. The study was approved by The Research Ethics Committee at The Fifth Affiliated Hospital of Sun Yat-Sen University (no.·ZDWY [2023] Lunzi no.·K254-1). Written informed consent was obtained from each participant before the initiation of study procedures.

A total of 16 HBeAg-negative HBV-CLD patients were enrolled at The Fifth Affiliated Hospital of Sun Yat-Sen University from February 2022 to March 2024, including 3 early stage of cirrhosis and 13 pre-cirrhosis. HBV-CLD patients on oral antivirals, which included entecavir (ETV), tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide fumarate (TAF) alone, for more than a year undetectable of serum levels of HBVDNA and those who consented for the IMT were included in the IMT arm. A total of 10 age-and BMI-matched Chinese healthy individuals were enrolled, The inclusion criteria were as follows: (a) alcohol free history; (b) smooth and soft stool that was sausage or snake shaped, and (c) voluntary participation in this study. Patients with co-infections with hepatitis C virus (HCV) or human immunodefciency virus (HIV), chronic kidney disease, hepatocellular carcinoma, underlying other comorbidities, and those unwilling to provide written informed consent for the IMT procedure were excluded. We also excluded patients with underlying cirrhosis.

Fecal microbiota capsules for the IMT group were obtained from healthy undergraduate donors (Guangdong Pharmaceutical University) and evaluated using comprehensive physical examinations. The preparation and the transplantation of fecal microbiota capsules were performed as previously described (Kao et al., 2017). Forty capsules were manufactured from 1 donation. All HBV-CLD patients received a total of 120 capsules, 6–10 capsules three times a day (according to the digestive reaction of patients, each dosage can be appropriately reduced), for a total course of about 6 days. Theoretically, there were four groups: Group A, HBV-CLD patients; group B, healthy controls; group C, pri-IMT; group D, post-IMT.

Clinical data collection

All patients who underwent IMT were evaluated clinically daily while taking the fecal microbiota capsules for the development of any new symptoms, assessment of side-effects post-procedure. Adverse events in the form of eight gastrointestinal symptom parameters were observed within 7 days following IMT. Liver function tests, blood routine examinations, HBsAg, and HBVDNA levels were measured at baseline and weeks 8, 24. All 16 patients completed blood tests, 13 patients completed pri-IMT stool 16S rRNA sequencing, and 7 patients completed post-IMT 16S rRNA sequencing.

16S rRNA sequencing and bioinformatic analysis

Stool samples were collected before IMT and 1 month after IMT. Study participants were given a stool sample kit at each in-person visit to collect the stool sample. Explicit instructions were given to the participants on how to collect the samples. Once collected (within seven days of an inperson visit), the samples were frozen at −80°C until sequencing. Libraries were generated by amplification of the V3–V4 region of the 16S ribosomal RNA gene of DNA extracted from gut mucosal biopsies and from stool samples, and sequenced on the Illumina

MiSeq platform (Illumina, San Diego, California). For 16S analysis, QIIME (version 1.9.1) was used to demultiplex and quality-filter the raw FASTQ files. Next, operational taxonomic units (OTUs) were generated and clustered using a 97% threshold and Usearch (version 7.1). After aligning with the gold database (v20110519), UCHIME (4.2.40) was used to filter out chimaeric sequences, and use arch_global was used to quantify the OTU abundances for each sample. For taxonomic analysis of each representative OTU, the Greengenes database (v201305) was used based on the RDP classifier (Version 2.2) with a 0.8 confidence value. OTUs were then assigned to different hierarchical levels and taxonomic relative abundance profiles were summarized.

Metabolomic profiling

To understand the roles and interactions of proteins, metabolites, and genes in biological systems, the KEGG database was built. In this study, we used DIAMOND (v0.9.7) to annotate the representative sequences of gene sets with the KEGG database. To obtain the functional abundance at each level, we used the BLAST comparison parameters with an expectation of E < 1e−5. To find the metabolic pathway enrichment results for metabolites, enrichment analysis was carried out using their KEGG IDs. Pathway entries that were considerably enriched in significantly differently expressed metabolites relative to the background were identified using hypergeometric testing.

Statistical analysis

To analyze differences in continuous variables, we employed the paired-samples t-test or the Wilcoxon signed-rank test. Pearson correlation analysis for gut microbiota post IMT, FDR for multiple correction. Microbial features (species and metabolites) found in fewer than 20% of the samples were eliminated prior to statistical analysis of the relative abundance data. Spearman's rank correlation was used to examine associations between gut microbiota and metabolites, which were then adjusted for significance using the Benjamini-Hochberg approach. Linear discriminant analysis effect size (LEfSe) was used to assess groups for statistically significant species and functional differences. The nonparametric factorial Kruskal-Wallis test, Wilcoxon rank sum test, and LDA were used to identify biological and functional markers that were enriched for differences between multiple metadata categories. We performed statistical analyses using SPSS 22.0 (IBM Corporation, Armonk, New York, United States) and GraphPad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA, United States). Unless otherwise stated, all statistical tests are two-tailed, and $p < 0.05$ was deemed statistically significant.

Results

Evaluation of clinical comprehensive curative effect of IMT on HBV-CLD patients

Between December 2023 and March 2024, 30 participants were screened and 26 were enrolled in this study: 16 HBV-CLD patients accepted IMT, 10 healthy controls. Two HBV-CLD patients were excluded for they did not complete the treatment course of IMT. Fourteen HBV-CLD patients received full course of IMT, while two patients did not complete blood tests.

All of the patients had undetectable HBVDNA at baseline and continued on their previous antiviral medication while undergoing IMT. 78.5% of patients had hypothrombocytemia, with a median of 75×10^9/L. Plaque increased considerably after IMT at 8 weeks ($p = 0.001$) and 24 weeks ($p < 0.001$) (Figure 1B). Additionally, 78.5% had hypoproalbuminemia, with a median of 145 g/L 8 weeks ($p = 0.259$), 24 weeks ($p = 0.007$) (Figure 1F), HBsAg showed a significant decrease after IMT: 8 weeks ($p = 0.026$), 24 weeks ($p = 0.116$) (Figure 1C), and total bile acid (TBA) showed a significant decrease after IMT: 8 weeks ($p = 0.024$), 24 weeks ($p = 0.001$) (Figure 1E). Some individuals with hypoleukocytoemia had an increase in white blood cells following transplantation (Figure 1A). Nonetheless, the alanine transaminase (ALT) (Table 1) and aspartate aminotransferase (AST) levels were mostly maintained in the normal range following IMT.

Alteration of gut microbiota composition in HBV-CLD patients pri-IMT and post-IMT

Alpha diversity indexes indicating community richness, diversity, evenness and coverage were assessed via Chaol, Shannon, Simpson, ACE and Coverage indexes. Therein, there was no difference in the Shannon index and Simpson index, but the Chao1 index was higher in the patient group. But, Chao1 places a strong emphasis on the presence of rare species within a community, with a primary focus on species richness or the total number of different species present. However, its drawback lies in the fact that it does not take into account the distribution abundance and evenness of species within the community. Both qualitative PCoA and PCA analysis were performed to evaluate β diversity. No significant changes in β diversity was observed in the comparison between HBV-CLD patients and healthy controls (Supplementary Figure S1).

There was significant difference in the Shannon and Simpson indexes between prior to IMT (pri-IMT) and post-IMT (Figures 2E,F; Shannon $p = 0.034$, Simpson $p = 0.011$), indicating that the impaired abundance of the gut microbiota had been improved after IMT in HBV-CLD patients. No significant changes in β diversity was observed in the comparison between pri-IMT and post-IMT (Supplementary Figure S2). In addition, we conducted pearson correlation analysis of individual microflora post-IMT. Although there was still a strong correlation between the microflora before and after transplantation, individual evolution occurred to different degrees (Table 2).

To further explore the taxonomic features and composition differences of gut microbiota associated with HBV-CLD patients, the raw macrogenome data were checked by quality control, filtered, and assembled to construct abundance profiles at the corresponding taxonomic levels. And we compared bacteria abundance before IMT and after IMT to further demonstrate the microbiota shifts in HBV-CLD patients. In LEfSe analysis ($p < 0.05$, $q < 0.1$, LDA > 2.0), compared with the HCs, the c. Bacilli, o. Lactobacillales, f. Streptococcaceae, g. *Streptococcus*, g. Paeniclostridium, g. Veillonella, were further diminished in CHB patients (Figure 3). While f. Rikenellaceae, f. Lachnospiraceae, f. Barnesiellaceae, g. Megamonas, g. Alistipes, g. Erysipelatoclostridium, were more enriched in CHB patients (Figures 4A,B).

After IMT, compared with pri-IMT, the o. Coriobacteriales f. Enterobacteriaceae, g. Collinsella, g. Lachnoclostridium, g.

10.3389/fmicb.2024.1458754

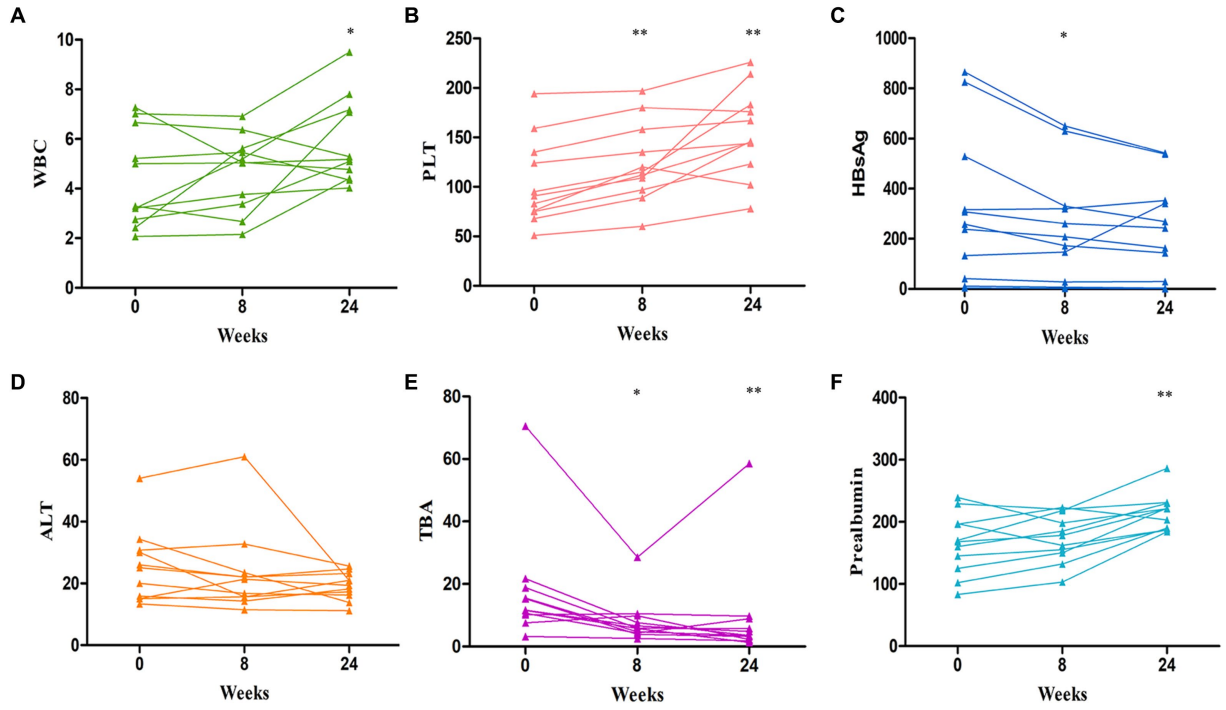


FIGURE 1
Changes curve in laboratory parameters over time post IMT. **(A)** the change curve of white blood cells (WBC); **(B)** the change curve of platelet (PLT); **(C)** the change curve of HBsAg; **(D)** the change curve of alanine aminotransferase (ALT); **(E)** the change curve of total bile acid (TBA); **(F)** the change curve of prealbumin. *$p < 0.05$, **$p < 0.01$.

TABLE 1 Changes in laboratory parameters over time post IMT.

	Pri-IMT	Post-IMT 8 weeks	*p* value	Post-IMT 24 weeks	*p* value
WBC (×10^9/L)	4.3 ± 1.9	4.6 ± 1.5	0.495	5.8 ± 1.7	0.072
PLT (×10^9/L)	104.6 ± 43.4	116.9 ± 44.5	0.001	124.3 ± 42.7	<0.001
ALT (U/L)	25.3 ± 11.9	23.3 ± 13.7	0.307	19.2 ± 4.4	0.097
TBA (μmol/L)	17.8 ± 18.1	8.3 ± 7.2	0.024	9.4 ± 16.5	0.001
Prealbumin (mg/L)	164.9 ± 49.3	174.9 ± 38.7	0.259	214.7 ± 29.8	0.007
HBsAg (IU/ML)	320.7 ± 302.4	250.4 ± 224.5	0.026	238.8 ± 193.9	0.116

Data are presented as mean (SD). WBC, white blood cells; PLT, platelet; ALT, alanine aminotransferase; TBA, total bile acid.

Caproiciproducens, were significantly decreased in post-IMT patients (Figure 5).

Alteration of gut metabolites in HBV-CLD patients pri-IMT and post-IMT

To determine if the functional pathways involved in gut metabolites and microbiota are consistently changed, we ran pathway enrichment analysis on the KEGG IDs of metabolites and obtained pathway enrichment findings. Representatively various metabolites would be examined.

Compared to the HCs, amino acid metabolism, including alanine, aspartate, glutamate and tryptophan metabolism, was significantly decreased in CHB patients. In addition, the nicotinate and nicotinamide metabolism, starch and sucrose metabolism, steroid biosynthesis, and vitamins metabolism, were significantly lower in CHB patients than HCs. It is worth noting that oxidative phosphorylation and nucleotide excision repair were further depleted in CHB patients. Whereas p53 signaling pathway was more exuberantly in CHB patients than HCs (Figure 4C).

Following IMT, PICRUST analysis showed that the relative abundance of t the metabolites listed above were partially restored. In addition, the PPAR signaling pathway and pathogenic *Escherichia coli* infection significantly lower in post-IMT than pri-IMT (Figure 4D).

Discussion

In this study, we investigated the effectiveness of IMT on HBV-CLD patients and characterized gut microbiota in HBV-CLD progression by combining microbiome with metabolome pri-IMT and post-IMT. HBV-CLD patients in our study presented with hypoproalbuminemia, hypersplenism such as leukopenia and

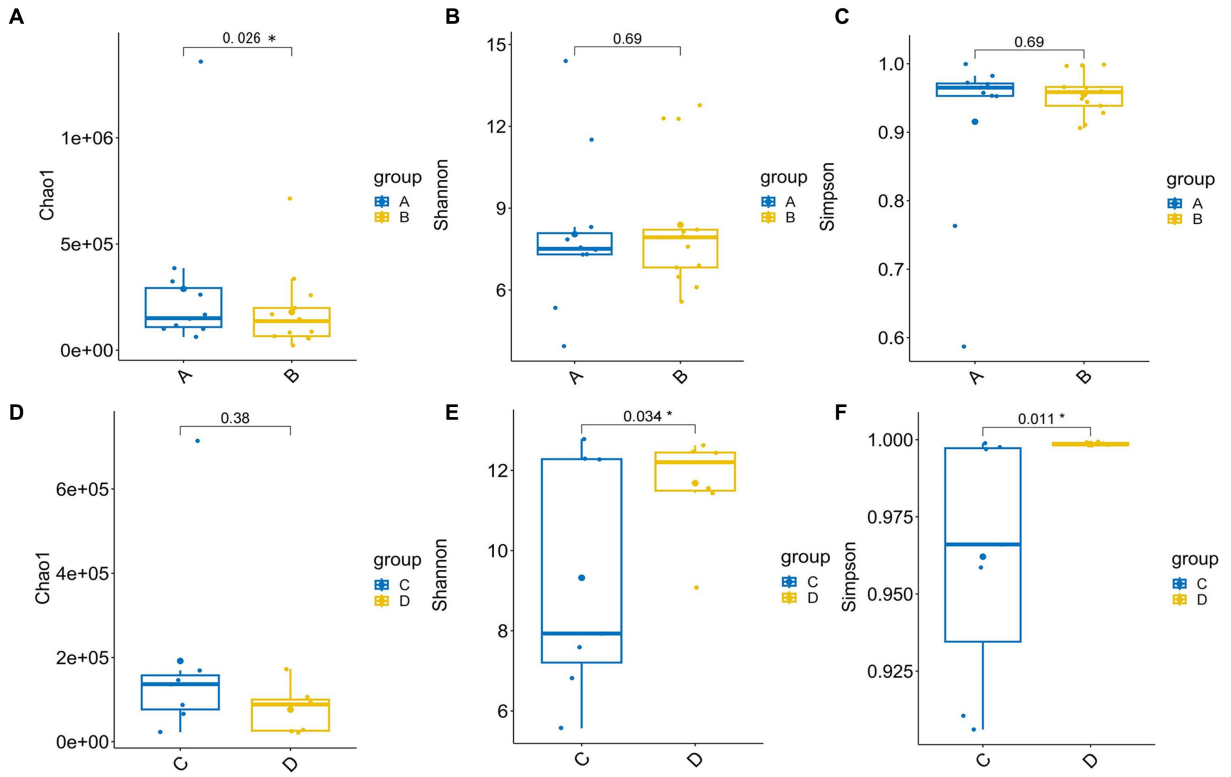


FIGURE 2
The α diversity of gut microbiota. The α diversity for HBV-CLD patients (group A) compared with healthy controls (group B) in Chaol indexes **(A)**, Shannon indexes **(B)**, Simpson indexes **(C)**, the α diversity for pri-IMT (group C) compared with post-IMT (group D) in Chaol indexes **(D)**, Shannon indexes **(E)**, Simpson indexes **(F)**. *$p < 0.05$.

TABLE 2 **Pearson correlation analysis of gut microbiota post IMT.**

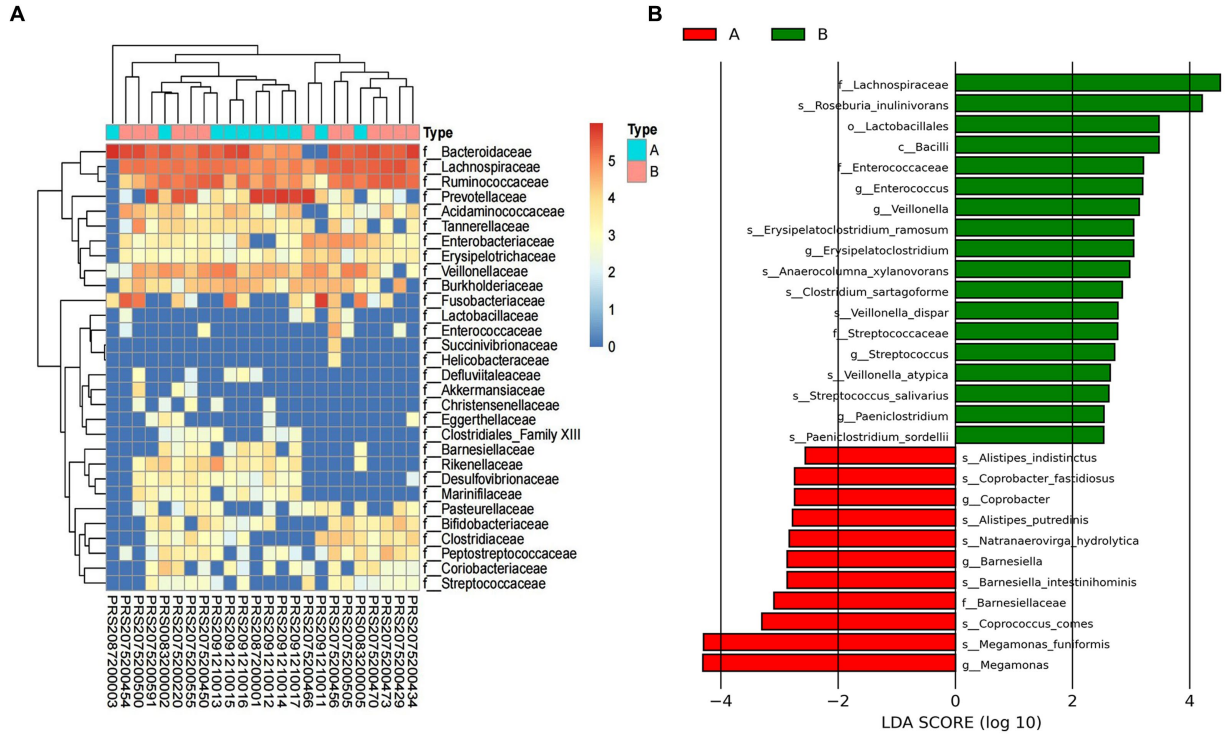
Number	Pri-IMT	Number	Post-IMT	Cor	*p* value
PRS2075200220	N1-pri IMT	PRS2075200475	N1-post IMT	0.78	0.00
PRS2075200473	N2-pri IMT	PRS2075200458	N2-post IMT	0.45	0.00
PRS2075200591	N3-pri IMT	PRS2075200433	N3-post IMT	0.8	0.00
PRS2075200470	N4-pri IMT	PRS2075200402	N4-post IMT	0.52	0.00
PRS2075200450	N5-pri IMT	PRS2075200404	N5-post IMT	0.66	0.00
PRS2075200454	N6-pri IMT	PRS2075200589	N6-post IMT	0.11	0.06
PRS2075200505	N7-pri IMT	PRS2075200482	N7-post IMT	0.54	0.00

Cor: Pearson correlation coefficient, FDR for multiple correction.

thrombocytopenia, but no sequelae from decompensated cirrhosis. Long-term antiviral medication did not ameliorate these problems.

In this study, a decrease in HBsAg was observed in E antigen-negative patients post-IMT 8 weeks. There were reported that IMT appeared to be safe and potentially effective in terms of viral suppression and HBeAg clearance in patients with HBeAg-positive CHB, but achieved no HBsAg clearance (Chauhan et al., 2021; Ren et al., 2017). IMT has the potential to modulate the host's immune response and alter its susceptibility to HBV infection (Wang et al., 2022).

Oral IMT capsules were safe and well-tolerated in patient with cirrhosis and recurrent HE; besides, gut microbial function in cirrhosis is beneficially affected by capsular FMT, with improved inflammation and cognition (Wang et al., 2022; Bajaj et al., 2019). For patients with HBV-CLD in this study, increase in platelets and white blood cells could be observed after 24 weeks. It has been proven that gut flora (including Bacteroidetes, Parabacteroides, and Proteobacteria) were restored to levels comparable to those reported in healthy persons following splenectomy, which was followed by an increase in WBC and PLT counts in liver cirrhosis patients (Liu et al., 2018). Furthermore, significant improvements in proalbumin could be observed post-IMT. PICRUST analysis revealed that IMT restored the relative abundance of KEGG pathways involved in amino acid metabolism. That might be contribute by the improved composition of the gut microbiota by IMT. Furthermore, IMT might contribute largely to slow the progress of liver impairment.


A
Type
f__Bacteroidaceae
f__Lachnospiraceae
f__Ruminococcaceae
f__Prevotellaceae
f__Acidaminococcaceae
f__Tannerellaceae
f__Enterobacteriaceae
f__Erysipelotrichaceae
f__Veillonellaceae
f__Burkholderiaceae
f__Fusobacteriaceae
f__Lactobacillaceae
f__Enterococcaceae
f__Succinivibrionaceae
f__Helicobacteraceae
f__Defluviitaleaceae
f__Akkermansiaceae
f__Christensenellaceae
f__Eggerthellaceae
f__Clostridiales_Family XIII
f__Barnesiellaceae
f__Rikenellaceae
f__Desulfovibrionaceae
f__Marinifilaceae
f__Pasteurellaceae
f__Bifidobacteriaceae
f__Clostridiaceae
f__Peptostreptococcaceae
f__Coriobacteriaceae
f__Streptococcaceae
PRS207520043
PRS207520042
PRS207520047
PRS207520045
PRS207520050
PRS207520049
PRS207520046
PRS207520050
PRS008320000
PRS207520055
PRS207520045
PRS209121001
PRS209121001
PRS209121001
PRS209121001
PRS209121001
PRS207520046
PRS207520045
PRS008320000
PRS207520047
PRS207520042
PRS207520043
PRS207520045
PRS207520049

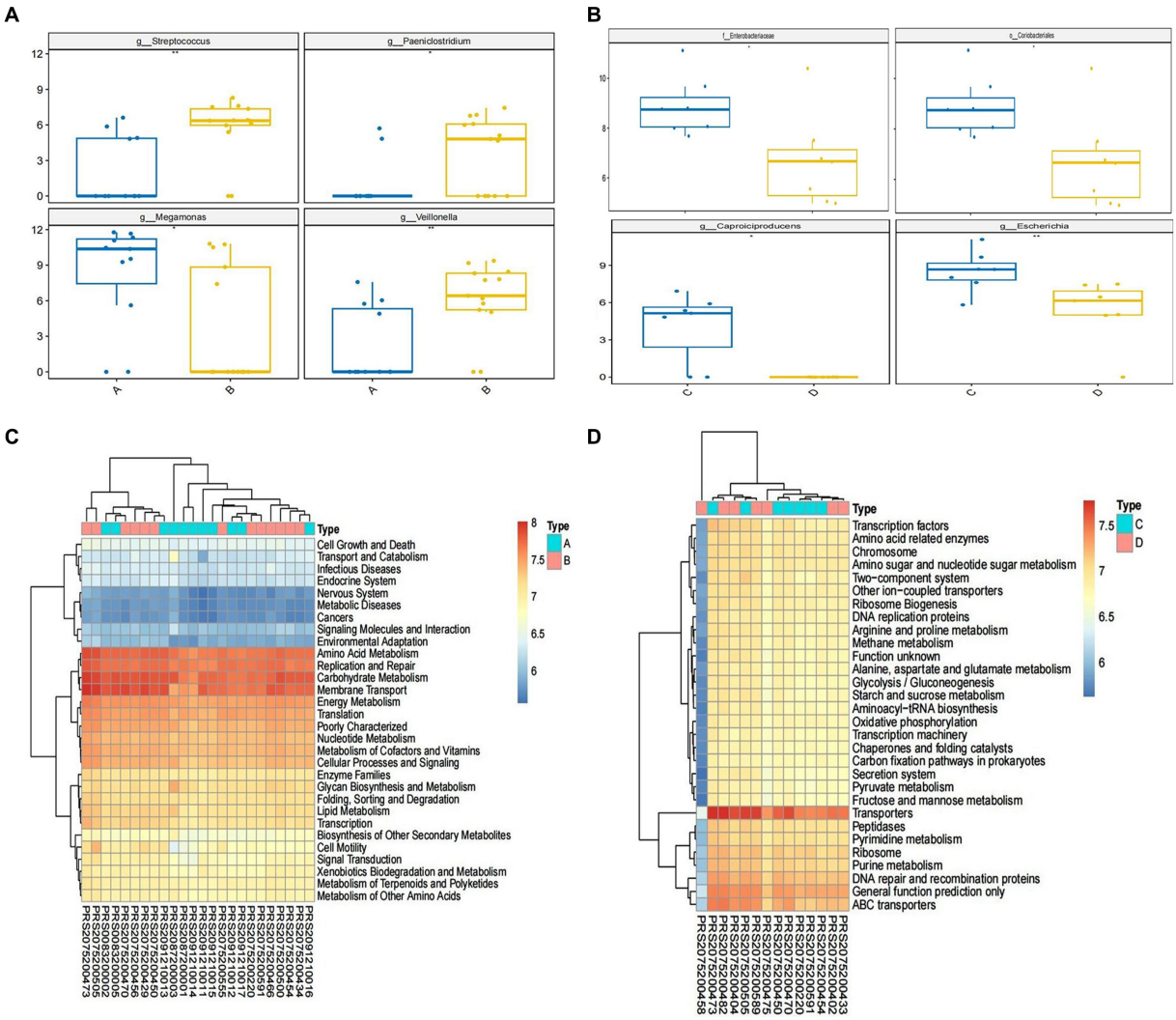


FIGURE 4
Alternation of microbiome and the predicted microbial functional composition with PICRUST analysis. The relative abundance of microbiome in genus level between group A and group B **(A)**; the relative abundance of microbiome in species level between group C and group D **(B)**. The predicted microbial functional composition with PICRUST analysis for HBV-CLD patients (level II) **(C)**. The KEGG pathways (level III) with PICRUST analysis after IMT **(D)**. Group A: HBV-CLD patients, group B: healthy controls, group C: pri-IMT, group D: post-IMT.

Conclusion

In conclusion, HBV-CLD patients who underwent IMT showed improvements in liver function as well as a recovery of the composition and metabolism of the gut microbiota in this study. IMT results in decreased relative abundance of pathogenic bacteria such as Enterobacteriaceae and Megamonas. Furthermore, a increase in prealbumin and metabolomics of amino acid (including Eaa, tryptophan). The underlying mechanism still needs further study. Therefore, the regulation of gut microbiota by IMT may provide a new clinical approach for the treatment of HBV-CLD.

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

The studies involving humans were approved by the Research Ethics Committee at The Fifth Affiliated Hospital of Sun Yat-Sen University. 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

LD: Formal analysis, Writing – original draft, Writing – review & editing, Conceptualization, Investigation, Supervision, Methodology, Project administration, Resources, Software, Visualization. XG: Data curation, Resources, Writing – original draft, Investigation, Supervision. JC: Investigation, Project administration, Resources, Writing – original draft. BL: Project administration, Writing – original

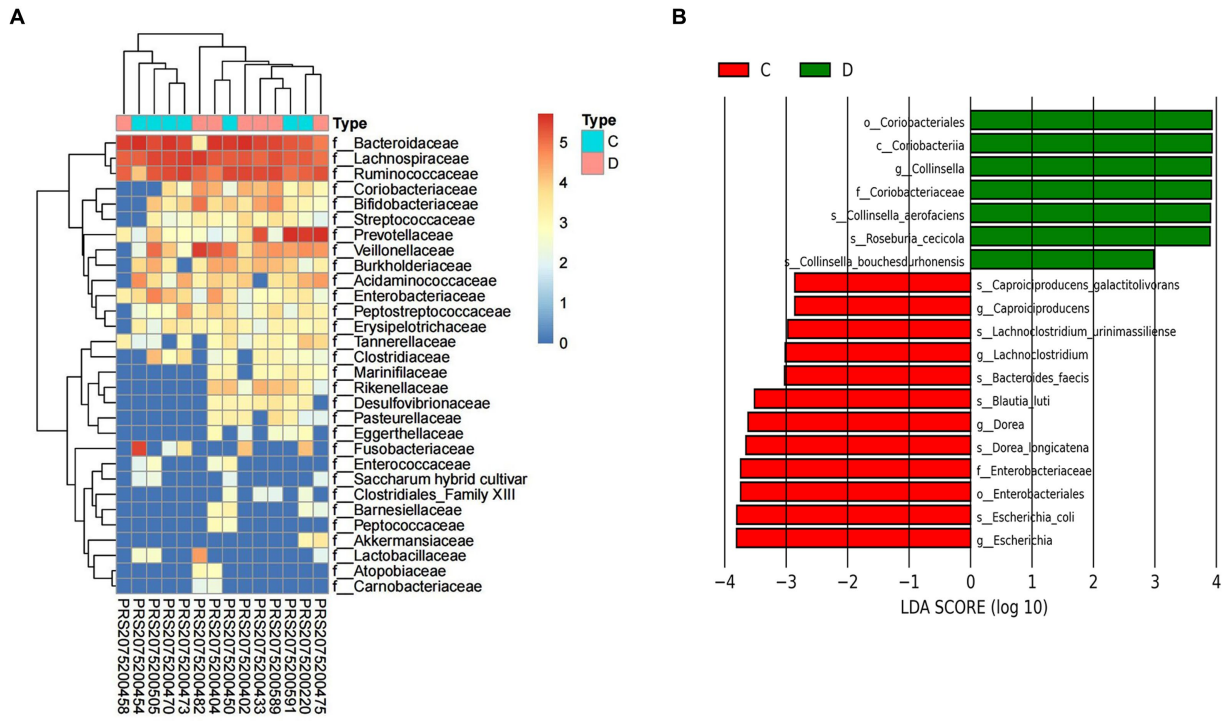


FIGURE 5
Alternation of gut microbiota composition after IMT. The relative abundance between group C and group D **(A)**. LEfSe analysis shows species with significant differences in abundance between group C and group D **(B)**. Group C: pri-IMT, group D: post-IMT.

draft. NL: Investigation, Validation, Writing – original draft. JX: Funding acquisition, Supervision, Writing – original draft. MO: Funding acquisition, Investigation, Supervision, Writing – review & editing. ZH: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. Funding for this study was provided by Start-up funds for scientific research of high-level talents, the Fifth Affiliated Hospital, Sun Yat-sen University.

Acknowledgments

We acknowledge all health-care workers involved in the diagnosis and treatment of patients in the Fifth Affiliated Hospital, Sun Yat-sen University and thank all patients and their families involved in the study.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1458754/full#supplementary-material

References

Albillos, A., de Gottardi, A., and Rescigno, M. (2020). The gut-liver axis in liver disease: pathophysiological basis for therapy. *J. Hepatol.* 72, 558–577. doi: 10.1016/j.jhep.2019.10.003

Arora, A., Singh, S. P., Kumar, A., Saraswat, V. A., Aggarwal, R., Bangar, M., et al. (2018). INASL position statements on prevention, diagnosis and Management of Hepatitis B Virus Infection in India: the Andaman statements. *J. Clin. Exp. Hepatol.* 8, 58–80. doi: 10.1016/j.jceh.2017.12.001

Bajaj, J. S., Salzman, N., Acharya, C., Takei, H., Kakiyama, G., Fagan, A., et al. (2019). Microbial functional change is linked with clinical outcomes after capsular fecal transplant in cirrhosis. *JCI Insight* 4:e133410. doi: 10.1172/jci.insight.133410

Cani, P. D., Van Hul, M., Lefort, C., Depommier, C., Rastelli, M., and Everard, A. (2019). Microbial regulation of organismal energy homeostasis. *Nat. Metab.* 1, 34–46. doi: 10.1038/s42255-018-0017-4

Cesaro, C., Tiso, A., Del Prete, A., Cariello, R., Tuccillo, C., Cotticelli, G, et al. (2011). Gut microbiota and probiotics in chronic liver diseases. *Dig. Liver Dis.* 43, 431–438. doi: 10.1016/j.dld.2010.10.015

Chauhan, A., Kumar, R., Sharma, S., Mahanta, M., Vayuuru, S. K., Nayak, B., et al. (2021). Fecal microbiota transplantation in hepatitis B e antigen-positive chronic hepatitis B patients: a pilot study. *Dig. Dis. Sci.* 66, 873–880. doi: 10.1007/s10620-020-06246-x

Chen, Y. C., Sheen, I. S., Chu, C. M., and Liaw, Y. F. (2002). Prognosis following spontaneous HBsAg seroclearance in chronic hepatitis B patients with or without concurrent infection. *Gastroenterology* 123, 1084–1089. doi: 10.1053/gast.2002.36026

Chen, C. J., and Yang, H. I. (2011). Natural history of chronic hepatitis B REVEALed. *J. Gastroenterol. Hepatol.* 26, 628–638. doi: 10.1111/j.1440-1746.2011.06695.x

Chen, Y., Yang, F., Lu, H., Wang, B., Chen, Y., Lei, D., et al. (2011). Characterization of fecal microbial communities in patients with liver cirrhosis. *Hepatology* 54, 562–572. doi: 10.1002/hep.24423

Chotiyaputta, W., and Lok, A. S. (2010). Endpoints of hepatitis B treatment. *J. Viral Hepat.* 17, 675–684. doi: 10.1111/j.1365-2893.2010.01369.x

de Groot, P. F., Frissen, M. N., de Clercq, N. C., and Nieuwdorp, M. (2017). Fecal microbiota transplantation in metabolic syndrome: history, present and future. *Gut Microbes* 8, 253–267. doi: 10.1080/19490976.2017.1293224

Dhiman, R. K., Rana, B., Agrawal, S., Garg, A., Chopra, M., Thumburu, K. K., et al. (2014). Probiotic VSL#3 reduces liver disease severity and hospitalization in patients with cirrhosis: a randomized, controlled trial. *Gastroenterology* 147, 1327–1337.e3. doi: 10.1053/j.gastro.2014.08.031

Jia, W., Xie, G., and Jia, W. (2018). Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. *Nat. Rev. Gastroenterol. Hepatol.* 15, 111–128. doi: 10.1038/nrgastro.2017.119

Kao, D., Roach, B., Silva, M., Beck, P., Rioux, K., Kaplan, G. G., et al. (2017). Effect of Oral capsule-vs colonoscopy-delivered fecal microbiota transplantation on recurrent *Clostridium difficile* infection: a randomized clinical trial. *JAMA* 318, 1985–1993. doi: 10.1001/jama.2017.17077

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From dietary Fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Li, S. Q., Shen, Y., Zhang, J., Weng, C. Z., Wu, S. D., and Jiang, W. (2023). Immune modulation of gut microbiota and its metabolites in chronic hepatitis B. *Front. Microbiol.* 14:1285556. doi: 10.3389/fmicb.2023.1285556

Liu, Y., Li, J., Jin, Y., Zhao, L., Zhao, F., Feng, J., et al. (2018). Splenectomy leads to amelioration of altered gut microbiota and metabolome in liver cirrhosis patients. *Front. Microbiol.* 9:963. doi: 10.3389/fmicb.2018.00963

Meighani, A., Alimirah, M., Ramesh, M., and Salgia, R. (2020). Fecal microbiota transplantation for Clostridioides difficile infection in patients with chronic liver disease. *Int. J. Hepatol.* 2020:1874570. doi: 10.1155/2020/1874570

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., et al. (2012). Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267. doi: 10.1126/science.1223813

Paik, Y. H., Schwabe, R. F., Bataller, R., Russo, M. P., Jobin, C., and Brenner, D. A. (2003). Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 37, 1043–1055. doi: 10.1053/jhep.2003.50182

Ren, Z., Li, A., Jiang, J., Zhou, L., Yu, Z., Lu, H., et al. (2019). Gut microbiome analysis as a tool towards targeted non-invasive biomarkers for early hepatocellular carcinoma. *Gut* 68, 1014–1023. doi: 10.1136/gutjnl-2017-315084

Ren, Y. D., Ye, Z. S., Yang, L. Z., Jin, L. X., Wei, W. J., Deng, Y. Y., et al. (2017). Fecal microbiota transplantation induces hepatitis B virus e-antigen (HBeAg) clearance in patients with positive HBeAg after long-term antiviral therapy. *Hepatology* 65, 1765–1768. doi: 10.1002/hep.29008

Riordan, S. M., and Williams, R. (2010). Gut flora and hepatic encephalopathy in patients with cirrhosis. *N. Engl. J. Med.* 362, 1140–1142. doi: 10.1056/NEJMe1000850

Sung, C. M., Lin, Y. F., Chen, K. F., Ke, H. M., Huang, H. Y., Gong, Y. N., et al. (2019). Predicting clinical outcomes of cirrhosis patients with hepatic encephalopathy from the fecal microbiome. *Cell. Mol. Gastroenterol. Hepatol.* 8, 301–318.e2. doi: 10.1016/j.jcmgh.2019.04.008

Surawicz, C. M., Brandt, L. J., Binion, D. G., Ananthakrishnan, A. N., Curry, S. R., Gilligan, P. H., et al. (2013). Guidelines for diagnosis, treatment, and prevention of *Clostridium difficile* infections. *Am. J. Gastroenterol.* 108:499. doi: 10.1038/ajg.2013.4

Vaughn, B. P., Rank, K. M., and Khoruts, A. (2019). Fecal microbiota transplantation: current status in treatment of GI and liver disease. *Clin. Gastroenterol. Hepatol.* 17, 353–361. doi: 10.1016/j.cgh.2018.07.026

Wang, J., Zhou, X., Li, X., Guo, W., Zhu, Q., Zhu, B., et al. (2022). Fecal microbiota transplantation alters the outcome of hepatitis B virus infection in mice. *Front. Cell. Infect. Microbiol.* 12:844132. doi: 10.3389/fcimb.2022.844132

Yachida, S., Mizutani, S., Shiroma, H., Shiba, S., Nakajima, T., Sakamoto, T., et al. (2019). Metagenomic and metabolomic analyses reveal distinct stage-specific phenotypes of the gut microbiota in colorectal cancer. *Nat. Med.* 25, 968–976. doi: 10.1038/s41591-019-0458-7

Zeng, Y., Chen, S., Fu, Y., Wu, W., Chen, T., Chen, J., et al. (2020). Gut microbiota dysbiosis in patients with hepatitis B virus-induced chronic liver disease covering chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. *J. Viral Hepat.* 27, 143–155. doi: 10.1111/jvh.13216

Zhao, M., Zhao, L., Xiong, X., He, Y., Huang, W., Liu, Z., et al. (2020). TMAVA, a metabolite of intestinal microbes, is increased in plasma from patients with liver steatosis, inhibits gamma-Butyrobetaine hydroxylase, and exacerbates fatty liver in mice. *Gastroenterology* 158, 2266–2281.e27. doi: 10.1053/j.gastro.2020.02.033

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 13 November 2024
DOI 10.3389/fmicb.2024.1451303

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Xuehua Wan,
Nankai University, China
Yiming Meng,
Cancer Hospital of Dalian University of Technology, China

*CORRESPONDENCE
Xiao-Qin Mu
muxiaoqin@ems.hrbmu.edu.cn
Shu-Lin Liu
slliu@hrbmu.edu.cn

RECEIVED 19 June 2024
ACCEPTED 24 October 2024
PUBLISHED 13 November 2024

CITATION
Han X, Zhang B-W, Zeng W, Ma M-L, Wang K-X, Yuan B-J, Xu D-Q, Geng J-X, Fan C-Y, Gao Z-K, Arshad M, Gao S, Zhao L, Liu S-L and Mu X-Q (2024) Suppressed oncogenic molecules involved in the treatment of colorectal cancer by fecal microbiota transplantation.
Front. Microbiol. 15:1451303.
doi: 10.3389/fmicb.2024.1451303

COPYRIGHT
© 2024 Han, Zhang, Zeng, Ma, Wang, Yuan, Xu, Geng, Fan, Gao, Arshad, Gao, Zhao, Liu and Mu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Suppressed oncogenic molecules involved in the treatment of colorectal cancer by fecal microbiota transplantation

Xing Han[1,2,3], Bo-Wen Zhang[1,2,3], Wei Zeng[1], Meng-Lin Ma[1], Ke-Xin Wang[1], Bao-Juan Yuan[1,2,3], Dan-Qi Xu[1,2,3], Jia-Xin Geng[1,2,3], Chao-Yuan Fan[1,2,3], Zhan-Kui Gao[1,2,3], Muhammad Arshad[1,2,3], Shan Gao[4], Liangliang Zhao[5], Shu-Lin Liu[1,2,3,6,7]* and Xiao-Qin Mu[1,2,3,7]*

[1]Genomics Research Center (Key Laboratory of Gut Microbiota and Pharmacogenomics of Heilongjiang Province), College of Pharmacy, Harbin Medical University, Harbin, China, [2]National Key Laboratory of Frigid Zone Cardiovascular Diseases, Harbin Medical University, Harbin, China, [3]HMU-UCCSM Centre for Infection and Genomics, Harbin Medical University, Harbin, China, [4]Pathology Department, The Second Affiliated Hospital of Harbin Medical University, Harbin, China, [5]Department of Colorectal Surgery, First Affiliated Hospital of Harbin Medical University, Harbin, China, [6]Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, AB, Canada, [7]Translational Medicine Research and Cooperation Center of Northern China, Heilongjiang Academy of Medical Sciences, Harbin, China

Dysbiosis of the intestinal microbiota is prevalent among patients with colorectal cancer (CRC). This study aims to explore the anticancer roles of the fecal microbiota in inhibiting the progression of colorectal cancer and possible mechanisms. The intestinal microbial dysbiosis in CRC mice was significantly ameliorated by fecal microbiota transplantation (FMT), as indicated by the restored ACE index and Shannon index. The diameter and number of cancerous foci were significantly decreased in CRC mice treated with FMT, along with the restoration of the intestinal mucosal structure and the lessening of the gland arrangement disorder. Key factors in oxidative stress (TXN1, TXNRD1, and HIF-1α); cell cycle regulators (IGF-1, BIRC5, CDK8, HDAC2, EGFR, and CTSL); and a critical transcription factor of the innate immune signal pathway (IRF5) were among the repressed oncogenic targets engaged in the FMT treatment of CRC. Correlation analysis revealed that their expressions were positively correlated with uncultured_bacterium_o_Mollicutes_RF39, Rikenellaceae_RC9_gut_group, and negatively correlated with *Bacillus*, *Marvinbryantia*, *Roseburia*, *Angelakisella*, *Enterorhabdus*, *Bacteroides*, *Muribaculum*, and genera of uncultured_bacterium_f_Eggerthellaceae, uncultured_bacterium_f_Xanthobacteraceae, Prevotellaceae_UCG-001, uncultured_bacterium_f_Erysipelotrichaceae, uncultured_bacterium_f_Lachnospiraceae, uncultured_bacterium_f_Ruminococcaceae, Eubacterium_coprostanoligenes_group, Ruminococcaceae_UCG-005, and uncultured_bacterium_f_Peptococcaceae. This study provides more evidence for the application of FMT in the clinical treatment of CRC.

KEYWORDS

colorectal cancer, intestinal microbiota, intestinal homeostasis, fecal microbiota transplantation, oncogenic molecules

1 Introduction

The incidence and mortality of colorectal cancer (CRC) are 9.3 and 9.6%, respectively, in 2022, according to data on the global cancer burden provided by the International Agency for Research on Cancer. Conventional cancer treatments include radiation, chemotherapy, and surgery (Ossibi et al., 2022). However, 66% of CRC patients who have undergone radical surgical resection have developed recurrent and metastatic carcinoma (Plazas et al., 2015). Patients' survival rates can be greatly increased by adjuvant chemotherapy, which includes fluorouracil, oxaliplatin, capecitabine, irinotecan, and panitumumab. On the other hand, the adverse effects—which might include heart toxicity, gastrointestinal upset, and suppression of the bone marrow—can be extremely painful (Chen et al., 2021; Gobran et al., 2020; Huang et al., 2016; Lam and Lee, 2012). Investigating a secure and efficient CRC treatment strategy is therefore essential.

The human gut is home to over a thousand different types of bacteria that coexist in a dynamic balance and are an essential component of the physiological ecosystem (Jiang et al., 2020). Intestinal microbiota is responsible for preserving the homeostasis of the internal milieu, fostering immune system maturation, preventing the incursion of harmful microbes, and providing the host with biochemical metabolic pathways and enzymes absent in humans (Jiang et al., 2020; Monreal et al., 2005). However, it is evident that nutrition, exercise, and stress all have an impact on the diversity and abundance of the intestinal microbiota (Gubert et al., 2020). Once the balance of the gut microbiota is disturbed, inflammation is induced and carcinogenic pathways are activated, which leads to a disordered intestinal microecology and the development of colorectal cancer (Tilg et al., 2018). Therefore, maintaining the balance of the intestinal microbiota might be an effective strategy for slowing the progression of CRC.

Fecal microbiota transplantation (FMT) offers significant potential for treating CRC by reversing intestinal microbial dysbiosis (Kaźmierczak-Siedlecka et al., 2020). FMT provides *Lactobacillus rhamnosus* and *Lactobacillus plantarum*, which stimulate mucin production and enhance intestinal barrier function (Fong et al., 2020). Short-chain fatty acids (SCFAs) are important microbial metabolites in the gut and are mainly composed of acetate, propionate, and butyrate. FMT regulates the production of SCFAs by inhibiting NF-κB reporter activity and thus alleviating CRC (Zhang et al., 2020). FMT increases the abundance of *Bacteroidetes thetaiotaomicron*, which subsequently induces immune responses in dendritic cells and mediates gut homeostasis (Huang et al., 2022).

Many studies have demonstrated the importance of FMT in restoring the balance of the gut microbiota, but little is known on its anticancer properties. In this study, we screened pathways that were reported to be associated with carcinogenesis, identified 10 potential targets for FMT and predicted the gut bacteria associated with each target. This study provides support to the application of FMT in CRC treatment.

2 Materials and methods

2.1 The CRC mouse model

Male Balb/c mice (8-week-old) were purchased from Beijing Vital River Lab. All animals had free access to food and water while living in ventilated cages with 12-h light/dark cycles. The CRC mouse model was established by using the carcinogen azoxymethane (AOM, Sigma) and the proinflammatory agent dextran sulfate sodium (DSS, MP Biomedicals) as previously described (Yu et al., 2023). To put it briefly, AOM (10 mg/kg) was injected intraperitoneally into the mice during the first week of model establishment. The mice were allowed to drink water containing 2.5% DSS during the second week. The mice were provided unrestricted access to sterile water without DSS throughout the third and fourth weeks. Two more cycles of the treatments from week 2 to week 4 were conducted. Following the establishment of the CRC model, mice were randomized into two experimental groups (AOM/DSS and AOM/DSS + FMT), along with one control group without treatment with AOM/DSS and FMT. Each group of mice had five cages with four mice per cage, all of which were raised at random. The experiments were approved by the Ethics Committee of Harbin Medical University (HMUIRB20180013).

2.2 Fecal microbiota transplantation

Fresh faeces from mice in the normal control group were collected, mixed with PBS (200 mg/mL), and centrifuged at 1,500 rpm for 1 min to obtain the microbial supernatant. Each recipient mouse in the FMT-treated group was given 1 mL of fecal slurry by enema twice a week beginning in the second week of model establishment and continuing until the end of the study.

2.3 16S rDNA amplicon sequencing

Two tubes of feces were collected in each cage, and a total of 10 tubes were collected for each experimental group. The total genomic DNA was isolated from feces by using TIANamp Stool DNA Kit (Tiangen). 16S rDNA sequencing of the fecal microbiota was conducted by Beijing Biomarker Biotechnologies. The V3 and V4 regions of the 16S rDNA were amplified with the specific primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and sequenced on the Illumina HiSeq 2500. Sequencing data for six replicates was obtained in each experimental group and submitted to NCBI (PRJNA 949363). Taxonomy was assigned to the OTUs using the SILVA database (v.123) with the RDP classifier at a 70% confidence threshold. The alpha diversity indicated by the ACE index and Shannon index was calculated using QIIME2 software (Version 2020.8). Beta diversity analysis was performed to investigate the structural variation in microbial communities across samples using non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) by QIIME-1.8.0, and visualized by R 3.1.1. Statistical significance was determined by one-way analysis of variance. Spearman rank correlation analysis was employed to identify data with correlation coefficient larger than 0.1 and p-value smaller than 0.05, which were used to construct the network.

2.4 HE staining

After being extracted, colorectal tissue was fixed with polyformaldehyde and embedded in paraffin blocks. Sections of 3 mm

thickness were cut for staining with hematoxylin for 2 min and with eosin for 1 min. The slices were finally sealed with neutral resin and then imaged.

2.5 Immunohistochemistry

After dewaxing in xylene and hydrating in gradient ethanol, the paraffin slices were treated with 3% H_2O_2 for 10 min to block endogenous peroxidase. Antigen retrieval was conducted in a sodium citrate solution at high power for 2 min and then at low power for 10 min in the microwave, respectively. Tissues were then blocked at room temperature for 1 h using 50% goat serum. Slices were incubated at 4°C overnight with primary antibodies for ki67 (1:200, 12202, Cell Signaling Technology), cleaved caspase-3 (1:1000, 9661, Cell Signaling Technology), IGF-1 (1:100, A12305, Abclonal), BIRC5 (1:100, GB11177, Servicebio), CDK8 (1:100, A9654, Abclonal), HDAC2 (1:100, A2084, Abclonal), EGFR (1:100, GB111504, Servicebio), TXN1 (1:100, GB11993, Servicebio), TXNRD1 (1:100, A16631, Abclonal), HIF1-α (1:100, A16873, Abclonal), IRF5 (1:100, A16388, Abclonal), or CTSL (1:100, A12200, Abclonal). After washing with PBS, the tissues were incubated with HRP-labeled secondary antibody (1:200, AS014, Abclonal) at 37°C for 1 h. The staining was visualized with a DAB peroxidase substrate kit (Origene). The slices were finally dehydrated in ethanol and xylene and sealed with resin. The tissues were photographed using Olympus microscope and the yellow-brown area indicates a positive expression.

2.6 PCR array

The total RNA of the entire colorectal tissue was extracted by using the rneasy plus mini kit (Qiagen) and then converted to cDNA by using the RT2 first strand kit (Qiagen). RT2 profiler PCR array (PAMM-507ZA, Qiagen) was employed to detect the mouse cancer drug targets, including *Abcc1*, *Akt1*, *Akt2*, *Atf2*, *Aurka*, *Aurkb*, *Aurkc*, *Bc12*, *Birc5*, *Cdc25a*, *Cdk1*, *Cdk2*, *Cdk4*, *Cdk5*, *Cdk7*, *Cdk8*, *Cdk9*, *Ctsb*, *Ctsd*, *Ctsl*, *Ctss*, *Egfr*, *Erbb2*, *Erbb3*, *Erbb4*, *Esr1*, *Esr2*, *Figf*, *Flt1*, *Flt4*, *Grb2*, *Gstp1*, *Hdac1*, *Hdac11*, *Hdac2*, *Hdac3*, *Hdac4*, *Hdac6*, *Hdac7*, *Hdac8*, *Hif1a*, *Hras*, *Hsp90aa1*, *Hsp90b1*, *Igf1*, *Igf1r*, *Igf2*, *Irf5*, *Kdr*, *Kit*, *Kras*, *Mdm2*, *Mdm4*, *Mtor*, *Nfkb1*, *Nras*, *Ntn3*, *Parp1*, *Parp2*, *Parp4*, *Pdgfra*, *Pdgfrb*, *Pgr*, *Piksc2a*, *Pik3c3*, *Pik3ca*, *Plk1*, *Plk2*, *Plk3*, *Plk4*, *Prkca*, *Prkcb*, *Prkcd*, *Prkce*, *Ptgs2*, *Rhoa*, *Rhob*, *Tert*, *Tnks*, *Top2a*, *Top2b*, *Trp53*, *Txn1*, *Txnrd1*.

2.7 Western blot

To get the protein-containing supernatant, the entire colorectal tissue was homogenized in RIPA lysis buffer supplemented with protease inhibitor PMSF. Tissue proteins were separated through SDS-PAGE electrophoresis and transferred onto a PVDF membrane, followed by blocking with fast blocking buffer (Biosharp) for 10 min, and then incubated overnight at 4°C with primary antibodies: IGF-1 (1:500, A12305, Abclonal), BIRC5 (1:500, GB11177, Servicebio), CDK8 (1:500, A9654, Abclonal), HDAC2 (1:500, A2084, Abclonal), EGFR (1:500, GB111504, Servicebio), TXN1 (GB11993, Servicebio), TXNRD1 (A16631, Abclonal), HIF1-α (1:500, A16873, Abclonal), IRF5 (1:500, A16388, Abclonal), CTSL (1:500, A12200, Abclonal), GAPDH (1:2000, A19056, Abclonal) or β-Tubulin (1:2000, AC008, Abclonal). After washing with PBS, membranes were incubated at room temperature for 2 h with HRP goat anti-rabbit IgG (H + L) (1:5000, AS014, Abclonal). Images were acquired by using Tanon-5200 and Tanon MP.

2.8 Statistical analysis

Statistical analysis was performed by Tukey's test, and the statistical comparison was performed by GraphPad Prism 8.0. All data were expressed as mean ± SD. A two-tailed value of $p < 0.05$ was taken to indicate statistical significance. All experiments were repeated independently at least three times.

3 Results

3.1 FMT reduces the burden of CRC

Following a 10-week period of establishment of the CRC mouse model, a significant number of malignant foci were identified in the intestines of CRC mice, with an average of 11 and an average diameter of 3.8 mm. Additionally, the expression of ki67 was raised in these foci when compared to the normal control animals. The average number of foci in CRC mice treated with FMT dropped to 4, and their average diameter was 1.9 mm. Increased expression of cleaved caspase-3 indicated that cancer cells were more likely to undergo apoptosis, which was substantially responsible for the reduction of cancer foci (Figures 1A–D,G). Morphologically, the mucosal layer's structure was damaged in CRC mice, along with the goblet and crypt cells' absence, a significant infiltration of inflammatory cells, and an abnormal glandular tube. In mice treated with FMT, the lesions were greatly ameliorated, with less destruction of crypt and goblet cells and the glands being regularly arranged (Figure 1F). Body weight indirectly reflects intestinal functions. Compared with mice in the normal control group, at the sixth week of inducing the CRC model, CRC mice began to show significant body weight reduction, while the trend of weight loss was significantly inhibited by FMT (Figure 1E).

3.2 FMT reverses intestinal microbial dysbiosis in CRC mice

We used 16S rDNA high-throughput sequencing to detect the diversity of gut microbiota in order to determine the differences in gut microbiota between CRC mice and normal control mice, as well as whether fecal microbiota transplantation may correct these differences. The 16S rDNA of the intestinal microbiota was sequenced using Illumina HiSeq 2500. Following quality control and assembly, a total of 1,777,674 clean reads were collected from 18 samples. The typical length ranges from 415 bp to 422 bp, making it possible to encompass the 16S rDNA's V3 and V4 sections. The clean reads number for each sample and rarefaction curves were shown in (Supplementary Table 1 and Supplementary Figures 1A,B). OTU clustering was done with a 97% similar sequence. A total of 17 phyla,

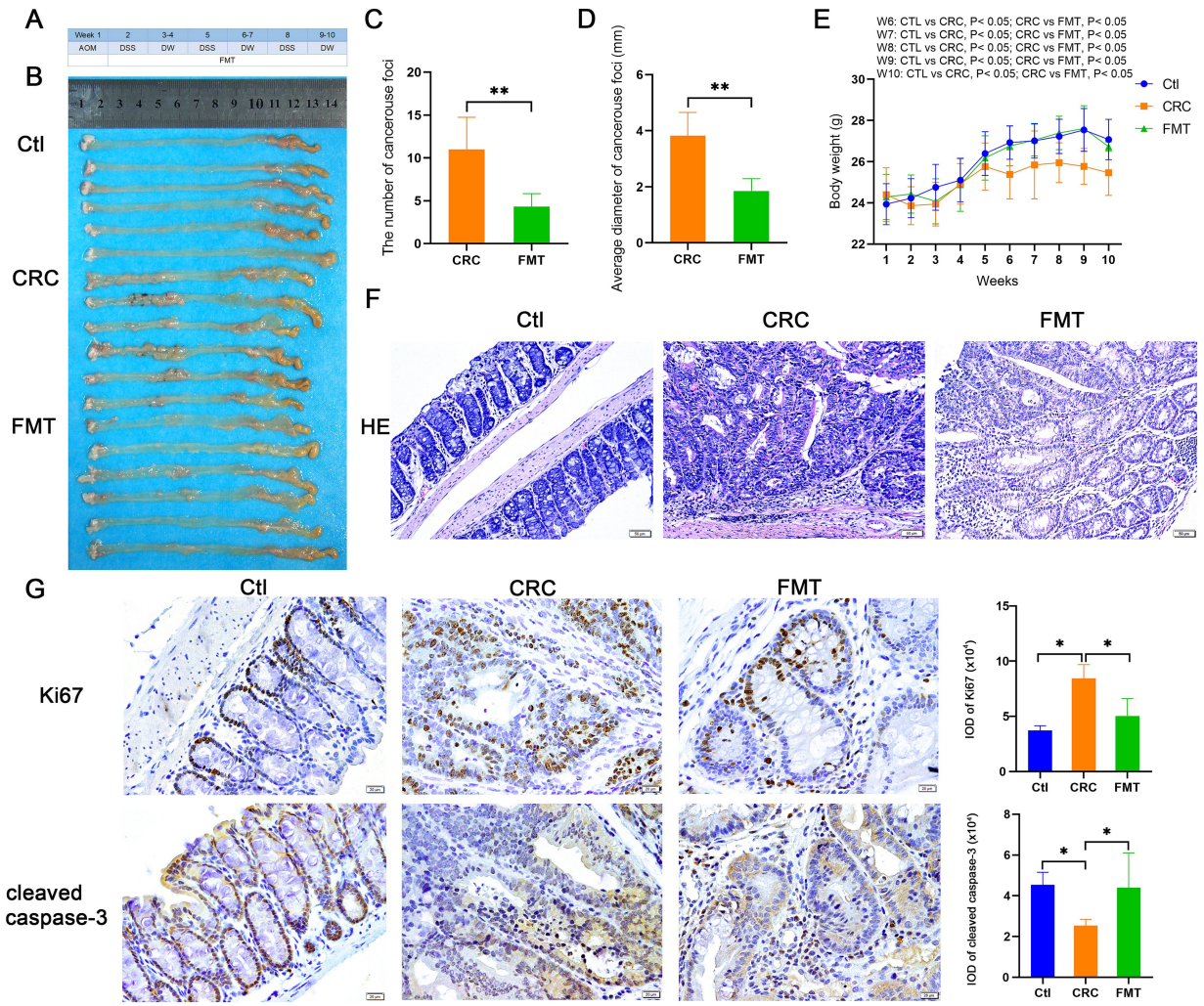


FIGURE 1
Inhibitory effect of FMT on CRC progression. **(A)** Design of the experiment. **(B)** Pictures of intestinal tissue. Ctl, normal control mice; CRC, colorectal cancer mice; FMT, colorectal cancer mice receiving fecal microbiota transplantation. **(C,D)** The average number and diameter of cancerous foci. $^{**}p < 0.01$ (Ctl, $n = 6$; CRC, $n = 6$; FMT, $n = 6$). **(E)** The average body weight of mice in each week. $^{*}p < 0.05$ (Ctl, $n = 19$; CRC, $n = 15$; FMT, $n = 18$). **(F)** HE staining of intestinal tissue. **(G)** Immuno-histochemical staining of Ki67 and cleaved caspase-3 in intestinal tissue. $^{*}p < 0.05$, $n = 6$.

29 classes, 57 orders, 94 families, 183 genera, and 199 species were identified among the intestinal bacteria.

The relative abundance of intestinal bacteria in CRC mice was significantly different at the phylum, family, genus and species levels compared to the normal control mice (Figures 2A–D). We first examined the predominant microbes of each group at the genus level. Intestinal bacteria enriched in normal control mice were genera of uncultured_bacterium_f_Muribaculaceae (28.21%), *Alloprevotella* (12.68%), *Alistipes* (9.81%), uncultured_bacterium_f_Lachnospiraceae (8.69%), *Bacteroides* (5.60%), Prevotellaceae_UCG-001 (4.68%), Ruminococcaceae_UCG-014 (4.10%), Lachnospiraceae_NK4A136_group (2.79%), uncultured_bacterium_f_Ruminococcaceae (2.43%), *Muribaculum* (1.09%), and *Enterorhabdus* (1.06%). Some of these microbes, such as *Alloprevotella* (8.31%), *Alistipes* (4.92%), *Bacteroides* (3.81%), Prevotellaceae_UCG-001 (2.18%), uncultured_bacterium_f_Ruminococcaceae (1.97%), *Muribaculum* (0.62%), and *Enterorhabdus* (0.69%) showed a notable decline in percentage in CRC mice. Furthermore, as shown in Figure 2C, the intestinal bacteria Rikenellaceae_RC9_gut_group (1.70%), *Blautia* (1.28%), uncultured_bacterium_o_Mollicutes_RF39 (0.85%), and *Intestinimonas* (0.80%) were significantly increased in CRC mice. The FMT therapy significantly reversed the abundance of *Bacteroides* (4.54%), Prevotellaceae_UCG-001 (3.93%), uncultured_bacterium_f_Ruminococcaceae (2.56%), *Enterorhabdus* (1.03%), and uncultured_bacterium_f_Erysipelotrichaceae (0.83%). Certain intestinal bacteria were completely identified at the species level as follows with the percentage of normal controls mice and CRC mice: the increased species of *Lactobacillus_gasseri* (0.10 to 2.19%), *Lachnospiraceae_bacterium_609* (0.48 to 1.16%), *Clostridiales_bacterium_CIEAF_020* (0.008 and 0.014%), *Phaseolus_acutifolius_tepary_bean* (0.009 and 0.013%), and the decreased species of *Bacteroides_acidifaciens* (5.30 to 2.13%), *Lachnospiraceae_bacterium_10-1* (0.26 to 0.21%), *Mucispirillum_schaedleri_ASF457* (0.08 to 0.04%), *Clostridium_sp* (0.03 to 0.01%), *Burkholderiales_bacterium_YL45* (0.02 to 0.01%), and *Lachnospiraceae_bacterium_COE1* (0.08 to 0.01%). The use of FMT reversed most of the alterations, such as *Lactobacillus_gasseri* (0.31%),

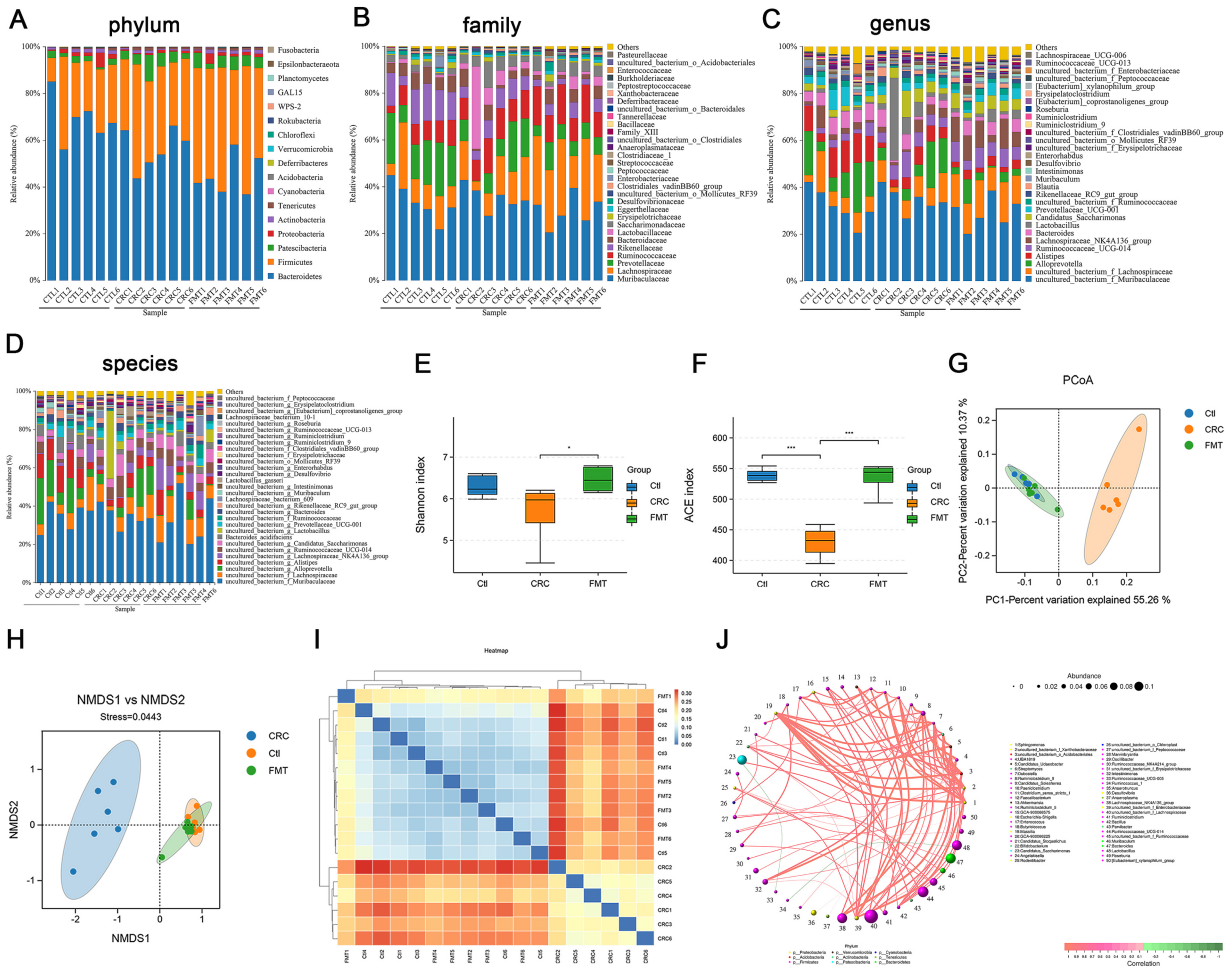


FIGURE 2
FMT reverses the microbial disturbance in CRC mice. **(A–D)** The distribution of gut microbiota at the levels of phylum, family, genus and species. **(E,F)** Alpha diversity indicated by Shannon index **(E)** and ACE index **(F)**, $*p < 0.05$ and $***p < 0.001$, $n = 6$. **(G,H)** Beta diversity indicated by PCoA analysis **(G)** and NMDS analysis **(H)**. **(I)** Cluster analysis of samples. **(J)** Bacterial co-occurrence network.

Bacteroides_acidifaciens (3.34%), *Mucispirillum_schaedleri_ASF457* (0.05%), *Clostridium_sp* (0.04%), *Lachnospiraceae_bacterium_COE1* (0.12%).

In addition to examining the dominant microbiota, we assessed the alpha diversity of gut microbiota, including the Shannon index and ACE index, to confirm the improvement of the entire gut microbiota in CRC mice following FMT. In CRC mice, there was a considerable decrease in the Shannon index and ACE index, which indicate the richness and diversity of the microbial population. However, the declining trend was reversed by FMT (Figures 2E,F). To confirm if FMT could effectively lessen the difference in gut microbiota diversity between groups and reverse the disorder of the gut microbiota, we conducted β diversity analysis using non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA). The analysis of PCoA and NMDS revealed that the microbial structure of normal control mice and FMT-treated mice was similar but considerably different from that of the CRC mice (Figures 2G,H). Furthermore, sample clustering analysis of unweighted unifrac demonstrated that the phylogenetic relationship of the intestinal bacteria between mice in the normal control and FMT groups was more closely linked compared to the CRC mice (Figure 2I). These results indicated that FMT considerably reduced the dysbiosis of the gut microbiota, shaping the gut microbiota of CRC mice to resemble that of normal controls. Additionally, through complex network relationships, intestinal bacteria supported or inhibited one another, working together to preserve the homeostasis of intestinal microbiota (Figure 2J).

3.3 The suppressed oncogenic targets in the treatment of CRC by FMT

First, to find possible targets for FMT to halt the progression of CRC, a genetic screening was done first. Results showed that 17 genes significantly differed in expression between the FMT-treated and non-treated groups, including genes involved in oxidative stress *Ptgs2*, *Txn1*, *Txnrd1*, drug resistance *Abcc1*, transcription factors *Atf2*, *Hif1a*, *Irf5*, apoptosis gene *Birc5*, gene in the cell cycle *Cdk8*, cathepsins *Ctsl*, growth factors and receptors *Egfr*, *Figf*, *Flt4*, *Igf1*, histone deacetylases *Hdac2*, poly ADP-ribose polymerases *Parp4*, protein kinases *Plk2* (Figure 3A).

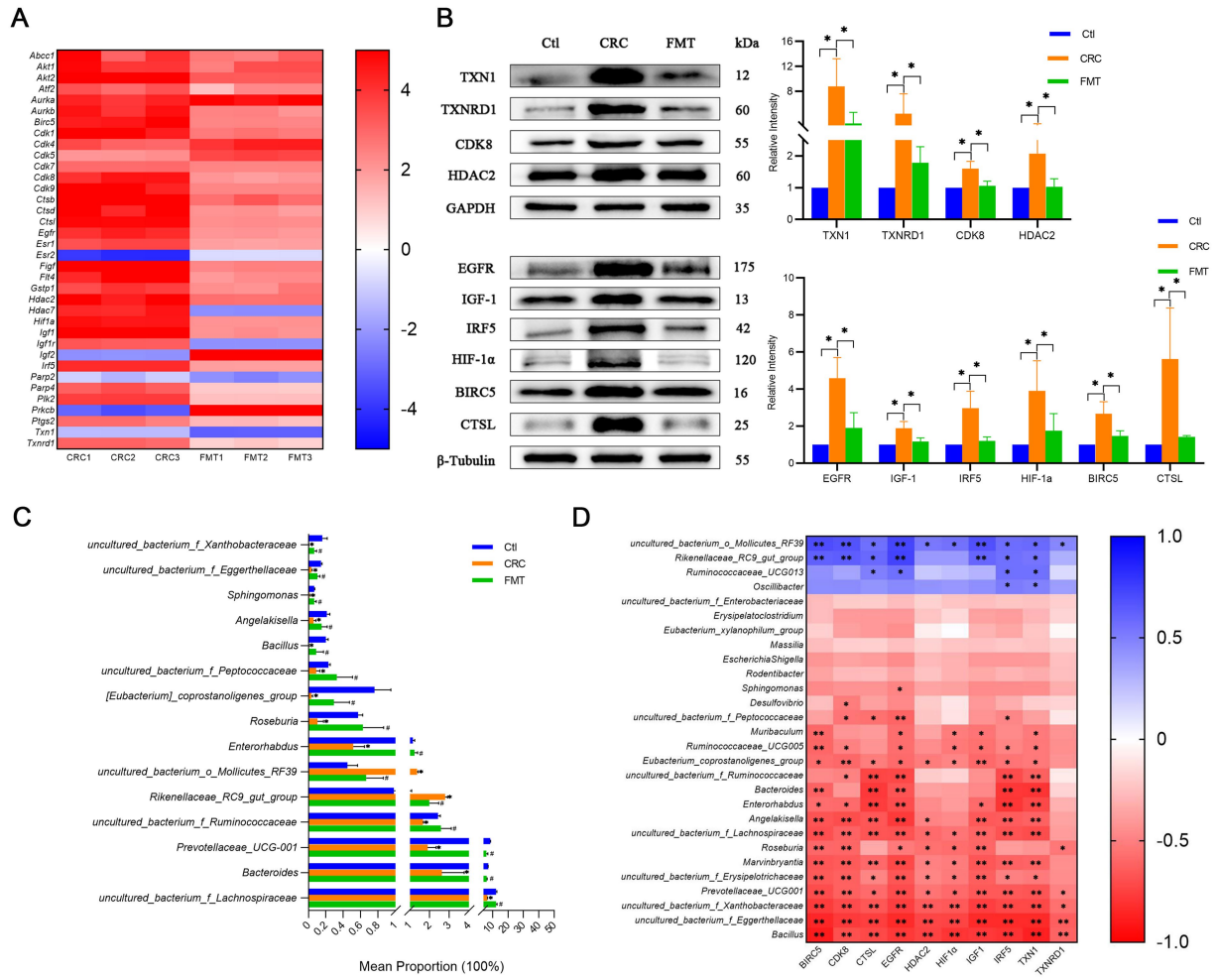


FIGURE 3
FMT inhibits the expression levels of cancer-promoting molecules. **(A)** Differentially expressed genes associated with CRC development. **(B)** Protein levels of the cancer-promoting molecules detected by western blot. *$p < 0.05$, $n = 3$. **(C)** The abundance of intestinal microbes related to the cancer-promoting factors. *$p < 0.05$, Ctl vs. CRC; #$p < 0.05$, CRC vs. FMT, $n = 3$. **(D)** Correlation analysis between the intestinal bacteria and the molecules screened out. *$p < 0.05$ and **$p < 0.01$, $n = 3$.

At the protein level, the expression of these genes was further investigated. The thioredoxin (TXN)/thioredoxin reductase (TXNRD) system is one of the major redox control mechanisms (Holmgren and Lu, 2010). Cancer cells that express high levels of TXN1 and TXNRD1 are better able to withstand the effects of oxidative stress, proliferate, and even invade and metastasize (Zhang et al., 2017). In comparison to normal controls mice, there was a notable increase in TXN1 and TXNRD1 expression in CRC mice. Their protein expression was substantially lower in mice administered with FMT, indicating that alterations in the gut microbiota affected their expression as well as their carcinogenic characteristics (Figure 3B).

The activation of the epidermal growth factor receptor (EGFR) signaling pathway promotes the secretion of insulin-like growth factor-1 (IGF-1), which in turn induces tumor-associated macrophage polarization. Tumor-associated macrophages have been shown to accelerate the development of CRC (Zhang W. et al., 2016). An inflammatory macrophage phenotype has been demonstrated to be promoted by interferon regulatory factor 5 (IRF5), a potential intrinsic regulator of the intestinal macrophage hallmark (Corbin et al., 2020). Moreover, the IRF5-induced inflammatory response may enhance the production of hypoxia-inducible factor 1-α (HIF-1α) in dendritic cells (Hammami et al., 2015). HIF-1α is a pivotal modulator in metabolic reprogramming, which occurs in hypoxic cancer cells (Infantino et al., 2021). We then validated the protein level in CRC mice and found obvious elevations of IRF5, EGFR, IGF-1, and HIF-1α in colon tissues compared with normal mice. FMT treatment dramatically lowered their levels of protein expression (Figure 3B).

Baculoviral inhibitor of apoptosis repeat containing 5 (BIRC5) is closely linked with high-grade cancer and differentiation (Wang et al., 2022). Cyclin-dependent kinase 8 (CDK8) has been identified as an oncogene that is associated with a reduced survival rate in colorectal cancers (Broude et al., 2015). Cathepsin L (CTSL) encodes a lysosomal cysteine proteinase that regulates cancer progression. Cancer patients with up-regulated CTSL have a worse prognosis and are more likely to have aggressive metastases (Zhang L. et al., 2022). In this study, CRC mice showed considerably higher amounts of BIRC5, CDK8, and CTSL protein than normal control mice did. FMT intervention significantly reduced their elevated protein expression. This suggests that the gut

microbiota influences the expression of these proteins, which in turn affects the differentiation, metastasis, and prognosis of CRC (Figure 3B).

Histone deacetylase 2 (HDAC2) has been linked to numerous biological processes, including inflammation, cancer initiation and progression, cell signaling, cellular proliferation, and gene expression control (Jo et al., 2023). In comparison to normal controls mice, HDAC2 expression was obviously up-regulated in CRC mice, as Figure 3B illustrates, and this raised trend was dramatically suppressed by using FMT.

The abundance of gut bacteria linked to these cancer-promoting targets showed significant shifts (Figure 3C). The results of the correlation analysis demonstrated a positive correlation between the expression of BIRC5, CDK8, CTSL, EGFR, HDAC2, HIF-1α, IGF1, IRF5, TXN1, and TXNRD1 and uncultured_bacterium_o_Mollicutes_RF39, Rikenellaceae_RC9_gut_group, and a negative correlation with *Bacillus*, *Marvinbryantia*, *Roseburia*, *Angelakisella*, *Enterorhabdus*, *Bacteroides*, *Muribaculum* and the genera of uncultured_bacterium_f_Eggerthellaceae, uncultured_bacterium_f_Xanthobacteraceae, Prevotellaceae_UCG-001, uncultured_bacterium_f_Erysipelotrichaceae, uncultured_bacterium_f_Lachnospiraceae, uncultured_bacterium_f_Ruminococcaceae, Eubacterium_coprostanoligenes_group, Ruminococcaceae_UCG-005, and uncultured_bacterium_f_Peptococcaceae (Figure 3D).

3.4 Localization of oncogenic targets in intestinal tissues

Structurally, the intestinal tissue is mainly composed of the mucosa, submucosa, muscularis propria, and serosal layer. Perhaps as a result of the mucosa's direct exposure to the intricate interior environment of the intestine, the majority of CRC cases arise in this area. This cancer-prone site is further subdivided into the mucosal epithelium, lamina propria, and muscularis mucosa.

The mucosal epithelium consists of epithelial cells that secrete mucus to maintain the mucosal surface moist and shield the intestinal tissue from injury, irritation, and infection of the intestinal environment while also absorbing nutrients and water. Numerous blood and lymphatic capillaries that supply nutrients, oxygen, and aid in the removal of metabolic waste are abundant in the lamina propria. Mucosal immune protection is also supported by the lymphoid tissue and immune cells that are dispersed here. Muscularis mucosae is composed of smooth muscles, the contraction and relaxation of which contribute to the digestion and propulsion of food and regulate the volume and pressure of the mucosa. In this study, immunohistochemical staining revealed that BIRC5, TXN1, TXNRD1, CDK8, HIF-1α, IGF-1, HDAC2, CTSL, IRF5, and EGFR were not only strongly expressed within the mucosal epithelium and lamina propria but also present in a small proportion of cells within the muscularis mucosae (Figure 4). In comparison to normal control mice, the intestinal tissue of CRC animals showed disruptions in immune defense, barrier, and nutritional and metabolic pathways due to the overexpression of these molecules in the mucosa. After restoring the balance of the gut microbiota by FMT, the overexpression of these targets was significantly inhibited. Through regulating the expression of these oncogenic chemicals, the gut microbiota plays an important role in the regulation of intestinal tissue function. Sustaining the equilibrium of the gut microbiota contributes to the preservation of the typical functioning of intestinal tissue.

4 Discussion

In a previous work, we discovered that by disrupting the intestinal immune microenvironment, the gut microbiota significantly contributes to the regulation of CRC progression. The disruption of the intestinal microbiota in CRC mice was corrected by FMT intervention, and immune cells and inflammatory cytokines were also regulated in the intestinal tissue (Yu et al., 2023). Nevertheless, this investigation revealed that changes in the diversity and abundance of gut bacteria also impacted the expression of oncogenic molecules at both gene and protein levels, such as TXN1, TXNRD1, IRF5, EGFR, IGF-1, HIF-1α, BIRC5, CDK8, CTSL, and HDAC2. This suggests that the intestinal microbiota influences CRC development in numerous aspects and holds significant promise for its therapeutic management.

In this study, the fecal microbiota transplanted from healthy mice effectively inhibited microbial dysbiosis in CRC mice. The FMT therapy of CRC mice resulted in a considerable increase in the richness and variety of the microbial population. Principal coordinate analysis and phylogenetic analysis of the microbial sequencing data revealed that FMT intervention caused the CRC mice's gut microbiota structure to resemble that of the normal control group.

The short-chain fatty acid butyrate, which is mostly produced by *Eubacterium*, *Roseburia*, *Lachnospiraceae*, and *Ruminococcaceae*, inhibits the growth and proliferation of cancer cells by decreasing the activity of HDACs in immune cells and colon cells and causing the expression of CDK inhibitors, which leads to cell cycle arrest in the G1 phase (Gu et al., 2023; Li and Seto, 2016; Louis et al., 2014). According to this study, FMT dramatically raised the low abundance of *Eubacterium* and *Ruminococcaceae* in CRC mice, which may encourage butyrate synthesis and in turn inhibit the activation and expression of HDAC2 and CDK8. Propionate, produced by bacteria from *Roseburia*, *Bacteroides*, and *Lachnospiraceae*, inhibits cancer progression by inducing apoptosis and cell cycle arrest through down-regulating BIRC5 expression (Louis et al., 2014; Kim et al., 2019; Bilotta and Cong, 2019). The study's findings demonstrated that FMT restored the bacteria's abundance in CRC mice to levels comparable to those in control mice, perhaps assisting in the downregulation of BIRC5 expression in cancer treatment-related scenarios.

Hypoxic conditions are a common characteristic of the cancer microenvironment. In these circumstances, the metabolism of cancer cells is reprogrammed, thus promoting the survival and proliferation of cancer cells and ensuring cancer progression (Infantino et al., 2021). HIF-1a is a member of the hypoxia-inducible factors family that is triggered by the gut bacteria *Yersinia enterocolitica*. It inhibits mitochondrial oxidative metabolism by lowering oxygen consumption, ensuring oxygen homeostasis in cancer tissues under hypoxia (Gardlik, 2014). TXNRD, a component of the antioxidant defense system involved in regulating the redox balance and sensing the oxidative stress state, is activated by LPS and regulated by Keap1-Nrf2/ho-1, the main regulator of various antioxidant enzymes (Li et al., 2021; Yu et al., 2021). Its primary substrate, TXN1, was

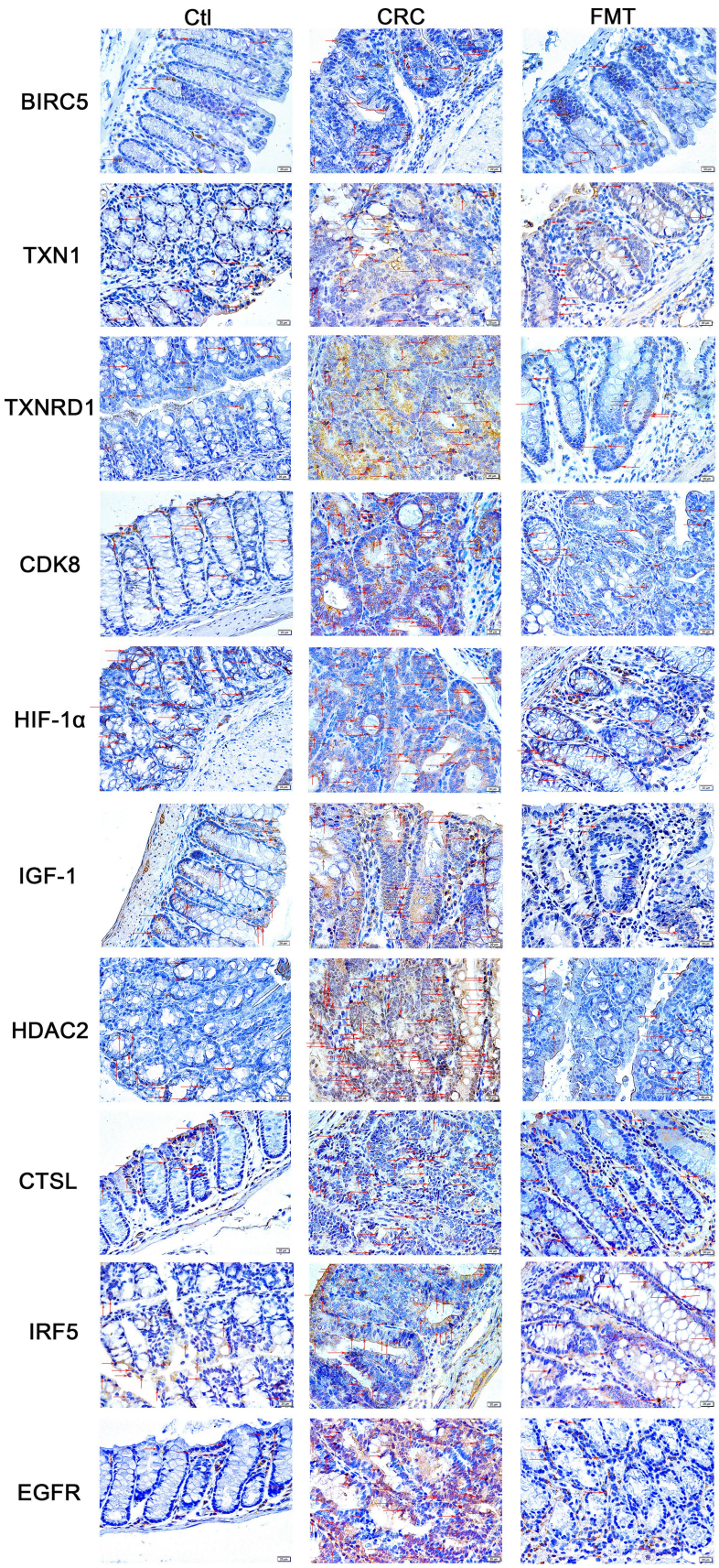


FIGURE 4
Locations of the oncogenic targets in the intestinal tissue. BIRC5, TXN1, TXNRD1, CDK8, HIF-1α, IGF-1, HDAC2, CTSL, IRF5, and EGFR were distributed in all sub-structures of the mucosa, including the mucosal epithelium, amina propria, and muscularis mucosae.

likewise diminished in cells devoid of TXNRD1 (Arnér, 2021). Therefore, lowering the number of bacteria that produce LPS with FMT may aid in blocking the actions of TXNRD1 and TXN1, encourage oxidative stress-induced damage to cancer cells, and thereby stop the onset and progression of colorectal cancer.

Chronic inflammation is a major risk factor for CRC (Lucas et al., 2017). Numerous pro-inflammatory cytokines have been reported to be positively associated with the incidence of colorectal adenomas (Kim et al., 2008). Inflammatory cells have the ability to activate a significant amount of lysosomal protease CTSL, which may facilitate invasion and metastasis and result in a poor prognosis (Gomes et al., 2020). The expression of HDAC2 in macrophages induced by LPS has a positive effect on inducing proinflammatory cytokine expression (Wu et al., 2018). Inflammation in the intestinal tract increases EGFR expression (Hardbower et al., 2017). The activation of the EGFR signaling pathway enhances IGF-1 secretion and directly drives M2 macrophage polarization, resulting in an immunosuppressor effect and promoting the development of cancer (Yang et al., 2021). One of the key players in inflammation among the IRF family members is IRF5. IRF5 is temporarily expressed by inflammatory stimuli in macrophages, and IRF5 overexpression causes proinflammatory cytokines like IL-6, IL-12, IL-23, and TNF-α to be produced (Krausgruber et al., 2011). This increases inflammation, accelerates the development of CRC, and interferes with regular bowel movements. On the other hand, guanosine produced by *Roseburia* and *Enterorhabdus* has an anti-inflammatory impact by lowering the expression of TNF-α, IL-1β, and IL-6 (Zhang H. et al., 2022; Zizzo et al., 2018). Additionally, uncultured bacteria f_Peptococcaceae metabolize tryptophan to produce indole acrylic acid, a sign of an anti-inflammatory immune response (Wlodarska et al., 2017). *Bacillus subtilis* administration alleviates systemic inflammation in mice by balancing pro- and anti-inflammatory factors in the gut (Zhang H.-L. et al., 2016). Changes to these oncogenic molecules are associated with the process by which intestinal inflammation expedites the progression of CRC, as demonstrated by the correction of these intestinal bacterial abnormalities by FMT in our study.

Based on the previously mentioned findings and examination, applying FMT to reverse intestinal dysbiosis in CRC mice and maintain intestinal environment homeostasis resulted in a reduction in the expression of oncogenic molecules. These noteworthy anti-cancer effects offered a theoretical foundation for the treatment and prevention of CRC. The strong relationships between the expression of some carcinogenic chemicals and the quantity of various bacteria were also examined in this work; further research is needed to determine the mechanisms underlying FMT's inhibition of CRC progression.

Data availability statement

The data on intestinal microbiota 16S rDNA are available at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) with the following accession number: PRJNA 949363. The raw data supporting the conclusion of this article will be made available by the authors without undue reservation.

Ethics statement

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

Author contributions

XH: Formal analysis, Investigation, Writing – original draft. B-WZ: Formal analysis, Writing – original draft. WZ: Investigation, Writing – original draft. M-LM: Investigation, Writing – original draft. K-XW: Investigation, Writing – original draft. B-JY: Formal analysis, Writing – original draft. D-QX: Formal analysis, Writing – original draft. J-XG: Formal analysis, Writing – original draft. C-YF: Formal analysis, Writing – original draft. Z-KG: Formal analysis, Writing – original draft. MA: Formal analysis, Writing – original draft. SG: Methodology, Writing – review & editing. LZ: Funding acquisition, Investigation, Writing – review & editing. S-LL: Funding acquisition, Supervision, Writing – review & editing. X-QM: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Natural Science Foundation of China (NSFC81903631, NSFC82020108022) and the Heilongjiang Provincial Natural Science Foundation of China (LH2020H036).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1451303/full#supplementary-material

References

Arnér, E. S. J. (2021). Effects of mammalian thioredoxin reductase inhibitors. *Handb. Exp. Pharmacol.* 264, 289–309. doi: 10.1007/164_2020_393

Bilotta, A. J., and Cong, Y. (2019). Gut microbiota metabolite regulation of host defenses at mucosal surfaces: implication in precision medicine. *Precis. Clin. Med.* 2, 110–119. doi: 10.1093/pcmedi/pbz008

Broude, E. V., Győrffy, B., Chumanevich, A. A., Chen, M., Mcdermott, M. S., Shtutman, M., et al. (2015). Expression of CDK8 and CDK8-interacting genes as potential biomarkers in breast cancer. *Curr. Cancer Drug Targets* 15, 739–749. doi: 10.2174/1568009615081510011058l4

Chen, W., Ye, J., Tan, X., Zhang, Y., Liang, J., and Huang, M. (2021). Analysis of 154 T4 colorectal cancer patients with peritoneal carcinomatosis treated by surgery. *Int. J. Clin. Med.* 12, 61–70. doi: 10.4236/ijcm.2021.122008

Corbin, A. L., Gomez-Vazquez, M., Berthold, D. L., Attar, M., Arnold, I. C., Powrie, F. M., et al. (2020). IRF5 guides monocytes toward an inflammatory $CD11c^{+}$ macrophage phenotype and promotes intestinal inflammation. *Sci. Immunol.* 5:eaax6085. doi: 10.1126/sciimmunol.aax6085

Fong, W., Li, Q., and Yu, J. (2020). Gut microbiota modulation: a novel strategy for prevention and treatment of colorectal cancer. *Oncogene* 39, 4925–4943. doi: 10.1038/s41388-020-1341-1

Gardlik, R. (2014). A non-specific effect of orally administered *Escherichia coli*. *Bioeng. Bugs* 2, 288–290. doi: 10.4161/bbug.2.5.16115

Gobran, N. S., Kelany, M. R., and Fathy, M. A. (2020). Chemotherapy toxicity profile in adjuvant treated colorectal carcinoma patients. *J. Cancer Ther.* 11, 74–87. doi: 10.4236/jct.2020.112007

Gomes, C. P., Fernandes, D. E., Casimiro, F., Da Mata, G. F., Passos, M. T., Varela, P., et al. (2020). Cathepsin L in COVID-19: from pharmacological evidences to genetics. *Front. Cell. Infect. Microbiol.* 10:589505. doi: 10.3389/fcimb.2020.589505

Gu, M., Yin, W., Zhang, J., Yin, J., Tang, X., Ling, J., et al. (2023). Role of gut microbiota and bacterial metabolites in mucins of colorectal cancer. *Front. Cell. Infect. Microbiol.* 13:1119992. doi: 10.3389/fcimb.2023.1119992

Gubert, C., Kong, G., Renoir, T., and Hannan, A. J. (2020). Exercise, diet and stress as modulators of gut microbiota: implications for neurodegenerative diseases. *Neurobiol. Dis.* 134:104621. doi: 10.1016/j.nbd.2019.104621

Hammami, A., Charpentier, T., Smans, M., and Stäger, S. (2015). IRF-5-mediated inflammation limits $CD8^{+}$ T cell expansion by inducing HIF-1α and impairing dendritic cell functions during *Leishmania* infection. *PLoS Pathog.* 11:e1004938. doi: 10.1371/journal.ppat.1004938

Hardbower, D. M., Coburn, L. A., Asim, M., Singh, K., Sierra, J. C., Barry, D. P., et al. (2017). EGFR-mediated macrophage activation promotes colitis-associated tumorigenesis. *Oncogene* 36, 3807–3819. doi: 10.1038/onc.2017.23

Holmgren, A., and Lu, J. (2010). Thioredoxin and thioredoxin reductase: current research with special reference to human disease. *Biochem. Biophys. Res. Commun.* 396, 120–124. doi: 10.1016/j.bbrc.2010.03.083

Huang, R., Quan, Y., Huang, A., and Min, Z. (2016). Role of osteopontin in the carcinogenesis and metastasis of colorectal cancer. *J. Cancer Ther.* 7, 729–740. doi: 10.4236/jct.2016.710074

Huang, J., Zheng, X., Kang, W., Hao, H., Mao, Y., Zhang, H., et al. (2022). Metagenomic and metabolomic analyses reveal synergistic effects of fecal microbiota transplantation and anti-PD-1 therapy on treating colorectal cancer. *Front. Immunol.* 13:874922. doi: 10.3389/fimmu.2022.874922

Infantino, V., Santarsiero, A., Convertini, P., Todisco, S., and Iacobazzi, V. (2021). Cancer cell metabolism in hypoxia: role of HIF-1 as key regulator and therapeutic target. *Int. J. Mol. Sci.* 22:5703. doi: 10.3390/ijms22115703

Jiang, Y., Chen, X., and Fu, S. (2020). Advances in the correlation between intestinal microbiota and breast cancer development. *J. Cancer Ther.* 11, 758–771. doi: 10.4236/jct.2020.1112066

Jo, H., Shim, K., Kim, H. U., Jung, H. S., and Jeoung, D. (2023). HDAC2 as a target for developing anti-cancer drugs. *Comput. Struct. Biotechnol. J.* 21, 2048–2057. doi: 10.1016/j.csbj.2023.03.016

Kaźmierczak-Siedlecka, K., Daca, A., Fic, M., van de Wetering, T., Folwarski, M., and Makarewicz, W. (2020). Therapeutic methods of gut microbiota modification in colorectal cancer management—fecal microbiota transplantation, prebiotics, probiotics, and synbiotics. *Gut Microbes* 11, 1518–1530. doi: 10.1080/19490976.2020.1764309

Kim, S., Keku, T. O., Martin, C., Galanko, J., Woosley, J. T., Schroeder, J. C., et al. (2008). Circulating levels of inflammatory cytokines and risk of colorectal adenomas. *Cancer Res.* 68, 323–328. doi: 10.1158/0008-5472.CAN-07-2924

Kim, K., Kwon, O., Ryu, T., Jung, C. R., Kim, J., Min, J. K., et al. (2019). Propionate of a microbiota metabolite induces cell apoptosis and cell cycle arrest in lung cancer. *Mol. Med. Rep.* 20, 1569–1574. doi: 10.3892/mmr.2019.10431

Krausgruber, T., Blazek, K., Smallie, T., Alzabin, S., Lockstone, H., Sahgal, N., et al. (2011). IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat. Immunol.* 12, 231–238. doi: 10.1038/ni.1990

Lam, K.-O., and Lee, V. H.-F. (2012). Metastatic colorectal cancer: optimizing treatment with anti-EGFR monoclonal antibody. *J. Cancer Ther.* 3, 902–911. doi: 10.4236/jct.2012.326116

Li, J., Lu, K., Sun, F., Tan, S., Zhang, X., Sheng, W., et al. (2021). Panaxydol attenuates ferroptosis against LPS-induced acute lung injury in mice by Keap1-Nrf2/HO-1 pathway. *J. Transl. Med.* 19:96. doi: 10.1186/s12967-021-02745-1

Li, Y., and Seto, E. (2016). HDACs and HDAC inhibitors in Cancer development and therapy. *Cold Spring Harb. Perspect. Med.* 6:a026831. doi: 10.1101/cshperspect.a026831

Louis, P., Hold, G. L., and Flint, H. J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. *Nat. Rev. Microbiol.* 12, 661–672. doi: 10.1038/nrmicro3344

Lucas, C., Barnich, N., and Nguyen, H. (2017). Microbiota, inflammation and colorectal Cancer. *Int. J. Mol. Sci.* 18:1310. doi: 10.3390/ijms18061310

Monreal, M. T. F. D., Pereira, P. C. M., and de Magalhães Lopes, C. A. (2005). Intestinal microbiota of patients with bacterial infection of the respiratory tract treated with amoxicillin. *Braz. J. Infect. Dis.* 9, 292–300. doi: 10.1590/s1413-86702005000400005

Ossibi, P. E., Julie Note Madzele, M. E., Motoula Latou, N. H., Miabaou, D. M., Avala, P. P., Mimiesse-Monamou, J. F., et al. (2022). Lymphadenectomy for colorectal Cancer: experience of the Brazzaville teaching hospital. *Surg. Sci.* 13, 79–90. doi: 10.4236/ss.2022.133011

Plazas, J. G., Martinez, E. A., Ore, M., Cotes, A., Ricote, G., Lozano, I., et al. (2015). Colorectal cancer surgery in extreme elderly population. *J. Clin. Oncol.* 33:738. doi: 10.1200/jco.2015.33.3_suppl.738

Tilg, H., Adolph, T. E., Gerner, R. R., and Moschen, A. R. (2018). The intestinal microbiota in colorectal cancer. *Cancer Cell* 33, 954–964. doi: 10.1016/j.ccell.2018.03.004

Wang, N., Huang, X., and Cheng, J. (2022). BIRC5 promotes cancer progression and predicts prognosis in laryngeal squamous cell carcinoma. *PeerJ* 10:e12871. doi: 10.7717/peerj.12871

Wlodarska, M., Luo, C., Kolde, R., d'Hennezel, E., Annand, J. W., Heim, C. E., et al. (2017). Indoleacrylic acid produced by commensal *Peptostreptococcus* species suppresses inflammation. *Cell Host Microbe* 22, 25–37.e6. doi: 10.1016/j.chom.2017.06.007

Wu, C., Li, A., Hu, J., and Kang, J. (2018). Histone deacetylase 2 is essential for LPS-induced inflammatory responses in macrophages. *Immunol. Cell Biol.* 97, 72–84. doi: 10.1111/imcb.12203

Yang, X., Guo, Y., Chen, C., Shao, B., Zhao, L., Zhou, Q., et al. (2021). Interaction between intestinal microbiota and tumour immunity in the tumour microenvironment. *Immunology* 164, 476–493. doi: 10.1111/imm.13397

Yu, H., Li, X.-X., Han, X., Chen, B.-X., Zhang, X.-H., Gao, S., et al. (2023). Fecal microbiota transplantation inhibits colorectal cancer progression: reversing intestinal microbial dysbiosis to enhance anti-cancer immune responses. *Front. Microbiol.* 14:1126808. doi: 10.3389/fmicb.2023.1126808

Yu, C., Xiao, J.-H., and Rupasinghe, H. P. V. (2021). The Keap1-Nrf2 system: a mediator between oxidative stress and aging. *Oxid. Med. Cell. Longev.* 2021, 1–16. doi: 10.1155/2021/6635460

Zhang, W., Chen, L., Ma, K., Zhao, Y., Liu, X., Wang, Y., et al. (2016). Polarization of macrophages in the tumor microenvironment is influenced by EGFR signaling within colon cancer cells. *Oncotarget* 7, 75366–75378. doi: 10.18632/oncotarget.12207

Zhang, H., Hui, X., Wang, Y., Wang, Y., and Lu, X. (2022). Angong Niuhuang pill ameliorates cerebral ischemia/reperfusion injury in mice partly by restoring gut microbiota dysbiosis. *Front. Pharmacol.* 13:1001422. doi: 10.3389/fphar.2022.1001422

Zhang, J., Li, X., Han, X., Liu, R., and Fang, J. (2017). Targeting the thioredoxin system for cancer therapy. *Trends Pharmacol. Sci.* 38, 794–808. doi: 10.1016/j.tips.2017.06.001

Zhang, H.-L., Li, W.-S., Xu, D.-N., Zheng, W.-W., Liu, Y., Chen, J., et al. (2016). Mucosa-reparing and microbiota-balancing therapeutic effect of *Bacillus subtilis* alleviates dextrate sulfate sodium-induced ulcerative colitis in mice. *Exp. Ther. Med.* 12, 2554–2562. doi: 10.3892/etm.2016.3686

Zhang, L., Wei, C., Li, D., He, J., Liu, S., Deng, H., et al. (2022). COVID-19 receptor and malignant cancers: association of CTSL expression with susceptibility to SARS-CoV-2. *Int. J. Biol. Sci.* 18, 2362–2371. doi: 10.7150/ijbs.70172

Zhang, W., Zou, G., Li, B., Du, X., Sun, Z., Sun, Y., et al. (2020). Fecal microbiota transplantation (FMT) alleviates experimental colitis in mice by gut microbiota regulation. *J. Microbiol. Biotechnol.* 30, 1132–1141. doi: 10.4014/jmb.2002.02044

Zizzo, M. G., Caldara, G., Bellanca, A., Nuzzo, D., Di Carlo, M., and Serio, R. (2018). Preventive effects of guanosine on intestinal inflammation in 2, 4-dinitrobenzene sulfonic acid (DNBS)-induced colitis in rats. *Inflammopharmacology* 27, 349–359. doi: 10.1007/s10787-018-0506-9

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 15 November 2024
DOI 10.3389/fmicb.2024.1458923

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Jianquan He,
Xiamen University, China
Yefei Zhu,
Tongji University, China

*CORRESPONDENCE
Seok-Hwan Kim
kjxh7@cnuh.co.kr
Bon-Sang Koo
porco9@kribb.re.kr

RECEIVED 03 July 2024
ACCEPTED 29 October 2024
PUBLISHED 15 November 2024

CITATION
Kang P, Bae G-S, Jeon E, Choi J, Hwang E-H, Kim G, Baek SH, Shim K, An YJ, Lim KS, Kim Y, Oh T, Hong JJ, Lee W-K, Kim S-H and Koo B-S (2024) Comprehensive effects of fecal microbiota transplantation on cynomolgus macaques across various fecal conditions. *Front. Microbiol.* 15:1458923. doi: 10.3389/fmicb.2024.1458923

COPYRIGHT
© 2024 Kang, Bae, Jeon, Choi, Hwang, Kim, Baek, Shim, An, Lim, Kim, Oh, Hong, Lee, Kim and Koo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Comprehensive effects of fecal microbiota transplantation on cynomolgus macaques across various fecal conditions

Philyong Kang[1], Gyu-Seo Bae[2], Eunsu Jeon[2], Jeonghwa Choi[2,3], Eun-Ha Hwang[2], Green Kim[2], Seung Ho Baek[2], Kyuyoung Shim[2,3], You Jung An[2], Kyung Seob Lim[1], Yujin Kim[2], Taehwan Oh[2], Jung Joo Hong[2,3], Wan-Kyu Lee[4], Seok-Hwan Kim[5]* and Bon-Sang Koo[2,3]*

[1]Futuristic Animal Resource & Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Republic of Korea, [2]National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju, Republic of Korea, [3]KRIBB School of Bioscience, Korea University of Science & Technology (UST), Daejeon, Republic of Korea, [4]Bacteriology Laboratory, College of Veterinary Medicine, Chungbuk National University, Cheongju, Republic of Korea, [5]Department of Surgery, College of Medicine, Chungnam National University, Daejeon, Republic of Korea

Fecal microbiota transplantation (FMT) and probiotics therapies represent key clinical options, yet their complex effects on the host are not fully understood. We evaluated the comprehensive effects of FMT using diarrheal or normal feces, as well as probiotic therapies, on multiple anatomical sites in healthy cynomolgus macaques through colonoscopy and surgery. Our research revealed that FMT led to a partial microbiome transplantation without exhibiting the donor's fecal clinical characteristics. Notably, FMT increased insulin and C-peptide levels in each animal according time series, regardless of fecal conditions. Immunologically, a reduction in neutrophil-to-lymphocyte ratio were exclusively observed in femoral veins of FMT group. In blood chemistry analyses, reductions in aspartate aminotransferase, blood urea nitrogen, and creatinine were observed in the femoral veins, while elevated levels of alanine aminotransferase and calcium were exclusively detected in the portal veins. These changes were not observed in the probiotic group. Also, short chain fatty acids were significantly higher increase in portal veins rather than femoral veins. Transcriptome analysis of liver tissues showed that metabolic pathways were primarily affected by both FMT and probiotics therapies. In summary, FMT therapy significantly influenced metabolic, immunologic and transcriptomic responses in normal macaque models, regardless of fecal conditions. Also, these macaque models, which utilize surgery and colonoscopy, serve as a human-like preclinical platform for evaluating long-term effects and anatomically specific responses to gut-targeted interventions, without the need for animal sacrifice.

KEYWORDS

probiotics, fecal microbiota transplantation, cynomolgus monkeys, immune response, metabolic response, insulin, short chain fatty acid

1 Introduction

The gut microbiome has been extensively studied across various animal species, including humans. The gut is home to trillions of microbes, consisting of diverse bacteria, viruses, fungi, and yeast. Over 1,000 bacterial species have been identified in the gastrointestinal (GI) tract using next-generation sequencing technology (Qin et al., 2010). The characteristics of these microbiomes vary significantly from one individual to another (Lloyd-Price et al., 2016). Nevertheless, a healthy human microbiome contains a core set of microbes that play a pivotal role in maintaining the stability and resilience of the gut microbial community (Turnbaugh et al., 2007). At the phylum level, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* together make up over 99% of the microbiome found in the GI tract (Eckburg et al., 2005). The development of this microbiome is shaped in early life by exposure to external microbes, which is crucial for establishing a stable microbial community (Enav et al., 2022; Yatsunenko et al., 2012). Once a stable and resilient microbiome is established in later life stages, harmful non-resident microbes struggle to colonize the gastrointestinal tract because of selective pressures imposed by existing gut microbes and the host's immune system (Lee and Chang, 2021; Lozupone et al., 2012).

The symbiotic relationship between commensal microbes and their host has been extensively elucidated through microbial-derived molecules such as short-chain fatty acids (SCFAs), tryptophan, and secondary bile acids (Canfora et al., 2015; Gheorghe et al., 2019; Wahlstrøm et al., 2016). SCFAs, including acetate, butyrate, and propionate, are produced from the fermentation of indigestible plant fibers by specific bacterial species like *Faecalibacterium prausnitzii* and *Roseburia* (Machiels et al., 2014). SCFAs serve as an energy source for the colonic epithelium, enhance gut barrier integrity by reducing gut permeability, maintain the mucosal immune system, particularly regulatory T cells (Treg cells), and increase the activity of the sympathetic nervous system while regulating lipid and glucose metabolism (He et al., 2020). The production of SCFAs can be influenced by dietary intake and the microbial composition in the GI tract. Mucolytic bacteria, especially *Akkermansia muciniphilia*, can protect the GI mucin layer, preventing bacterial invasion (Zhang et al., 2019).

Dysbiosis refers to a disturbance in gut microbes, characterized by reduced diversity, an increase in harmful bacteria, and a decrease in beneficial bacteria within the GI tract. Factors that lead to dysbiosis include antibiotic overuse, inappropriate diets (notably high-fat diets), exposure to foodborne toxins, elevated systemic inflammation, and organic pollutants (Napolitano and Covasa, 2020). Microbial imbalance has been linked to various human diseases, including neurological, hepatic, metabolic, autoimmune, cardiovascular, oncogenic, infectious, and gastrointestinal conditions (Carding et al., 2015). The intricate relationship between gut microbes and various physiological systems can be understood through the lens of multiple axes: the gut-brain, gut-liver, gut-skin, gut-immune, gut-heart, and gut-kidney axes (Anand and Mande, 2022; Sinha et al., 2021; Chen et al., 2019; Shi et al., 2017). However, direct evidence establishing whether dysbiosis is a cause or a consequence of specific diseases remains elusive. While disruption of the microbiome may not be the sole driver behind these conditions, it appears to play a contributory role in their progression via diverse mechanisms. Hence, changes in the microbiome can exert profound and complex effects on human physiology and diseases, although the precise outcomes remain largely undetermined.

Microbiome research has been actively conducted through *in vitro* and *in vivo* experiments, as well as human clinical studies. A variety of preclinical animal models, including humanized mice, honeybees, zebrafish and non-human primates have been used for these investigations (Douglas, 2019; Park and Im, 2020; Brenchley and Ortiz, 2021). The selection of animal models is often dictated by specific research objectives. However, these species possess distinct characteristics in terms of anatomy, physiology, genetics, metabolism, and immunology that set them apart from humans. For human clinical trials, time-series longitudinal studies are challenging to conduct due to the lack of data prior to disease onset (Lee and Chang, 2021). Additionally, examining tissue-specific microbiomes can be difficult in human subjects. Typically, human patients can provide only fecal samples, which primarily reflect the luminal microbiome of the descending colon (Yasuda et al., 2015). Furthermore, significant inter-individual variations exist among humans, making the interpretation of cross-sectional human microbiome studies complex (Ley et al., 2006). Currently, among preclinical animal models, old world macaque species most closely mirror the human microbiomes (Amato et al., 2019).

Within the realm of GI-targeted therapies, fecal microbiota transplantation (FMT) and probiotic therapies stand out as two prominent clinical treatment options (Suez et al., 2018). Probiotic therapy has been widely adopted for both healthy individuals and those with medical conditions. FMT presents a potential solution for otherwise intractable diseases, such as antibiotic-resistant *Clostridium difficile* infections and inflammatory bowel diseases (Drekonja et al., 2015; Colman and Rubin, 2014). Despite the evident impact of microbiome alterations on various aspects of human health, the comprehensive and direct effects of these therapies remain to be fully elucidated. In this study, we used the old world macaque, the preclinical animal model most similar to humans, to comprehensively analyze the effects of FMT and probiotic treatments from various perspectives.

2 Materials and methods

2.1 Animals

Nine female cynomolgus monkeys (*Macaca fascicularis*) aged between 6 and 10 years, were sourced from Cambodia and raised under specific pathogen-free conditions at the National Primate Research Center within the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Each monkey was housed individually in indoor cages and provided with a commercial monkey feed (2050 Teklad Global 20% Protein Primate Diet, Harlan, Envigo, United Kingdom) composed of 20% crude protein, 5.4% fat, 8.1% crude fiber, and 40.1% carbohydrates. In addition to this diet, they were given seasonal fruits and had unrestricted access to water. The housing conditions adhered strictly to the minimal requirements outlined in 'The Guide for the Care and Use of Laboratory Animals' as proposed by the Institute for Laboratory Animal Research (ILAR) in 2010 (National Research Council, 2010). The breeding environment was automatically regulated to maintain a temperature of 24°C ± 2°C, a relative humidity of 50% ± 5%, light intensity ranging

from 150 to 300 Lux, ventilation cycling 10–20 times per hour, and a 12-h light/dark cycle. Institutional non-human primate veterinary specialists confirmed that all monkeys were in good health conditions except for one monkey suffering idiopathic chronic diarrhea. Molecular tests confirmed that all animals were negative for infections with enteric pathogens, including *Campylobacter jejuni*, *Clostridium difficile*, *Salmonella* spp., *Shigella* spp., and *Yersinia enterocolitica*. Routine fecal smear examinations also showed no evidence of fecal parasite infections.

2.2 Animal experimental design

Nine healthy animals were categorized into three distinct groups. FMT + D group consisted of four animals that underwent FMT using diarrheal feces via colonoscopy. These feces were obtained from a donor monkey diagnosed with idiopathic chronic diarrhea based on clinical signs, medical imaging, and dysbiosis, without evidence of specific enteric pathogens (Koo et al., 2020). FMT + N group included two animals that received a transplant of normal feces. FMT group, which included both the FMT + D and FMT + N groups, was also assessed. Lastly, probiotics group comprised three animals who were inoculated with a six-week probiotic therapy via nasogastric tubes, using a 1ml blend of four human-origin lactic acid bacteria: *L. fermentum, L. plantarum, Leuconostoc mesenteroides*, and *Bifidobacterium breve* with over 10^9 cfu (Table 1). Fecal sampling occurred at the outset, then at 2 and 6 weeks post-initiation of FMT and probiotic therapy. Samples, including blood from both the portal and femoral veins and liver tissues, were collected at 6 weeks post-initiation of therapy. Surgical interventions, which were executed at the beginning and 6 weeks following the FMT and probiotic treatments, facilitated the collection of liver tissue and blood from the portal veins. A Bovie electrosurgical unit was utilized during the abdominal incision and tissue collection to mitigate bleeding risks. Surgical and postoperative care were overseen by a surgical specialist alongside a non-human primate veterinary expert. Detailed specifics of the experimental design were described in Figure 1. Clinical signs were monitored daily by the breeders, and in some instances, health monitoring was performed periodically by non-human primate veterinary specialists.

2.3 FMT therapy and surgery

We conducted FMT therapy on cynomolgus monkeys via colonoscopy using fresh feces sourced from monkeys housed in the same facility. Prior to FMT, monkeys in the designated FMT groups were treated with a combined regimen of vancomycin (15 mg/kg, IM, bid) and enrofloxacin (10 mg/kg, IM, bid) for a duration of 7 days. As a preparatory step, we administered an enema, introducing 50ml of a polyethylene glycol solution into the monkeys via nasogastric tubes on two separate occasions. We aseptically collected two distinct fecal types: normal and diarrheal. The normal fecal samples were randomly obtained from healthy monkeys and subsequently pooled in sterile plastic bags. In contrast, the diarrheal feces were obtained from a monkey exhibiting severe idiopathic chronic diarrhea, characterized by recurrent episodes of watery, bloody, and mucus-laden feces (Supplementary Figure S2). Notably, this monkey had previously been identified as having a disturbed microbiome in an earlier study (Koo et al., 2020). All fecal samples were collected within a 2-h window post-defecation. After collection, the 25g feces were combined with a sterile 40 mL PBS solution. This fecal mixture was then passed through a moistened 5-layer sterile gauze positioned in a funnel and subsequently filtered using a 70μm pore size filter. The resulting solution was administered into the transverse colon of the recipient monkeys, who were placed in a right recumbency position during the procedure. Post-transplantation, we ensured that the monkeys remained in this position for an additional 30 min to ensure optimal uptake. The entire process, spanning fecal collection to FMT therapy, was completed within a 4–6 h timeframe.

2.4 ^{18}F-fluorodeoxyglycose (FDG) positron emission tomography (PET)/computed tomography (CT) analysis

Medical images were obtained using a PET/CT scanner (Siemens Biograph-mCT) and 18F-fluorodeoxyglucose (18F-FDG) was supplied from DuChemBio Co., Ltd. All monkeys fasted for at least 12 h before and were anesthetized with 2% isoflurane, administered via a respiratory anesthesia machine (Royal Medical) at a flow rate of 2 L/min for radiotracer injection and scan. They were given with 5.0 ± 0.5 MBq of 18F-FDG intravenously and after 1h, whole-body PET/CT scan was performed to visualize inflammation in the GI tracts for 10min/bed (5 bed) in a supine position. PET images were reconstructed to 200 × 200 matrics and 3.4 mm × 3.4 mm pixel sizes with a 3.0 mm slice thickness using a OSEM iterative algorithm (3 iterations and 21 subsets) with time of flight.

2.5 Microbiome analysis

Fecal samples were collected from each monkey at 0, 2, and 6 weeks following FMT and probiotic therapies. After defecation, fecal

TABLE 1 Detailed information of animal history and experimental designs.

Group	Animal			Experiments	
	Species	Sex	Number	Therapies	Constituents
FMT + D	*Macaca fascicularis*	Female	4	FMT	Diarrheal fresh stool
FMT + N	*Macaca fascicularis*	Female	2	FMT	Normal fresh stool
Probiotics	*Macaca fascicularis*	Female	3	Probiotics	A combination of four lactic acid bacteria

A total of nine animals were divided into three groups: FMT + D, FMT + N, and the probiotics groups. Lactic acid bacteria included *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Bifidobacterium breve*. FMT, fecal microbiota transplantation.

10.3389/fmicb.2024.1458923

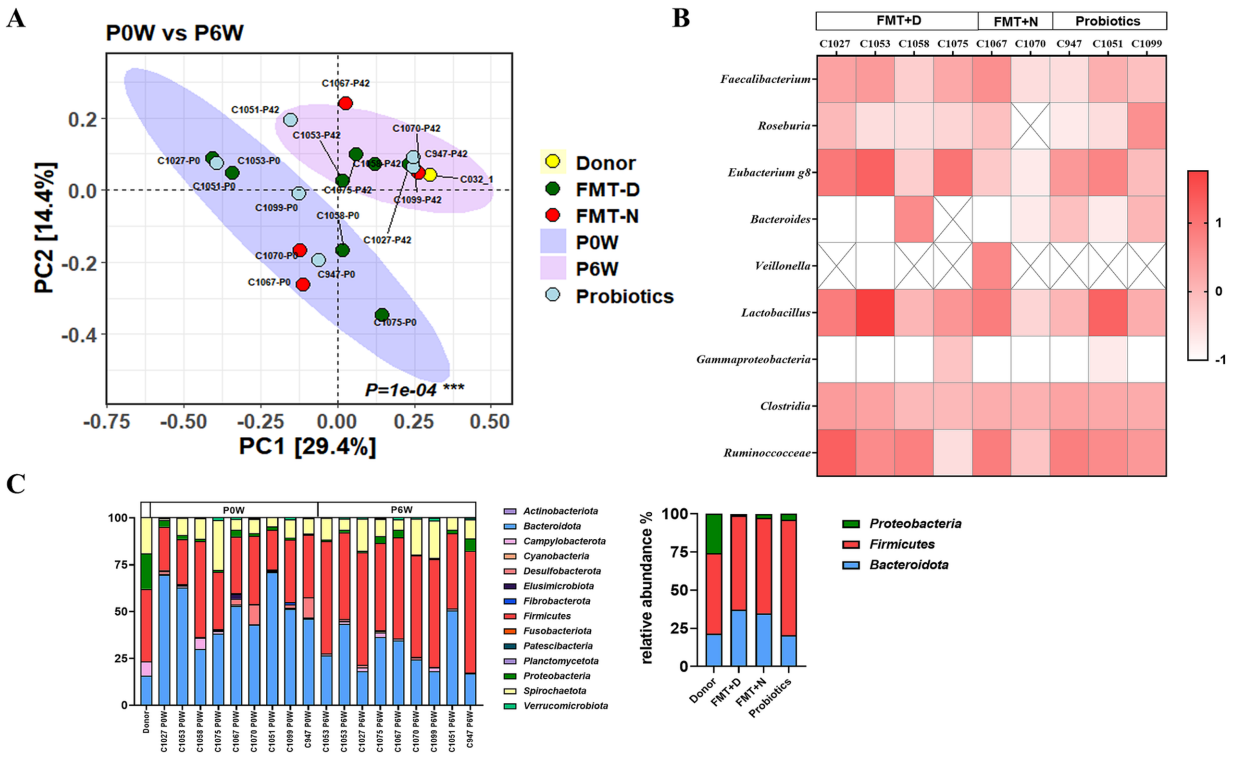


FIGURE 1
Impact of fecal Microbiota transplantation and probiotics on fecal microbial composition in recipient macaques. (A) Beta diversity analysis shows significant changes in the fecal microbiome of monkeys in both the FMT and probiotics groups between pre-and post-treatment. (B) Relative change rates of specific bacterial taxa before and after treatment. (C) Relative bacterial composition in fecal microbiome at the phylum level.

samples were collected within a 2-h window and promptly transported to the laboratory on ice. Total DNA was extracted from samples using the QIAamp Fast DNA Stool Mini kit (Qiagen). The V3 and V4 hypervariable regions of the 16S bacterial rRNA gene were amplified from the extracted DNA using PCR. Sequence similarity thresholds for taxonomy classifications were as follows: genus (97 > X > 94.5), family (94.5 > X > 86.5), order (86.5 > X > 82), class (82 > X > 78.5), and phylum (78.5 > X > 75). The metagenomic processing data were assessed using the EZBioCloud Database, which is supported by CJ Bioscience. Bacterial composition values were examined from the phylum to genus level. Alpha diversity metrics included evenness, valid reads, operational taxonomic units (OTUs), abundance-based coverage estimator (ACE) and Chao1, Jackknife, Shannon, and Simpson indices. Beta diversity, featuring PCO analysis, was used for longitudinal comparisons between the pre-and post-treatment groups. The core microbiome was characterized as bacterial phyla accounting for at least 0.1% of the entire microbiome. We performed a comparative analysis of bacterial taxa between groups using the ANCOMBC2 package. All sequencing and analytical procedures were performed by CJ Bioscience (Korea).

2.6 Hematology, blood chemistry and hormone assay

Blood samples were drawn from the femoral and portal veins. Blood was collected from the femoral and portal veins at 0 and 6 weeks through a surgical procedure. Hematological values were assessed using the HEMAVET 950FS hematology analyzer (Drew Scientific Ltd.). Blood biochemical analyses were conducted using the Dri-Chem 7000i biochemistry analyzer (Fujifilm). The values for pancreatic and reproductive hormones were measured using a 96-well multiplex magnetic bead panel in the Milliplex MAP Non-Human Primate Pituitary Magnetic Bead Panel 1 (NHPPT1MG-46K) and the Milliplex MAP Non-Human Primate Metabolic Magnetic Bead Panel (NHPMHMAG-45K). Detailed information on hematological, chemistry, and hormonal values in the cynomolgus macaque are provided in Supplementary Table S1.

2.7 SCFAs measurement

Plasma samples from the femoral and portal veins were treated with methanol and then incubated at 30°C for 3 h in a shaking incubator. After centrifuging at 3,000 rpm for 20 min, the supernatant was passed through a 0.45-μm filter. A 1 μL aliquot of the filtered sample was injected into an Agilent GC-FID system fitted with an HP-FFAP column for chromatographic analysis. The concentrations of three SCFAs (acetic acid, butyric acid, and propionic acid) were determined using a Flame Ionization Detector. Nitrogen gas served as the carrier gas and was maintained at a consistent flow rate of 1.0 mL/min. The entire procedure was conducted by the Korea Polymer Testing and Research Institute.

2.8 Flow cytometry

Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) was conducted using the BD LSR Fortessa flow cytometer (BD Biosciences). PBMCs were harvested from blood samples drawn from both femoral and portal veins using Ficoll–Hypaque density gradient (Lymphoprep, Axis-Shield). Red blood cells were lysed by incubating with ACK lysis buffer (Gibco) at room temperature for 5 min. Subsequently, cell viability was assessed by staining with Fixable Viability Stain 575V (BD Biosciences) for 20 min at room temperature. For surface staining, cells were treated with the panel of antibodies for 30 min at 4°C. Post-staining, cells were rinsed with the permeabilization wash buffer and fixed using 1% paraformaldehyde. Data acquisition was executed using the LSRFortessa system (BD Bioscience) and analyzed using FlowJo v10.7.1.

2.9 Transcriptome analysis

Total RNA was extracted from liver tissues using TRIZOL reagent (Invitrogen) following the manufacturer's protocol. Library construction was achieved using the TrueSeq Stranded Total RNA LT Sample Prep Kit (Gold) from Illumina Inc. following the manufacturer's guidelines. High-throughput sequencing was conducted using paired-end sequencing (2 × 101 nt) on the NovaSeq 6000 system (Macrogen, Seoul, Korea). For functional annotation and gene-set enrichment analyses, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used. All RNA sequencing procedures and subsequent analyses were performed by Macrogen Inc. (Seoul, Korea).

2.10 Statistical analysis

Statistical significance was determined using a paired t-test for paired samples. A *p*-value of less than 0.05 indicated statistical significance. All statistical analyses were conducted using GraphPad Prism software, version 8.4.3 (GraphPad Software).

2.11 Ethical statements

All experimental procedures were sanctioned by the Institutional Animal Care and Use Committee at Korea Research Institute of Bioscience and Biotechnology (Approval No. KRIBB-AEC-21324).

3 Results

3.1 Clinical and vital signs, and ^{18}FDG-PET CT analysis

Throughout the experimental period, which extended from week 0 to week 6 after the initiation of therapies, no clinical signs were observed in any of the monkeys across all groups receiving FMT with either diarrheal (FMT + D group) or normal feces (FMT + N group), as well as probiotics (Probiotics group) (Figures 1A,B). Despite using feces with significant watery and mucous consistency from a donor monkey for the FMT in FMT + D group (Supplementary Figure S2), none of the four recipient monkeys displayed any gastrointestinal disturbances, including diarrhea, throughout the study period. No notable changes in body weight or temperature were observed in any of the monkeys during the study (Supplementary Figure S3). Consistent with the clinical observations, the ^{18}FDG-PET CT analysis did not indicate any FDG uptake indicative of intestinal inflammation (Supplementary Figure S4). However, in the donor monkey suffering idiopathic chronic diarrhea (C032), extensive FDG uptake was observed throughout the entire large intestine area, including the colon and rectum (Supplementary Figure S2C) (Koo et al., 2020). All animals exhibited normal health conditions after the completion of experiments, based on clinical signs, hematology, and blood chemistry results by yearly health monitoring by Institutional veterinarians.

3.2 Microbiome analysis

Fecal microbiome was analyzed from fecal samples collected 0, 2, and 6 weeks post-treatment in all monkeys. In principal coordinate (PCO) analysis, statistically significant changes in the fecal microbiome were observed in both the FMT and probiotics groups between pre-and post-treatment (Figure 1A). However, full transplantation was not ultimately confirmed in any of the monkeys in FMT + D group. Also, significant differences in bacterial taxa and beta diversity among the groups were not verified (Figure 1). No significant changes were detected in alpha diversity indices, including ACE, Chao1, Jackknife, NPShannon, Shannon, or Simpson, across the FMT and probiotics groups (Supplementary Figure S5). *Bifidobacterium* spp., *Akkermansia* spp., *Butyricicoccus* spp. and *Citrobacter* spp. were rarely detected in feces of these monkeys.

3.3 Hematology and flow cytometry analysis

Hematologic values and their subsets were assessed in all monkeys in samples from both femoral and portal veins using hematology and flow cytometry analysis. Differences were observed in hematologic values between the two anatomical sites. Hematological assessments revealed a statistically significant decrease in WBC counts in the FMT + D group, but this decrease was observed only in samples from the portal veins (Figure 2). Furthermore, reduced neutrophil counts were identified in samples from both portal and femoral veins of the FMT group which included FMT + D and FMT + N groups (Figure 3). In contrast, elevated lymphocyte counts were observed in samples from the femoral veins of the FMT group. In the Probiotic group, an increase in monocyte counts was detected only in samples from the portal veins (Figure 2, and Supplementary Figure S9). Among the lymphocyte subsets, $CD8^+$, $CD8^+$ T_{CM} and $CD8^+$ T_{EM} cells showed a significant increase in the FMT groups (Figure 3, and Supplementary Figure S7). For B lymphocyte subsets, no notable differences were found between the FMT and Probiotic groups except for plasmablast cells (Figure 3, and Supplementary Figure S8). In the FMT groups, the counts of natural killer cells increased, but this was limited to samples from the femoral veins (Figure 3). The neutrophil to lymphocyte ratio (NLR) in peripheral blood showed a significant decrease exclusively in the femoral veins of the FMT groups

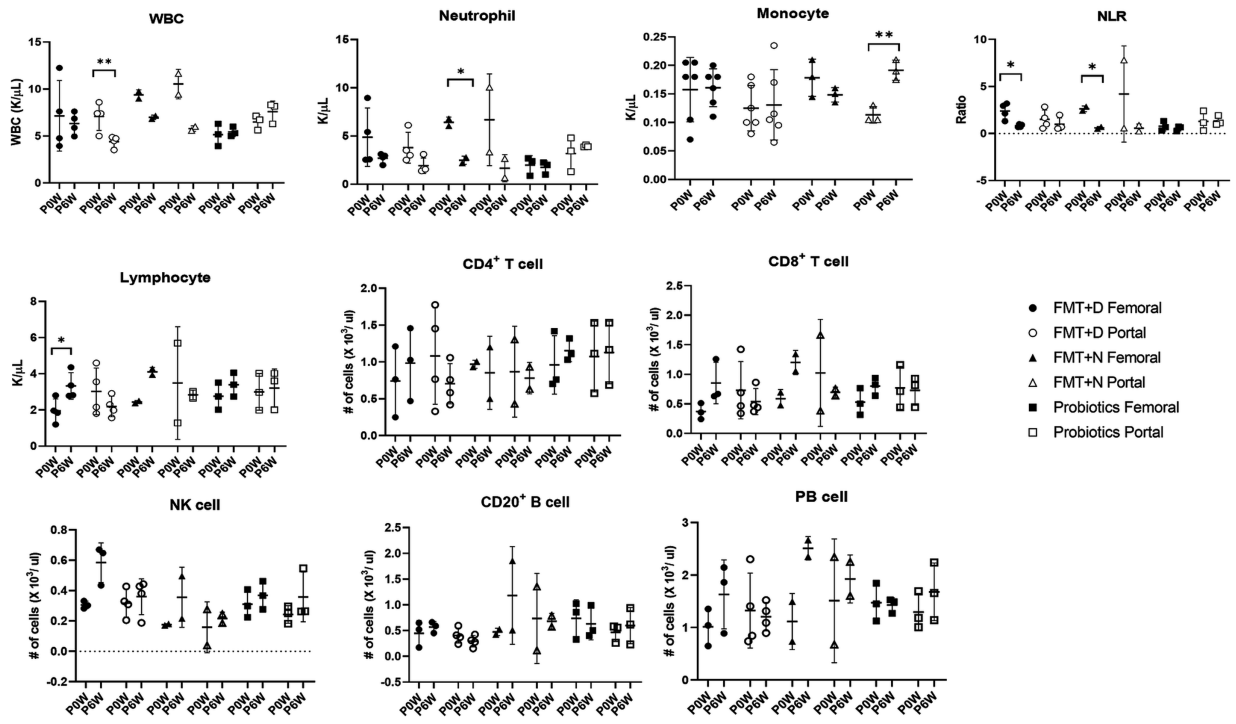


FIGURE 2
Comparative analysis of immune cell populations in femoral and portal veins of monkeys undergoing fecal microbiota transplantation with diarrheal (FMT + D) and normal (FMT + N) feces as well as probiotic groups. Monkeys undergoing fecal microbiota transplantation with diarrheal (FMT + D) and normal (FMT + N) feces, as well as those in the probiotic groups. P0W and P6W represent 0 weeks and 6 weeks post-FMT, respectively. Femoral and portal refer to the femoral and portal veins, which are anatomical sites for blood collection. The lines in the graphs indicate the mean values with standard deviation (SD). Statistically significance were determined by *t* test and indicated by *p*-values. $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***). NLR, the neutrophil to lymphocyte ratio; WBC, white blood cells; NK cell, natural killer cell; PB cell, plasmablast.

(Figures 2, 3). Additionally, a rise in dendritic cell counts was identified solely in samples from the femoral veins of the FMT + D group (Supplementary Figure S9). The values of RBC, platelet and hemoglobin were not changed in all groups.

3.4 Hormonal response

Pancreatic and reproductive hormones were measured in samples from both femoral and portal veins. In the FMT group, levels of insulin and C-peptide increased in samples from both veins, whereas no such increase was observed in the Probiotic group. Additionally, an elevated concentration of monocyte chemoattractant-1 (MCP-1) was found solely in samples from the portal veins of monkeys in the FMT group (Figure 4). There were no observed changes in the levels of gastric inhibitory polypeptide, glucagon, or pancreatic polypeptide (Supplementary Figure S10). In the FMT groups, a decrease in adrenocorticotropic hormone levels and an increase in growth hormone levels were observed in samples from the portal vein. In contrast, no reproductive hormonal changes were detected in the Probiotic group (Supplementary Figure S11).

3.5 Blood chemistry and SCFAs evaluation

Changes in blood chemistry and SCFAs values were observed in samples from both femoral and portal veins. In the FMT groups, aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CRE) levels decreased in samples from femoral veins. Conversely, alanine aminotransferase (ALT) and calcium (Ca) levels increased, while inorganic phosphorus (IP) levels decreased in samples from portal veins (Figure 5). For the FMT + D group, there was an increase in AST and decrease in TG values in samples from the femoral veins. Additionally, ALT values increased and albumin (ALB) values decreased in samples from the portal veins (Supplementary Figure S12). In the Probiotic group, AST values in samples from the portal veins increased, while there was a statistically significant rise in glucose (GLU) levels in samples from the femoral veins (Figure 5). All SCFAs, including acetic acid, butyric acid and propionic acid, showed increased levels in samples from both femoral and portal veins in the Probiotic group. The SCFA values were significantly higher in samples from portal veins than in those from femoral veins (Figure 6).

3.6 Transcriptome analysis in liver tissue

Hierarchical clustering and multi-dimensional scaling plots showed distinct clustering of RNA transcripts in liver tissues following FMT (Figure 7A) and probiotic treatments (Figure 7B). Metabolic pathways, as illustrated by KEGG pathway analyses, were predominantly influenced by both FMT and probiotics. Notably, the number of genes exhibiting up-and down-regulation varied

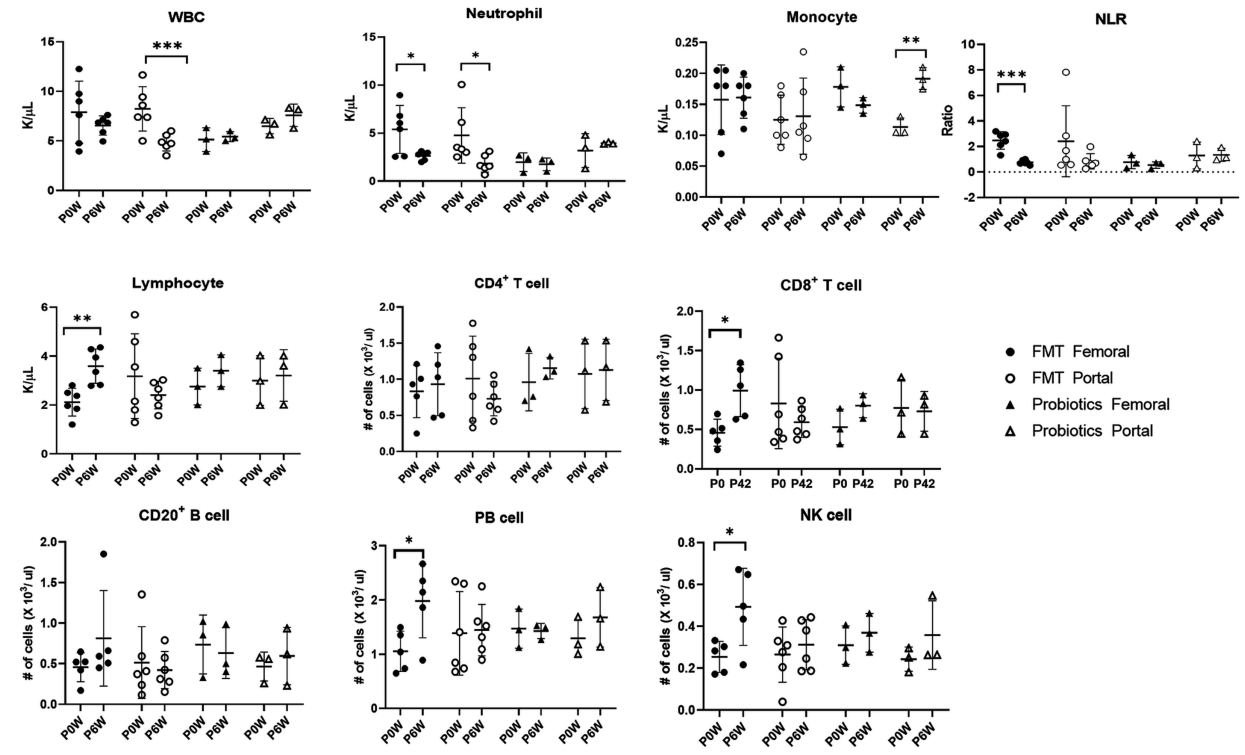


FIGURE 3
Comparative analysis of immune cell populations in femoral and portal veins of monkeys in groups of fecal microbiota transplantation (FMT) and probiotic therapies. Data in FMT group combined results from both the FMT + D and FMT + N groups. Monkeys undergoing fecal microbiota transplantation with diarrheal (FMT + D) and normal (FMT + N) feces, as well as those in the probiotic groups. P0W and P6W represent 0 weeks and 6 weeks post-FMT, respectively. Femoral and portal refer to the femoral and portal veins, which are anatomical sites for blood collection. The lines in the graphs indicate the mean values with standard deviation (SD). Statistically significance were determined by *t* test and indicated by *p*-values. $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***). NLR, the neutrophil to lymphocyte ratio; WBC, white blood cells; NK cell, natural killer cell; PB cell, plasmablast.

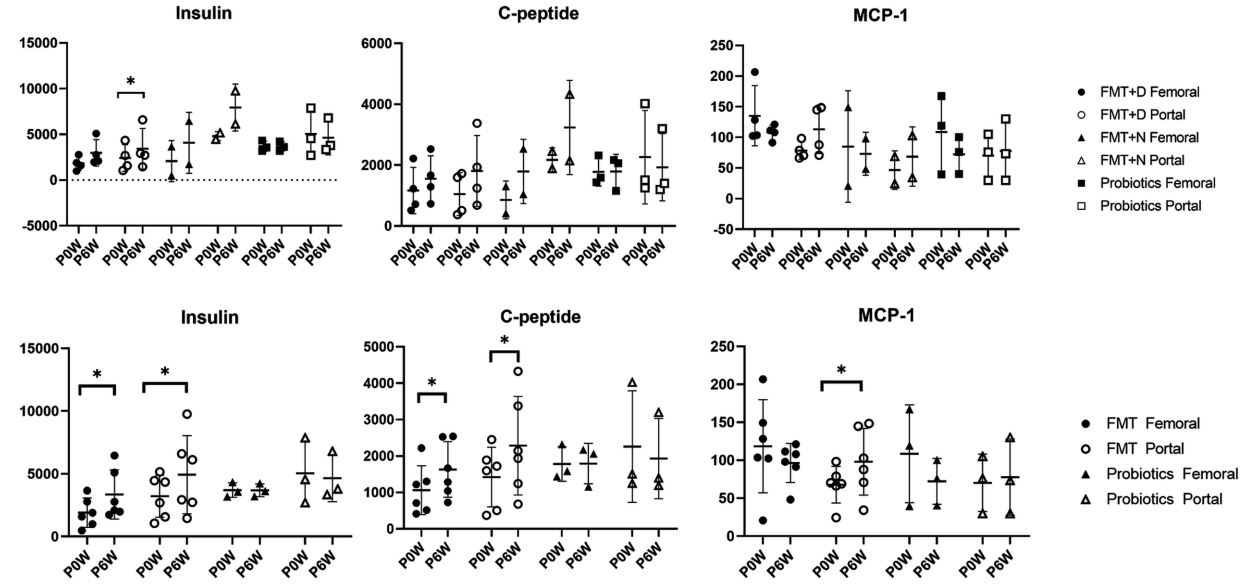


FIGURE 4
Metabolic hormone changes in femoral and portal veins of monkeys undergoing fecal microbiota transplantation and probiotic therapy. Comparative evaluations were based on differences among three groups (FMT + D, FMT + N and probiotics) and two groups (FMT and probiotics). Data in FMT group combined results from both the FMT + D and FMT + N groups. Monkeys undergoing fecal microbiota transplantation with diarrheal (FMT + D) and normal (FMT + N) feces, as well as those in the probiotic groups. P0W and P6W represent 0 weeks and 6 weeks post-FMT, respectively. Femoral and portal refer to the femoral and portal veins, which are anatomical sites for blood collection. The lines in the graphs indicate the mean values with standard deviation (SD). Statistically significance were determined by *t* test and indicated by *p*-values. $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$. MCP-1, monocyte chemoattractant protein-1; C-peptide, connecting peptide.

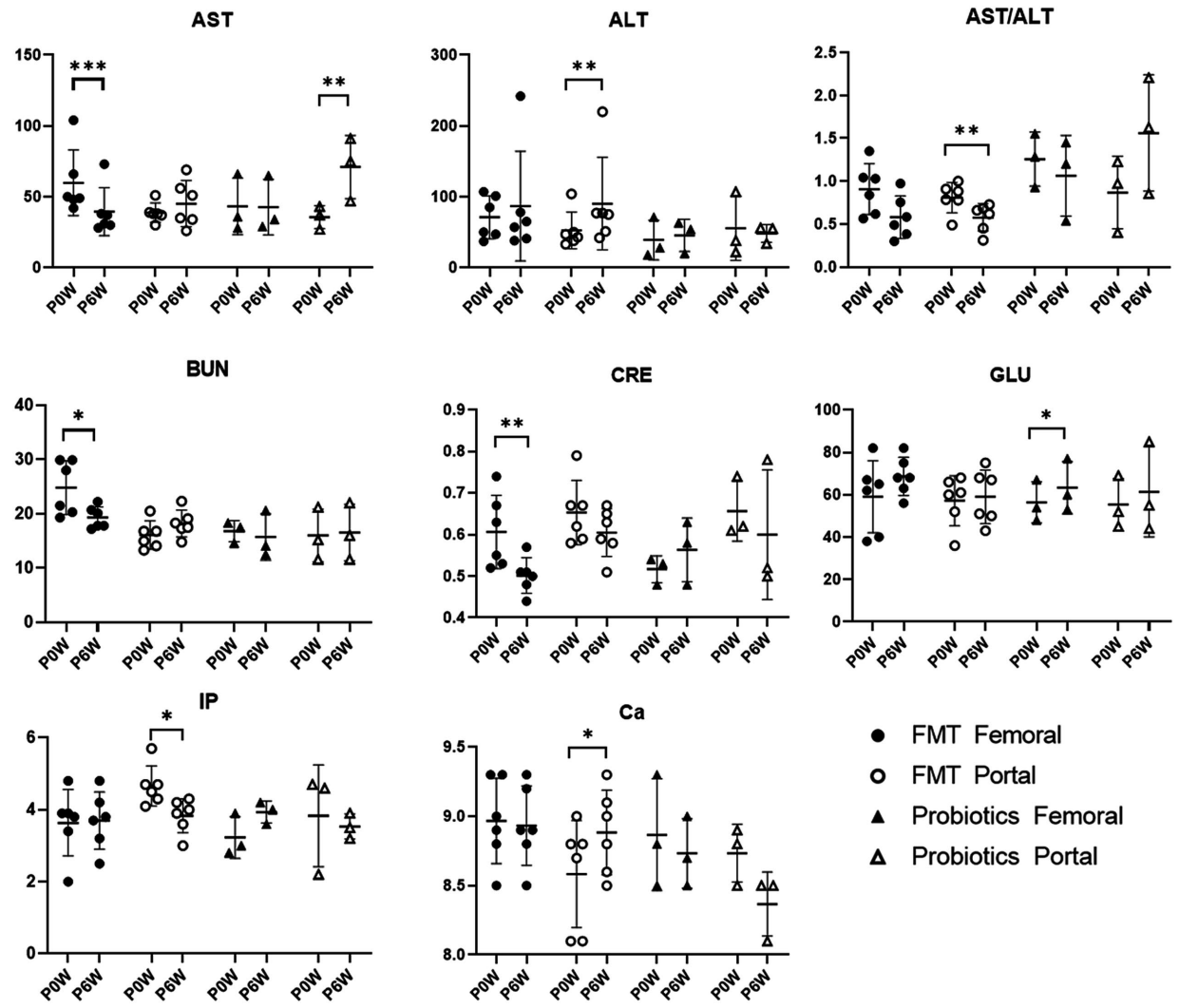


FIGURE 5
Comparative analysis of Blood chemistry results in femoral and portal veins of monkeys undergoing fecal microbiota transplantation (FMT) and probiotic therapy. Data in FMT group combined results from both the FMT + D and FMT + N groups. Monkeys undergoing fecal microbiota transplantation with diarrheal (FMT + D) and normal (FMT + N) feces, as well as those in the probiotic groups. P0W and P6W represent 0 weeks and 6 weeks post-FMT, respectively. Femoral and portal refer to the femoral and portal veins, which are anatomical sites for blood collection. The lines in the graphs indicate the mean values with standard deviation (SD). Statistically significance were determined by *t* test and indicated by *p*-values. $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***). AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; GLU, glucose; IP, inorganic phosphate; Ca, calcium.

significantly among individuals, irrespective of whether they were in the FMT or probiotic groups.

4 Discussion

The microbiome is recognized for its influence on both health and disease in humans. However, understanding of the intricate and complicated effects of FMT and probiotics on the healthy host remains limited. In clinical practice, feces with diverse microbiome have been used for FMT practices due to variations among individual donor stool samples. In this study, we aimed to comprehensively evaluate the effects of FMT using feces under significant different conditions, as well as probiotic therapy, across various anatomical sites in cynomolgus monkeys. This macaque model, which employs clinical techniques such as surgery and colonoscopy, could elucidate the compound effects of these gut-targeted therapies according to anatomical sites in individuals.

Changes in the microbiome have been associated with various diseases, particularly inflammatory bowel diseases (IBD) (Lee and Chang, 2021). Clinical signs were not observed in macaques until 6 weeks after the administration of FMT using diarrheal contents from a monkey with spontaneous idiopathic chronic diarrhea (Koo et al., 2020). The six-week time point was chosen because clear therapeutic effects of FMT in human patients are typically observed around 4 weeks post-treatment (Cheng et al., 2019; Fang et al., 2021). Statistically significant changes and reduced variation in the fecal microbiome were observed in both the FMT and probiotics groups between pre-and post-treatment (Figure 1A). However, full transplantation was not ultimately confirmed in any of the monkeys

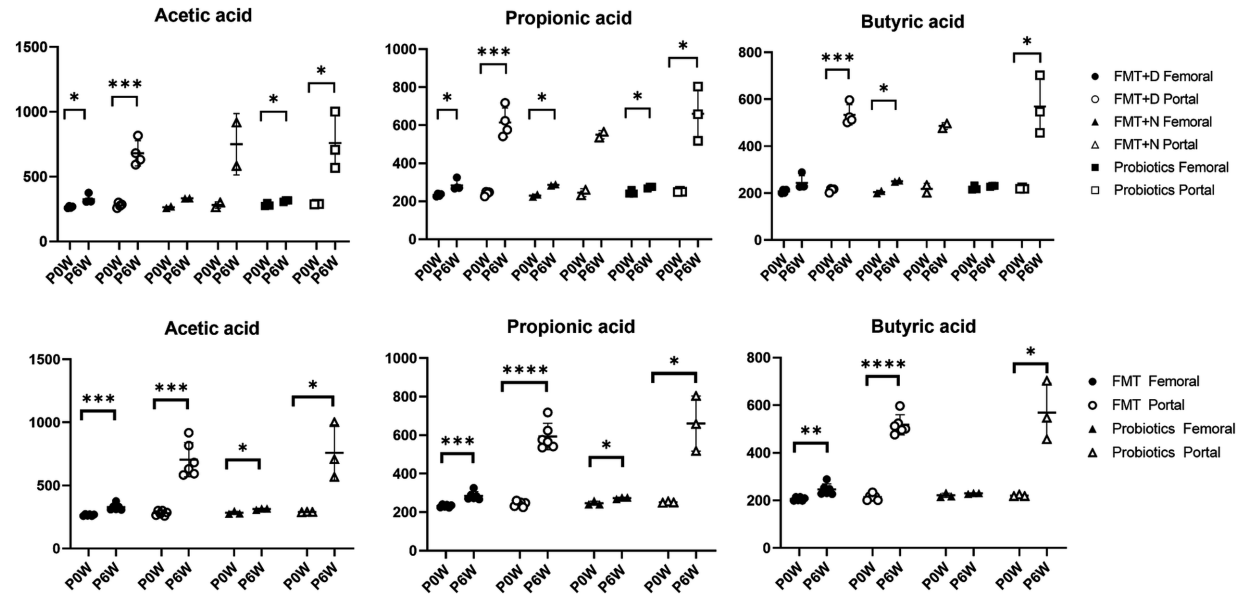


FIGURE 6
The changes of short chain fatty acids in femoral and portal veins of monkeys undergoing fecal microbiota transplantation and probiotic therapy. Short chain fatty acids include acetic acid, propionic acid, and butyric acid. Monkeys undergoing fecal microbiota transplantation with diarrheal (FMT + D) and normal (FMT + N) feces, as well as those in the probiotic groups. Comparative evaluations were based on differences among three groups (FMT + D, FMT + N and probiotics) and two groups (FMT and probiotics). Data in FMT group combined results from both the FMT + D and FMT + N groups. P0W and P6W represent 0 weeks and 6 weeks post-FMT, respectively. Femoral and portal refer to the femoral and portal veins, which are anatomical sites for blood collection. The lines in the graphs indicate the mean values with standard deviation (SD). Statistically significance were determined by *t* test and indicated by *p*-values. $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); $p \leq 0.0001$.

in FMT + D group. Also, significant differences in bacterial taxa and beta diversity among the groups were not verified (Figure 1). This finding aligns with the beta diversity results reported in human FMT studies, where fecal microbiome transplants also exhibited high variability between individuals, and none of the human patients reached the endpoint (Sokol et al., 2020). In adults, the microbiome demonstrates both stability and resilience, which may contribute to partial transplantation and inter-individual variations in the microbiome in recipient monkeys, even in the face of a high concentration of feces in the FMT (Lozupone et al., 2012; Sommer et al., 2017). To overcome the stability of gut microbiome, consequence FMT procedures were conducted for human clinical trials and significantly increased the therapeutics effects compared to single FMT procedure (Baunwall et al., 2020). Also, repeated FMT procedures were conducted for three monkeys, and no pathological signs were observed based on clinical assessments and medical diagnostics using the ^{18}FDG-PET CT analysis (unpublished data). Consequently, our findings suggest that the only fecal microbiota is not a sole trigger for idiopathic chronic diarrhea in monkeys that showed similar microbiome characteristics with IBD in human (Koo et al., 2020). However, this observation does not imply that the intestinal microbiome is not a contributing factor in chronic diarrhea in both human and monkeys. It remains unclear what role the microbiome may play when combined with other factors.

Following FMT therapy, we observed a statistically significant decline in neutrophil levels in samples from both the femoral and portal veins, while elevated lymphocyte values were detected exclusively in samples from the femoral veins indicating a decrease in NLR values in healthy monkeys (Figure 3). The NLR value is a well-established prognostic biomarker for a variety of diseases, including cancer, infectious diseases, sepsis, and cardiovascular diseases (Buonacera et al., 2022). In human IBD patients, heightened neutrophil activity and extensive inflammation within the GI tracts have been identified (Herrero-Cervera et al., 2022). The observation that both decreased neutrophil levels and increased lymphocyte levels were noted in healthy individuals receiving FMT suggests that FMT may be a viable preventative strategy against various diseases in healthy populations (Goloshchapov et al., 2019). Interestingly, these preventive benefits of FMT appear to occur irrespective of the donor's fecal conditions.

Endocrine analysis showed significant elevations in insulin and c-peptide proteins in monkeys in the FMT groups (Figure 4). These increases were evident in samples from both the femoral and portal veins, irrespective of the fecal conditions. While prior studies have highlighted the benefits of FMT therapy for both type 1 and 2 diabetes in humans, our study is the first to demonstrate in a preclinical model that FMT boosts insulin levels on healthy individuals regardless of fecal state (De Groot et al., 2021; Wu et al., 2023). The rise in MCP-1 was solely detected in samples from the portal vein. While the result was not statistically significant, we observed a trend towards declining MCP-1 values in femoral veins (Figure 4). The escalation in MCP-1 might signal an active inflammatory response and has links to various diseases (Singh et al., 2021). The observed variation in MCP-1 levels across different anatomical sites exhibited a pattern similar to blood chemistry results, where increased ALT values were noted in the portal veins, and decreased AST values were observed in the femoral veins. Consequently, FMT may be advantageous for systemic anti-inflammatory responses, yet potentially detrimental in terms of liver damage in normal people.

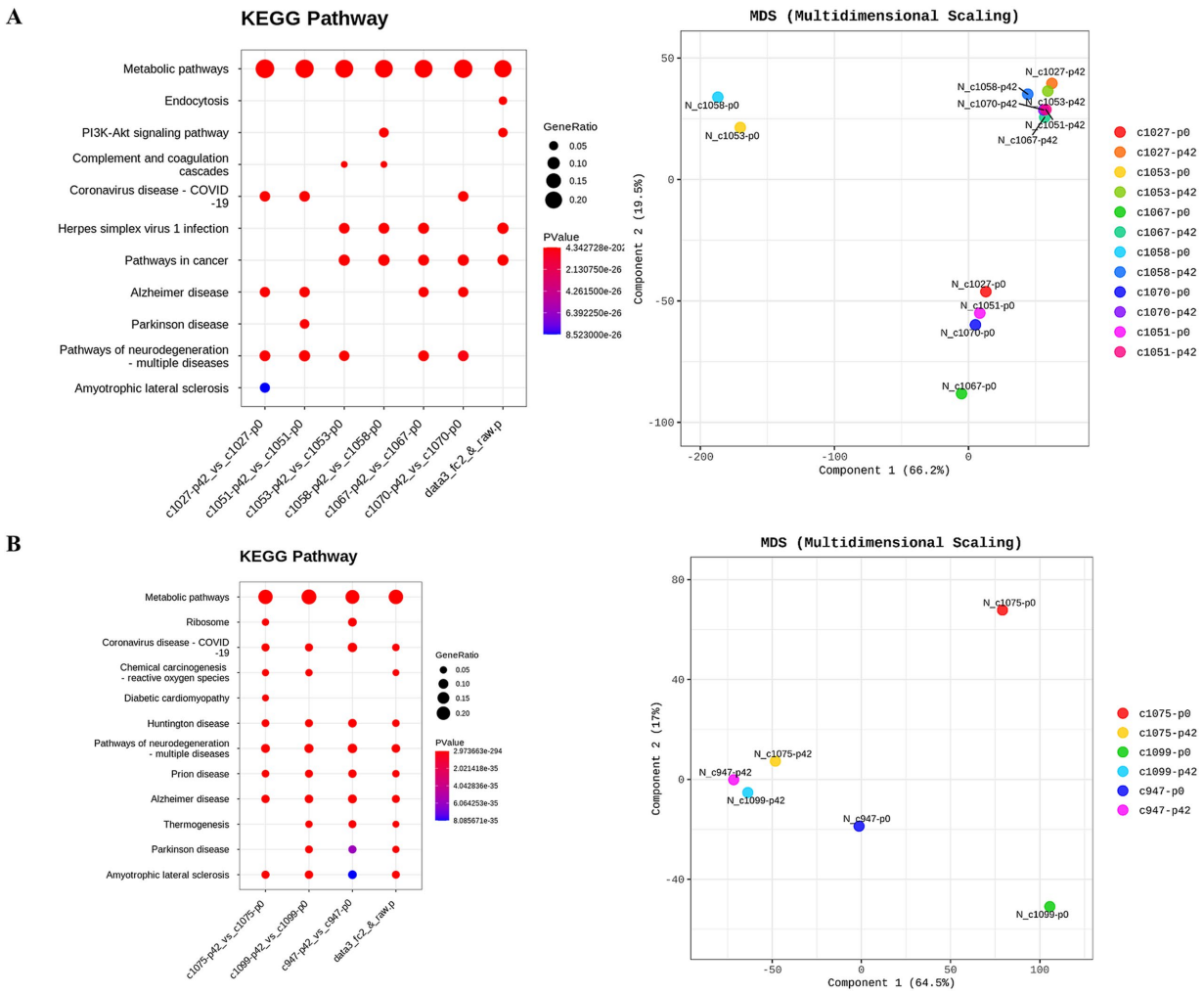


FIGURE 7
Investigation of transcriptional changes in liver tissues of monkeys undergoing fecal microbiota transplantation and probiotic therapy. Transcriptional changes were evaluated based on KEGG pathway database and multidimensional scaling analysis. (A) FMT + D group comprised C1027, C1053, C1075 and C1058 while (B) FMT + N group included C1067 and C1070, and probiotics group. P0 and P6 represent 0 weeks and 6 weeks post-FMT, respectively.

In both the FMT and probiotics groups, there was a dramatic increase in all SCFAs including acetate, butyrate, and propionate in samples from blood veins. In human cases, elevated levels of SCFAs have been observed following FMT procedures in patients with IBD (El-Salhy et al., 2021). Notably, the rise of SCFAs in samples from portal veins was significantly higher than those in femoral veins (Figure 6). SCFAs are generated in the colon by gut microbiota during the fermentation of non-digestible fibers and are subsequently absorbed by colonocytes and bloodstreams via portal vein to liver tissues (Bloemen et al., 2009). Due to these anatomical and physiological characteristics, the portal vein is more directly affected by changes in SCFA levels compared to the femoral vein. Therefore, the portal vein is the primary vessel directly influenced by gut microbiome–targeted treatments like FMT and probiotics (Ohtani and Hara, 2021). We predict more pronounced effects of SCFAs on liver functions through FMT and probiotics therapy via gut-liver axis, as we expected based on the results from peripheral blood. SCFAs significantly impact inflammation, immune response, anti-obesity, anti-diabetes, cardiovascular protection, hepatic protection, carcinogenesis and IBD (Visekruna and Luu, 2021; Xiong et al., 2022). Notably, butyrate promotes the differentiation of Treg cells, which play a key role in controlling inflammation (Furusawa et al., 2013). Therefore, these gut-targeted interventions may prove to be beneficial for human health from multiple aspects. These findings coincide with hepatic transcriptomic data indicating that various metabolic pathways were significantly altered following FMT and probiotic therapies (Figure 7).

The level of AST decreased in samples from the femoral veins, while ALT levels increased in samples from the portal veins (Figure 5). ALT levels are direct indicators of liver damage and are primarily found in the liver tissues (Goorden et al., 2013). On the other hand, significant concentrations of AST are not only present in the liver but also in the kidneys, skeletal muscles, and myocardium (Goorden et al., 2013). Therefore, considering anatomical and physiological characteristics of the two enzymes, ALT values from the portal vein might be the most sensitive indicator for diagnosing liver damage. We should closely monitor liver-related side effects in patients administered with FMT. With the observed simultaneous decline in CRE and BUN levels, FMT therapy could contribute to kidney health. Compared with FMT, the effects of probiotic therapy on monkeys

were relatively minor. FMT has been shown to improve not only liver function in human patients with non-alcoholic fatty liver disease, alcoholic liver disease, hepatitis B virus infection, and primary sclerosing cholangitis, but also renal function in patients with chronic kidney disease (CKD) (Zhao et al., 2023; Arteaga-Muller et al., 2024). Both the therapeutic and harmful effects of FMT on liver diseases have been reported in human patients, possibly linked to increased levels of SCFAs, which can be induced by FMT and probiotics (Pant et al., 2023). Additionally, SCFAs may alleviate CKD in human patients by modulating inflammation, oxidative stress, and cellular autophagy (He et al., 2024). Notably, we observed a statistically significant increase in monocyte values in the femoral veins of monkeys treated with probiotics, an effect absent in other groups (Figure 3). *In vitro* studies have shown that *Lactobacillus(L) rhamnosus* can enhance the expression of surface molecules related to monocyte activation, thereby potentially stimulating an immune response to probiotic therapy (Rückle et al., 2023).

In this study, we investigated the systemic and multifaceted effects of FMT and probiotic therapies on various anatomical sites in healthy individuals. FMT therapy has the potential to beneficially affect immunologic and hormonal responses and SCFAs, regardless of specific fecal conditions. These host responses have also been observed in human patients undergoing the FMT procedure. Given the anatomical differences, evaluating samples from the portal veins may provide more direct insights into the interventions on the liver through the gut-liver axis. Although some invasive procedures were essential for conducting more in-depth anatomical and physiological evaluations, all animals have been maintained in normal health with appropriate veterinary care following the procedures. In conclusion, these macaque models, which utilize surgery and colonoscopy, serve as a human-like preclinical platform for evaluating longitudinal effects and anatomically specific responses to gut-targeted interventions, without the need for animal sacrifice. This report has some limitations. The effects of probiotic therapy were measured with a focus on its short-term impact. In clinical practice, probiotic therapy is typically administered over a longer period than FMT, limiting the applicability of the current findings. Furthermore, the small sample size constrains the generalizability of the results, particularly for microbiome data, due to high inter-individual variability. Future research will involve a larger number of primates to validate these findings, explore underlying mechanisms, and assess the long-term effects of the therapies. Additionally, the therapeutic potential of these interventions needs to be evaluated in primate disease models.

Data availability statement

The raw dataset in this study are available into the NCBI sequence read archive (SRA) database under accession number PRJNA1182038.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee at Korea Research Institute of Bioscience and Biotechnology (Approval No. KRIBB-AEC-21324). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PK: Methodology, Validation, Writing – original draft, Software, Writing – review & editing. G-SB: Investigation, Methodology, Writing – review & editing. EJ: Investigation, Methodology, Writing – review & editing. JC: Writing – original draft, Data curation, Methodology. E-HH: Investigation, Writing – review & editing. GK: Methodology, Visualization, Writing – review & editing. SB: Data curation, Investigation, Writing – review & editing. KS: Data curation, Methodology, Visualization, Writing – review & editing. YA: Project administration, Writing – review & editing. KL: Investigation, Resources, Writing – review & editing. YK: Methodology, Writing – review & editing. TO: Methodology, Writing – review & editing. JH: Validation, Writing – review & editing. W-KL: Resources, Writing – review & editing. S-HK: Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Validation. B-SK: Conceptualization, Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science and ICT, MSIT) (2020R1C1C1003990, RS-2024-00343326), the grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (HR20C0025), and the grant of Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Programs (KGM4572431).

Acknowledgments

We thank breeders and veterinarians (National Primate Research Center) who contributed to NHPs care.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1458923/full#supplementary-material

References

Amato, K. R., Mallott, E. K., Mcdonald, D., Dominy, N. J., Goldberg, T., Lambert, J. E., et al. (2019). Convergence of human and Old World monkey gut microbiomes demonstrates the importance of human ecology over phylogeny. *Genome Biol.* 20, 1–12. doi: 10.1186/s13059-019-1807-z

Anand, S., and Mande, S. S. (2022). Host-microbiome interactions: gut-liver axis and its connection with other organs. *NPJ Biofilms Microbiomes* 8:89. doi: 10.1038/s41522-022-00352-6

Arteaga-muller, G. Y., Flores-trevióo, S., Bocanegra-ibarias, P., Robles-espino, D., Garza-Gonzãlez, E., Fabela-valdez, G. C., et al. (2024). Changes in the progression of chronic kidney disease in patients undergoing fecal microbiota transplantation. *Nutrients* 16:1109. doi: 10.3390/nu16081109

Baunwall, S. M. D., Lee, M. M., Eriksen, M. K., Mullish, B. H., Marchesi, J. R., Dahlerup, J. F., et al. (2020). Faecal microbiota transplantation for recurrent Clostridioides difficile infection: an updated systematic review and meta-analysis. *EClinicalMedicine* 29-30:100642. doi: 10.1016/j.eclinm.2020.100642

Bloemen, J. G., Venema, K., Van De Poll, M. C., Damink, S. W. O., Buurman, W. A., and Dejong, C. H. (2009). Short chain fatty acids exchange across the gut and liver in humans measured at surgery. *Clin. Nutr.* 28, 657–661. doi: 10.1016/j.clnu.2009.05.011

Brenchley, J. M., and Ortiz, A. M. (2021). Microbiome studies in non-human primates. *Curr. HIV/AIDS Rep.*, 18, 527–537. doi: 10.1007/s11904-021-00584-9

Buonacera, A., Stancanelli, B., Colaci, M., and Malatino, L. (2022). Neutrophil to lymphocyte ratio: an emerging marker of the relationships between the immune system and diseases. *Int. J. Mol. Sci.* 23:3636. doi: 10.3390/ijms23073636

Canfora, E. E., Jocken, J. W., and Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat. Rev. Endocrinol.* 11, 577–591. doi: 10.1038/nrendo.2015.128

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., and Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microb. Ecol. Health Dis.* 26:26191. doi: 10.3402/mehd.v26.26191

Chen, Y.-Y., Chen, D.-Q., Chen, L., Liu, J.-R., Vaziri, N. D., Guo, Y., et al. (2019). Microbiome–metabolome reveals the contribution of gut–kidney axis on kidney disease. *J. Transl. Med.* 17, 1–11. doi: 10.1186/s12967-018-1756-4

Cheng, Y.-W., Phelps, E., Ganapini, V., Khan, N., Ouyang, F., Xu, H., et al. (2019). Fecal microbiota transplantation for the treatment of recurrent and severe *Clostridium difficile* infection in solid organ transplant recipients: a multicenter experience. *Am. J. Transplant.* 19, 501–511. doi: 10.1111/ajt.15058

Colman, R. J., and Rubin, D. T. (2014). Fecal microbiota transplantation as therapy for inflammatory bowel disease: a systematic review and meta-analysis. *J. Crohn's Colitis* 8, 1569–1581. doi: 10.1016/j.crohns.2014.08.006

De Groot, P., Nikolic, T., Pellegrini, S., Sordi, V., Imangaliyev, S., Rampanelli, E., et al. (2021). Faecal microbiota transplantation halts progression of human new-onset type 1 diabetes in a randomised controlled trial. *Gut* 70, 92–105. doi: 10.1136/gutjnl-2020-322630

Douglas, A. E. (2019). Simple animal models for microbiome research. *Nat. Rev. Microbiol.* 17, 764–775. doi: 10.1038/s41579-019-0242-1

Drekonja, D., Reich, J., Gezahegn, S., Greer, N., Shaukat, A., Macdonald, R., et al. (2015). Fecal microbiota transplantation for *Clostridium difficile* infection: a systematic review. *Ann. Intern. Med.* 162, 630–638. doi: 10.7326/M14-2693

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638. doi: 10.1126/science.1110591

El-salhy, M., Valeur, J., Hausken, T., and Gunnar Hatlebakk, J. (2021). Changes in fecal short-chain fatty acids following fecal microbiota transplantation in patients with irritable bowel syndrome. *Neurogastroenterol. Motil.* 33:e13983. doi: 10.1111/nmo.13983

Enav, H., Bæckhed, F., and Ley, R. E. (2022). The developing infant gut microbiome: a strain-level view. *Cell Host Microbe* 30, 627–638. doi: 10.1016/j.chom.2022.04.009

Fang, H., Fu, L., Li, X., Lu, C., Su, Y., Xiong, K., et al. (2021). Long-term efficacy and safety of monotherapy with a single fresh fecal microbiota transplant for recurrent active ulcerative colitis: a prospective randomized pilot study. *Microb. Cell Factories* 20, 1–14. doi: 10.1186/s12934-021-01513-6

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504, 446–450. doi: 10.1038/nature12721

Gheorghe, C. E., Martin, J. A., Manriquez, F. V., Dinan, T. G., Cryan, J. F., and Clarke, G. (2019). Focus on the essentials: tryptophan metabolism and the microbiome-gut-brain axis. *Curr. Opin. Pharmacol.* 48, 137–145. doi: 10.1016/j.coph.2019.08.004

Goloshchapov, O. V., Olekhnovich, E. I., Sidorenko, S. V., Moiseev, I. S., Kucher, M. A., Fedorov, D. E., et al. (2019). Long-term impact of fecal transplantation in healthy volunteers. *BMC Microbiol.* 19, 1–13. doi: 10.1186/s12866-019-1689-y

Goorden, S., Buffart, T. E., Bakker, A., and Buijs, M. M. (2013). Liver disorders in adults: ALT and AST. *Ned. Tijdschr. Geneeskd.* 157:A6443.

He, M., Wei, W., Zhang, Y., Xiang, Z., Peng, D., Kasimumali, A., et al. (2024). Gut microbial metabolites SCFAs and chronic kidney disease. *J. Transl. Med.* 22:172. doi: 10.1186/s12967-024-04974-6

He, J., Zhang, P., Shen, L., Niu, L., Tan, Y., Chen, L., et al. (2020). Short-chain fatty acids and their association with signalling pathways in inflammation, glucose and lipid metabolism. *Int. J. Mol. Sci.* 21:6356. doi: 10.3390/ijms21176356

Herrero-Cervera, A., Soehnlein, O., and Kenne, E. (2022). Neutrophils in chronic inflammatory diseases. *Cell. Mol. Immunol.* 19, 177–191. doi: 10.1038/s41423-021-00832-3

Koo, B. S., Baek, S. H., Kim, G., Hwang, E. H., Oh, H., Son, Y., et al. (2020). Idiopathic chronic diarrhea associated with dysbiosis in a captive cynomolgus macaque (*Macaca fascicularis*). *J. Med. Primatol.* 49, 56–59. doi: 10.1111/jmp.12447

Lee, M., and Chang, E. B. (2021). Inflammatory bowel diseases (IBD) and the microbiome—searching the crime scene for clues. *Gastroenterology* 160, 524–537. doi: 10.1053/j.gastro.2020.09.056

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Human gut microbes associated with obesity. *Nature* 444, 1022–1023. doi: 10.1038/4441022a

Lloyd-Price, J., Abu-Ali, G., and Huttenhower, C. (2016). The healthy human microbiome. *Genome Med.* 8, 1–11. doi: 10.1186/s13073-016-0307-y

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Machiels, K., Joossens, M., Sabino, J., De Preter, V., Arijs, I., Eeckhaut, V., et al. (2014). A decrease of the butyrate-producing species Roseburia hominis and *Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut* 63, 1275–1283. doi: 10.1136/gutjnl-2013-304833

Napolitano, M., and Covasa, M. (2020). Microbiota transplant in the treatment of obesity and diabetes: current and future perspectives. *Front. Microbiol.* 11:590370. doi: 10.3389/fmicb.2020.590370

National Research Council (2010). Guide for the care and use of laboratory animals. Washington, DC: National Academies Press.

Ohtani, N., and Hara, E. (2021). Gut-liver axis-mediated mechanism of liver cancer: a special focus on the role of gut microbiota. *Cancer Sci.* 112, 4433–4443. doi: 10.1111/cas.15142

Pant, K., Venugopal, S. K., Pisarello, M. J. L., and Gradilone, S. A. (2023). The role of gut microbiome-derived short chain fatty acid butyrate in hepatobiliary diseases. *Am. J. Pathol.* 193, 1455–1467. doi: 10.1016/j.ajpath.2023.06.007

Park, J. C., and Im, S.-H. (2020). Of men in mice: the development and application of a humanized gnotobiotic mouse model for microbiome therapeutics. *Exp. Mol. Med.* 52, 1383–1396. doi: 10.1038/s12276-020-0473-2

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Rückle, X., Rþhle, J., Judd, L., Hebel, J., Dietz, S., Poets, C. F., et al. (2023). Different probiotic strains alter human cord blood monocyte responses. *Pediatr. Res.* 94, 103–111. doi: 10.1038/s41390-022-02400-5

Shi, N., Li, N., Duan, X., and Niu, H. (2017). Interaction between the gut microbiome and mucosal immune system. *Mil. Med. Res.* 4, 1–7. doi: 10.1186/s40779-017-0122-9

Singh, S., Anshita, D., and Ravichandiran, V. (2021). MCP-1: function, regulation, and involvement in disease. *Int. Immunopharmacol.* 101:107598. doi: 10.1016/j.intimp.2021.107598

Sinha, S., Lin, G., and Ferenczi, K. (2021). The skin microbiome and the gut-skin axis. *Clin. Dermatol.* 39, 829–839. doi: 10.1016/j.clindermatol.2021.08.021

Sokol, H., Landman, C., Seksik, P., Berard, L., Montil, M., Nion-Larmurier, I., et al. (2020). Fecal microbiota transplantation to maintain remission in Crohn's disease: a pilot randomized controlled study. *Microbiome* 8, 1–14. doi: 10.1186/s40168-020-0792-5

Sommer, F., Anderson, J. M., Bharti, R., Raes, J., and Rosenstiel, P. (2017). The resilience of the intestinal microbiota influences health and disease. *Nat. Rev. Microbiol.* 15, 630–638. doi: 10.1038/nrmicro.2017.58

Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., Dori-Bachash, M., Bashiardes, S., et al. (2018). Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell* 174, 1406–1423. doi: 10.1016/j.cell.2018.08.047

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R., and Gordon, J. I. (2007). The human microbiome project. *Nature* 449, 804–810. doi: 10.1038/nature06244

Visekruna, A., and Luu, M. (2021). The role of short-chain fatty acids and bile acids in intestinal and liver function, inflammation, and carcinogenesis. *Front. Cell Dev. Biol.* 9:703218. doi: 10.3389/fcell.2021.703218

Wahlstrøm, A., Sayin, S. I., Marschall, H.-U., and Bæckhed, F. (2016). Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab.* 24, 41–50. doi: 10.1016/j.cmet.2016.05.005

Wu, Z., Zhang, B., Chen, F., Xia, R., Zhu, D., Chen, B., et al. (2023). Fecal microbiota transplantation reverses insulin resistance in type 2 diabetes: a randomized, controlled, prospective study. *Front. Cell. Infect. Microbiol.* 12:1929. doi: 10.3389/fcimb.2022.1089991

Xiong, R.-G., Zhou, D.-D., Wu, S.-X., Huang, S.-Y., Saimaiti, A., Yang, Z.-J., et al. (2022). Health benefits and side effects of short-chain fatty acids. *Food Secur.* 11:2863. doi: 10.3390/foods11182863

Yasuda, K., Oh, K., Ren, B., Tickle, T. L., Franzosa, E. A., Wachtman, L. M., et al. (2015). Biogeography of the intestinal mucosal and lumenal microbiome in the rhesus macaque. *Cell Host Microbe* 17, 385–391. doi: 10.1016/j.chom.2015.01.015

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Zhang, T., Li, Q., Cheng, L., Buch, H., and Zhang, F. (2019). *Akkermansia muciniphila* is a promising probiotic. *Microb. Biotechnol.* 12, 1109–1125. doi: 10.1111/1751-7915.13410

Zhao, Y., Gong, C., Xu, J., Chen, D., Yang, B., Chen, Z., et al. (2023). Research progress of fecal microbiota transplantation in liver diseases. *J. Clin. Med.* 12:1683. doi: 10.3390/jcm12041683

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 29 January 2025
DOI 10.3389/fmicb.2025.1452423

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Munis Dundar,
Erciyes University, Türkiye
Lemonica Koumbi,
International Hellenic University, Greece

*CORRESPONDENCE
Lihui Wu
✉ jaemny@163.com

RECEIVED 22 July 2024
ACCEPTED 15 January 2025
PUBLISHED 29 January 2025

CITATION
Lu J, Jiang M, Chai D, Sun Y and Wu L (2025) Integrative analysis of intestinal flora and untargeted metabolomics in attention-deficit/hyperactivity disorder.
Front. Microbiol. 16:1452423.
doi: 10.3389/fmicb.2025.1452423

COPYRIGHT
© 2025 Lu, Jiang, Chai, Sun and Wu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Integrative analysis of intestinal flora and untargeted metabolomics in attention-deficit/hyperactivity disorder

Jiamin Lu[1], Maoying Jiang[2], Dingyue Chai[1], Yuzi Sun[1] and Lihui Wu[1]*

[1]Departments of Basic Medicine and Forensic Medicine, Hangzhou Medical College, Hangzhou, China, [2]Behavioral Pediatric Department and Child Primary Care Department, Hangzhou Children's Hospital, Hangzhou, China

Attention Deficit Hyperactivity Disorder (ADHD) is a clinically common neurodevelopmental disorder of the brain. In addition to genetic factors, an imbalance in gut flora may also play a role in the development of ADHD. Currently, it is critical to investigate the function of gut flora and related metabolites, which may form the fundamental basis of bidirectional cross-linking between the brain and the gut, in addition to focusing on the changed gut flora in ADHD. This study aimed to investigate the possible relationship between changes in gut flora and metabolites and ADHD by analyzing metagenome and untargeted metabolomics of fecal samples from ADHD patients. Specifically, we attempted to identify key metabolites and the metabolic pathways they are involved in, as well as analyze in detail the structure and composition of the gut flora of ADHD patients. In order to further investigate the relationship between gut flora and ADHD symptoms, some behavioral studies were conducted following the transplantation of gut flora from ADHD patients into rats. The results of the metagenome analysis revealed several distinct strains, including *Bacteroides cellulosilyticus*, which could be important for diagnosing ADHD. Additionally, the ADHD group showed modifications in several metabolic pathways and metabolites, including the nicotinamide and nicotinic acid metabolic pathways and the metabolite nicotinamide in this pathway. The behavioral results demonstrated that rats with ADHD gut flora transplants displayed increased locomotor activity and interest, indicating that the onset of behaviors such as ADHD could be facilitated by the flora associated with ADHD. This research verified the alterations in gut flora and metabolism observed in ADHD patients and provided a list of metabolites and flora that were significantly altered in ADHD. Simultaneously, our findings revealed that modifications to the microbiome could potentially trigger behavioral changes in animals, providing an experimental basis for comprehending the function and influence of gut flora on ADHD. These results might provide new perspectives for the development of novel treatment strategies.

KEYWORDS

attention deficit hyperactivity disorder, intestinal flora, metagenomics, metabolomics, fecal microbial transplantation

1 Introduction

The gut is not only a key component of the digestive system but also a crucial component of the immune system. An independent neurological system called the enteric nervous system exists in the gastrointestinal tract. Millions of neurons make up the enteric nervous system, which is closely connected to the brain and controls the activity, secretory function, and blood circulation of the gut (Dabke et al., 2019; Margolis et al., 2021). Gut microbiome refers to the vast population of bacteria that reside in the human gut (Backhed et al., 2005). The microbiota create a complex ecosystem that is essential for controlling metabolism in addition to aiding in food digestion, detoxifying medications and poisons, and maintaining the health of the immune system (Farzi et al., 2018; Frisbee and Petri, 2020; Zeineldin et al., 2018). There may be a significant metabolic potential for the bacteria in the gut microbiota as their number is similar to that of human cells (Sender et al., 2016). In addition, gut microbes can reduce inflammation, modify gene expression, and affect the synthesis of neurotransmitters - particularly the latter as they have a direct bearing on behavior and mood (Sasso et al., 2023). Clinical problems such as obesity, diabetes, cardiovascular disease, and allergies are significantly linked to an imbalance in the gut microbiota (Fukuda et al., 2011; Kelly et al., 2016; Orivuori et al., 2015; Safari et al., 2019). Furthermore, gut microbial dysbiosis has been linked to several types of neurological conditions, including attention deficit/hyperactivity disorder (ADHD), depression, anxiety disorders, and autism spectrum disorders (Aizawa et al., 2016; Bundgaard-Nielsen et al., 2020; Cheng et al., 2020; Kang et al., 2013; Reimherr et al., 2017; Rosenfeld, 2015; Sharon et al., 2019). Research has indicated that altering the composition of the gut microbiota may be a useful strategy for both treating and preventing certain neurological conditions. Thus, designing novel therapeutic approaches requires a thorough understanding of the gut microbiota and its roles.

ADHD, a highly heritable neurodevelopmental disorder, is characterized by distractibility, hyperactivity, and impulsivity (Martinez-Badia and Martinez-Raga, 2015; Wustner et al., 2019). Patients with ADHD experience profound consequences in social and emotional management as well as in their academic and professional lives (Usami, 2016). Numerous elements, including pathophysiology, neurobiology, etiology, and genetics, are involved in the complicated pathogenesis of ADHD (Hinshaw, 2018). There is currently no solid evidence regarding the precise causes of ADHD. According to recent research, changes in gut flora may be linked to the onset of ADHD.

Three main pathways explain the relationship between gut microbiota and ADHD: the microbes directly produce metabolites and neurotransmitters that affect the nervous system (Logsdon et al., 2018), they act on the vagus nerve to affect the brain (Carabotti et al., 2015), and they stimulate peripheral immune cells to affect the blood–brain barrier and other nervous system factors (Amini-Khoei et al., 2019; Fung et al., 2017). There is a notable variation in the intestinal flora composition of ADHD patients when compared to the healthy population, according to an investigation of their gut flora (Wan et al., 2020). The overall composition and relative abundance of the flora reflect this variation; however, there is currently inconclusive research in this area. Focusing on particular microbiota, it was discovered that Bifidobacterium, Odorobacterium, Enterococcus, Neisseria, and Clostridium showed an increasing trend in relative abundance when studied in ADHD, while Microbacterium, Lachnoclostridium, Faecalibacterium, and Parabacterium showed a decreasing trend in relative abundance (Ming et al., 2018; Wang et al., 2020). Szopinska-Tokov showed that the Ruminococcaceae_UGC_004 and inattentive symptoms of ADHD are related (Szopinska-Tokov et al., 2020). This underscores the possibility that alterations in gut flora are linked to the neuropathological mechanisms of ADHD. These findings add to our knowledge of the pathogenic mechanisms underlying ADHD and may provide key hints for the creation of novel therapeutic strategies. Nevertheless, more thorough research is required to gain a more precise understanding of the connection between gut flora and ADHD.

While the interaction between the brain and gut microorganisms is a topic of current research, a complete understanding of the mechanism involved may not always be revealed by concentrating only on the gut germs. As the foundation for two-way communication between the brain and gut flora, attention must also be given to the impact of gut flora on metabolite production. According to some research, the brain-gut axis affects the brain by regulating the generation and function of neurotransmitters via vagal stimulation, immune system reactions, bacterial metabolites, or activation of the hypothalamic–pituitary–adrenal (HPA) axis (Bonaz et al., 2019; De Punder and Pruimboom, 2015; Eicher and Mohajeri, 2022; Fu et al., 2023). Simultaneously, the brain can modify gut secretion, peristalsis, and sensation, which in turn can affect the makeup and activity of the gut bacteria (Margolis et al., 2021). In the case of ADHD, for instance, an increase in certain gut microorganisms such as Bifidobacterium may raise blood tryptophan levels (Desbonnet et al., 2008). This can then be converted to 5-hydroxytryptophan in the brain, which has an impact on mood and cognitive performance. Additionally, phenylalanine, a precursor of the neurotransmitters dopamine and norepinephrine (Aarts et al., 2017), which are essential for mood regulation and cognitive function, is increased by Bifidobacterium (Coleman et al., 2019). Disturbances in these neurotransmitters may be linked to pathomechanisms related to ADHD. Thus, variations in the gut microbiota's composition may result in variations in neurotransmitter production, which may then play a role in the emergence of neurological diseases like ADHD. The aforementioned results highlight the significance of gaining a more comprehensive understanding of the function of gut flora and metabolites in disease preventive and treatment approaches.

In summary, the properties of the gut microbiota and metabolites in ADHD, as well as their interactions, are not well understood. The relationship between alterations in gut microbiota and metabolites and ADHD has not been clearly established by previous research. In order to detect specific alterations in microbiota and metabolites in the ADHD gut and investigate the relationship between them, our study evaluated shotgun metagenomic and untargeted metabolomics from ADHD patients and healthy controls in tandem. To investigate the relationship between gut microbiota and symptoms of ADHD, we also transplanted microbiota from ADHD patients into Sprague–Dawley (SD) rats and assessed the impact these microbiota produced in the rats. We hope to provide new perspectives for the development of novel ADHD treatment plans.

2 Methods

2.1 Participant selection

During July and August of 2020, five children who had been initially diagnosed with ADHD were chosen from Hangzhou Children's Hospital. The following criteria had to be met for a subject to be included: (1) participants had to meet the DSM-V criteria for ADHD diagnosis (Austerman, 2015; Posner et al., 2020); (2) participants could not have serious organic diseases affecting the neurological, hepatic, cardiovascular, or hematopoietic systems; (3) participants could not have a history of digestive or other chronic diseases; (4) participants could not have an allergy such as allergic rhinitis or asthma; (5) participants' body mass index could not be less than 20 kg/m2; and (6) participants had to be free of probiotics, related traditional Chinese medicine, psychostimulant drugs, etc. and other combined treatment methods such as electroencephalography biofeedback. The healthy control (HC) group consisted of eight children from various households who were chosen at the same time and under the same inclusion criteria, with the exception that the HCs did not have an ADHD diagnosis based on the DSM-V criteria. The Conners Parent Rating Scale was utilized by the parents of each participant involved to evaluate the degree of symptoms associated with ADHD. Feces samples were obtained between 8:00 and 12:00 a.m. Before testing, the stool samples were transported at 4°C and stored at −80°C. The parents or legal guardians of the subjects provided verbal informed consent.

2.2 Shotgun metagenome sequencing of fecal samples

DNA was extracted from human feces samples using the hexadecyl trimethyl ammonium bromide method. DNA degradation degree, potential contamination and DNA concentration were measured using an Agilent 5,400. Sequencing libraries were generated using the NEBNext@ UltraTM DNA Library Prep Kit for Illumina. The library preparations were sequenced on an Illumina Novaseq 6,000 platform and paired-end reads were generated. The raw data of bacteria, fungi and viruses in human feces samples were obtained by metagenomic sequencing using the Illumina Novaseq high-throughput sequencing platform. In order to ensure the reliability of data, the raw sequencing data was preprocessed using Kneaddata software. Kraken2 and the self-build microbial database were used to identify the species contained in the samples, and then Bracken was used to predict the actual relative abundance of species in the samples. The clean reads after quality control and de-host were used to obtain a blast database (UniRef90) using HUMAnN2 software, and the annotation information and relative abundance table from each functional database were obtained according to the corresponding relationship between Uniref90 ID and each database. Based on the species abundance table and functional abundance table, abundance clustering analysis, Principal Coordinates Analysis (PCoA) analysis (species only) and sample clustering analysis were performed. When grouping information was available, Linear discriminant analysis Effect Size (LEfSe) Biomarker and Dunn test analyses were performed to excavate differences in species composition and functional composition between samples.

2.3 Untargeted metabolomics of fecal samples

Analysis was performed using an ultra-high performance liquid chromatography coupled to a quadrupole time-of-flight (TOF) mass spectrometer. For hydrophilic interaction chromatography separation, samples were analyzed using a 2.1 mm × 100 mm ACQUIY UPLC BEH Amide 1.7 μm column. In both ESI positive and negative modes, the mobile phase contained A = 25 mM ammonium acetate and 25 mM ammonium hydroxide in water and B = acetonitrile. The gradient was 95% B for 0.5 min and was linearly reduced to 65% in 6.5 min, and then reduced to 40% in 1 min and kept for 1 min, and then increased to 95% in 0.1 min, with a 3 min re-equilibration period employed. The ESI source conditions were set as follows: Ion Source Gas1 (Gas1) as 60, Ion Source Gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature: 600°C, IonSpray Voltage Floating (ISVF) ± 5,500 V. In mass spectrometry (MS) only acquisition, the instrument was set to acquire over the m/z range 60–1,000 Da, and the accumulation time for the TOF MS scan was set at 0.20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25–1,000 Da, and the accumulation time for product ion scan was set at 0.05 s/spectra. The product ion scan was acquired using information dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was fixed at 35 V with ±15 eV; declustering potential (DP), 60 V (+) and − 60 V (−); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10. The raw MS data were converted to MzXML files using ProteoWizard MSConvert. For peak picking, the following parameters were used: centWave m/z = 10 ppm, peakwidth = c (10, 60), prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, and minfrac = 0.5 were used. CAMERA (Collection of Algorithms of Metabolite pRofile Annotation) was used for annotation of isotopes and adducts. In the extracted ion features, only the variables having more than 50% of the nonzero measurement values in at least one group were kept. Compound identification of metabolites was performed by comparing the accuracy of m/z value (<10 ppm), and MS/MS spectra with an in-house database established with available authentic standards.

2.4 Fecal microbial transplantation and behavioral experiments

2.4.1 Animals

Male SD rats were purchased from Beijing Viton Lihua Laboratory Animal Co. All the rats were given free access to food and water and were gradually acclimated to the laboratory conditions (12-h light/dark cycle, 22 ± 1°C, and 55 ± 5% relative humidity). To reduce animal suffering, the number of animals utilized in the study was kept to a minimum.

2.4.2 Fecal microbial transplantation

In order to eliminate individual differences, fecal samples from five ADHD patients and eight HCs were processed and mixed in sterile tubes. To obtain the final bacterial solution for usage, 1 g of feces was combined with 10 mL of sterile PBS, vortexed for approximately 10 min, and then filtered through 200, 400, and 800 mesh in that order. The configured bacterial solution was stored at

−80°C after adding glycerol, and the proportion of bacterial solution to glycerol was 1:1. The solution was thawed in a 37°C water bath and vortexed once more before use.

Figure 1 depicts the experiment timeframe. Four distinct animal groupings were established: Blank (no disposition), HC.T, and ADHD.T, Transoral gavage was used to populate the microbiota from HCs and ADHD patients, respectively, and sterile PBS was used to set up a sham-operated group. Rats underwent microbial colonization on days 1, 2, 14, 15, 22, and 23 of the experiment to guarantee that their gut microbiome remained consistent.

2.4.3 Behavioral experiments

2.4.3.1 Open field test

The experimental box for the OFT was 100 cm × 100 cm × 40 cm. The bottom surface was uniformly divided into 16 compartments, with the central area consisting of the middle four compartments and the peripheral area consisting of the remaining 12 compartments. The rats were positioned in the open field's lower left corner at the start of the experiment, and they were allowed to wander around freely for 5 min. The system then automatically logged the data. Calculations were made to determine how long the rat spent engaging in activities in the central zone, how many times it entered the zone overall, how far it went in 5 min, and how many times it stood straight up. The total distance traveled and the activity time in the central area reflected the locomotor activity of the rats, and the number of times they stood upright reflected their curiosity and interest in exploring the outside world.

2.4.3.2 Novel object recognition

The experimental equipment was a square laboratory box of 50 cm × 50 cm × 45 cm. Three objects were used: A, B, and C. Rectangles A and B were the same, while C was a colored cylinder. During the training phase, A and B were placed in the center of the experimental box. The rats were placed in the experimental box with their backs to these objects and it was ensured that the rats' noses were equidistant from these two objects, and the rats remained in the experimental box for 10 min. In the test phase 24 h later, B was replaced by C, and the rat was placed in the same direction and distance for 5 min. Every encounter the rats had with these objects was meticulously documented, down to the number of times they made contact and the amount of time they spent moving within two to three centimeters of them. Static activities, such as maintaining a position on the object, were not regarded as exploratory behaviors; instead, actions such as patting with the paw, smelling with the nose, or licking the object with the mouth were documented as such. By comparing the frequency and duration of exploring new and old things, the cognitive ability of rats was deduced; a higher frequency and longer duration of exploring new objects indicates normal cognition. The Recognition Index (RI) was calculated as RI = Explore New Objects/ (Explore New Objects + Explore Old Objects) × 100%.

2.4.3.3 Marble burying test

An identical rat cage that was used to house the rats was used as the experimental box. The experimental box was lined with padding approximately 5 cm thick. Glass marbles were gently placed on top of the padding, and were arranged neatly using the 4 × 6 rule. The rats were carefully positioned in a corner of the cage and did not touch the glass marbles when the experiment began. The observer left the test room after placement. The rats were allowed to move freely in the cage without disturbance for 30 min, during which time they were not given water or food. Thirty minutes later, the rats were taken out of the cage, taking care not to move the glass marbles. The number of marbles buried by the rats (marbles with 1/2 or more of their surface area covered were considered buried). The number of buried marbles reflected the impulsiveness of the rats, with more buried marbles indicating greater impulsiveness.

2.5 Statistical analyses

All sequencing data were analyzed at the Wekemo Bioincloud online site. The concept of amplicon was borrowed for intergroup diversity analysis of the metagenome. PCoA analysis based on Bray Curtis distance was performed to test the similarity of bacterial community structure between samples. Intergroup LEfSe analysis was performed to determine biomarkers from phylum to species level (LDA > 2, $p < 0.05$). Orthogonal Partial Least Squares Discrimination

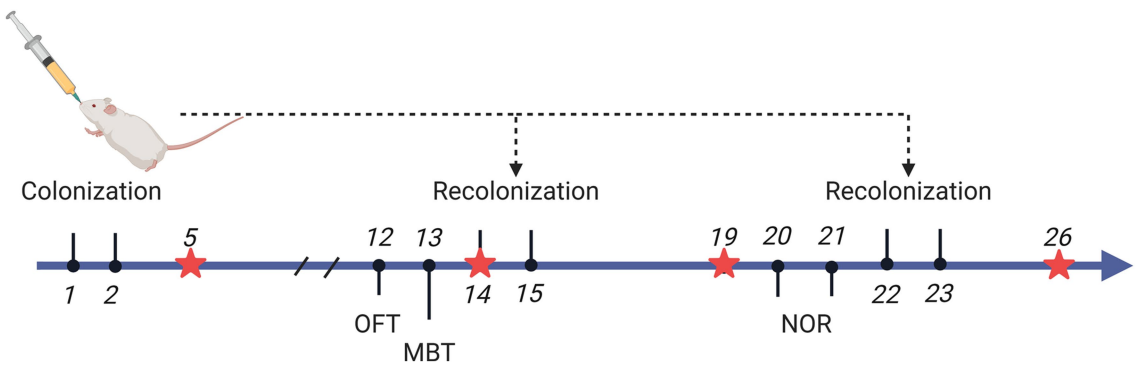


FIGURE 1
Experimental Timeline. Rats underwent microbial colonization on days 1, 2, 14, 15, 22, and 23 of the experiment, while fecal samples were collected on days 5, 14, 19, and 26. On day 14, fecal collection was performed prior to colonization. Behavioral experiments were conducted on day 12 (open field test), day 13 (marble burying test), and days 20 and 21 (training and testing for the novel object recognition test). Created with BioRender.com.

Analysis (OPLS-DA) was performed to determine metabolite features between groups (VIP > 1, $p < 0.05$). Other data were analyzed using GraphPad statistical software. The Shapiro–Wilk test was performed on the measures first, and then the unpaired t-test (normal distribution) or Kruskal-Wallis test (non-normal distribution) was used to compare the differences between the groups. Values of $p < 0.05$ were considered to be statistically significant. Finally, the statistical power (1-β) of the experimental design was calculated using the G*Power software, with a one-way ANOVA: Fixed effects, omnibus test applied.

3 Results

3.1 Participants characteristics

According to the evaluation criteria, eight healthy control children and five patients with ADHD were included. The individuals in both groups were male thus excluding the influence of sex. Table 1 displays the metadata for the two groups. The mean age of the ADHD group was (9.1 ± 0.9) years, while the mean age of the HC group was (8.9 ± 0.8) years. There was no statistically significant difference in age between the two groups ($p = 0.74$), indicating that age did not influence the study's findings. The Conners Scale, Weiss's Functional Degree of Impairment Scale, and the SNAP-IV Teacher Rating Scale were used to diagnose the ADHD patients.

3.2 Differences in microbiota composition between ADHD and healthy controls

All samples underwent shotgun metagenomic sequencing. Nine distinct species types were identified in the two sets of samples, with bacteria accounting for the largest share at 87.16%. In total, 1,195 different bacterial species were found in the two sample groups; 318 of these species were unique to the ADHD group, while 193 species were unique to the HC group as shown in Figure 2A. There was no discernible difference between the ADHD and HC groups in terms of the bacterial groups' alpha and beta diversity (Figure 2B). Examination of the sample composition showed that Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria had the largest relative abundances in both groups at the phylum level (Figure 2C). As shown in Figure 2E, the relative abundances of *Bacteroidetes*, *Bacteroidia*, *Bacteroidalestes*, *Bacteroidaceae*, and *Bacteroides* were higher in the ADHD group than in the HC group, but no significant difference was observed (all $p > 0.05$).

TABLE 1 Donor metadata.

Donor ID	Group	Sex	Age	Diagnosis
HC-1	HC	M	9	NA
HC-2	HC	M	9	NA
HC-3	HC	M	8	NA
HC-4	HC	M	8.5	NA
HC-5	HC	M	9	NA
HC-6	HC	M	10	NA
HC-7	HC	M	8	NA
HC-8	HC	M	10	NA
ADHD-1	ADHD	M	9.9	ADHD
ADHD-2	ADHD	M	8.4	ADHD
ADHD-3	ADHD	M	8.9	ADHD
ADHD-4	ADHD	M	8.4	ADHD
ADHD-5	ADHD	M	10	ADHD

M, male; NA, not diagnosed.

LEfSe analysis can be used to identify biological markers with substantial variations between subgroups. The LEfSe analysis results indicated that a total of 5 families, 7 genera, and 23 species had different relative abundances between the two groups (LDA score > 2.0, $p < 0.05$), as indicated in Table 2. Detailed results of all differential bacteria with p values are provided in Supplementary Table S1. Compared to the HC group, the ADHD group had a greater relative abundance of nine species, such as *Bacteroides cellulosilyticus* and *Enterococcus saigonensis*. Furthermore, the ADHD group had 61 characteristic KO genes. ADHD-specific bacterial species are shown in Figure 3. Column lengths represent the Linear discriminant analysis (LDA) values; the larger the LDA value, the larger the difference.

3.3 Higher activity and curiosity were observed in SD rats receiving microbiota colonization from ADHD patients

It is unknown how alterations in the microbiota of ADHD patients relate to symptoms of the disease, even though shotgun metagenomic sequencing data have shown that the gut microbiota of ADHD patients differed from that of healthy persons. In order to investigate this relationship further, we conducted several behavioral tests on SD rats after isolating and transplanting the gut microbiota from ADHD patients and HCs. Furthermore, two sham-operated groups were established: one with PBS intervention and the other blank control group receiving no treatment at all. In contrast to the other three groups, the OFT results demonstrated a significant increase in the rat^{ADHD} group's total distance traveled in terms of activity and the number of uprights in the open field. This suggested that the rat^{ADHD} group exhibited higher levels of movement and a stronger interest in external exploration, which is consistent with behavior that is similar to ADHD. The other measures in the OFT, such as the number of entries into the central zone, the distance traveled, and the amount of time spent in the central zone did not differ substantially across the groups, as depicted in Figure 4.

In the training portion of the NOR experiment, which evaluates memory, all rats were equally able to investigate two similar objects. The rat^{ADHD} group did not exhibit a trend to lower the discrimination index when compared to the control groups throughout the 24-h test phase. However, they did investigate new objects less frequency and fewer times overall than they did during the training phase. The MBT can be used to identify obsessive or impulsive behaviors in animals. Our research revealed no discernible variation in the number of marbles buried in the rat^{ADHD} group when compared to the other rat groups. All data and analyses from the behavioral experiments are presented in Supplementary Tables S2, S3.

To evaluate the validity of the experimental design and its ability to detect group differences, a statistical power analysis was performed on the key behavioral indicators mentioned above. The results presented in Table 3 were obtained with a significance level of $\alpha = 0.05$, a total sample size of 40, and a group size of four. The analysis revealed

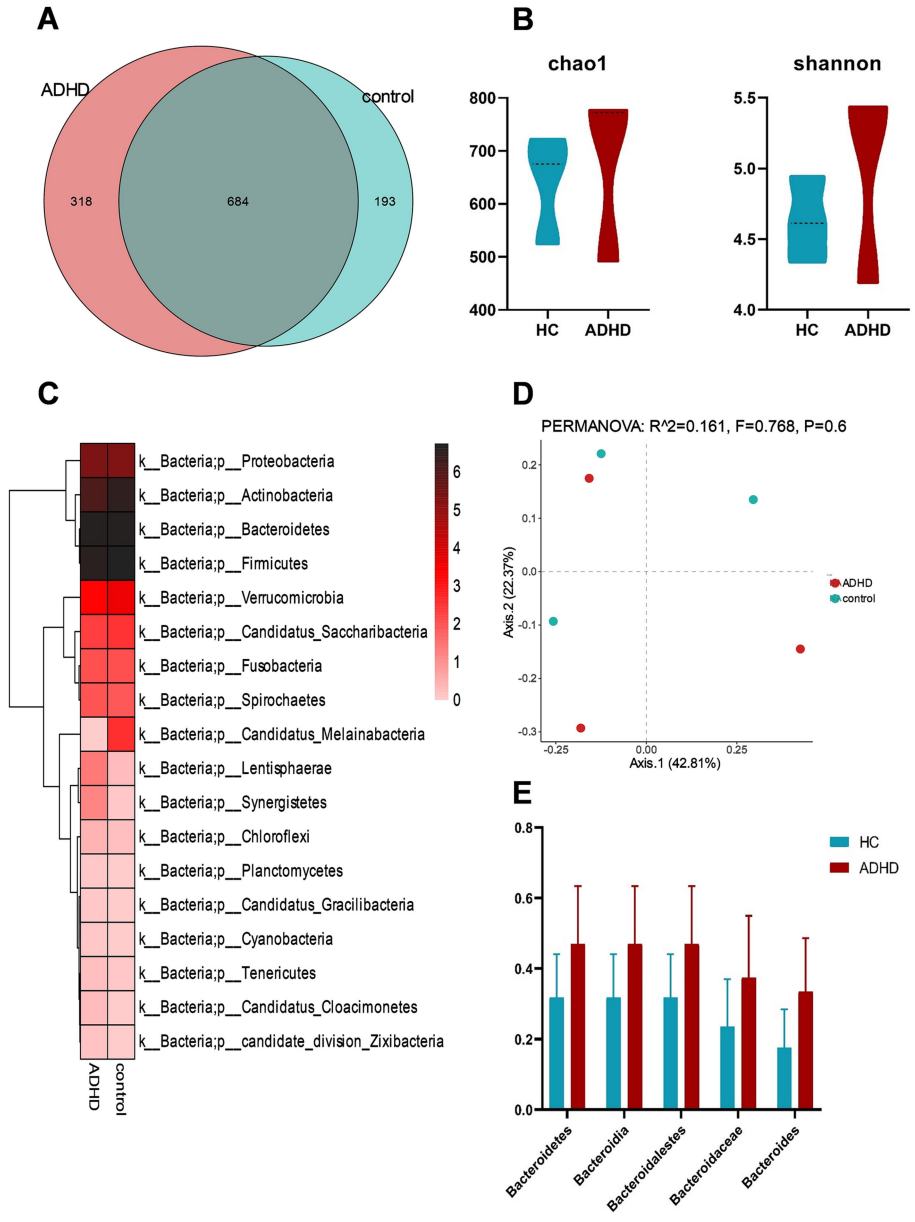


FIGURE 2
Analysis of bacterial diversity and composition. **(A)** Comparison of bacterial counts between the two groups; **(B)** Estimation of α-diversity using the Shannon and Chao1 indices (Shannon, $p = 0.51$; Chao1, $p = 0.51$); **(C)** Comparison of bacterial phyla levels between the two groups, with the highest relative abundances at the phylum level in both groups observed for *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*; **(D)** PCoA analysis of β-diversity based on Bray-Curtis distance; **(E)** Comparison of relative abundances of Bacteroidetes phylum, class, order, family, and genus between the two groups, with all comparisons showing $p > 0.05$.

that the test power for the total distance traveled in the OFT and the number of uprights in the OFT was high, indicating a reliable capacity to detect group differences. In contrast, the test power for the NOR experiment and the number of burials in the MBT was low, suggesting a risk of insufficient power to detect meaningful differences.

3.4 Metabolite nicotinamide may be correlated with ADHD

Metabolites in feces were further analyzed to assess whether there were differences between ADHD and HC metabolite groups. The characteristics that differed most between subgroups were chosen using OPLS-DA. The association between metabolites expression and grouping was modeled by OPLS-DA, providing improved access to intergroup differences and enabling prediction of sample grouping, which could not be accomplished using PCA. The ADHD and HC metabolite clusters were clearly distinguished as shown in Figure 5A, where the horizontal coordinates show differences between groups, and the vertical coordinates show differences between samples within groups. The positive ion model plot is depicted in Figure 5B; R^2 and Q^2 represent the variables that the model explained and the degree of predictability, respectively. These values allow for the differentiation of the model's advantages and disadvantages. Figure 5B illustrates that

TABLE 2 Overview of significant microbiota.

	Decrease in ADHD	Increase in ADHD
Family level	Candidatus Nanogingivalaceae	Tannerellaceae Acidaminococcaceae Rhizobiaceae Nitrobacteraceae
Genus level	Peptacetobacter Flavonifractor	Parabacteroides Sphingorhabdus Phascolarctobacterium Bradyrhizobium Enterocloster
Species level	Bifidobacterium_pseudocatenulatum Bifidobacterium_longum Ligilactobacillus_ruminis Bifidobacterium_catenulatum Clostridium_bornimense Flavonifractor_plautii TM7_phylum_sp__oral_taxon_349 Bifidobacterium_angulatum Actinomyces_sp__oral_taxon_848 Peptacetobacter_hiranonis Limosilactobacillus_oris Candidatus_Nanogingivalaceae_bacterium Sutterella_wadsworthensis Clostridium_sp__AF37_7	Bacteroides_cellulosilyticus Alistipes_sp__dk3624 Enterococcus_saigonensis Parabacteroides_goldsteinii Enterocloster_bolteae Klebsiella_aerogenes Sphingorhabdus_lacus Haemophilus_influenzae Bacteroides_zhangwenhongii

the positive ion model accepted both R^2 and Q^2. Eight hundred fifty-six positive ion metabolites were found in the positive ion model; of these, 26 were considerably up-regulated ($p < 0.05$, VIP > 1) in ADHD compared to HCs, and 10 were significantly down-regulated ($p < 0.05$, VIP > 1), as shown in Figure 5C.

KEGG enrichment analysis and pathway annotation of metabolites from both groups were used to further examine the metabolic effects of ADHD. The Oracle database was utilized to identify the KEGG pathways that were significantly enriched for the particular set of metabolites that were obtained from OPLS-DA analysis. The topological influence was then computed, which served as an anchor for the significantly enriched KEGG pathways (Figures 5D, E). The findings demonstrated some metabolic pathways such as nicotinamide and nicotinic acid metabolism in the positive ionic pattern, as well as the four negative ionic metabolic pathways: cofactor biosynthesis, ubiquinone and other terpene quinone biosynthesis, pyrimidine metabolism, and caffeine metabolism. It was possible that the development of disease was correlated with certain metabolic pathways. When the findings of the metagenomic sequencing analysis of colony function were integrated, three distinct genes were identified, namely *pncB* (K00763), *nadD* (K00969), and *E6.3.5.1* (K01950), that exhibited comparatively lower expression in the ADHD group in the nicotinic acid and nicotinamide metabolic pathways. The expression of the positive ionic metabolite nicotinamide was substantially down-regulated in the ADHD group compared to the HC group ($p = 0.03$, VIP = 1.87), which is consistent with the findings of untargeted metabolomics (Figure 6).

4 Discussion

ADHD is a common neurodevelopmental disorder. While many studies have shown that the gut microbiota of patients with ADHD differed from that of healthy individuals, it is still unclear exactly how gut flora contribute to the pathophysiology of ADHD, and it is impossible to identify the specific changes that occur in the gut flora of ADHD patients based on previous research. The majority of research has employed 16S RNA sequencing to detect variations in the gut microbiome between healthy and ADHD patient groups; however, intergroup differences in gut flora cannot be examined at the species level using 16S sequencing. This study focused on the species-level composition and abundance of bacteria and examined the functional differences between them by applying shotgun metagenomic sequencing to investigate the structural and compositional features of the gut flora in both healthy populations and ADHD patients. All clinical samples in this study were collected from male patients, as previous literature indicates significantly higher rates of pediatric ADHD diagnoses in males compared to females (Faraone et al., 2015; Reuben and Elgaddal, 2024). Notably, the male-to-female diagnosis ratio in epidemiological studies has risen from 3–4:1 to 7–8:1, underscoring the predominance of males in ADHD diagnoses (Thapar and Cooper, 2016). Consequently, our findings may primarily reflect the characteristics of ADHD in male patients. According to the shotgun metagenomic sequencing data, there was no discernible change in α-diversity and β-diversity between the two sample groups, which is consistent with some earlier research findings (Jiang et al., 2018; Wan et al., 2020; Wang et al., 2022, 2023). However, other research has demonstrated notable distinctions between ADHD patients and healthy individuals in terms of gut microbiota α-diversity (Jung et al., 2022) or β-diversity (Szopinska-Tokov et al., 2020). Geographical location, eating practices, sample selection, and other environmental factors could all contribute to these variations. For instance, dietary intake of dairy products, nuts, and legumes in ADHD patients showed a significant association with *S. stercoricanis* (Wang et al., 2020). The development of ADHD may include the gut microbiota in a complex way, and the main markers that set apart children with ADHD from healthy children may not be α-diversity or β-diversity.

Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria were the phylum-level bacteria with the highest relative abundance in both groups, according to our data. As the two main bacterial groups participating in the pathways leading to the metabolism of sugar and transporters, respectively, Firmicutes and Bacteroidetes are known to exist (Meehan and Beiko, 2012). The ratio of Firmicutes to Bacteroidetes, or F/B, is a crucial indicator for measuring the extent of gut microbiota disorder. The abundance of Bacteroidetes to Bacteroides in ADHD was higher than that in HCs, according to the results of sample composition analysis at each biological level. This suggested that F/B was trending downward in ADHD, indicating that the gut microbiota in ADHD was different to that in HCs, albeit not significantly. Based on the intergroup LEfSe study, the ADHD group had a higher relative abundance of *Bacteroides cellulosilyticus* than the

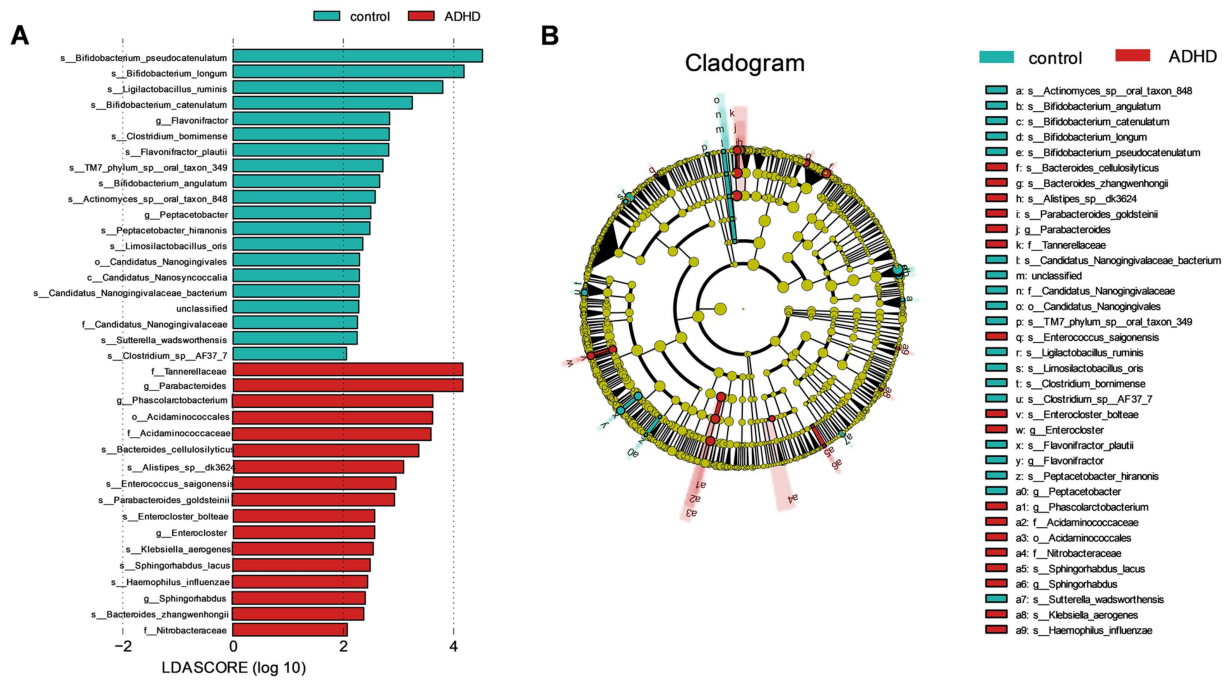


FIGURE 3
Significantly different bacteria. **(A)** Histogram of LEfSe analysis showing significantly different bacteria between the groups, categorized by group; **(B)** Evolutionary branching diagram of significantly different bacteria between the groups, sorted by relative abundance. *B. cellulosilyticus* exhibited the highest relative abundance in the ADHD group.

HC group, and there were changes in bacterial abundance in three orders, five families, seven genera, and 23 species. Several studies have shown that patients with inattentive-ADHD had a higher relative abundance of *B. cellulosilyticus* when compared to HCs, consistent with the results of our study (Shirvani-Rad et al., 2022; Tas and Ulgen, 2023). Additionally, one report indicated that this bacterium was negatively correlated with symptoms of hyperactivity/impulsivity (DSM_HD scores) and attention deficit (DSM_AD scores) in ADHD patients ($p < 0.001$; Li et al., 2022), implying that *B. cellulosilyticus* may play a beneficial role in the gut microbiota of patients with ADHD. Imbalances in the gut microbiota are closely associated with the onset and progression of ADHD. Gut microbes can influence cognitive function through short-chain fatty acids (SCFAs) or tryptophan metabolism (Desbonnet et al., 2008; Selkrig et al., 2014). As one of the major metabolites of gut microbiota, SCFAs regulate host metabolism through multiple pathways, including regulating glucose and lipid metabolism, maintaining the intestinal barrier, and mitigating inflammatory responses (Koh et al., 2016). Plasma concentrations of SCFAs have been observed to decrease in ADHD patients (Yang et al., 2022), and Bacteroides is among the known producers of SCFAs (Koh et al., 2016). Therefore, we hypothesize that intermediate metabolites like SCFAs may serve as key breakthroughs in elucidating the link between behavioral alterations in ADHD and Bacteroides. The relative abundance of *B. cellulosilyticus* was found to be down-regulated in obese populations that were metabolically healthy (Chen et al., 2022) and in individuals who had atherosclerosis (Jie et al., 2023). The enrichment of *B. cellulosilyticus* was linked to healthy dietary practices that decreased the risk of cardiometabolic illnesses. This correlation may stem from the polysaccharide utilization loci (PUL) function of *B. cellulosilyticus*. The PULs in Bacteroides species are thought to include secreted glycosidases, uptake transporter proteins embedded in the cytoplasmic membrane, and cytoplasmic enzymes involved in carbohydrate metabolism (Zafar and Saier, 2021). These intricate PUL systems enable many Bacteroides species to initiate and carry out the utilization and metabolism of carbohydrates. Therefore *B. cellulosilyticus* may be beneficial in preventing or treating obesity and disorders associated with obesity. Furthermore, it is believed that *B. cellulosilyticus* results in diminished anti-inflammatory actions because some Bacteroides species may participate in intestinal immune responses through mechanisms such as protein mimicry, protein export and regulation of surface polysaccharides (Arnolds et al., 2023; Wang et al., 2021). None of the currently identified changes in bacterial taxa fully explain their precise relationship with ADHD symptoms, highlighting an area that warrants further investigation in future research.

We aimed to further verify whether there was a connection between gut flora and ADHD-like behaviors based on the findings of shotgun metagenomic sequencing analysis that could detect changes in the gut microbiota between ADHD patients and HCs. FMT is frequently employed to confirm the existence and development of gut flora in a range of illnesses (Antushevich, 2020). Thus, this study used FMT to confirm the link between gut flora and ADHD behaviors. After transplanting fecal flora from both the HC population and ADHD patients into SD rats, we simultaneously established two control groups and conducted a series of behavioral experiments to determine whether the rats that received colonization displayed distinct behavioral responses. According to the findings, SD rats colonized with ADHD gut microbiota exhibited higher locomotor activity and curiosity than the other three groups, which is consistent with behaviors similar to ADHD. This suggests that ADHD flora may

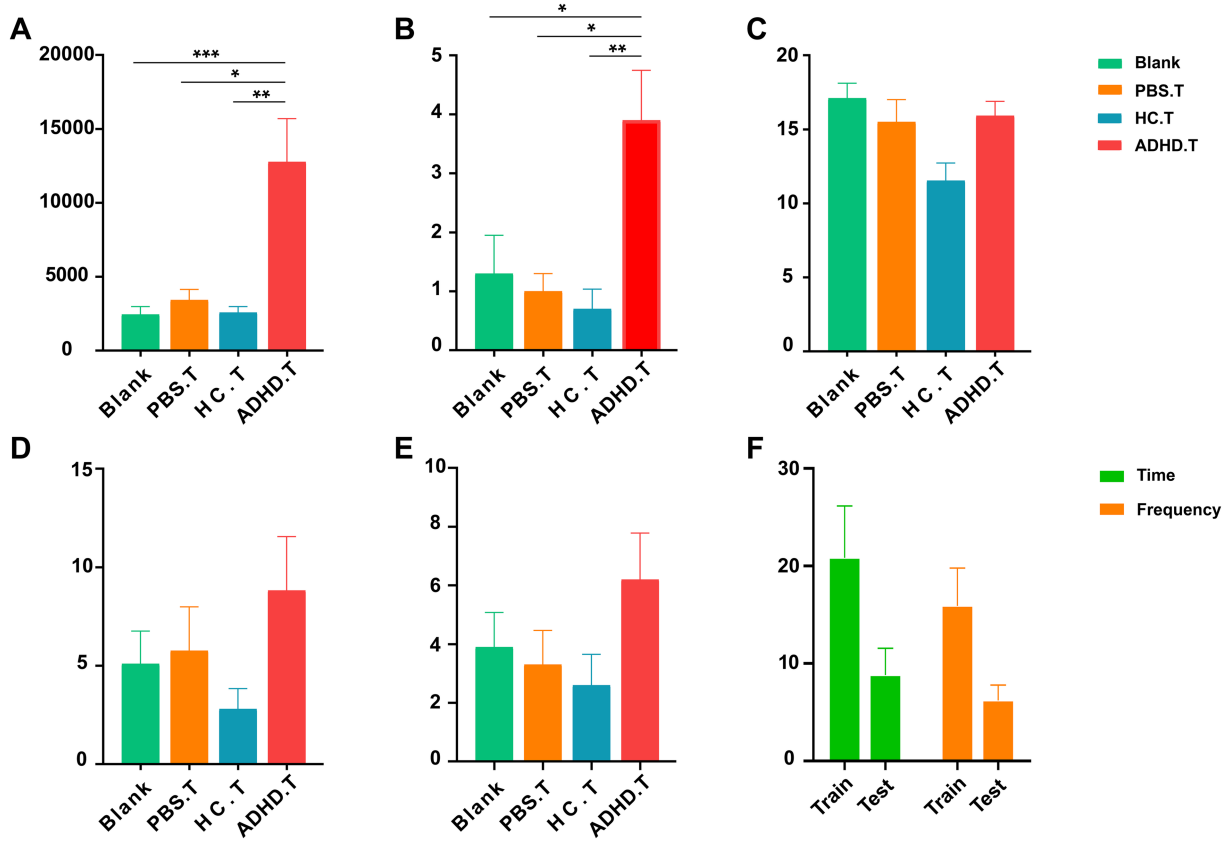


FIGURE 4
Behavioral results. **(A)** The total distance traveled in the OFT among the four groups. Significant differences were observed between the three control groups and the ratADHD group (Blank vs. ADHD.T: $p = 0.0008$; PBS.T vs. ADHD.T: $p = 0.0333$; HC.T vs. ADHD.T: $p = 0.0037$). **(B)** The number of times the animals stood upright in the OFT among the four groups. Significant differences were found between the three control groups and the ratADHD group (Blank vs. ADHD.T: $p = 0.0197$; PBS.T vs. ADHD.T: $p = 0.0379$; HC.T vs. ADHD.T: $p = 0.0050$). **(C)** Comparison of the number of marbles buried among the four groups of animals in the MBT. **(D)** Time spent exploring objects among the four groups of animals in the NOR. **(E)** Number of explorations of objects among the four groups of animals in the NOR. **(F)** Comparison between the training and testing phases of the NOR in the ADHD.T group. Blank: blank control group; HC.T, Health Control colony transplantation group; ADHD.T, ADHD colony transplantation group; PBS.T, sham operation group. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

TABLE 3 Statistical power of behavioral experimental indicators.

	SD within each group	Effect size f	Power (1-β err prob)
OFT distance	4871.84	0.888	0.997
OFT uprights	1.83	0.696	0.952
NOR time	6.39	0.336	0.363
NOR trials	3.99	0.339	0.368
MBT burials	4.11	0.437	0.581

contribute to the development of behaviors similar to ADHD. As other results (Tengeler et al., 2020) do not agree with ours, it was theorized that this might be because of the vast variations in animal individuality and the many transplantation techniques. To evaluate the validity of the behavioral experimental design and the reliability of the data, this study reported high test efficacy for the OFT total distance traveled and number of uprights through statistical efficacy analyses. These results highlight the significance of between-group differences and the efficiency of the experimental design. This indicates that in experiments investigating animal activity and curiosity, the current sample size and design were sufficient to effectively capture treatment effects and ensure both reliability and statistical significance. The relatively lower test efficacy observed in other experiments may stem from individual animal differences and the varying sensitivity of behavioral indicators. Compared to the OFT, which directly reflects the animal's state, NOR and MBT involve more complex cognitive and behavioral patterns that may require larger sample sizes or more refined experimental conditions to minimize data variability. Nonetheless, despite the lower test efficacy, the between-group means for exploration time and number of explorations in NOR exhibited observable trends, offering valuable directions for further investigation into related behavioral mechanisms.

Researching the relationship between gut microorganisms and disease alone is insufficient as the gut contains a large number of metabolites, most of which require the assistance of the gut flora for production and breakdown. Consequently, we carried out an untargeted metabolomics investigation. By combining OPLS-DA analysis and KEGG enrichment analysis, the metabolism of nicotinamide and niacin was found to be enriched in the positive ion mode. The metabolite nicotinamide was the highlight of this analysis. According to our study findings, ADHD patients had lower

10.3389/fmicb.2025.1452423

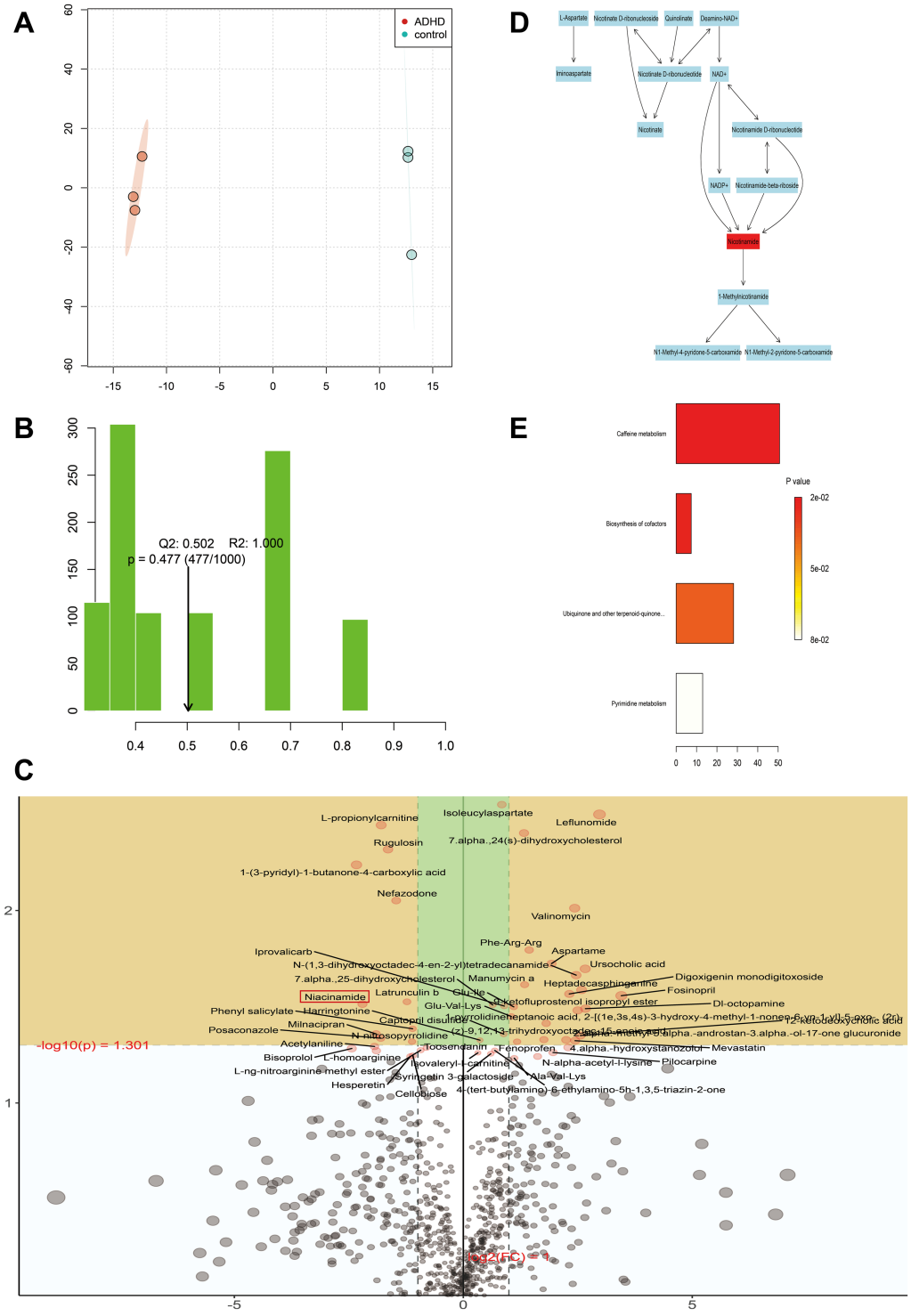


FIGURE 5
Metabolite analysis. **(A)** Metabolite cluster separation between the two groups is clearly defined. The horizontal axis represents differences between groups, while the vertical axis represents differences within samples of each group. **(B)** The R^2 and Q^2 values represent the variance explained by the model and the predictability of the model, respectively. Our model demonstrates high reliability and strong predictive ability. **(C)** Each point represents a specific metabolite. Metabolites located in the yellow area are significantly differentially expressed between the two groups. Points in the upper left indicate metabolites with downregulated relative differential expression in the ADHD group, while points in the upper right indicate metabolites with upregulated relative differential expression in the ADHD group. The closer a point is to the left, right, or top edges of the graph, the more significant the difference in expression. **(D)** Schematic of the reaction network for nicotinamide-related metabolites. Red markers highlight nicotinamide as a key metabolite requiring special attention. **(E)** Negative ion mode ORA enrichment results suggest that these metabolic pathways may also warrant further focus.

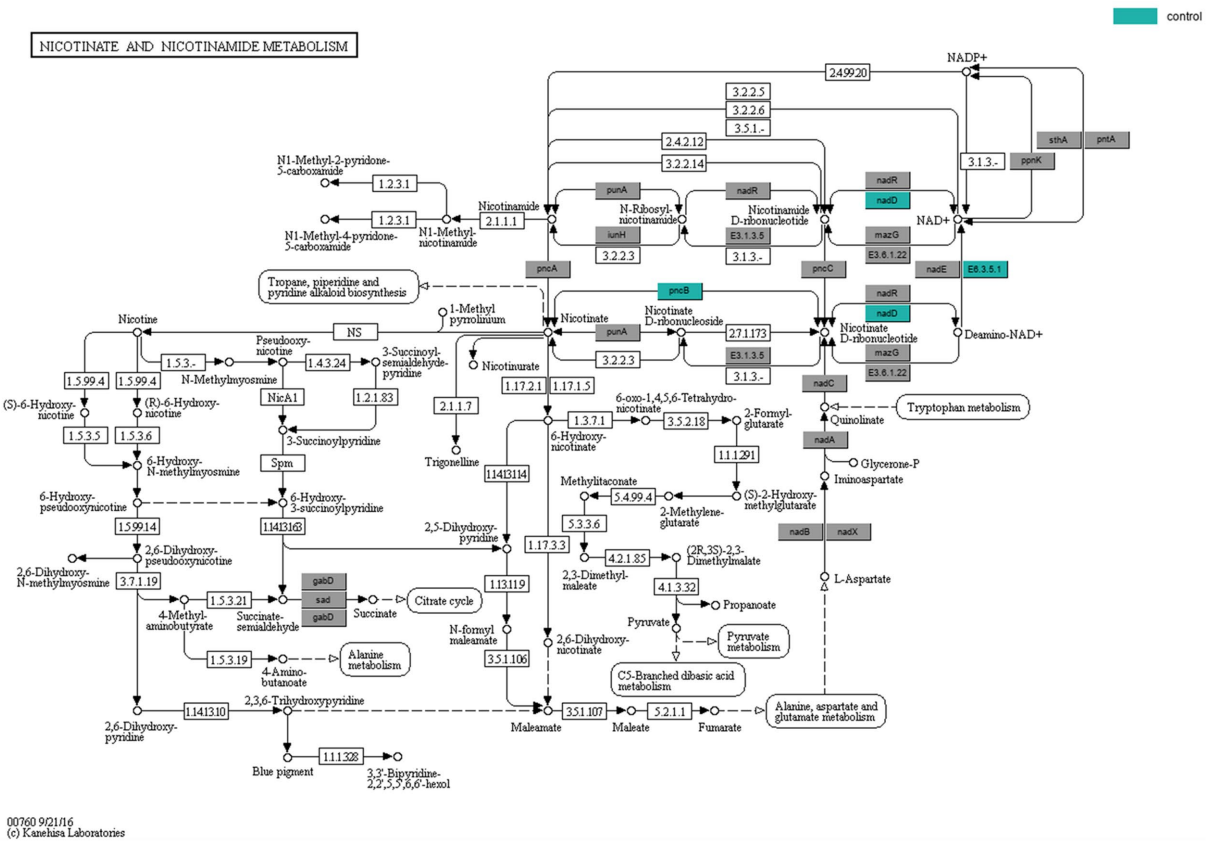


FIGURE 6
Nicotinic acid and nicotinamide metabolic pathway. The color-coded genes, nadD and pncB, represent critical sites in the metabolic pathway.

nicotinamide levels than HCs. Nicotinamide, a form of vitamin B3, has been linked to the growth, survival, and function of neurons in the central nervous system and may be beneficial in the prevention and improvement of neurocognitive function (Rennie et al., 2015), as well as in conditions such as depression, ischemia, stroke, and schizophrenia (Fricker et al., 2018; Song et al., 2019). Monoamine neurotransmitter levels are maintained in cells by nicotinamide (Song et al., 2019). Nicotinamide is a precursor of nicotinamide adenine dinucleotide (NAD+), and the level of NAD+ may be involved in one of the possible processes of nicotinamide's actions. NAD+ plays critical roles in DNA repair, cell signaling, and cellular energy metabolism (Dhuguru et al., 2023; Niño-Narvión et al., 2023). Its concentration particularly impacts nerve cell survival and function in the neurological system. Dysfunction of neural cells resulting from abnormal NAD+ metabolism may influence cognitive and behavioral processes (Rennie et al., 2015). The health of neurons may be significantly impacted by the harmonious interaction between nicotinamide and NAD+.

Reduced nicotinamide levels in ADHD may indicate a problem with NAD+ metabolism. Disproportions in NAD+ metabolism could be linked to symptoms of ADHD, given the significance of NAD+ in neurological function. For instance, imbalances in NAD+ metabolism may impact neurotransmitter synthesis and release, nerve cell energy metabolism and communication (Chellappa et al., 2022; Shats et al., 2020). NAD+ metabolism is regulated by both nicotinate-nucleotide adenylyltransferase (*nadD*) and nicotinate phosphoribosyltransferase (*pncB*), the two characteristic genes whose relative expression was found to be decreased in ADHD in the functional analysis of macrosequencing. Overexpression of the *pncB* gene in a strain resulted in an increase in NAD+ production (Wu et al., 2021). *NadD* is a crucial enzyme in the NAD+ metabolic pathway (Ajunwa et al., 2022; Rodionova et al., 2015). Modulating the NAD+ metabolic pathway may become a viable therapeutic strategy if the anomalies of nicotinamide and NAD+ metabolism in ADHD can be further proven. Increasing NAD+ production by supplementing with nicotinamide may help reduce some of the symptoms associated with ADHD. Furthermore, one study (Mallya et al., 2023) that examined the use of dietary supplements and herbs to treat ADHD noted that dietary supplements are helpful for the disorder and that nicotinamide may be a useful supplement. Additionally, nicotinamide has been used to treat behavioral disorders, migraine headaches, and depression (Tian et al., 2013).

In conclusion, our study provided a comprehensive list of the metabolites and fecal bacteria that were changed in ADHD, especially the species-level microbes found. Our study's strength was that, in comparison to single-omics studies, the use of a combined multi-omics analysis provided a fuller and complementary understanding of ADHD, assisting researchers in better understanding the condition. Further research is necessary to ascertain the possible influence of gut microbiota and metabolites on ADHD, given the advancement of gut microecological assays. This study has several limitations that should be acknowledged. The clinical sample size was relatively small and the geographic selection

area was restricted, which may limit the diversity of the participant population. Additionally, the imbalance in the male-to-female ratio could have influenced the observed differences in the intestinal microbiota. As the findings predominantly reflect the characteristics of ADHD in male patients, future studies should strive to include a more balanced representation of genders to enhance the generalizability and robustness of the results. Furthermore, to validate and expand upon our findings, it is essential to conduct larger-scale and more diverse studies. Such efforts could help overcome current limitations by investigating factors such as environmental and lifestyle influences. Overall, this multi-omics study methodically examined the possible relationship between alterations in gut flora, fecal metabolites and their enrichment pathways with ADHD. This study has the potential to offer an experimental foundation for future investigations into the functions and impacts of gut microbiota and metabolites in ADHD.

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/sra, BioProject ID PRJNA1222598. The associated BioSample IDs are as follows: SAMN46785179, SAMN46785180, SAMN46785181, SAMN46785182, SAMN46785183, and SAMN46785184.

Ethics statement

The studies involving humans were approved by Hangzhou Medical College Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because the parents or legal guardians of the subjects provided verbal informed consent. The animal study was approved by Hangzhou Medical College Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JL: Resources, Visualization, Writing – original draft, Validation. MJ: Investigation, Writing – review & editing, Resources. DC: Data curation, Writing – review & editing, Validation. YS: Data curation, Methodology, Writing – review & editing. LW: Writing – review & editing, Conceptualization, Project administration.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Natural Science Foundation of China (No. 82171168).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1452423/full#supplementary-material

References

Aarts, E., Ederveen, T., Naaijen, J., Zwiers, M. P., Boekhorst, J., Timmerman, H. M., et al. (2017). Gut microbiome in adhd and its relation to neural reward anticipation. *PLoS One* 12:e183509. doi: 10.1371/journal.pone.0183509

Aizawa, E., Tsuji, H., Asahara, T., Takahashi, T., Teraishi, T., Yoshida, S., et al. (2016). Possible association of bifidobacterium and lactobacillus in the gut microbiota of patients with major depressive disorder. *J. Affect. Disord.* 202, 254–257. doi: 10.1016/j.jad.2016.05.038

Ajunwa, O. M., Odeniyi, O. A., Garuba, E. O., Nair, M., Marsili, E., and Onilude, A. A. (2022). Evaluation of extracellular electron transfer in *Pseudomonas aeruginosa* by co-expression of intermediate genes in nad synthetase production pathway. *World J. Microbiol. Biotechnol.* 38:90. doi: 10.1007/s11274-022-03274-9

Amini-Khoei, H., Haghani-Samani, E., Beigi, M., Soltani, A., Mobini, G. R., Balali-Dehkordi, S., et al. (2019). On the role of corticosterone in behavioral disorders, microbiota composition alteration and neuroimmune response in adult male mice subjected to maternal separation stress. *Int. Immunopharmacol.* 66, 242–250. doi: 10.1016/j.intimp.2018.11.037

Antushevich, H. (2020). Fecal microbiota transplantation in disease therapy. *Clin. Chim. Acta* 503, 90–98. doi: 10.1016/j.cca.2019.12.010

Arnolds, K. L., Yamada, E., Neff, C. P., Schneider, J. M., Palmer, B. E., and Lozupone, C. A. (2023). Disruption of genes encoding putative zwitterionic capsular polysaccharides of diverse intestinal bacteroides reduces the induction of host anti-inflammatory factors. *Microb. Ecol.* 85, 1620–1629. doi: 10.1007/s00248-022-02037-1

Austerman, J. (2015). Adhd and behavioral disorders: assessment, management, and an update from dsm-5. *Cleve. Clin. J. Med.* 82, S2–S7. doi: 10.3949/ccjm.82.s1.01

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

Bonaz, B., Sinniger, V., and Pellissier, S. (2019). Vagus nerve stimulation at the interface of brain-gut interactions. *Cold Spring Harb. Perspect. Med.* 9:199. doi: 10.1101/cshperspect.a034199

Bundgaard-Nielsen, C., Knudsen, J., Leutscher, P., Lauritsen, M. B., Nyegaard, M., Hagstrom, S., et al. (2020). Gut microbiota profiles of autism spectrum disorder and attention deficit/hyperactivity disorder: a systematic literature review. *Gut Microbes* 11, 1172–1187. doi: 10.1080/19490976.2020.1748258

Carabotti, M., Scirocco, A., Maselli, M. A., and Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Ann. Gastroenterol.* 28, 203–209

Chellappa, K., McReynolds, M. R., Lu, W., Zeng, X., Makarov, M., Hayat, F., et al. (2022). Nad precursors cycle between host tissues and the gut microbiome. *Cell Metab.* 34, 1947–1959.e5. doi: 10.1016/j.cmet.2022.11.004

Chen, H., Tang, N., Ye, Q., Yu, X., Yang, R., Cheng, H., et al. (2022). Alternation of the gut microbiota in metabolically healthy obesity: an integrated multiomics analysis. *Front. Cell. Infect. Microbiol.* 12:1012028. doi: 10.3389/fcimb.2022.1012028

Cheng, S., Han, B., Ding, M., Wen, Y., Ma, M., Zhang, L., et al. (2020). Identifying psychiatric disorder-associated gut microbiota using microbiota-related gene set enrichment analysis. *Brief. Bioinform.* 21, 1016–1022. doi: 10.1093/bib/bbz034

Coleman, J. A., Yang, D., Zhao, Z., Wen, P. C., Yoshioka, C., Tajkhorshid, E., et al. (2019). Serotonin transporter-ibogaine complexes illuminate mechanisms of inhibition and transport. *Nature* 569, 141–145. doi: 10.1038/s41586-019-1135-1

Dabke, K., Hendrick, G., and Devkota, S. (2019). The gut microbiome and metabolic syndrome. *J. Clin. Invest.* 129, 4050–4057. doi: 10.1172/JCI129194

De Punder, K., and Pruimboom, L. (2015). Stress induces endotoxemia and low-grade inflammation by increasing barrier permeability. *Front. Immunol.* 6:223. doi: 10.3389/fimmu.2015.00223

Desbonnet, L., Garrett, L., Clarke, G., Bienenstock, J., and Dinan, T. G. (2008). The probiotic bifidobacteria infantis: an assessment of potential antidepressant properties in the rat. *J. Psychiatr. Res.* 43, 164–174. doi: 10.1016/j.jpsychires.2008.03.009

Dhuguru, J., Dellinger, R. W., and Migaud, M. E. (2023). Defining nad(p)(h) catabolism. *Nutrients* 15:3064. doi: 10.3390/nu15133064

Eicher, T. P., and Mohajeri, M. H. (2022). Overlapping mechanisms of action of brain-active bacteria and bacterial metabolites in the pathogenesis of common brain diseases. *Nutrients* 14:661. doi: 10.3390/nu14132661

Faraone, S. V., Asherson, P., Banaschewski, T., Biederman, J., Buitelaar, J. K., Ramos-Quiroga, J. A., et al. (2015). Attention-deficit/hyperactivity disorder. *Nat. Rev. Dis. Primers* 1:15020. doi: 10.1038/nrdp.2015.20

Farzi, A., Frohlich, E. E., and Holzer, P. (2018). Gut microbiota and the neuroendocrine system. *Neurotherapeutics* 15, 5–22. doi: 10.1007/s13311-017-0600-5

Fricker, R. A., Green, E. L., Jenkins, S. I., and Griffin, S. M. (2018). The influence of nicotinamide on health and disease in the central nervous system. *Int. J. Tryptophan Res.* 11:71858. doi: 10.1177/1178646918776658

Frisbee, A. L., and Petri, W. J. (2020). Considering the immune system during fecal microbiota transplantation for clostridioides difficile infection. *Trends Mol. Med.* 26, 496–507. doi: 10.1016/j.molmed.2020.01.009

Fu, X., Wang, Y., Zhao, F., Cui, R., Xie, W., Liu, Q., et al. (2023). Shared biological mechanisms of depression and obesity: focus on adipokines and lipokines. *Aging (Albany NY)* 15, 5917–5950. doi: 10.18632/aging.204847

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 469, 543–547. doi: 10.1038/nature09646

Fung, T. C., Olson, C. A., and Hsiao, E. Y. (2017). Interactions between the microbiota, immune and nervous systems in health and disease. *Nat. Neurosci.* 20, 145–155. doi: 10.1038/nn.4476

Hinshaw, S. P. (2018). Attention deficit hyperactivity disorder (ADHD): controversy, developmental mechanisms, and multiple levels of analysis. *Annu. Rev. Clin. Psychol.* 14, 291–316. doi: 10.1146/annurev-clinpsy-050817-084917

Jiang, H. Y., Zhou, Y. Y., Zhou, G. L., Li, Y. C., Yuan, J., Li, X. H., et al. (2018). Gut microbiota profiles in treatment-naive children with attention deficit hyperactivity disorder. *Behav. Brain Res.* 347, 408–413. doi: 10.1016/j.bbr.2018.03.036

Jie, Z., Zhu, Q., Zou, Y., Wu, Q., Qin, M., He, D., et al. (2023). A consortium of three-bacteria isolated from human feces inhibits formation of atherosclerotic deposits and lowers lipid levels in a mouse model. *Iscience* 26:106960. doi: 10.1016/j.isci.2023.106960

Jung, T. H., Hwang, H. J., and Han, K. S. (2022). Correlation of attention deficit hyperactivity disorder with gut microbiota according to the dietary intake of korean elementary school students. *PLoS One* 17:e275520. doi: 10.1371/journal.pone.0275520

Kang, D. W., Park, J. G., Ilhan, Z. E., Wallstrom, G., Labaer, J., Adams, J. B., et al. (2013). Reduced incidence of prevotella and other fermenters in intestinal microflora of autistic children. *PLoS One* 8:e68322. doi: 10.1371/journal.pone.0068322

Kelly, T. N., Bazzano, L. A., Ajami, N. J., He, H., Zhao, J., Petrosino, J. F., et al. (2016). Gut microbiome associates with lifetime cardiovascular disease risk profile among Bogalusa heart study participants. *Circ. Res.* 119, 956–964. doi: 10.1161/CIRCRESAHA.116.309219

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Backhed, F. (2016). From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* 165, 1332–1345. doi: 10.1016/j.cell.2016.05.041

Li, Y., Sun, H., Huang, Y., Yin, A., Zhang, L., Han, J., et al. (2022). Gut metagenomic characteristics of adhd reveal low *Bacteroides ovatus*-associated host cognitive impairment. *Gut Microbes* 14:2125747. doi: 10.1080/19490976.2022.2125747

Logsdon, A. F., Erickson, M. A., Rhea, E. M., Salameh, T. S., and Banks, W. A. (2018). Gut reactions: how the blood-brain barrier connects the microbiome and the brain. *Exp. Biol. Med.* 243, 159–165. doi: 10.1177/1535370217743766

Mallya, R., Naik, B., and Momin, M. (2023). Application of herbs and dietary supplements in adhd management. *CNS Neurol. Disord. Drug Targets* 22, 950–972. doi: 10.2174/1871527321666220720103923

Margolis, K. G., Cryan, J. F., and Mayer, E. A. (2021). The microbiota-gut-brain axis: from motility to mood. *Gastroenterology* 160, 1486–1501. doi: 10.1053/j.gastro.2020.10.066

Martinez-Badia, J., and Martinez-Raga, J. (2015). Who says this is a modern disorder? The early history of attention deficit hyperactivity disorder. *World J. Psychiatry* 5, 379–386. doi: 10.5498/wjp.v5.i4.379

Meehan, C. J., and Beiko, R. G. (2012). Lateral gene transfer of an abc transporter complex between major constituents of the human gut microbiome. *BMC Microbiol.* 12:248. doi: 10.1186/1471-2180-12-248

Ming, X., Chen, N., Ray, C., Brewer, G., Kornitzer, J., and Steer, R. A. (2018). A gut feeling: a hypothesis of the role of the microbiome in attention-deficit/hyperactivity disorders. *Child Neurol. Open* 5:6799. doi: 10.1177/2329048X18786799

Niño-Narvión, J., Rojo-López, M. I., Martinez-Santos, P., Rossell, J., Ruiz-Alcaraz, A. J., Alonso, N., et al. (2023). Nad+ precursors and intestinal inflammation: therapeutic insights involving gut microbiota. *Nutrients* 15:2992. doi: 10.3390/nu15132992

Orivuori, L., Mustonen, K., de Goffau, M. C., Hakala, S., Paasela, M., Roduit, C., et al. (2015). High level of fecal calprotectin at age 2 months as a marker of intestinal inflammation predicts atopic dermatitis and asthma by age 6. *Clin. Exp. Allergy* 45, 928–939. doi: 10.1111/cea.12522

Posner, J., Polanczyk, G. V., and Sonuga-Barke, E. (2020). Attention-deficit hyperactivity disorder. *Lancet* 395, 450–462. doi: 10.1016/S0140-6736(19)33004-1

Reimherr, F. W., Marchant, B. K., Gift, T. E., and Steans, T. A. (2017). Adhd and anxiety: clinical significance and treatment implications. *Curr. Psychiatry Rep.* 19:109. doi: 10.1007/s11920-017-0859-6

Rennie, G., Chen, A. C., Dhillon, H., Vardy, J., and Damian, D. L. (2015). Nicotinamide and neurocognitive function. *Nutr. Neurosci.* 18, 193–200. doi: 10.1179/1476830514Y.0000000112

Reuben, C., and Elgaddal, N. (2024). Attention-deficit/hyperactivity disorder in children ages 5-17 years: United States, 2020-2022. *NCHS Data Brief* 499, 1–9

Rodionova, I. A., Zuccola, H. J., Sorci, L., Aleshin, A. E., Kazanov, M. D., Ma, C. T., et al. (2015). Mycobacterial nicotinate mononucleotide adenylyltransferase: structure, mechanism, and implications for drug discovery. *J. Biol. Chem.* 290, 7693–7706. doi: 10.1074/jbc.M114.628016

Rosenfeld, C. S. (2015). Microbiome disturbances and autism spectrum disorders. *Drug Metab. Dispos.* 43, 1557–1571. doi: 10.1124/dmd.115.063826

Safari, Z., Monnoye, M., Abuja, P. M., Mariadassou, M., Kashofer, K., Gerard, P., et al. (2019). Steatosis and gut microbiota dysbiosis induced by high-fat diet are reversed by 1-week chow diet administration. *Nutr. Res.* 71, 72–88. doi: 10.1016/j.nutres.2019.09.004

Sasso, J. M., Ammar, R. M., Tenchov, R., Lemmel, S., Kelber, O., Grieswelle, M., et al. (2023). Gut microbiome-brain alliance: a landscape view into mental and gastrointestinal health and disorders. *ACS Chem. Neurosci.* 14, 1717–1763. doi: 10.1021/acschemneuro.3c00127

Selkrig, J., Wong, P., Zhang, X., and Pettersson, S. (2014). Metabolic tinkering by the gut microbiome: implications for brain development and function. *Gut Microbes* 5, 369–380. doi: 10.4161/gmic.28681

Sender, R., Fuchs, S., and Milo, R. (2016). Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. *Cell* 164, 337–340. doi: 10.1016/j.cell.2016.01.013

Sharon, G., Cruz, N. J., Kang, D., Gandal, M. J., Wang, B., Kim, Y., et al. (2019). Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. *Cell* 177, 1600–1618.e17. doi: 10.1016/j.cell.2019.05.004

Shats, I., Williams, J. G., Liu, J., Makarov, M. V., Wu, X., Lih, F. B., et al. (2020). Bacteria boost mammalian host nad metabolism by engaging the deamidated biosynthesis pathway. *Cell Metab.* 31, 564–579.e7. doi: 10.1016/j.cmet.2020.02.001

Shirvani-Rad, S., Ejtahed, H. S., Ettehad, M. F., Taghavi, M., Sharifi, F., Arzaghi, S. M., et al. (2022). The role of gut microbiota-brain axis in pathophysiology of adhd: a systematic review. *J. Atten. Disord.* 26, 1698–1710. doi: 10.1177/10870547211073474

Song, S. B., Park, J. S., Chung, G. J., Lee, I. H., and Hwang, E. S. (2019). Diverse therapeutic efficacies and more diverse mechanisms of nicotinamide. *Metabolomics* 15:137. doi: 10.1007/s11306-019-1604-4

Szopinska-Tokov, J., Dam, S., Naaijen, J., Konstanti, P., Rommelse, N., Belzer, C., et al. (2020). Investigating the gut microbiota composition of individuals with attention-deficit/hyperactivity disorder and association with symptoms. *Microorganisms* 8:406. doi: 10.3390/microorganisms8030406

Tas, E., and Ulgen, K. O. (2023). Understanding the adhd-gut axis by metabolic network analysis. *Meta* 13:592. doi: 10.3390/metabo13050592

Tengeler, A. C., Dam, S. A., Wiesmann, M., Naaijen, J., van Bodegom, M., Belzer, C., et al. (2020). Gut microbiota from persons with attention-deficit/hyperactivity disorder affects the brain in mice. *Microbiome* 8:44. doi: 10.1186/s40168-020-00816-x

Thapar, A., and Cooper, M. (2016). Attention deficit hyperactivity disorder. *Lancet* 387, 1240–1250. doi: 10.1016/S0140-6736(15)00238-X

Tian, Y. J., Li, D., Ma, Q., Gu, X. Y., Guo, M., Lun, Y. Z., et al. (2013). Excess nicotinamide increases plasma serotonin and histamine levels. *Sheng Li Xue Bao* 65, 33–38

Usami, M. (2016). Functional consequences of attention-deficit hyperactivity disorder on children and their families. *Psychiatry Clin. Neurosci.* 70, 303–317. doi: 10.1111/pcn.12393

Wan, L., Ge, W., Zhang, S., Sun, Y., Wang, B., and Yang, G. (2020). Case-control study of the effects of gut microbiota composition on neurotransmitter metabolic pathways in children with attention deficit hyperactivity disorder. *Front. Neurosci.* 14:127. doi: 10.3389/fnins.2020.00127

Wang, L. J., Li, S. C., Li, S. W., Kuo, H. C., Lee, S. Y., Huang, L. H., et al. (2022). Gut microbiota and plasma cytokine levels in patients with attention-deficit/hyperactivity disorder. *Transl. Psychiatry* 12:76. doi: 10.1038/s41398-022-01844-x

Wang, L. J., Li, S. C., Yeh, Y. M., Lee, S. Y., Kuo, H. C., and Yang, C. Y. (2023). Gut mycobiome dysbiosis and its impact on intestinal permeability in attention-deficit/hyperactivity disorder. *J. Child Psychol. Psychiatry* 64, 1280–1291. doi: 10.1111/jcpp.13779

Wang, L. J., Yang, C. Y., Chou, W. J., Lee, M. J., Chou, M. C., Kuo, H. C., et al. (2020). Gut microbiota and dietary patterns in children with attention-deficit/hyperactivity disorder. *Eur. Child Adolesc. Psychiatry* 29, 287–297. doi: 10.1007/s00787-019-01352-2

Wang, C., Zhao, J., Zhang, H., Lee, Y. K., Zhai, Q., and Chen, W. (2021). Roles of intestinal bacteroides in human health and diseases. *Crit. Rev. Food Sci. Nutr.* 61, 3518–3536. doi: 10.1080/10408398.2020.1802695

Wu, S., Zhao, P., Li, Q., and Tian, P. (2021). Intensifying niacin-based biosynthesis of nad(+) to enhance 3-hydroxypropionic acid production in *Klebsiella pneumoniae*. *Biotechnol. Lett.* 43, 223–234. doi: 10.1007/s10529-020-03011-y

Wustner, A., Otto, C., Schlack, R., Holling, H., Klasen, F., and Ravens-Sieberer, U. (2019). Risk and protective factors for the development of adhd symptoms in children and adolescents: results of the longitudinal bella study. *PLoS One* 14:e214412. doi: 10.1371/journal.pone.0214412

Yang, L. L., Stiernborg, M., Skott, E., Gillberg, T., Landberg, R., Giacobini, M., et al. (2022). Lower plasma concentrations of short-chain fatty acids (scfas) in patients with adhd. *J. Psychiatr. Res.* 156, 36–43. doi: 10.1016/j.jpsychires.2022.09.042

Zafar, H., and Saier, M. J. (2021). Gut bacteroides species in health and disease. *Gut Microbes* 13, 1–20. doi: 10.1080/19490976.2020.1848158

Zeineldin, M., Aldridge, B., and Lowe, J. (2018). Dysbiosis of the fecal microbiota in feedlot cattle with hemorrhagic diarrhea. *Microb. Pathog.* 115, 123–130. doi: 10.1016/j.micpath.2017.12.059

frontiers | Frontiers in Microbiology

TYPE Review
PUBLISHED 25 March 2025
DOI 10.3389/fmicb.2025.1556827

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Raphael R. Fagundes,
Leiden University Medical Center (LUMC), Netherlands
Giuseppe Guido Maria Scarlata,
University Magna Graecia of Catanzaro, Italy

*CORRESPONDENCE
Zihan Yu
yuzihan@tmu.edu.cn
Xin Chen
xchen03@tmu.edu.cn

†These authors have contributed equally to this work and share first authorship

RECEIVED 07 January 2025
ACCEPTED 07 March 2025
PUBLISHED 25 March 2025

CITATION
Liu Y, Li X, Chen Y, Yao Q, Zhou J, Wang X, Meng Q, Ji J, Yu Z and Chen X (2025) Fecal microbiota transplantation: application scenarios, efficacy prediction, and factors impacting donor-recipient interplay. *Front. Microbiol.* 16:1556827. doi: 10.3389/fmicb.2025.1556827

COPYRIGHT
© 2025 Liu, Li, Chen, Yao, Zhou, Wang, Meng, Ji, Yu and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbiota transplantation: application scenarios, efficacy prediction, and factors impacting donor-recipient interplay

Yaxin Liu[1,2†], Xinru Li[1,2†], Yuchao Chen[1,2†], Qinyan Yao[1,2], Jinjie Zhou[1,2], Xiaoxuan Wang[1,2], Qingguo Meng[1,2], Jiaxuan Ji[1,2], Zihan Yu[1,2*] and Xin Chen[1,2*]

[1]Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, Tianjin, China, [2]Tianjin Institute of Digestive Disease, Tianjin Medical University General Hospital, Tianjin, China

Fecal microbiota transplantation (FMT) represents a therapeutic approach that directly regulates the gut microbiota of recipients, normalizes its composition and reaping therapeutic rewards. Currently, in addition to its general application in treating *Clostridium difficile* (*C. difficile*) infection (CDI), FMT treatment has also been extended to the fields of other gastrointestinal diseases, infections, gut-liver or gut-brain axis disorders, metabolic diseases and cancer, etc. Prior to FMT, rigorous donor screening is essential to reduce the occurrence of adverse events. In addition, it is imperative to evaluate whether the recipient can safely and effectively undergo FMT treatment. However, the efficacy of FMT is influenced by the complex interactions between the gut microbiota of donor and recipient, the degree of donor microbiota engraftment is not necessarily positively related with the success rate of FMT. Furthermore, an increasing number of novel factors affecting FMT outcomes are being identified in recent clinical trials and animal experiments, broadening our understanding of FMT treatment. This article provides a comprehensive review of the application scenarios of FMT, the factors influencing the safety and efficacy of FMT from the aspects of both the donors and the recipients, and summarizes how these emerging novel regulatory factors can be combined to predict the clinical outcomes of patients undergoing FMT.

KEYWORDS

gut microbiota, fecal microbiota transplantation, donor-recipient interplay, prediction model, safety and efficiency

1 Introduction

The gut microbiota is composed of trillions of different microorganisms, bacteria, archaea, phages, and protozoa, which forms a solid organ weighing ~2 kg (Ruan et al., 2020). The gut microbiome contributes to the homeostasis in human, which is responsible for nutrient digestion, metabolism regulation, resistance to external pathogens, and modulation of host immune responses (Ghani et al., 2024). The value of live biotherapeutic products, which act directly in the gastrointestinal tract and alter microbial community of the recipient, has been identified in the treatment of microbiome disruption-related diseases (Goldsmith et al., 2024). Fecal microbiota transplantation (FMT) is a therapy performed by oral, enteral, or colonic administration of donor feces containing natural microbiota, restoring the disrupted microbial community into a healthy one (Gupta and Khanna, 2017). At present, FMT has been recognized as an innovative therapeutic

approach from the two main aspects: its curative effect in the treatment of *Clostridioides difficile* infection (CDI) and the expanded comprehension of the intricate interplay between the gut microbiota and human wellbeing (Afzaal et al., 2022). In addition to the delightful treatment outcomes observed in CDI, FMT has also been investigated for a wide range of diseases related to dysbiosis, including inflammatory bowel disease (IBD) (Costello et al., 2017), irritable bowel syndrome (IBS) (Johnsen et al., 2018), metabolic syndrome (MetS) (Proença et al., 2020), neuropsychiatric diseases (Settanni et al., 2021) and autoimmune diseases (Huang et al., 2022), etc. As the interest in FMT continues to surge, a great number of clinical studies are currently employing FMT for the treatment of more than 80 diseases, showing promising safety and effectiveness (Wang Y. et al., 2022; Karimi et al., 2024).

Nevertheless, variations in the therapeutic efficacy of FMT are observed when treating different patients with the same disease, suggesting that a multitude of factors influence the effectiveness of FMT. In terms of the donor, it involves donor screening, baseline characteristics of the donor, such as health condition (presence or absence of underlying diseases), lifestyle, body weight, gender, age, genetic relatedness, etc, as well as the microorganism constituents within the donor's gut, including the microbiota, virus and fungal. Several recent guidelines have been published to direct the treatment process for FMT (Cammarota et al., 2017; Lopetuso et al., 2023), however, part of the restrictions for donors are not mandatory, and several factors are not explicitly stated in current guidelines, suggesting that we should consider the importance of these factors in FMT from multiple perspectives. Regarding the recipients, firstly, the indication of FMT should be considered. Besides the extended diseases mentioned above, which have the potential to be treated by FMT, other baseline status of the recipients are required to be considered, for example, the immune system function of the recipient, to reduce the occurrence of adverse events (Shogbesan et al., 2018). Furthermore, from the aspect of efficiency of FMT, factors such as bowel preparation, drug usage, diet and lifestyle, etc, are taken into account. The severity of the patient's disease also affects the efficacy of FMT, more severe or recurrent conditions may require repeated FMT procedures or be exhibit no response to FMT.

It is worth noting that the interplay between the donors and the recipients is a dominant factor of FMT, defined as "donor-recipient compatibility". For example, the potential impact of sex-discordant FMT and age-discrepant FMT warrants more attention (Benítez-Páez et al., 2022; Sehgal et al., 2024). More importantly, mechanistically, as a therapy based on gut microbiota, the gut microorganisms from the donor are introduced into the gut of recipients after FMT. With the invasion of exogenous microorganisms, the baseline microbial community is disturbed, subsequent alteration of gut microbiota occurs within recipient's gut, and ultimately, a newly constituted equilibrium state of microbiota is reached. Generally, the engraftment of donor-derived microorganisms is the mostly considered physiological process post-FMT, however, the succession of gut microbiota following FMT extends far beyond the mere microbial engraftment. Various possible ecological scenarios within the gut of recipients could be observed, which depends on the baseline gut microbiota of both colonizers and residents, further emphasizing the importance of donor-recipient interplay (Schmidt et al., 2022; Wilson et al., 2021). Moreover, studies also showed that engraftment of microbial components from the donor may not be necessarily correlated with clinical improvement (Browne et al., 2021). It is of significant importance to clarify the patterns of post-FMT microbial changes within the recipient's gut, and how the ultimate microbial community and the subsequent long-term physiological changes affect the prognosis of diseases.

To sum up, the success of FMT therapy is determined by a complex regulatory network involving multiple factors. Thus, the therapeutic efficacy-related factors mentioned above can be extended to two application scenarios: (1) predicting the outcomes of FMTs that have been conducted; {2) selecting the optimal donors according to donor-recipient matching scheme prior to FMT. Recently, however, more and more researchers are exploring the treatment effect of FMT after carefully donor screening, while several studies resulted in contradictory conclusions (Caenepeel et al., 2024; Zhang Y. et al., 2024). This confusing phenomenon reminds us that still numerous unknown factors involved in this complex regulatory process, acting as promotors and inhibitors in the FMT process. There is still a considerable journey ahead to elucidate the optimal regimen for FMT therapy.

Innovatively, recent animal experiments are contributing to revealing potential participants in the FMT process, which could be partially explained by the difference of microbiota, and the results of these studies are gradually being extended to randomized controlled trials for validation. Furthermore, researchers are committing to the development predictive models for the efficacy of FMT (He et al., 2022), which integrate a variety of variables, including general characteristics and microbiome features of individuals, etc., which contributes to further refining more standardized procedures of FMT. In the future, the illumination of regulatory mechanisms will also be conducive to enhance the explainablity and transparency of predictive models.

In our review, we first summarized the current therapeutic status of FMT in various diseases, the microbial alterations in these FMT-targeting diseases and potential microorganism-related mechanisms. Moreover, our review systematically integrated the latest results of clinical researches and animal experiments in the field of FMT, which aims to summarize the currently recognized factors which should be paid considerable attention in the process of FMT, emphazing the factors which have always been overlooked, and discussing the values of newly discovered factors affecting the success rate of FMT.

2 Current applications of FMT

Many diseases are characterized by functional changes in composition and gut microbiota. When gut homeostasis is disrupted, normal body functions are impaired and gastrointestinal and extra-gastrointestinal disorders arise, including recurrent CDI (rCDI), IBD, metabolic disorders, cancer, etc. Dysbiosis of 479 gut microorganisms has been reported to be associated with 117 gastrointestinal and extra-gastrointestinal diseases (Zhang X. et al., 2023). Microbial dysbiosis-related diseases have the potential to be alleviated via FMT.

The safety and efficacy of FMT in rCDI have been extensively evaluated. In a systematic review that included 317 patients treated in 27 case series and reports, 92% of the individuals showed disease remission (Gough et al., 2011). Early FMT improves survival in severe CDI. A retrospective cohort study described 111 patients with severe CDI, including 66 patients treated with FMT and 45 patients who did not receive FMT, and the three-month mortality rate after diagnosis of severe CDI in the FMT-treated patients was 12% (8/66), compared with 42% (19/45) in the standard-treatment group, $p < 0.001$ (Hocquart et al., 2018). Six clinical studies, enrolling 320 subjects, found that the use of FMT in immunocompetent rCDI participants may lead to a substantial increase in the remission of rCDI in the FMT group compared to the control group (Minkoff et al., 2023). FMT also appears to be beneficial for CDI subtypes. For severe or fulminant CDI, the overall successful remission rate for a single FMT was 0.88 (Song et al., 2022). Notably, however, severe CDI, severe-complicated indication, number of previous CDI-related hospitalizations and inpatient status are independent predictors of failure in single FMT in patients with rCDI (Beran et al., 2023; Fischer et al., 2016; Ianiro et al., 2017). Level of fecal calprotectin concentration of CDI patients before first FMT procedure is associated with the need of repeat FMT (Gallo et al., 2020).

Crohn's disease (CD) and ulcerative colitis (UC) are defined as IBD, which are chronic inflammatory diseases that affect parts of the gastrointestinal tract and may even manifest with additional intestinal symptoms. A growing body of evidence supports the role of gut microbiota in the pathogenesis of IBD. Pooled results from four RCTs showed FMT to be superior to placebo in active UC, with the endpoint defined as endoscopic remission (Costello et al., 2017), which is also supported by several other researches (Paramsothy et al., 2017; Tang et al., 2020). In an animal study, FMT from healthy human donor reduced the susceptibility to colitis in dextran sulfate sodium (DSS)-induced germ-free mice, while UC bacteria promoted the expression of inflammatory markers and pro-inflammatory factors (Yang et al., 2022). According to the international Rome consensus conference on gut microbiota and FMT in IBD, FMT may be effective in the induction of remission for mild to moderate UC, while there is insufficient evidence to recommend FMT as a treatment for UC in routine clinical practice (Lopetuso et al., 2023). Investigations for pouchitis and CD are ongoing (Lee and Chang, 2021). However, there is insufficient evidence on the safety and efficiency to recommend FMT as a treatment for CD and pouchitis in clinical practice (Lopetuso et al., 2023). In short, FMT is a promising alternative or adjunct to current therapies for patients with UC and CD. More extensive clinical trials are pending to confirm its long-term efficacy and safety.

The results of current studies vary on the efficacy of FMT in IBS. In a RCT examining the effects of FMT delivered via colonoscopy in patients with IBS, the IBS severity scores 3 months after single donor FMT was lower than that after autologous FMT, indicating the effectiveness of FMT on IBS (Johnsen et al., 2018). Other studies have shown that FMT can effectively improve the abdominal symptoms, fatigue and quality of life of IBS patients. For example, the response rates of FMT groups after 3 months were significantly higher than that of the placebo group, and the improvement of symptoms was correlated with the change of intestinal microbiota, which further confirmed the positive effect of FMT on IBS (El-Salhy et al., 2020). However, in a randomized, double-blind, placebo-controlled trial that included patients with moderate to severe IBS, a significant difference in improvement in IBS-severity scoring system (IBS-SSS) scores was observed 3 months after treatment in favor of placebo, but not FMT (Halkjær et al., 2018). Additionally, in another clinical trial, two FMTs 4 weeks apart did not significantly reduce IBS-SSS scores, although the improment of abdominal distension was more observed in patients receiving FMT and was associated with a reduction in hydrogen sulfide-producing bacteria (Yau et al., 2023). These findings suggests that changes in the gut microbiota may not be sufficient to fully achieve clinical improvement of IBS, and further in-depth studies on the mechanism of action of FMT and optimization of treatment regimens are needed to improve its therapeutic efficacy in IBS.

Generally, studies pertaining to FMT has predominantly focused on the aforementioned gastrointestinal diseases. Overall, the AGA Clinical Practice Guideline on FMT for select gastrointestinal diseases provided recommendations on the use of FMT in adults with rCDI, severe to fulminant CDI, IBD (including pouchitis) and IBS, adhering to the perspective that FMT is recommended in CDI under certain conditions, while this therapy cannot yet be recommended in other gastrointestinal conditions (Peery et al., 2024).

FMT also has potential applications in other diseases. More than 200 clinical trials have been conducted on the use of FMT in the treatment of various inflammatory and autoimmune diseases and cancer. FMT has emerged as a potential treatment for severe colitis associated with graft-vs.-host disease (GvHD) after hematopoietic stem cell transplantation (Zhang et al., 2021). In cancer, studies have shown that FMT improves immune checkpoint inhibitor (ICI)-associated colitis in cancer patients, accompanied by reconstitution of the intestinal flora and a relative increase in the proportion of regulatory T cells in the colonic mucosa (Wang et al., 2018). In a non-randomized clinical trial, after FMT in two enemas one week apart, five individuals subjectively reported improvement in immune-mediated dry eye 3 months after FMT (Watane et al., 2022). Germ-free mice transplanted with microbiota from MS patients exhibited a reduced proportion of IL-10 Tregs compared to mice with microbiota from healthy controls. Further investigation of immunomodulatory mechanisms suggests that multiple sclerosis (MS)-associated bacterial species reduce Tregs and increase Th1 lymphocyte differentiation *in vitro*, while exacerbating disease severity (Berer et al., 2017). To date, participants with psoriatic arthritis (PsA) have found FMT to be acceptable and safe. A double-blind, randomized, placebo-controlled trial (NCT03058900) is underway to determine whether FMT is more effective than placebo in reducing disease activity in patients with PsA and active peripheral arthritis treated with weekly subcutaneous methotrexate injections (Kragsnaes et al., 2021). A study in mice determined that FMT alleviates the severity of lupus by repairing antibiotic-induced dysbiosis in the gut flora, suggesting that manipulation of the gut microbiota is a logical and promising novel therapeutic strategy for systemic lupus erythematosus (SLE) (Zhang et al., 2020). The first clinical trial of FMT in patients with active SLE

(ChiCTR2000036352) suggested that FMT may be a feasible, safe, and potentially effective short-term treatment for patients with SLE, which effectively shifted the gut microbiota to anti-inflammatory and improved clinical parameters (Huang et al., 2022). In type 1 diabetes (T1DM), a RCT in Netherlands showed that FMT could stabilized residual β-cell function of patients (de Groot et al., 2021).

Moreover, the value of FMT in metabolic dysfunction-associated fatty liver disease (Abenavoli et al., 2024), spesis (Keskey et al., 2020), anti-aging, neuropsychiatric disorders (Bruggeman et al., 2024), obesity and metabolic syndrome (Zhang Z. et al., 2024), etc, are also being explored in both human cohort trails and animal experiments. A systematic literature review including 782 studies investigated the clinical FMT uses in 85 specific diseases, showing the promising future of FMT for dysbiosis-related diseases within and beyond the gut (Wang Y. et al., 2022).

3 The fundamental patterns of changes in the gut microbiota post-FMT

As a therapy based on gut microbiome, the introduction of donor-derived microorganisms would lead to the alteration of recipients' microbiota (Figure 1). The trend of alterations in microbial community is influenced by the baseline microbial features within donor feces and recipient intestine, including the relative abundance of certain bacteria at phylum, family and genus taxonomic levels, and the diversity of gut microbiota, which is generally accessed by alpha-diversity and beta-diversity. Alpha-diversity refers to the richness and evenness of the microbial community, and beta-diversity is defined as the compositional dissimilarity among the microbiome community (Barandouzi et al., 2020). Moreover, the microbial interaction between new colonizers and resident microbiota in the gut of the recipient plays an essential role in the determination of FMT outcome. It is crucial to understand the general patterns of microbial succession following FMT. Generally speaking, the process of the succession of gut microbiota toward the ideal state is supported by the administration of supraphysiologic numbers of strains per species, which increases recipient strain richness, which then gradually converges back to the population average over time after dosing is ceased. In recipients, there is a significant correlation between the strain richness 8 weeks and 5 years post-FMT, showing the durably effect of FMT on the microbial structure of recipients (Chen-Liaw et al., 2024). Simply put, timing is a significant determining factor in FMT.

The ecological scenarios post-FMT in recipient's gut includes no replacement, replace with novel, replace and retain, temporary replacement, gain and retain, temporary gain, and loss of strain. Recipient strains with lower initial relative abundances were more susceptible to replacement than strains that were at higher initial relative abundance (Wilson et al., 2021). Engraftment of strains from relatively abundant species were more likely, and predicted oral, oxygen-tolerant, and gram-positive species had a reduced chance of engraftment in FMT, indicating the importance of microbial adaptation to the gut environment (Podlesny et al., 2022). Another study proved that there was a greater possibility of colonizing in the gut of recipients if a species is among the baseline microbiota of recipients (Li et al., 2016). The dissimilarity in the baseline gut microbiota of donors and recipients is negatively-related with the impact on gut microbiota structure and benefits post-FMT (Benítez-Páez et al., 2022). Loss of baseline strains indicated the niche replacement and out-competition induced by the engraftment of exogenous strains. Interestingly, there is also a study showing that the engraftment of strains from donors follows an "all or nothing" way, which means that the strains are either completely maintained or completely replaced by donor strains post-FMT (Smillie et al., 2018). After FMT, the gaining of new strains which recipients did not possess at baseline occurred more frequently than strain replacement (Wilson et al., 2021). The low baseline SR of species in the gut of recipients would lead to the replacement of recipient strains by therapeutic strains of the same species or engraftment failure, while high baseline strain richness of species provides a microbiota that is more conducive to the engraftment of the same species without replacement (Chen-Liaw et al., 2024). The post-FMT novel strains which could not be detected both in the baseline microbiota of donors and recipients showed strain instability, which may be due to the influence of the environment, the limited detection threshold and undetected secondary strains (Wilson et al., 2021). Inter-individual oscillation could be observed for the appearance of either donor or recipient fecal strain dominance, thus leading to various outcomes in different individuals post-FMT, providing new insights into the dynamics of the microbial community interactions with the recipients post-FMT (Koo and Morrow, 2022). Special microbial interactions can also be detected post-FMT. Recombination could occur between the donor and recipient strains (Koo and Morrow, 2022). Horizontal gene transfer (HGT) is an agent of adaptive evolution enabling the transmission of DNA outside of direct ancestral lineages. FMT does not influence the basal rate of horizontal gene transfer (HGT), which means the transmission of DNA outside of direct ancestral lineages (Behling et al., 2024).

Following the aforementioned patterns, the long-term re-construction of microbiota is performed, and the identities of elevated and decreased strains could be analyzed after FMT, which will be profoundly discussed in the following sections.

4 Characteristics of gut microbiota community pre- and post-FMT in various diseases

As have been mentioned above, FMT therapy has been widely used in various diseases associated with microbial dysbiosis. Furthermore, it should be noticed that these diseases exhibit different characteristics of the baseline microbial community, which are generally manifested as the alteration of the abundance of certain strains, or the overall impairment of microbial community after the long-term re-construction of microbiota post-FMT. Mechanistically, the changes in gut bacterial ecology post-FMT can correct the microbiota disorder from the impaired baseline state in certain diseases. For example, FMT can reduce multidrug-resistant organism (MDRO) colonization in a conspecific strain competition

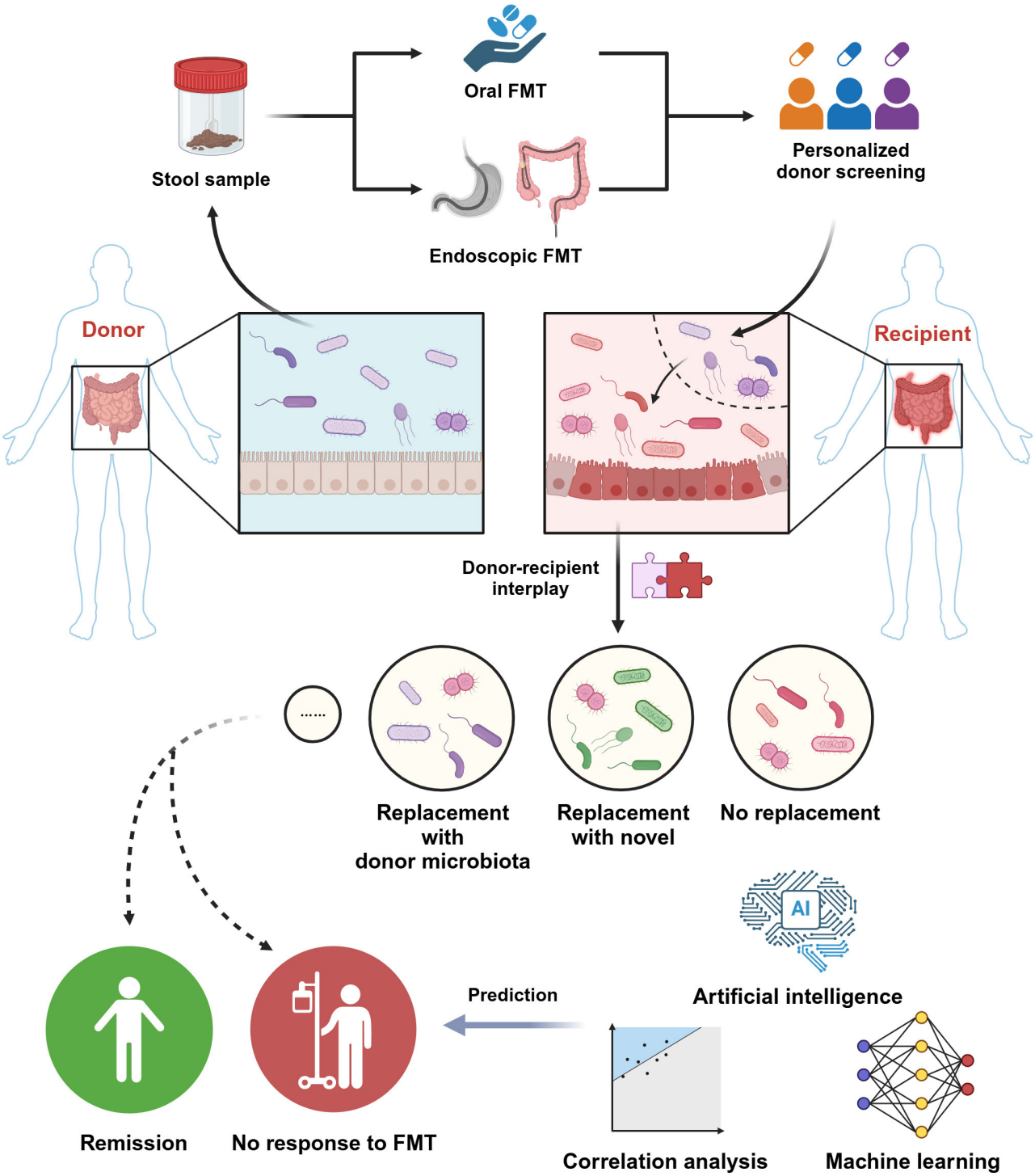


FIGURE 1
Overview of FMT process, possible ecological scenarios within the gut of recipients post-FMT and the prediction of therapeutic efficacy.

manner (Woodworth et al., 2023). Moreover, changes in signaling pathways may be caused by the variations in specific strains.

Considering these specificities, FMT should be more targeted and personalized. Therefore, it can be preliminarily inferred that microbial characteristics post-FMT that more resemble those of healthy donors, along with the restoration of specific microbial members, may have profound influence in disease recovery. Here, we summarize the baseline impairment and reconstruction of microbial community observed in patients with FMT-targeting diseases, providing a method for the evaluation of therapeutic efficacy in diseases. Moreover, we introduce the alteration of key molecular expressions and metabolic characteristics in the onset and recovery of microbial disorder-related diseases, revealing the value of FMT at a mechanistic

level and laying a foundation for future improvement of therapeutic options.

4.1 CDI

CDI can be classified into subtypes including severe CDI and fulminant CDI (FCDI), whose definitions have been stated in ESCMID guidelines and IDSA guidelines (Yakout et al., 2024). In addition, rCDI is defined as a relapse of CDI within 8 weeks of treatment or at least two episodes of severe CDI with hospitalization and significant morbidity (Levy et al., 2024).

16S rRNA gene sequencing showed that the dysbiotic state in rCDI patients is characterized by a large expansion of *Proteobacteria* (Weingarden et al., 2014). A review exploring the link between gut microbiota and CDI development showed that hospitalized elderly individuals with CDI had significantly lower abundances of Lachnospiraceae, Ruminococcaceae, Blautia spp., *Prevotella spp.*, Dialister spp., Bifidobacterium spp., Roseburia spp., Anaerostipes spp., *Faecalibacterium spp.* and *Coprococcus spp.*, compared with healthy controls, and higher *Enterococcaceae* and *Enterococcus spp.*. While asymptomatic colonization (AC) patients with *C. difficile* showed decreased abundances of *Prevotella*, *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Dorea*, *Coprococcus*, and *Roseburia* (Martinez et al., 2022). Another study showed that the relative bacterial abundances of *Negativicutes* (*Firmicutes*), *Gammaproteobacteria* (*Proteobacteria*), and *Fusobacteria* (*Fusobacteria*) were high in CDI patients (Fujimoto et al., 2021).

After performing FMT, increased *Bacteroidetes* and decreased *Proteobacteria* were observed in CDI patients (Seekatz et al., 2014). Another study also proved the importance of *Bacteroidetes* abundance in CDI treatment, indicating that FMT is related with the increased *Bacteroidetes* to *Firmicutes* ratio (Weingarden et al., 2014). *Bacteroidetes* and *Actinobacteria* species showed higher engraftment than *Firmicutes* in recipients' feces in rCDI treatment. Another study showed that the proportions of *Clostridia* (*Firmicutes*), *Erysipelotrichia* (*Firmicutes*) and *Bacteroidia* (*Bacteroidetes*) increased significantly, the richness and diversity of the bacterial species were significantly higher in recipients than those before FMT, and the *bacteriomes* of the recipients tended to approach those of the donors after FMT (Fujimoto et al., 2021). Genomic analyses also showed longitudinal persistent enrichment of *Trichosporonaceae* and *Ruminalococcaceae* bacteria after FMT in CDI patients (Gupta et al., 2016; Ramos et al., 2022). In addition, in a single-center study, an increase in the normal abundance of *Mycobacterium avium* and *Mycobacterium thick-walled* and a decrease in *Mycobacterium aspergillus* were found in the feces of rCDI patients treated with FMT, along with a reduction in the number and diversity of antimicrobial resistance genes in the feces of the patients (Millan et al., 2016).

Functionally, fecal samples from rCDI patients pre-FMT contain high concentrations of primary bile acids and bile salts, instead of secondary bile acids, while post-FMT fecal samples contain mostly secondary bile acids. The identification of bacterial and viral gene functions pre- and post-FMT also revealed improved secondary bile acid biosynthesis, which inhibit the germination of C. difficile spores (Fujimoto et al., 2021; Gupta et al., 2016; Ramos et al., 2022; Weingarden et al., 2014). Enhanced fluorobenzoate degradation can also be detected in rCDI patients post-FMT (Fujimoto et al., 2021). A recent study constructs a mice model and shows that the interaction between *C. difficile*, intestinal commensal microorganisms and the host immune system via inter-related arginine-ornithine metabolism influences the pathogenesis of CDI and the improvement provided by FMT (Yang et al., 2024). Microbial interaction patterns influencing *C. difficile* growth and toxin production is influenced by different nutrient landscapes within the intestine of CDI patients, which helps clarify the therapeutic effect of FMT and improve the effectiveness of anti-CDI strategies (Sulaiman et al., 2024).

4.2 IBD

The alpha and beta diversities of microbial populations at the strain level was significantly reduced in UC subjects compared to control. A trail published in Nature followed 132 patients with IBD for 1 year and the increase in facultative anaerobes at the expense of obligate anaerobes and molecular disruptions in microbial transcription (for example, among *Clostridia*) were observed in this study (Lloyd-Price et al., 2019). A recent integrative analysis drew a conclusion that the relative abundance of 117 strains were significantly different between UC and control microbiomes, manifested as decreased *Faecalibacterium prausnitzii* and increased *Ruminococcus gnavus* in UC microbiomes (Zhu J. et al., 2024).

When performing FMT, high bacterial richness and high alpha-diversity in donor fecal is linked with the efficiency of FMT in UC patients (Kump et al., 2018; Rees et al., 2022). The presence of *Bacteroides* (Paramsothy et al., 2019; Rees et al., 2022), *Clostridium clusters IV* and *XIVa* (Paramsothy et al., 2019; Rees et al., 2022), *Akkermansia muciniphila* (Kump et al., 2018), *unclassified Ruminococcaceae* and *Ruminococcus spp.* (Kump et al., 2018; Zhang Z. et al., 2024), *Bifidobacterium* (Nishida et al., 2017) and *Lachnospiraceae* (Moayyedi et al., 2015) in donor stool are correlated with the remission in FMT recipients. The feces of "super-donor" in UC treatment was enriched in *Ruminococcaceae* and *Lachnospiraceae* families (Moayyedi et al., 2015). Whereas *Streptococcus* species (Paramsothy et al., 2019), *Lactobacillales* (Nishida et al., 2017) in donor stool are linked to a lack of response to FMT.

In terms of the FMT recipients with UC, patients in remission post-FMT have enrichment of *Eubacterium hallii*, *Roseburia inulivorans* (Paramsothy et al., 2019), *Ruminococcaceae* and *Lachnospiraceae* (Pinto et al., 2024), and *Clostridium clusters IV* and *XIVa* compared to patients with no remission post-FMT. Conversely, *Fusobacterium gonidiaformans*, *Sutterella wadsworthensis*, *Escherichia* species and *Prevotellaceae* in patients are associated with poor alleviation of UC post-FMT (Paramsothy et al., 2019; Pinto et al., 2024; Wilson et al., 2019). The relapse and poor sustained response of UC post-FMT is correlated with *Proteobacteria* and *Bacteroidetes* (Fuentes et al., 2017). Nevertheless, there are also paradoxes when investigating the significance of microbiota in donor feces, for example, higher *Clostridium clusters IV* level was also observed in the donor feces for nonresponders (Nishida et al., 2017).

Functionally, a cross-cohort integrative analysis enrolling 9 metagenomic and 4 metabolomics cohorts of IBD from different populations proves that essential gene of "Two-component system" pathway, linked to fecal calprotectin, is related with IBD. Moreover, metabolomics analysis shows 36 identified metabolites with significant differences in IBD, and highlights gut microbial biotransformation deficiencies and significant alterations in aminoacyl-tRNA synthetases in IBD patients (Ning et al., 2023). Overabundance of proteases originated from *Bacteroides vulgatus* is associated with UC, which was proved via multi-omics approach (Mills et al., 2022). Another multi-omics study identified impaired metabolism of acylcarnitines, bile acids, SCFAs and levels of antibodies in host serum during IBD activity (Lloyd-Price et al., 2019). The reduction of *Faecalibacterium prausnitzii* and increase of *Ruminococcus gnavus* in UC patients leads to the unique mode of metabolic pathways, characterized by attenuated glycan degradation, fermentation and amino acid metabolism subsystems, while increased citric acid cycle, simple sugars, lipid metabolism, and vitamin and cofactor metabolism subsystems (Zhu J. et al., 2024). For IBD diagnosis, bacterial-associated metabolites including SCFAs, medium-chain fatty acids, tryptophan-derivatives, bile acids and sphingolipids are regarded as metabolism-related biomarkers in clinical practice (Vich Vila et al., 2024). A recent study searches for biomarkers of UC via machine learning and metabolomics, showing the difference of serum levels of tridecanoic acid, pelargonic acid and asparaginyl valine in different subtypes of UC (Ge et al., 2025). In terms of FMT treatment, sustained remission of IBD is associated with overall increased butyrate production capacity (Fuentes et al., 2017), while IBD patients who did not achieve remission post-FMT shows increased levels of heme and lipopolysaccharide biosynthesis (Paramsothy et al., 2019). The baseline levels of xanthine and oleic acid in recipients with IBD are significantly lower than that of the donors and increase after FMT, and putrescine and 5-aminovaleric acid are lower post-FMT compared (Nusbaum et al., 2018).

4.3 IBS

IBS could be classified into four subtypes: IBS with predominant constipation (IBS-C), IBS with predominant diarrhea (IBS-D), IBS with mixed bowel habits (IBS-M) or IBS, unsubtyped (Lacy and Patel, 2017). Dysbiosis of intestinal microbiota plays an important role in the pathogenesis of IBS. In a randomized, double-blind, placebo-controlled trial published in Gut that included 52 adult patients with moderate to severe IBS, 11 OTUs established in FMT recipients, 6 of which were classified in the *Clostridiales* order and 4 of which were classified in the *Bacteroidales* order. The IBS-SSS was negatively-correlated with *Blautia* genus of the *Clostridiales* order, which is associated with a healthy gut-microbiome (Halkjær et al., 2018). *Dorea*, *Lactobacillus* and *Ruminococcaceae spp.* in recipients' feces are associated with higher success rate of FMT when treating IBS (El-Salhy et al., 2020).

The characteristics of baseline microbiota impairments in IBS subtypes differ. In a meta-analysis of a randomized controlled study showed that IBS-C patients had higher fecal *Bacteroides* level, while no significant increase in *Bifidobacterium*, *Lactobacillus*, *Enterobacteriaceae*, or *Enterococcus* were found (Shukla et al., 2015). In another randomized controlled study enrolling 27 individuals with IBS-D, 7 bacterial genera (*Gemella*, *Roseburia*, *Acidovorax*, *Lactobacillus*, *Weissella*, *Klebsiella* and *Parvimonas*) were associated with differences in the pre- and post-treatment IBS-SSS score, and *Gemella*, *Acidovorax* and *Klebsiella* might be involved in the development of the clinical symptoms of IBS-D (Zhang Y. et al., 2024). The level of *Faecalibacterium prausnitzii* has a potential relation with IBS-M (Soldi et al., 2019).

For the mechanism, in IBS patients, the links between gut microbiota and fecal metabolites are observed, revealing that *Odoribacter splanchnicus*, *Escherichia coli* and *Ruminococcus gnavus* are strongly associated with the low abundance of dihydropteroic acid. Furthermore, tryptophan/serotonin metabolism disorder is related with IBS depression comorbidity (Han et al., 2022). Through analyzing the longitudinal multi-omics data of IBS patients, the potential effect of purine metabolism associated with microorganisms in IBS is identified (Mars et al., 2020). The global alterations in microbiome composition in IBS patients also result in increased tyramine, upregulation of fructose and glucan metabolism, succinate pathway of carbohydrate fermentation, and decreased gentisate and hydrocinnamate. Moreover, IBS-D and IBS-C shows different characteristics in the metatranscriptome and metabolome, implying the importance of focusing on the subtypes of diseases (Jacobs et al., 2023). DESI-MSI shows that 6 medium-chain and long-chain fatty acids are determined to be most overrepresented in the IBS-D group, becoming potential indicators to distinguish IBS patients and healthy population (Zhang Y. et al., 2023). FMT increases the fecal SCFA levels in IBS patients, which are related with improved clinical symptoms of patients (El-Salhy et al., 2021).

4.4 Other potential FMT-targeting diseases

In metabolic diseases, successful FMT has been shown to significantly increase the abundance of SCFA-producing species such as *Roseburia intestinalis* and *Akkermansia muciniphila*, as well as various *Clostridium spp* (Kootte et al., 2017). In the treatment of severe obesity and metabolic syndrome, lower relative abundance of *Prevotella*, greater bacterial richness and more consistent engraftment of donor-specific bacteria ASVs (amplicon sequence variants) are associated with better treatment response (Zhang Z. et al., 2024). In obesity treatment, multi-donor FMT showed the efficiency of sustainably altering the microbiome of recipients, with 2 of the 4 donors dominating the microbial engraftment to the recipient. Exploring the gut microbiome characteristics of the two primary microbial strain providers, results showed high ratio of *Prevotella* to *Bacteroidetes* (P/B) dominated the engraftment and almost all FMT recipients with a low P/B ratio at baseline transitioned to a high P/B ratio (Wilson et al., 2021).

In immune-related diseases, a non-randomized clinical trial enrolling 10 individuals with immune-mediated dry eye, after administration of FMT in two enemas one week apart, subjects were found to have decreased abundance of *Enterococcus faecalis spp.*, *Prevotella spp.*, and *Ruminalococcus spp.*, and increased

abundance of the genera *Alistipes*, *Streptococcus*, and *Blautia*, as compared to the donors (Watane et al., 2022). In GvHD treatment, an increase in the richness and diversity of the intestinal bacterial group was found in subjects with GvHD who received two consecutive FMTs, and three major species were detected in the subjects' feces, including *Alistipes putredinis*, *Clostridium nexile*, and *Ruminococcus gnavu* (Zhang et al., 2021).

In neurologically related disorders, for example, when treating autism spectrum disorders (ASD), decreased *Collinsella* level is found in responded FMT recipients, and the relationship between other common bacterial strains and the treatment effect has also been elucidated (Chen et al., 2024). Meanwhile, in ASD patients, the pre-treatment abundance of *Eubacterium coprostanoligenes* was lower in responders, which was also negatively correlated with the improvement of gastrointestinal symptoms and the concentration of serum γ-amino acid (GABA), indicating its potential modulating role in the treatment of ASD by FMT (Li et al., 2021).

5 Definition of successful FMT: microbial engraftment vs. clinical outcomes

The characteristics of microbial changes in patients who successfully recovered from various disease, as listed above, suggest that we can to some extent assess the efficacy of FMT by analyzing the microbiota of recipient post-FMT. The changes which meet expectations in the recipient's gut microbiota following FMT can provide optimistic signals in a success therapy to some extent.

Generally, researchers define the success of FMT as the shift in the gut microbiome profile of recipient toward that of the donor and further augmentation of the local commensal community (Wilson et al., 2019). However, fundamentally, as a clinical therapeutic approach, it is the restoration of health after treatment that become the most important criterion for demonstrating the value of FMT. As expected, some studies suggest that considerable engraftment of donor strains is equivalent to high therapeutic efficacy in FMT. Donor microbial profile similarity in recipient post-FMT can be regarded as a predictor at response (Rees et al., 2022), for example, the microbiota of responders post-FMT was similar to that of their healthy donors in UC treatment (Rossen et al., 2015). Moreover, if we could identify the patient's impairment of microbiota and metabolic characteristics which are specific to their disorder, the donor-recipient matching approach may efficiently help recipients in reconstructing disrupted physiological conditions in a targeted manner (Wilson et al., 2019). As a result, ulteriorly, the screening of donors with certain structure of microbial community may help improve the efficacy of FMT.

Recently, the screening of "super-donor" before FMT is becoming more and more popular, which points to donors whose stool samples results in significantly more successful FMT outcomes than that of other donors (Wilson et al., 2019). In a randomized controlled trial of patients with obesity, multi-donor FMT was able to sustainably alter the patient microbiome, with two of four donors dominating the microbial engraftment of the recipient, which were characterized by high *Prevotella* to *Bacteroidetes* ratio, showing the tendency toward being a super donor (Wilson et al., 2021). In a mice gut colonization model, researchers identified a super-donor consortium, which could effectively induce the engraftment of microbiota into recipients. In FMT induced by these super-donors, we could observe a rapid engraftment by early colonizers within 72 h, followed by a slower engraftment by late colonizers over 15-30 days. Spatial transcriptomics has revealed that the microorganisms introduced into recipients are distributed in distinct niches over time, which partially summarized the mechanism of super-donor colonization (Urtecho et al., 2024). In a randomized controlled study, donor-recipient-matched FMT significantly improved the clinical symptoms, quality of life and anxiety scores of the patients with IBS-D than random-donor FMT (Zhang Y. et al., 2024). In short, the screening for super-donors adheres to the fundamental principle of microbiota modulation, and is directed toward achieving better clinical outcomes.

However, some studies did not support the necessary connection between microbial engraftment and clinical efficacy. A randomized controlled trial performed rigorous donor selection based on microbial cell count, enterotype and the abundance of specific genera. Unexpectedly, the trail has been halted for futility (Caenepeel et al., 2024). Moreover, although the persistent engraftment of strains from selected donors has been detected in the recipient, it might also end in a failed treatment, the engraftment of fecal dominant donor microbes of the donor is not necessarily correlated with clinical improvement (Browne et al., 2021; Koo and Morrow, 2021).

Therefore, a successful FMT should not be merely defined as the influence of the donor and the alterations in the gut microbiota. The exact mechanisms of "why FMT works" still remains to be explored in future researches.

6 Objective assessment tools for FMT efficacy: prognostic models

As mentioned above, donor screening based on microbiota characteristics may not necessarily lead to better prognosis, implying the presence of potential determining factors. In addition, due to the specificity of intestinal microecology, the complex interplay between microbial members leads to the difficulty to be exactly identified and included in the strategy of donor screening. Therefore, the construction of prognostic models could provide us with comprehensive insights into the factors influencing FMT efficacy. Specially, through the mining and analysis of a vast amount of data, machine learning algorithms are capable of precisely capturing the intricate correlations among FMT and disease treatment outcomes, thus providing novel perspectives and methodologies for disease diagnosis, treatment, and prognosis evaluation.

Wei et al. successfully fitted a random forest model to predict the treatment outcome 8 weeks post-FMT in rCDI patients, in which the factors that have the greatest impact on the output of the model are the abundance of *Enterobacteriaceae* and *Lachnospiraceae* at week 1 in recipients (Wei et al., 2022). Another study's application of random forest modeling in patients with rCDI found that bacterial abundance, classification and time after FMT were important predictive factors (Smillie et al., 2018).

Besides random forest models, a regression tree-based model could effectively predict the outcome (response and recurrence) of cap-FMT in treating rCDI, which includes taxa significantly related to clinical response (Staley et al., 2018). While Xiao et al. present an ecological framework using rCDI as a prototype disease to predict the taxonomic diversity of the diseased state, the impact of host-dependent microbial dynamics, and each level of host-dependency of the microbial dynamics on FMT efficacy, which provides innovative ideas for follow-up research (Xiao et al., 2020). Using metagenomic sequencing and machine learning, Kazemian N et al. proved that in rCDI treatment, the presence of baseline *Clostridioides spp.*, *Desulfovibrio spp.*, *Odoribacter spp.* and *Oscillibacter spp.*, etc, in donors and the absence of baseline *Yarrowia spp.* and *Wigglesworthia spp.* in recipients prior to FMT could predict FMT success (Kazemian et al., 2020).

In UC patients, Sood A et al. develpoed a nomogram predicting the response to FMT, which was defined as the achievement of corticosteroid free clinical remission at week 30. The factors associated with clinical remission includes younger age, disease extent E2 and endoscopic mayo score 2 (Sood et al., 2020). A LASSO logistic regression model was constructed using *Enterococcus*, *Rothia*, and *Colidextribacter* as predictors of UC response, and the AUC of the constructed model amounted to 0.84 (Kang et al., 2022). On the prediction of FMT treatment outcomes in patients with UC, Wu X. et al. utilized the random forest classifier to construct a model based on 20 serum metabolites screened by the Boruta method, in order to predict the clinical remission of UC patients after FMT treatment. The analysis revealed that the model had good predictive performance, with an AUC of 0.963, and good performance in terms of accuracy, sensitivity, and specificity (Wu X. et al., 2023). Zhang S. et al. used multiple machine learning models to construct an integrated model for predicting the clinical response of UC patients after one month of WMT treatment. After internal and external validation, it was found that the different integrated models had their own advantages and disadvantages, and that the vector machine was more stable and reliable (Zhang S. et al., 2024). Zou et al. applied the random forest algorithm and used its classification model to predict metagenomic OUT linkage groups (mOTU) presence and regression model to predict mOTU abundance, it can predict the gut microbiota composition of post-FMT recipients (Zou et al., 2020). Another machine learning model can predict the presence or absence of strains in recipients post-FMT in UC recipients (Ianiro et al., 2022).

To track strains in FMT, Aggarwala et al. assembled a collection of over different bacterial strains from the fecal samples of 22 FMT donors and recipients, and further developed a statistical approach named Strainer, which, in combination with culture and sequencing data, was rigorously benchmarked to accurately detect and quantify the colonization of donor strains in recipients and the retention of original strains in recipients (Aggarwala et al., 2021). To predict the outcome of FMT transplantation, the iMic algorithm was developed, based on the microbiological characteristics of human fecal donor samples, combined with multiple machine learning models, and evaluated with multiple metrics after data preprocessing. iMic was found to perform well in predicting the microbiome characteristics of the recipients and the clinical outcomes, and the demographic information of the donors could improve the prediction results (Shtossel et al., 2023).

To investigate how donor-derived bacteria affect FMT efficacy in both CDI and IBD patients, He R et al. recruited 2 longitudinal IBD cohorts of 103 FMT recipients and further analyzed 1,280 microbiota datasets from 14 public CDI and IBD studies. This research propose a new parameter defined as the ratio of colonizers to residents after FMT (C2R) to evaluate the engraftment of donor microbiota in recipients. An enterotype-based donor selection (EDS) statistical model based on enterotype (RCPT/E dominated by *Enterobacteriaceae* and RCPT/B dominated by *Bacteroides*) has been constructed to predict the level of donor-recipient matching (He et al., 2022). More comprehensively, Schmidt TSB et al. analyzed metagenomes from 316 FMTs for the treatment of 10 different disease indications, thus constructed a LASSO-regularized regression model that could predict the recipient strain turnover, recipient resilience, donor colonization and donor takeover with considerable accuracy. In this study, more clearly, the variables could be divided into ex ante variables, which are knowable pre-FMT, and *post hoc* variables, which are measurable post-FMT (Schmidt et al., 2022). On the whole, the above-mentioned researches extended the prediction model to various diseases, in other words, broke down the barriers between diseases and proposed a set of universal conclusions about the microbial community post-FMT, challenging the hypothesis of "super donor" and underscoring the importance of a multifactorial prediction of FMT efficacy, including recipient factors.

In short, current studies have achieved remarkable results in predicting the treatment efficacy of FMT for diseases related to the gut microbiota. The application of various algorithms, ranging from logistic regression-based nomogram models, machine learning models and newly developed statistical approaches or algorithms, has continuously deepened our understanding of the relationship between the microbiota and FMT (Table 1). By customizing the most optimal donor for each recipient, it provides strong support for precision medicine. In the future, mechanisms of the regulatory effects provided by the factors involved in the prediction models will be revealed, enhancing the explainablity and transparency of predictive models.

7 New players of microorganisms other than bacteria in FMT

As the development of research on the transplantation of the virome and fungal to recipients, the significant role of these new players other than bacteria in human health and the FMT process is gradually being unveiled.

The human gut virome is dominated by bacteriophages, which are a major component of the human gut microbiota. Emerging evidence suggests that gut bacteriophages play important roles in the intricate dynamics with bacteria and their transfer may be associated with the efficacy of FMT. Bacteriophage transfer might also be of substantial mechanistic importance in FMT because of their ability to maintain microbiome ecology equilibrium with bacteria (Liu et al., 2022). In the study of FMT for the treatment of patients with rCDI, successful FMT donors were found to have higher phage alpha diversity and lower relative abundance, suggesting that FMT with increased phage alpha diversity is more likely to successfully treat rCDI

TABLE 1 Prediction models for donor microbiota engraftment and clinical outcomes in FMT treatment.

References	Type of the prediction model	Disease under investigation	Population enrolled	Dominant predictors of the model	Prediction target	Area under the curve (AUC)
Wei et al. (2022)	Random forest model	rCDI	64	The taxa at week 1 post-FMT at genus level • Enterobacteriaceae (Escherichia, negatively correlated with response) • Lachnospiraceae (Blautia, positively correlated with response) • Ruminococcaceae	Treatment outcome at week 8	0.79
Smillie et al. (2018)	Random forest model	rCDI, metabolic syndrome	19-rCDI 5-metabolic syndrome	• The abundance of bacteria • The phylogeny of bacteria	Composition of the gut microbiota in patients after FMT	0.84-rCDI 0.82-metabolic syndrome
Staley et al. (2018)	Chi-squared automatic interaction detection (CHAID)-based regression tree model	rCDI		The abundances of members of the families at 7 days after cap-FMT • Lachnospiraceae • Ruminococcaceae • Bacteroidaceae • Porphyromonadaceae • Enterobacteriaceae	The eventual recurrence of CDI following cap-FMT	
Xiao et al. (2020)	Lotka-Volterra model	rCDI		• The impact of host-dependent microbial dynamics • Each level of host-dependency of the microbial dynamics • Taxonomic diversity of the diseased state	The FMT efficacy	
Kazemian et al. (2020)	Random forest model	rCDI	17	The gut microbiome of donor and recipients pre-FMT (1 week prior to FMT); Desulfovibrio, Filifactor, Bacillus, Yarrowia, Odoribacter, Wigglesworthia, Oscillibacter, Intestinimonas and Clostridioides	Treatment outcome	0.98
Sood et al. (2020)	Logistic regression-based nomogram	Active UC	93 patients who completed the multi-session FMT protocol	• Age • Disease severity • Disease duration • Disease extent • Endoscopic mayo score	Response to FMT (achievement of corticosteroid free clinical remission at week 30)	
Kang et al. (2022)	LASSO-regularized regression model	UC	10	• Enterococcus • Rothia • Colidextribacter	Successful response (defined as partial Mayo score and CRP reduction)	0.84
Wu X. et al. (2023)	Random forest model	UC	44	20 metabolic markers • Glycerophosphocholines • Glycerophospholipids • Glycerophosphoethanolamine	Clinical remission after 3 months of FMT in UC patients	0.96

(Continued)

10.3389/fmicb.2025.1556827

TABLE 1 (Continued)

References	Type of the prediction model	Disease under investigation	Population enrolled	Dominant predictors of the model	Prediction target	Area under the curve (AUC)
Zhang S. et al. (2024)	Voting machine Logistic regression Random forests Adaptive Boosting Light gradient boosting machine Support vector machine	UC	366 (210-training and internal validation; 156-external validation)	• Age • Defecation frequency • Mayo score • Platelet distribution width • Platelet Large Cell Ratio • γ-glutamyl transpeptidase	Clinical response	0.78; 0.614- external validation
Zou et al. (2020)	Random forest model	IBD	15	• The presence of each mOTU • The abundance of each mOTU	Gut microbiota mOTU profiles	0.74
Ianiro et al. (2022)	Random forest model			• Taxonomy • Microbial abundances • α-diversity • Microbial prevalence	The presence or absence of Species post-FMT	0.77
Aggarwala et al. (2021)	Strainer (Statistical methods)	rCDI	13	• Types of strains • Number of strains	Proportional Engraftment of Donor Strains(PEDS) Proportional Persistence of Recipient Strains(PPRS)	0.86
Shtossel et al. (2023)	iMic (image microbiome) Random forest model	IBD, CDI, IBS		• Microbiome characterization of the donor • Donor demographics: Age, sex, weight	Microbiome Characterization of Post-FMT Recipients Improvement in clinical symptoms	0.71-WGS cohorts 0.69-the 16S cohort 0.71-recipient-based learning 0.71- combined donor and recipient model
He et al. (2022)	Random forest model	IBD, CDI	286	• Enterotypes of recipients • Enterotypes of donors before FMT • Their corresponding microbial profiles	Outcome of FMT for each Recipient-donor pair	0.80
Schmidt et al. (2022)	LASSO-regularized regression model	rCDI, ESBL, MetS, IBD, etc	316	• Recipient resilience • Donor colonization • Donor takeover • Recipient strain turnover	Strain dynamics after FMT	0.62-recipient resilience 0.58-donor colonization 0.65-donor takeover 0.94-recipient strain turnover

10.3389/fmicb.2025.1556827

(Park et al., 2019). In a longitudinal study of healthy subjects and obese subjects treated with FMT, recipients with improved clinical outcomes had phage communities that shifted significantly toward healthy donors, with high abundance of phages HV39 and 84 associated with increased rates of glucose disappearance and better clinical outcomes (Manrique et al., 2021). Macrogenomic sequencing showed that FMT altered the characteristics of the enterovirome in post-FMT recipients compared to pre-FMT. For example, the proportion of microviridae in the recipients increased, and the behavior of most temperate phages paralleled that of host bacteria altered by FMT (Fujimoto et al., 2021). Fecal virome transplantation (FVT) has been applied in certain clinical contexts, however, it carries the risk of eukaryotic viral infections. Therefore, modified FVT characterized by removed or inactivated eukaryotic viruses in the viral component, as a efficiency method of safe bacteriophage-based therapies (Mao et al., 2024). Taken together, these findings highlight the association between gut phage and the clinical success of FMT therapies for various diseases. Recently, a growing number of studies have identified *Caudovirales phages* that may interact with host microorganisms and influence clinical outcomes after FMT. Accumulating data has demonstrated that *Caudovirales phages* may play a role in the efficacy of FMT in different diseases. CD and UC were found to be associated with significant amplification of *Caudovirales phages* (Fujimoto et al., 2021). Additionally, *Caudovirales phages* were found to be reduced in UC patients who responded to FMT compared to those who did not respond to the treatment (Gogokhia et al., 2019). Similarly, in a clinical trial, subjects with CDI demonstrated a significantly higher abundance of *Caudovirales phages* and a lower *Caudovirales* diversity, richness and evenness compared with healthy household controls. FMT treatment resulted in a significant decrease in the abundance of *Caudovirales* in CDI (Zuo et al., 2018a). Contrary to the findings of the previous two diseases, in a single case study exploring the use of FMT in a patient with severe gut GvHD, an increase in fecal virome diversity was observed after FMT, accompanied by increased *Caudovirales phages* and a reduction in the relative abundance of eukaryotic Torque teno viruses (Zhang et al., 2021).

Changes in fungal composition have also been associated with various diseases. In IBD patients, a decrease in *Saccharomyces cerevisiae* and *Filobasidium uniguttulatum* species and an increase in *Candida* (e.g., *Candida albicans*) were observed relative to healthy controls (Liguori et al., 2016). The presence of pre-FMT fungi (e.g., *Candida*) was also been found to be associated with increased bacterial diversity after FMT in UC patients (Leonardi et al., 2020). In rCDI patients, successful FMT was dependent on high abundance of *Saccharomyces* and *Aspergillus*, while *Candida* was negatively associated with successful FMT (Zuo et al., 2018b), in contrast to the results observed in UC patients (Leonardi et al., 2020). In the metagenomic analysis of fecal samples from donors and patients with UC receiving capsulized FMT, shifts in gut fungal diversity and composition were associated with capsulized FMT and validated in patients with active UC. Decreased levels of pathobionts, such as *Candida* and *Debaryomyces hansenii*, were associated with remission in patients receiving capsulized FMT (Chen et al., 2022).

8 Emphasizing FMT safety: focusing on infection sources and susceptible populations

8.1 Transmission of pathogens associated with donor feces

Despite its increasing popularity as a therapeutic intervention, FMT still faces numerous regulatory and safety challenges. Just as the engraftment of donor microbiota, potential pathogens may also be transmitted to the recipient's gut. MDROs including extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) can be detected in some of donor feces and a quarter of active donors were colonized with MDROs during participation in the donor programme (Vendrik et al., 2021). It was reported that two patients developed bacteremia due to ESBL-producing *E. coli* after receiving stool from the same donor for FMT and one of the patients died, emphasizing the importance of minimizing the transmission of potentially pathogenic microorganisms, thereby reducing the risk of adverse infectious events (DeFilipp et al., 2019). Stool donor colonized by Shiga toxin-producing *E. coli* (STEC) can also lead to adverse events after FMT (Zellmer et al., 2021). It is worth noting that the fecal carriage rate of ESBL-producing *Enterobacterales*, including *E. coli* and *Klebsiella pneumoniae*, and *diarrheagenic E. coli*, including EPEC, *enteroaggregative E. coli*, *enterotoxigenic E. coli* and STEC, were high (Chuang et al., 2023). Opportunistic infections induced by potential infected donors should be given increasing amount of attention, for the infection of these pathogens may not be symptomatic in IC donors but may lead to transmissible to immunosuppressed FMT recipients (Mehta et al., 2022).

Whole genome sequencing indicated strain transmission of procarcinogenic bacteria between donor and patient, and patients also exhibited clearance of procarcinogenic bacterial strains subsequent to negative donor in FMT (Drewes et al., 2019). Interestingly, a substantial proportion of recipients with potentially procarcinogenic polyketide synthase-negative (pks-) status who underwent FMT from pks+ donors remained pks-, while some pks- recipients treated with stool samples from pks- donors developed pks+ *E. coli* post-FMT (Khoruts, 2021). To sum up, although there is no exact sign of the donor-to-patient transmission of pks+ E. coli, the pks abundance and persistence of pks+ *E coli* of rCDI patients are associated with the pks+ donor (Nooij et al., 2021).

Specially, during the pandemic of infectious diseases, extra detection should be performed to avoid FMT-related transmission of pathogens. During the COVID-19 pandemic, the program of donor recruitment further included evaluation of clinical history and specific testing for the detection of SARS-CoV-2 including nasopharyngeal swab, reverse transcription polymerase chain reaction (RT-PCR) assay, serology and molecular stool testing, etc, showed significant effect in epidemic prevention (Ianiro et al., 2020).

A recent study provided an end-to-end donor screening program, which can effectively minimize the risk to patients receiving FMT. The following factors of donor screening should be valued: health history, physical exam, visual inspection of donations and laboratory testing. Failure modes and effects analysis

(FMEA)-based donor screening can be applied to avoid the risk of disease transmission from donors to recipients (Goldsmith et al., 2024).

8.2 Balancing the risks and benefits of FMT in immunosuppressed patients

In general, immunocompromised (IC) patients, including patients on immunosuppressant medications, with human immunodeficiency virus (HIV), inherited or primary immunodeficiency syndromes, cancer undergoing chemotherapy or organ transplant, were often excluded from FMT trials (Shogbesan et al., 2018). FMT is contraindicated in patients with significant primary and secondary immunodeficiencies, which is owe to the potential risk of bacterial translocation and the development of bacteremia, particularly with MDROs (Conover et al., 2023). However, FMT probably could be generally considered safe in IC patients in some conditions according to several studies. Research showed similar rates of serious adverse events when using FMT to IC patients compared to those with intact immune function (Shogbesan et al., 2018). When treating pediatric IC patients with rCDI by FMT, the success rate was similar to the treatment targeting IC adults and immunocompetent children (Conover et al., 2023; Rodig et al., 2023). A pilot placebo-controlled study showed that FMT is safe in HIV patients and led to no severe adverse events and attenuated HIV-associated dysbiosis (Serrano-Villar et al., 2021).

Patients in the following cases, who presented with varying degrees of immunosuppression, achieved alleviation of their primary conditions following FMT. A 59-year-old patient with common variable immunodeficiency accepted FMT to alleviate diarrhea after all therapeutic options had been exhausted, which turned out to be successful and led to no adverse effects (Napiórkowska-Baran et al., 2024). Three patients with refractory acute graft-vs.-host disease (GI-aGvHD) following allogeneic hematopoietic stem cell transplantation (allo-HSCT) also achieved sustained improvement after FMT (Spindelboeck et al., 2017). A single heart-kidney transplant recipient with rCDI, vancomycin-resistant *Enterococcus* (VRE) fecal dominance and recurrent VRE infections obtained relief from the above-mentioned infections via FMT (Stripling et al., 2015).

However, while using FMT in IC patients is accepted to a certain degree, we still need to pay more attention to this population to ensure their the safety and curative effect of FMT. From the aspect of success rate, repeated FMT or additional antibiotics may be needed to achieve improved outcomes in solid organ transplant (SOT) patients (Cheng et al., 2019). Most importantly, from the aspect of safety, several reported severe adverse effects of FMT in IC patients emphasizes the potential risks associated with FMT treatment to this typical population. Undetected opportunistic pathogens in stool samples might bring the risk of pathogenic transmission in immunosuppressed patients, including cytomegalovirus (CMV), Epstein Barr virus (EBV) and polyomaviruses, etc. (Mehta et al., 2022). Some researchers insist that although FMT therapy is not associated with the increased risk of severe adverse events according to several studies, the degree of immunosuppression should be accessed prior to the application of FMT, especially for patients with solid tumors receiving cytotoxic therapy, for the type and duration of chemotherapy varies, which may influence the safety and success rate of FMT. Specially, in addition to the function of individual's immune system, mucosal immunity is also an essential part in the resistance to pathogens. It should be noted that all reported FMT-related serious adverse events were observed in patients with mucosal barrier injury (Marcella et al., 2021). More case-by-case assessment of the benefit-to-risk ratio should be performed to guide the protocols of FMT for IC patients and help grasp the potential chances of therapy (Benech et al., 2024).

9 The potential influence of gender in the outcome of FMT

Sex differences in incidence rates of multiple diseases has been found, and gender factors are closely associated with the pathogenesis of some diseases. For example, a newly published review has discussed the effect of gender in IBD, which could help advance personalized medicine and improve the quality of life for people with IBD in a gendered point of view (Andersen et al., 2024). As a result, some studies insist that gender should be considered while donor screening to ensure the success of FMT (Benítez-Páez et al., 2022).

It is worth noting that researches have proposed the contribution of gender factors in the outcomes of FMT. Overall, gut microbiota differed in males and females, which could be partially explained by the impact of sex hormones (Yurkovetskiy et al., 2013). Individuals of different sexes possess unique physiological characteristics and less susceptibilities to certain diseases and these factors might be transferred to recipients post-FMT, whose mechanisms may involve that the gut bacterial community composition differs between male and female, and such difference can further modulate the metabolic processes and molecular expressions (Haro et al., 2016). As a result, in several diseases, stool sample from a certain gender might be considered as potential donors with higher quality. Improved outcome of ischemic stroke could be induced by female donor of FMT, for the gut microbiota of female is characterized by lower level of systemic proinflammatory cytokines (Wang J. et al., 2022). The susceptibilities to radiation toxicity (Cui et al., 2017) could also be transferred via FMT in a gender specific manner. During the process of FMT, change in the gender of either the donor or the recipient may lead to different clinical outcomes. The baseline characteristics of microbiota differs in donors of different sex, and the gender of recipients influences the strain colonization, the gut of mice of different genders prefers to accommodate microbiota differently (Wang et al., 2016). Post-inflammatory females with colitis could transfer visceral hyperalgesia to both males and females, but males could only transfer visceral hyperalgesia to individuals of the same sex (Arzamendi et al., 2024). Feces of young female mice could improve insulin sensitivity in females, while feces of aged male mice could increase insulin resistance in female mice, eliminating and enhancing sex differences in insulin sensitivity and metabolome, respectively (Sheng et al., 2021). FMT during lactation resulted in

long-term effects on the metabolism of male Wistar rats while no effect was observed in female rats (Pavanello et al., 2022).

In some cases, despite considering the potential benefits that certain gender may provide in FMT, attention must be paid to the risks associated with sex-discordant FMT. A recent research published in Gastroenterology proved that sex-concordant FMT contributes to fewer adverse events post-FMT, and sex-discordant FMT administrated while treating CDI has the potential to cause IBS (Sehgal et al., 2024). Another study showed that male recipients who underwent cross-sex FMT exhibited notably reduced testosterone levels in comparison to those who received same-sex FMT (Feješ et al., 2024), suggesting the disruption of gender-specific baseline physiological characteristics caused by sex-discordant FMT.

In conclusion, as an often overlooked demographic indicator, existing research has proposed the multidimensional impact of age on FMT outcomes, while we have not yet formed a detailed proposal to generalize to clinical applications. More research is imperative in the future.

10 The potential influence of age in the outcome of FMT

Gut microbial composition is considered to be associated with the age. Interestingly, a gut microbial age (MA) metric has been proposed to evaluate gut aging (Wang et al., 2024). As a result, the age of either the donor and the recipient may affect benefits provided by FMT due to age-related baseline microbial characteristics.

Generally, we advocate prioritizing young donors in donor screening. According to statements provided in European consensus conference on FMT in clinical practice, individuals aged <60 years should be preferred while selecting donors, while this age restriction is not must be strictly adhered to, the criteria may be appropriately relaxed under certain circumstances, for instance, the use of intimate healthy partners (Cammarota et al., 2017). In animal experiments, aged donor could lead to age-associated alteration of physiological activities and signaling pathways, resulting in the occurrence of major comorbidities associated with aging. Conversely, the transfer of young donor microbiota to old recipients could reverse aging-related alteration (Cheng et al., 2024; D'Amato et al., 2020; Parker et al., 2022). Aged mice is susceptibility to arrhythmia for the increased reactive oxygen species (ROS) level, which could be transmitted to young mice via FMT (Fu et al., 2024). Nevertheless, strikingly, under some circumstances, the older, the better. An aging-enriched enterotype was observed to conduce to improved immunotherapy outcomes in older patients with cancer, which was characterized by the up-regulation of exhausted and cytotoxic T cell markers in the tumor microenvironment and whose therapeutic effect in FMT has been proved in mice (Zhu X. et al., 2024). However, we still need to consider how this finding can be translated into clinical practice, as other physiological impairment caused by gut aging seem to be difficult to avoid.

The age of the recipient also has the potential to affect the efficiency of FMT. A prospective cohort study proved that patients with rCDI who aged over 65 years might be independently associated with a lower treatment effect from a single FMT (Baunwall et al., 2023). However, in another prospective study, genomic analysis showed that there was no significant difference in gut microbial diversity between donors aged ≥60 years and <60 years. The clinical efficacy of FMT in rCDI over 12 months was also not affected with advancing age (Anand et al., 2017). According to the second edition of joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines (2024), we should not refuse or delay FMT therapy due to any recipient risk factors, for example, recipient age over 75 years old (Mullish et al., 2024).

The age disparity between the donor and the recipient is of significant importance. When treating metabolic syndrome by FMT, the age difference of donor-recipient pairs is positively-related with the greater reversion of metabolic dysfunction (Benítez-Páez et al., 2022). Specially, significant age disparities between the donor and the recipient could occur in the FMT treatment of pediatric intestinal and extraintestinal diseases, it seems that pediatric patients have been also receiving transplant material from adult donors. The differences in gut microbiota between children and adults need to be taken seriously.

Specialized metabolic functions in pediatric recipients are observed while using adult fecal donors (Wu Q. et al., 2023). The match of adult donors and child recipients has the risk of atypical maturation of gut microbiota in pediatric patients (MacLellan et al., 2021). However, it needs to be noted that although there are challenges in the use of FMT in pediatric patients, it is necessary to overcome these difficulties and develop a more widely recognized standard, especially due to that the approvals of promising biotherapeutics are expected to be significantly delayed for children (Hourigan et al., 2021).

11 Metabolic status and lifestyle of donors and recipients

The metabolic status of the donor should be evaluated before FMT procedure, which could be regulated via lifestyle intervention. Diet is the main contributor to gut microbiota diversity, the proportional abundance of several taxa varies with the variation of diet patterns and body fat counterpart levels (Newman et al., 2021). Accompanied with the alteration of microbiota, different dietary patterns shape various metabolomic characteristics, manifested as the variation of 127 common metabolites including lipids, tri/di-glycerides and lyso/phosphatidylcholine, etc. (Tanaka et al., 2022). Specially, the Mediterranean diet intervention could lead to increased fiber-degrading *Faecalibacterium prausnitzii* and gene expression for microbial carbohydrate degradation associated with butyrate metabolism, fecal bile acid degradation and insulin sensitivity that co-varied with specific microorganisms (Meslier et al., 2020).

Animal experiments proved that increased total body and fat mass, as well as obesity-associated metabolic phenotypes were transmissible with FMT (Ridaura et al., 2013). Both leptin receptor knock-out obese and diabetic mice donor and diet-induced obese mice donor could result in elevated gut permeability, inflammation level and glucose metabolic dysfunctions in mice recipients, which could be partially explained by the impaired ethanolamine

metabolism (Mishra et al., 2023). Donor mice fed by high-fat, high-sucrose diet (HFHS) could induce disrupted glucose metabolism to recipient mice, while secondary adiposity was not observed in recipients (Zoll et al., 2020).

Conversely, donors who have a healthier habitus and adherence to healthy lifestyle are more likely to confer long-term health benefits to recipients. Within 6 weeks after FMT induced by lean donors, FMT resulted in significantly altered duodenal bacterial species including *Bifidobacterium pseudolongum*. Fecal bacterial species that were different between autologous and allogenic FMT from lean donors included the lactate-producing *Lactobacillus salivarius* and butyrate-producing *Butyrivibrio*, *Clostridium symbiosum*, and *Eubacterium* species, which were related to human metabolism (Kootte et al., 2017). Lean vegan donor could induce improvement of the outcome of recipients with hepatic steatosis via shaping microbial community and altering the expression of hepatic genes involved in inflammation and lipid metabolism (Witjes et al., 2020).

A randomized controlled trial published in Gastroenterology randomly divided abdominally obese or dyslipidemic individuals into three groups, in which participants followed healthy dietary guidelines, Mediterranean diet guidelines and green-Mediterranean diet guidelines (extra consumption of green tea and a Wolffia globosa green shake). After 6 months of weight-loss phase, participants underwent autologous FMT and those who was in green-Mediterranean diet group showed less weight regain, gut microbiota recovery and metabolic impairment. Mechanistically, the green-Mediterranean diet induced altered microbiome composition during the weight-loss phase, and prompted preservation of weight-loss-associated specific bacteria and microbial metabolic pathways including microbial sugar transport after autologous FMT (Rinott et al., 2021). Specially, although stool samples gained from metabolic syndrome donors (METS-D) induced decrease of insulin sensitivity, post-Roux-en-Y gastric bypass donors (RYGB-D) could improve insulin sensitivity and altered expression of metabolism related molecules (de Groot et al., 2020; Kootte et al., 2017).

Interestingly, populations traditionally considered as recipients for FMT may also possess the potential to serve as donors under certain circumstances. Healthy overweight or obese donors have been included to treat cachectic patients with advanced gastroesophageal cancer, although this FMT process prior to first-line chemotherapy did not improve cachexia, improved response and survival in patients with metastatic gastroesophageal cancer was observed (de Clercq et al., 2021).

Meanwhile, at the level of recipients, researchers are exploring the extra measures that recipients could adopt post-FMT to maximize the benefits of FMT treatment. The development of specific lifestyle or diet post-FMT may serve as a promoter for the development and maintenance of the intestinal type, which is favorable to disease recovery. Oral pectin intake for five consecutive days following FMT enhances the effect of FMT in UC by maintaining gut microbial diversity, which could be fermented into SCFAs (Wei et al., 2016). FMT plus lifestyle intervention resulted in greater efficacy of microbiota engraftment from donors in recipients with type 2 diabetes post-FMT (Ng et al., 2022). Patients with mild to moderate UC who accepted FMT and anti-inflammatory diet showed more profound deep remission than those who received stable baseline medications (Kedia et al., 2022). FMT coupled with dietary fiber intervention contributes to shape gut microbiota composition and improve the effectiveness of FMT in recipients (Zhong et al., 2021). This finding was further validated in a randomized double-blind trail, revealing that during FMT, supplementation of low-fermentable fiber, but not high-fermentable fiber, led to better outcome of obesity and metabolic syndrome (Mocanu et al., 2021). Specially, a microbiome-based artificial intelligence-assisted personalized diet significantly reduced IBS-SSS scores across all IBS subtypes, whose efficacy was even better than low-fermentable diet, revealing the prospect of artificial intelligence-assisted therapy in the field of FMT (Tunali et al., 2024).

In short, in the future, screening for the metabolic status and shaping lifestyle habits in donors and recipients should be further performed, which may lead to more stable clinical remission without accompanying metabolic disorders. Furthermore, we should not overlook the potential beneficial effects that the feces of "sub-healthy" individuals with specific metabolic characteristics may have on recipients.

12 Bowel preparation and medication application in recipients

As previously mentioned, the efficacy of FMT largely depends on the interaction between the gut microbiota of the donor and the recipient. Therefore, in addition to donor screening based on microbial community characteristics, pre-regulating the gut microbiota of recipients may contribute to the success rate of FMT.

Researches showed bowel cleansing could enhance donor microbiome engraftment, inadequate bowel preparation is an independent predictor of failure after single FMT in treating rCDI (Ianiro et al., 2017; Podlesny et al., 2022). While in addition to the use of intestinal preparation drugs such as polyethylene glycol, according to the international guidelines of FMT, patients with rCDI should be treated with antibiotics including vancomycin or fidaxomicin at least for 3 days pre-FMT to lower the abundance of intestinal *C. difficile*, and antibiotics should be stopped 12 to 48 h pre-FMT (Cammarota et al., 2017). However, in diseases other than rCDI, no exact recommendation has been proposed.

Current research on the use of antibiotics before FMT is preparing the groundwork for the development of more refined guidelines in the future. The reconstruction of gut microbiome occurs against intestinal antibiotic exposure, while due to the complex ecological network of intestine, the effect of antibiotics varies in different individuals. Overall, in terms of multi-species population, administration of antibiotics could influence the following factors of microbiota within patients' gut: taxonomic and resistome composition, nutrient availability and complexity, metabolic networks of cross-feeding or competition, and horzontal gene transfer of resistome elements, shaping the recipients' gut microbiota to better introduce those of the donors (Fishbein et al., 2023). A metagenomic systematic meta-analysis of 24 studies showed that antibiotics used pre-FMT was positively-associated with donor strain engraftment and clinical success (Ianiro et al., 2022). The administration of antibiotics pre-FMT in recipients showed better modification of gut microbiota and

increased xenomicrobiota colonization post-FMT, compared to performing bowel cleansing solution or no pretreatment (Ji et al., 2017). Species-level dysbiosis within the phylum *Bacteroidetes* could be found in the gut of UC patients, triple-antibiotic pre-treatment in these patients, including amoxicillin, fosfomycin and metronidazole, could promote the eradication of dysbiotic strains and further colonization of viable *Bacteroidetes* cells (Ishikawa et al., 2018). Another study also utilized a triple-antibiotic pre-treatment, including amoxicillin, doxycycline, and metronidazole, which also reached promising results (Haifer et al., 2022). The greater engraftment of strains in recipients provided by antibiotics used pre-FMT was also discovered in immunosuppressed patients, such as individuals with HIV (Serrano-Villar et al., 2021). In the safety study of SER-287, a spore-based microbiome therapeutic, in treating UC, the group vancomycin/SER-287 showed higher proportion of patients who achieved clinical remission than the placebo/SER-287 group (Henn et al., 2021).

Different therapeutic courses of certain antibiotics before FMT could result in distinct clinical outcomes post-FMT. An animal experiment proved that both frequency of dosing and duration of preparative antibiotic treatment influences strain engraftment post-FMT (Gopalakrishnan et al., 2021). In human, short duration of vancomycin pre-treatment is positively related with the positive C. difficile culture pre-FMT and early CDI recurrence (Groenewegen et al., 2024). In a retrospective study of rCDI treatment, oral vancomycin for standard duration of 10–14 days pre-FMT was not significantly associated with FMT success, while prolonged vancomycin taper for ≥6 weeks induced better outcome in FMT treatment. Mechanistically, standard therapeutic courses of vancomycin may reduce the stool concentrations of *C. difficile* and allow the persistence of disrupted microbiota. In contrast, the prolonged tapered course of oral vancomycin pre-FMT may result in partial microbiota replenishment and reducing the fecal concentration of vegetative C. difficile (Patron et al., 2017).

However, the application of antibiotics prior to FMT has, at times, been proved to be ineffective in enhancing treatment efficacy. In a randomized controlled trial, individuals with IBS-D received pre-FMT ciprofloxacin and metronidazole (CM-FMT) or rifaximin (R-FMT) conversely exhibited a lower rate of microbial strain engraftment compared to individuals with alone FMT (Singh et al., 2022). Moreover, paradoxically, the exposure to systemic antibiotic therapy might be related to the onset of dysbiosis-related chronic diseases including IBD (Nguyen et al., 2020). The elevated susceptibility to IBD is most frequently observed in population aged over 40. The highest risk of IBD onset is typically seen 1–2 years post-antibiotic administration and after the use of antibiotics commonly prescribed to treat gastrointestinal infections (Faye et al., 2023). Antibiotic exposure is also the highest risk for CDI (Pérez-Cobas et al., 2015). An animal experiment proved that short-term exposure to antibiotics could induce lowered microbial alpha and beta diversities, reduced complexity of gut molecular ecological networks, and impaired microbial metabolic pathways related with bacterial survival and physiological functions. These mentioned changes could not entirely recover over time. Although the antibiotic administration pre-FMT would reshape the impaired microecology to some degree, we still should not ignore the risk of long-term side effects induced by short-term antibiotics intervention (Hong et al., 2024). These findings remind us that further research is still warranted to confirm the benefits of antibiotic-based gut preparation pre-FMT. Caution must be exercised to regard the potential ineffectiveness of the treatment and the risk of illness aggravation antibiotics may pose.

Except for using antibiotics at the time of preparation before FMT, if antibiotics are administered at the wrong time point during the FMT process, they can also become ineffective or even become an obstacle to the effectiveness of the treatment. In the scope of pre-FMT, feces from human donors who administrated repeated, but not recent antibiotics might induce impaired mucus growth mucus barrier integrity in mice recipients, the microbiota community of which is characterized by enriched mucus-utilizing bacteria, including *Akkermansia muciniphila* and *Bacteroides fragilis*. As a result of altered microbiota composition, 10 metabolites, including adenine, adenosine and betaine, etc., showed significantly altered abundance in mice recipients (Krigul et al., 2024). While in the scope of post-FMT, non-CDI antibiotics during follow-up were all independently associated with a lower treatment effect from FMT in CDI treatment (Baunwall et al., 2023; Groenewegen et al., 2024). Antibiotic use within the first 8 weeks after FMT may disrupt microbial engraftment and lower the success rate of FMT (Allegretti et al., 2018).

In summary, the creation of a microenvironment conducive to microbial engraftment within the gut of recipients is of great importance pre-FMT, which could be achieving through the application of antibiotics pre-FMT. Specially, factors including the time point, duration, and type of antibiotics administration also influence clinical outcomes.

13 Conclusion and perspective

As a microbiome-based therapy, the primary change observed after FMT in recipients is the alteration of the gut microbiota. In this review, we summarized diseases that could be treated with FMT, which are characterized by microbial dysbiosis, with distinct baseline gut microbiome impairment, and the reshaping of the gut microbiota could be detected post-FMT. Some diseases have been widely recognized as targets for FMT treatment and are recommended in several conferences and guidelines, such as CDI. Some diseases still require further clinical trials to validate their application conditions and therapeutic value, such as IBD and IBS. Interestingly, some newly discovered potential diseases, which might have a response to FMT, are still in the initial stages of clinical trials or animal experiments. Therefore, FMT holds immense potential in the treatment of both gastrointestinal and extraintestinal diseases.

Furthermore, this article systematically summarized the critical factors affecting the efficacy of FMT at the donor and recipient levels. The interaction between donor and recipient microbiota is crucial in FMT, and our review outlined the fusion patterns of the gut microbiota post-FMT, which conduces to the understanding of different clinical outcomes. The interplay between the donr and the recipient is a complicated process, and researchers have developed various predictive models for FMT based on regression or machine learning methods, which have significant guidance value in clinical practice.

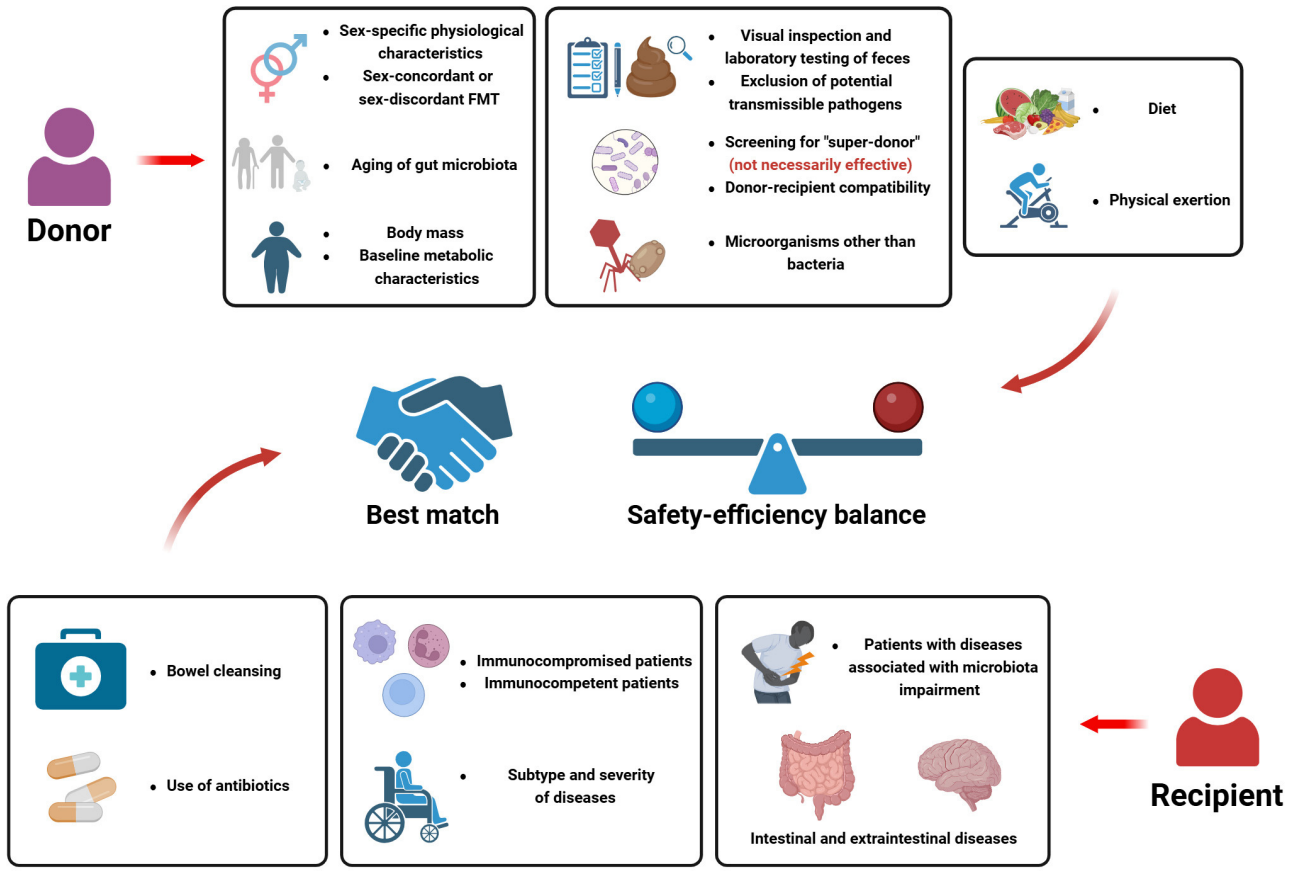


FIGURE 2
Potential factors influencing the efficacy of FMT from the perspective of donors and recipients.

In addition to gut microbiota-related factors, we have also listed other determinants of FMT safety and efficacy. Pre-testing of potential pathogens in donor feces is of great importance. There is also a need to assess the immune status of the recipient, although the recipient's immunocompromised state does not necessarily affect the conduct of FMT. The values of baseline characteristics of donors and recipients, including gender, age, clinical status, lifestyle and application of antibiotics, etc., have also been discussed in our review (Figure 2). The importance of these potential regulatory factors have been proved in clinical trails and animal experiments, while several researches have also provided opposite results.

In summary, this review comprehensively explored the application scenarios and conditions of FMT, expounded "what constitutes a successful FMT treatment", and how the interplay between donors and recipients affects treatment outcomes. More research is still needed to further guide the application of FMT, to improve the current situation where FMT is regarded as a "black box" to a certain extent, to enhance the explainablity and transparency of FMT treatment and related predictive models, and to extend the application scope of FMT.

Key-points of this review:

(1) We provide a systematical summary of the recognized or potential diseases that can be treated by FMT, and the microbial and metabolic characteristics of the diseases pre- and post-FMT.

(2) We introduce the microbial donor-recipient interplay and the lasting microbial succession within the gut post-FMT, which is influenced by the baseline microbial characteristics of both the donor and the recipient.

(3) We summarize the prognostic models on FMT efficacy to guide clinical decision-making.

(4) We analyze the potential role of several confounding factors (e.g., immune system function, age, gender, lifestyle, antibiotic use, etc) in the safety and efficiency of FMT from the perspectives of both donors and recipients. In this field, we include some early-stage studies, which have not yet reached clear conclusions or have obtained contradictory results, laying a foundation for future research.

(5) Combining the key points mentioned above, we clarify how to conduct a successful FMT at appropriate application scenarios.

Author contributions

YL: Visualization, Writing – original draft. XL: Visualization, Writing – original draft. YC: Visualization, Writing – original draft. QY: Conceptualization, Writing – review & editing. JZ: Conceptualization, Writing – review & editing. XW: Conceptualization, Writing – review & editing. QM: Conceptualization, Writing – review & editing. JJ: Conceptualization, Writing – review & editing. ZY: Conceptualization, Supervision, Writing – review & editing. XC: Conceptualization, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This project was supported by the Medjaden Academy & Research Foundation for Young Scientists (Grant No. MJR202510119).

Acknowledgments

Figures created with BioRender.com.

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 author(s) declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Abenavoli, L., Gambardella, M. L., Scarlata, G., Lenci, I., Baiocchi, L., and Luzza, F. (2024). The many faces of metabolic dysfunction-associated fatty liver disease treatment: from the mediterranean diet to fecal microbiota transplantation. *Medicina* 60:563. doi: 10.3390/medicina60040563

Afzaal, M., Saeed, F., Shah, Y. A., Hussain, M., Rabail, R., Socol, C. T., et al. (2022). Human gut microbiota in health and disease: unveiling the relationship. *Front. Microbiol.* 13:999001. doi: 10.3389/fmicb.2022.999001

Aggarwala, V., Mogno, I., Li, Z., Yang, C., Britton, G. J., Chen-Liaw, A., et al. (2021). Precise quantification of bacterial strains after fecal microbiota transplantation delineates long-term engraftment and explains outcomes. *Nat. Microbiol.* 6, 1309–1318. doi: 10.1038/s41564-021-00966-0

Allegretti, J. R., Kao, D., Sitko, J., Fischer, M., and Kassam, Z. (2018). Early antibiotic use after fecal microbiota transplantation increases risk of treatment failure. *Clin. Infect. Dis.* 66, 134–135. doi: 10.1093/cid/cix684

Anand, R., Song, Y., Garg, S., Girotra, M., Sinha, A., Sivaraman, A., et al. (2017). Effect of aging on the composition of fecal microbiota in donors for FMT and its impact on clinical outcomes. *Dig. Dis. Sci.* 62, 1002–1008. doi: 10.1007/s10620-017-4449-6

Andersen, V., Pingel, J., Søfelt, H. L., Hikmat, Z., Johansson, M., Pedersen, V. S., et al. (2024). Sex and gender in inflammatory bowel disease outcomes and research. *Lancet Gastroenterol. Hepatol.* 9, 1041–1051. doi: 10.1016/S2468-1253(24)00159-6

Arzamendi, M. J., Habibyan, Y. B., Defaye, M., Shute, A., Baggio, C. H., Chan, R., et al. (2024). Sex-specific post-inflammatory dysbiosis mediates chronic visceral pain in colitis. *Gut Microbes* 16, 2409207. doi: 10.1080/19490976.2024.2409207

Barandouzi, Z. A., Starkweather, A. R., Henderson, W. A., Gyamfi, A., and Cong, X. S. (2020). Altered composition of gut microbiota in depression: a systematic review. *Front. Psychiatry* 11:541. doi: 10.3389/fpsyt.2020.00541

Baunwall, S., Hansen, M. M., Andreasen, S. E., Eriksen, M. K., Rågård, N., Kelsen, J., et al. (2023). Donor, patient age and exposure to antibiotics are associated with the outcome of faecal microbiota transplantation for recurrent *Clostridioides difficile* infection: a prospective cohort study. *Aliment. Pharmacol. Ther.* 58, 503–515. doi: 10.1111/apt.17642

Behling, A. H., Wilson, B. C., Ho, D., Cutfield, W. S., Vatanen, T., O'Sullivan, et al. (2024). Horizontal gene transfer after faecal microbiota transplantation in adolescents with obesity. *Microbiome* 12:26. doi: 10.1186/s40168-024-01748-6

Benech, N., Cassir, N., Galperine, T., Alric, L., Scanzi, J., and Sokol, H. (2024). Fecal microbiota transplantation for recurrent *Clostridioides difficile* infection can be the best therapeutic option in severely immunocompromised patients depending on a case-by-case assessment of the benefit-to-risk ratio. *Gastroenterology* 167, 627–628. doi: 10.1053/j.gastro.2024.04.022

Benítez-Páez, A., Hartstra, A. V., Nieuwdorp, M., and Sanz, Y. (2022). Species- and strain-level assessment using rrn long-amplicons suggests donor's influence on gut microbial transference via fecal transplants in metabolic syndrome subjects. *Gut Microbes* 14:2078621. doi: 10.1080/19490976.2022.2078621

Beran, A., Sharma, S., Ghazaleh, S., Lee-Smith, W., Aziz, M., Kamal, F., et al. (2023). Predictors of fecal microbiota transplant failure in *Clostridioides difficile* infection: an updated meta-analysis. *J. Clin. Gastroenterol.* 57, 389–399. doi: 10.1097/MCG.0000000000001667

Berer, K., Gerdes, L. A., Cekanaviciute, E., Jia, X., Xiao, L., Xia, Z., et al. (2017). Gut microbiota from multiple sclerosis patients enables spontaneous autoimmune encephalomyelitis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10719–10724. doi: 10.1073/pnas.1711233114

Browne, P. D., Cold, F., Petersen, A. M., Halkjær, S. I., Christensen, A. H., Günther, S., et al. (2021). Engraftment of strictly anaerobic oxygen-sensitive bacteria in irritable bowel syndrome patients following fecal microbiota transplantation does not improve symptoms. *Gut Microbes* 13, 1–16. doi: 10.1080/19490976.2021.1927635

Bruggeman, A., Vandendriessche, C., Hamerlinck, H., De Looze, D., Tate, D. J., Vuylsteke, M., et al. (2024). Safety and efficacy of faecal microbiota transplantation in patients with mild to moderate Parkinson's disease (GUT-PARFECT): a double-blind, placebo-controlled, randomised, phase 2 trial. *EClinicalMedicine* 71:102563. doi: 10.1016/j.eclinm.2024.102563

Caenepeel, C., Deleu, S., Vazquez Castellanos, J. F., Arnauts, K., Braekeleire, S., Machiels, K., et al. (2024). Rigorous donor selection for fecal microbiota transplantation in active ulcerative colitis: key lessons from a randomized controlled trial halted for futility. *Clin. Gastroenterol. Hepatol.* 23, 621–631.e7. doi: 10.1016/j.cgh.2024.05.017

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanovi,ć, M., Kump, P., Satokari, R., et al. (2017). European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* 66, 569–580. doi: 10.1136/gutjnl-2016-313017

Chen, Q., Fan, Y., Zhang, B., Yan, C., Chen, Z., Wang, L., et al. (2022). Specific fungi associated with response to capsulized fecal microbiota transplantation in patients with active ulcerative colitis. *Front. Cell. Infect. Microbiol.* 12:1086885. doi: 10.3389/fcimb.2022.1086885

Chen, Q., Wu, C., Xu, J., Ye, C., Chen, X., Tian, H., et al. (2024). Donor-recipient intermicrobial interactions impact transfer of subspecies and fecal microbiota transplantation outcome. *Cell Host Microbe* 32, 349–365.e4. doi: 10.1016/j.chom.2024.01.013

Cheng, C. K., Gao, J., Kang, L., and Huang, Y. (2024). Fecal microbiota transfer from young mice reverts vascular aging hallmarks and metabolic impairments in aged mice. *Aging Dis.* doi: 10.14336/AD.2024.0384

Cheng, Y. W., Phelps, E., Ganapini, V., Khan, N., Ouyang, F., Xu, H., et al. (2019). Fecal microbiota transplantation for the treatment of recurrent and severe *Clostridium difficile* infection in solid organ transplant recipients: a multicenter experience. *Am. J. Transplant.* 19, 501–511. doi: 10.1111/ajt.15058

Chen-Liaw, A., Aggarwala, V., Mogno, I., Haifer, C., Li, Z., Eggers, J., et al. (2024). Gut microbiota strain richness is species specific and affects engraftment. *Nature* 637, 422–429. doi: 10.1038/s41586-024-08242-x

Chuang, C., Lee, K. C., Wang, Y. P., Lee, P. C., Chang, T. E., Huang, Y. H., et al. (2023). High carriage rate of extended-spectrum β-lactamase Enterobacterales and diarrheagenic *Escherichia coli* in healthy donor screening for fecal microbiota transplantation. *Eur. J. Clin. Microbiol. Infect. Dis.* 42, 1103–1113. doi: 10.1007/s10096-023-04644-3

Conover, K. R., Absah, I., Ballal, S., Brumbaugh, D., Cho, S., Cardenas, M. C., et al. (2023). Fecal microbiota transplantation for *Clostridioides difficile* infection in immunocompromised pediatric patients. *J. Pediatr. Gastroenterol. Nutr.* 76, 440–446. doi: 10.1097/MPG.0000000000003714

Costello, S. P., Soo, W., Bryant, R. V., Jairath, V., Hart, A. L., and Andrews, J. M. (2017). Systematic review with meta-analysis: faecal microbiota transplantation for the induction of remission for active ulcerative colitis. *Aliment. Pharmacol. Ther.* 46, 213–224. doi: 10.1111/apt.14173

Cui, M., Xiao, H., Li, Y., Zhou, L., Zhao, S., Luo, D., et al. (2017). Faecal microbiota transplantation protects against radiation-induced toxicity. *EMBO Mol. Med.* 9, 448–461. doi: 10.15252/emmm.201606932

D'Amato, A., Di Cesare Mannelli, L., Lucarini, E., Man, A. L., Le Gall, G., et al. (2020). Faecal microbiota transplant from aged donor mice affects spatial learning and memory via modulating hippocampal synaptic plasticity- and neurotransmission-related proteins in young recipients. *Microbiome* 8:140. doi: 10.1186/s40168-020-00914-w

de Clercq, N. C., van den Ende, T., Prodan, A., Hemke, R., Davids, M., Pedersen, H. K., et al. (2021). Fecal microbiota transplantation from overweight or obese donors in cachectic patients with advanced gastroesophageal cancer: a randomized, double-blind, placebo-controlled, phase II study. *Clin. Cancer Res.* 27, 3784–3792. doi: 10.1158/1078-0432.CCR-20-4918

de Groot, P., Nikolic, T., Pellegrini, S., Sordi, V., Imangaliyev, S., Rampanelli, E., et al. (2021). Faecal microbiota transplantation halts progression of human new-onset type 1 diabetes in a randomised controlled trial. *Gut* 70, 92–105. doi: 10.1136/gutjnl-2020-322630

de Groot, P., Scheithauer, T., Bakker, G. J., Prodan, A., Levin, E., Khan, M. T., et al. (2020). Donor metabolic characteristics drive effects of faecal microbiota transplantation on recipient insulin sensitivity, energy expenditure and intestinal transit time. *Gut* 69, 502–512. doi: 10.1136/gutjnl-2019-318320

DeFilipp, Z., Bloom, P. P., Torres Soto, M., Mansour, M. K., Sater, M., Huntley, M. H., et al. (2019). Drug-resistant *E. coli* bacteremia transmitted by fecal microbiota transplant. *N. Engl. J. Med.* 381, 2043–2050. doi: 10.1056/NEJMoa1910437

Drewes, J. L., Corona, A., Sanchez, U., Fan, Y., Hourigan, S. K., Weidner, M., et al. (2019). Transmission and clearance of potential procarcinogenic bacteria during fecal microbiota transplantation for recurrent *Clostridioides difficile*. *JCI Insight* 4:130848. doi: 10.1172/jci.insight.130848

El-Salhy, M., Hatlebakk, J. G., Gilja, O. H., Bråthen Kristoffersen, A., and Hausken, T. (2020). Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 69, 859–867. doi: 10.1136/gutjnl-2019-319630

El-Salhy, M., Valeur, J., Hausken, T., and Gunnar Hatlebakk, J. (2021). Changes in fecal short-chain fatty acids following fecal microbiota transplantation in patients with irritable bowel syndrome. *Neurogastroenterol. Motil.* 33:e13983. doi: 10.1111/nmo.13983

Faye, A. S., Allin, K. H., Iversen, A. T., Agrawal, M., Faith, J., Colombel, J. F., et al. (2023). Antibiotic use as a risk factor for inflammatory bowel disease across the ages: a population-based cohort study. *Gut* 72, 663–670. doi: 10.1136/gutjnl-2022-327845

Feješ, A., Belvončíková, P., Porcel Sanchis, D., Borbélyová, V., Celec, P., DŽunková, M., et al. (2024). The effect of cross-sex fecal microbiota transplantation on metabolism and hormonal status in adult rats. *Int. J. Mol. Sci.* 25:601. doi: 10.3390/ijms25010601

Fischer, M., Kao, D., Mehta, S. R., Martin, T., Dimitry, J., Keshteli, A. H., et al. (2016). Predictors of early failure after fecal microbiota transplantation for the therapy of *Clostridium difficile* infection: a multicenter study. *Am. J. Gastroenterol.* 111, 1024–1031. doi: 10.1038/ajg.2016.180

Fishbein, S., Mahmud, B., and Dantas, G. (2023). Antibiotic perturbations to the gut microbiome. *Nat. Rev. Microbiol.* 21, 772–788. doi: 10.1038/s41579-023-00933-y

Fu, Z. P., Ying, Y. G., Wang, R. Y., and Wang, Y. Q. (2024). Aged gut microbiota promotes arrhythmia susceptibility via oxidative stress. *iScience* 27:110888. doi: 10.1016/j.isci.2024.110888

Fuentes, S., Rossen, N. G., van der Spek, M. J., Hartman, J. H., Huuskonen, L., Korpela, K., et al. (2017). Microbial shifts and signatures of long-term remission in ulcerative colitis after faecal microbiota transplantation. *ISME J.* 11, 1877–1889. doi: 10.1038/ismej.2017.44

Fujimoto, K., Kimura, Y., Allegretti, J. R., Yamamoto, M., Zhang, Y. Z., Katayama, K., et al. (2021). Functional restoration of bacteriomes and viromes by fecal microbiota transplantation. *Gastroenterology* 160, 2089–2102.e12. doi: 10.1053/j.gastro.2021.02.013

Gallo, A., Cancelli, C., Ceron, E., Covino, M., Capoluongo, E., Pocino, K., et al. (2020). Fecal calprotectin and need of multiple microbiota trasplantation infusions in *Clostridium difficile* infection. *J. Gastroenterol. Hepatol.* 35, 1909–1915. doi: 10.1111/jgh.15072

Ge, C., Lu, Y., Shen, Z., Lu, Y., Liu, X., Zhang, M., et al. (2025). Machine learning and metabolomics identify biomarkers associated with the disease extent of ulcerative colitis. *J. Crohn's Colitis* 19:jjaf020. doi: 10.1093/ecco-jcc/jjaf020

Ghani, R., Chrysostomou, D., Roberts, L. A., Pandiaraja, M., Marchesi, J. R., and Mullish, B. H. (2024). Faecal (or intestinal) microbiota transplant: a tool for repairing the gut microbiome. *Gut Microbes* 16:2423026. doi: 10.1080/19490976.2024.2423026

Gogokhia, L., Buhrke, K., Bell, R., Hoffman, B., Brown, D. G., Hanke-Gogokhia, C., et al. (2019). Expansion of bacteriophages is linked to aggravated intestinal inflammation and colitis. *Cell Host Microbe* 25, 285–299.e8. doi: 10.1016/j.chom.2019.01.008

Goldsmith, J., Tomkovich, S., Auninš, J. G., McGovern, B. H., Mahoney, J. C., Hasson, B. R., et al. (2024). End-to-end donor screening and manufacturing controls: complementary quality-based strategies to minimize patient risk for donor-derived microbiome therapeutics. *Gut Microbes* 16:2402550. doi: 10.1080/19490976.2024.2402550

Gopalakrishnan, V., Dozier, E. A., Glover, M. S., Novick, S., Ford, M., Morehouse, C., et al. (2021). Engraftment of bacteria after fecal microbiota transplantation is dependent on both frequency of dosing and duration of preparative antibiotic regimen. *Microorganisms* 9:1399. doi: 10.3390/microorganisms9071399

Gough, E., Shaikh, H., and Manges, A. R. (2011). Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. *Clin. Infect. Dis.* 53, 994–1002. doi: 10.1093/cid/cir632

Groenewegen, B., van Lingen, E., Kovynev, A., van den Berg, A. J., Berssenbrugge, E., Sanders, I., et al. (2024). The presence of *Clostridioides difficile* in faeces before and after faecal microbiota transplantation and its relation with recurrent C. difficile infection and the gut microbiota in a Dutch cohort. *Clin. Microbiol. Infect.* doi: 10.1016/j.cmi.2024.12.003

Gupta, A., and Khanna, S. (2017). Fecal microbiota transplantation. *JAMA* 318:102. doi: 10.1001/jama.2017.6466

Gupta, S., Allen-Vercoe, E., and Petrof, E. O. (2016). Fecal microbiota transplantation: in perspective. *Therap. Adv. Gastroenterol.* 9, 229–239. doi: 10.1177/1756283X15607414

Haifer, C., Paramsothy, S., Kaakoush, N. O., Saikal, A., Ghaly, S., Yang, T., et al. (2022). Lyophilised oral faecal microbiota transplantation for ulcerative colitis (LOTUS): a randomised, double-blind, placebo-controlled trial. *Lancet Gastroenterol. Hepatol.* 7, 141–151. doi: 10.1016/S2468-1253(21)00400-3

Halkjær, S. I., Christensen, A. H., Lo, B., Browne, P. D., Günther, S., Hansen, L. H., et al. (2018). Faecal microbiota transplantation alters gut microbiota in patients with irritable bowel syndrome: results from a randomised, double-blind placebo-controlled study. *Gut* 67, 2107–2115. doi: 10.1136/gutjnl-2018-316434

Han, L., Zhao, L., Zhou, Y., Yang, C., Xiong, T., Lu, L., et al. (2022). Altered metabolome and microbiome features provide clues in understanding irritable bowel syndrome and depression comorbidity. *ISME J.* 16, 983–996. doi: 10.1038/s41396-021-01123-5

Haro, C., Rangel-Zúñiga, O. A., Alcalá-Díaz, J. F., Gómez-Delgado, F., Pérez-Martínez, P., Delgado-Lista, J., et al. (2016). Intestinal microbiota is influenced by gender and body mass index. *PLoS ONE* 11:e0154090. doi: 10.1371/journal.pone.0154090

He, R., Li, P., Wang, J., Cui, B., Zhang, F., and Zhao, F. (2022). The interplay of gut microbiota between donors and recipients determines the efficacy of fecal microbiota transplantation. *Gut Microbes* 14:2100197. doi: 10.1080/19490976.2022.2100197

Henn, M. R., O'Brien, E. J., Diao, L., Feagan, B. G., Sandborn, W. J., et al. (2021). A phase 1b safety study of SER-287, a spore-based microbiome therapeutic, for active mild to moderate ulcerative colitis. *Gastroenterology* 160, 115–127.e30. doi: 10.1053/j.gastro.2020.07.048

Hocquart, M., Lagier, J. C., Cassir, N., Saidani, N., Eldin, C., Kerbaj, J., et al. (2018). Early fecal microbiota transplantation improves survival in severe *Clostridium difficile* infections. *Clin. Infect. Dis.* 66, 645–650. doi: 10.1093/cid/cix762

Hong, Y., Li, H., Chen, L., Su, H., Zhang, B., Luo, Y., et al. (2024). Short-term exposure to antibiotics begets long-term disturbance in gut microbial metabolism and molecular ecological networks. *Microbiome* 12:80. doi: 10.1186/s40168-024-01795-z

Hourigan, S. K., Nicholson, M. R., Kahn, S. A., and Kellermayer, R. (2021). Updates and challenges in fecal microbiota transplantation for *Clostridioides difficile* infection in children. *J. Pediatr. Gastroenterol. Nutr.* 73, 430–432. doi: 10.1097/MPG.0000000000003229

Huang, C., Yi, P., Zhu, M., Zhou, W., Zhang, B., Yi, X., et al. (2022). Safety and efficacy of fecal microbiota transplantation for treatment of systemic lupus erythematosus: an EXPLORER trial. *J. Autoimmun.* 130:102844. doi: 10.1016/j.jaut.2022.102844

Ianiro, G., Mullish, B. H., Kelly, C. R., Kassam, Z., Kuijper, E. J., Ng, S. C., et al. (2020). Reorganisation of faecal microbiota transplant services during the COVID-19 pandemic. *Gut* 69, 1555–1563. doi: 10.1136/gutjnl-2020-321829

Ianiro, G., Punčochár, M., Karcher, N., Porcari, S., Armanini, F., Asnicar, F., et al. (2022). Variability of strain engraftment and predictability of microbiome composition after fecal microbiota transplantation across different diseases. *Nat. Med.* 28, 1913–1923. doi: 10.1038/s41591-022-01964-3

Ianiro, G., Valerio, L., Masucci, L., Pecere, S., Bibb,ò, S., Quaranta, G., et al. (2017). Predictors of failure after single faecal microbiota transplantation in patients with

recurrent *Clostridium difficile* infection: results from a 3-year, single-centre cohort study. *Clin. Microbiol. Infect.* 23, 337.e1–337.e3. doi: 10.1016/j.cmi.2016.12.025

Ishikawa, D., Sasaki, T., Takahashi, M., Kuwahara-Arai, K., Haga, K., Ito, S., et al. (2018). The microbial composition of bacteroidetes species in ulcerative colitis is effectively improved by combination therapy with fecal microbiota transplantation and antibiotics. *Inflamm. Bowel Dis.* 24, 2590–2598. doi: 10.1093/ibd/izy266

Jacobs, J. P., Lagishetty, V., Hauer, M. C., Labus, J. S., Dong, T. S., Toma, R., et al. (2023). Multi-omics profiles of the intestinal microbiome in irritable bowel syndrome and its bowel habit subtypes. *Microbiome* 11:5. doi: 10.1186/s40168-022-01450-5

Ji, S. K., Yan, H., Jiang, T., Guo, C. Y., Liu, J. J., Dong, S. Z., et al. (2017). Preparing the gut with antibiotics enhances gut microbiota reprogramming efficiency by promoting xenomicrobiota colonization. *Front. Microbiol.* 8:1208. doi: 10.3389/fmicb.2017.01208

Johnsen, P. H., Hilpüsch, F., Cavanagh, J. P., Leikanger, I. S., Kolstad, C., Valle, P. C., et al. (2018). Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. *Lancet Gastroenterol. Hepatol.* 3, 17–24. doi: 10.1016/S2468-1253(17)30338-2

Kang, G. U., Park, S., Jung, Y., Jee, J. J., Kim, M. S., Lee, S., et al. (2022). Exploration of potential gut microbiota-derived biomarkers to predict the success of fecal microbiota transplantation in ulcerative colitis: a prospective cohort in Korea. *Gut Liver* 16, 775–785. doi: 10.5009/gnl210369

Karimi, M., Shirsalimi, N., Hashempour, Z., Salehi Omran, H., Sedighi, E., Beigi, F., et al. (2024). Safety and efficacy of fecal microbiota transplantation (FMT) as a modern adjuvant therapy in various diseases and disorders: a comprehensive literature review. *Front. Immunol.* 15, 1439176. doi: 10.3389/fimmu.2024.1439176

Kazemian, N., Ramezankhani, M., Sehgal, A., Khalid, F. M., Kalkhoran, A., Narayan, A., et al. (2020). The trans-kingdom battle between donor and recipient gut microbiome influences fecal microbiota transplantation outcome. *Sci. Rep.* 10:18349. doi: 10.1038/s41598-020-75162-x

Kedia, S., Virmani, S., K., Vuyyuru, S., Kumar, P., Kante, B., et al. (2022). Faecal microbiota transplantation with anti-inflammatory diet (FMT-AID) followed by anti-inflammatory diet alone is effective in inducing and maintaining remission over 1 year in mild to moderate ulcerative colitis: a randomised controlled trial. *Gut* 71, 2401–2413. doi: 10.1136/gutjnl-2022-327811

Keskey, R., Cone, J. T., DeFazio, J. R., and Alverdy, J. C. (2020). The use of fecal microbiota transplant in sepsis. *Transl. Res.* 226, 12–25. doi: 10.1016/j.trsl.2020.07.002

Khoruts, A. (2021). Can FMT cause or prevent CRC? Maybe, but there is more to consider. *Gastroenterology* 161, 1103–1105. doi: 10.1053/j.gastro.2021.06.074

Koo, H., and Morrow, C. D. (2021). Incongruence between dominant commensal donor microbes in recipient feces post fecal transplant and response to anti-PD-1 immunotherapy. *BMC Microbiol.* 21:251. doi: 10.1186/s12866-021-02312-0

Koo, H., and Morrow, C. D. (2022). Time series strain tracking analysis post fecal transplantation identifies individual specific patterns of fecal dominant donor, recipient, and unrelated microbial strains. *PLoS ONE* 17:e0274633. doi: 10.1371/journal.pone.0274633

Kootte, R. S., Levin, E., Salojärvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., et al. (2017). Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. *Cell Metab.* 26, 611–619.e6. doi: 10.1016/j.cmet.2017.09.008

Kragsnaes, M. S., Kjeldsen, J., Horn, H. C., Munk, H. L., Pedersen, J. K., Just, S. A., et al. (2021). Safety and efficacy of faecal microbiota transplantation for active peripheral psoriatic arthritis: an exploratory randomised placebo-controlled trial. *Ann. Rheum. Dis.* 80, 1158–1167. doi: 10.1136/annrheumdis-2020-219511

Krigul, K. L., Feeney, R. H., Wongkuna, S., Aasmets, O., Holmberg, S. M., Andreson, R., et al. (2024). A history of repeated antibiotic usage leads to microbiota-dependent mucus defects. *Gut Microbes* 16:2377570. doi: 10.1080/19490976.2024.2377570

Kump, P., Wurm, P., Gröchenig, H. P., Wenzl, H., Petritsch, W., Halwachs, B., et al. (2018). The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of faecal microbiota transplantation in therapy refractory ulcerative colitis. *Aliment. Pharmacol. Ther.* 47, 67–77. doi: 10.1111/apt.14387

Lacy, B. E., and Patel, N. K. (2017). Rome Criteria and a Diagnostic Approach to Irritable Bowel Syndrome. *J. Clin. Med.* 6:99. doi: 10.3390/jcm6110099

Lee, M., and Chang, E. B. (2021). Inflammatory bowel diseases (IBD) and the microbiome-searching the crime scene for clues. *Gastroenterology* 160, 524–537. doi: 10.1053/j.gastro.2020.09.056

Leonardi, I., Paramsothy, S., Doron, I., Semon, A., Kaakoush, N. O., Clemente, J. C., et al. (2020). Fungal trans-kingdom dynamics linked to responsiveness to fecal microbiota transplantation (FMT) therapy in ulcerative colitis. *Cell Host Microbe* 27, 823–829.e3. doi: 10.1016/j.chom.2020.03.006

Levy, E. I., Dinleyici, M., Dinleyici, E., and Vandenplas, Y. (2024). *Clostridioides difficile* infections: prevention and treatment strategies. *Adv. Exp. Med. Biol.* 1449, 175–186. doi: 10.1007/978-3-031-58572-2_11

Li, N., Chen, H., Cheng, Y., Xu, F., Ruan, G., Ying, S., et al. (2021). Fecal microbiota transplantation relieves gastrointestinal and autism symptoms by improving the gut microbiota in an open-label study. *Front. Cell. Infect. Microbiol.* 11:759435. doi: 10.3389/fcimb.2021.759435

Li, S. S., Zhu, A., Benes, V., Costea, P. I., Hercog, R., Hildebrand, F., et al. (2016). Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science* 352, 586–589. doi: 10.1126/science.aad8852

Liguori, G., Lamas, B., Richard, M. L., Brandi, G., da Costa, G., Hoffmann, T. W., et al. (2016). Fungal dysbiosis in mucosa-associated microbiota of Crohn's disease patients. *J. Crohn's Colitis* 10, 296–305. doi: 10.1093/ecco-jcc/jjv209

Liu, Q., Zuo, T., Lu, W., Yeoh, Y. K., Su, Q., Xu, Z., et al. (2022). Longitudinal evaluation of gut bacteriomes and viromes after fecal microbiota transplantation for eradication of carbapenem-resistant enterobacteriaceae. *mSystems* 7:e0151021. doi: 10.1128/msystems.01510-21

Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., et al. (2019). Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 569, 655–662. doi: 10.1038/s41586-019-1237-9

Lopetuso, L. R., Deleu, S., Godny, L., Petito, V., Puca, P., Facciotti, F., et al. (2023). The first international Rome consensus conference on gut microbiota and faecal microbiota transplantation in inflammatory bowel disease. *Gut* 72, 1642–1650. doi: 10.1136/gutjnl-2023-329948

MacLellan, A. D., Finlay, B. B., and Appel-Cresswell, S. (2021). Age-matching in pediatric fecal matter transplants. *Front. Pediatr.* 9:603423. doi: 10.3389/fped.2021.603423

Manrique, P., Zhu, Y., van der Oost, J., Herrema, H., Nieuwdorp, M., de Vos, W. M., et al. (2021). Gut bacteriophage dynamics during fecal microbial transplantation in subjects with metabolic syndrome. *Gut Microbes* 13, 1–15. doi: 10.1080/19490976.2021.1897217

Mao, X., Larsen, S. B., Zachariassen, L., Brunse, A., Adamberg, S., Mejia, J., et al. (2024). Transfer of modified gut viromes improves symptoms associated with metabolic syndrome in obese male mice. *Nat. Commun.* 15:4704. doi: 10.1038/s41467-024-49152-w

Marcella, C., Cui, B., Kelly, C. R., Ianiro, G., Cammarota, G., and Zhang, F. (2021). Systematic review: the global incidence of faecal microbiota transplantation-related adverse events from 2000 to 2020. *Aliment. Pharmacol. Ther.* 53, 33–42. doi: 10.1111/apt.16148

Mars, R., Yang, Y., Ward, T., Houtti, M., Priya, S., Lekatz, H. R., et al. (2020). Longitudinal multi-omics reveals subset-specific mechanisms underlying irritable bowel syndrome. *Cell* 182, 1460–1473.e17. doi: 10.1016/j.cell.2020.08.007

Martinez, E., Taminiau, B., Rodriguez, C., and Daube, G. (2022). Gut microbiota composition associated with *Clostridioides difficile* colonization and infection. *Pathogens* 11:781. doi: 10.3390/pathogens11070781

Mehta, N., Wang, T., Friedman-Moraco, R. J., Carpentieri, C., Mehta, A. K., Rouphael, N., et al. (2022). Fecal microbiota transplantation donor screening updates and research gaps for solid organ transplant recipients. *J. Clin. Microbiol.* 60:e0016121. doi: 10.1128/JCM.00161-21

Meslier, V., Laiola, M., Roager, H. M., De Filippis, F., Roume, H., Quinquis, B., et al. (2020). Mediterranean diet intervention in overweight and obese subjects lowers plasma cholesterol and causes changes in the gut microbiome and metabolome independently of energy intake. *Gut* 69, 1258–1268. doi: 10.1136/gutjnl-2019-320438

Millan, B., Park, H., Hotte, N., Mathieu, O., Burguiere, P., Tompkins, T. A., et al. (2016). Fecal Microbial transplants reduce antibiotic-resistant genes in patients with recurrent *Clostridium difficile* infection. *Clin. Infect. Dis.* 62, 1479–1486. doi: 10.1093/cid/ciw185

Mills, R. H., Dulai, P. S., Vázquez-Baeza, Y., Sauceda, C., Daniel, N., Gerner, R. R., et al. (2022). Multi-omics analyses of the ulcerative colitis gut microbiome link *Bacteroides vulgatus* proteases with disease severity. *Nat Microbiol* 7, 262–276. doi: 10.1038/s41564-021-01050-3

Minkoff, N. Z., Aslam, S., Medina, M., Tanner-Smith, E. E., Zackular, J. P., Acra, S., et al. (2023). Fecal microbiota transplantation for the treatment of recurrent *Clostridioides difficile* (*Clostridium difficile*). *Cochrane Database Syst. Rev.* 4:CD013871. doi: 10.1002/14651858.CD013871.pub2

Mishra, S. P., Wang, B., Jain, S., Ding, J., Rejeski, J., Furdui, C. M., et al. (2023). A mechanism by which gut microbiota elevates permeability and inflammation in obese/diabetic mice and human gut. *Gut* 72, 1848–1865. doi: 10.1136/gutjnl-2022-327365

Moayyedi, P., Surette, M. G., Kim, P. T., Libertucci, J., Wolfe, M., Onischi, C., et al. (2015). Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology* 149, 102–109.e6. doi: 10.1053/j.gastro.2015.04.001

Mocanu, V., Zhang, Z., Deehan, E. C., Kao, D. H., Hotte, N., Karmali, S., et al. (2021). Fecal microbial transplantation and fiber supplementation in patients with severe obesity and metabolic syndrome: a randomized double-blind, placebo-controlled phase 2 trial. *Nat. Med.* 27, 1272–1279. doi: 10.1038/s41591-021-01399-2

Mullish, B. H., Merrick, B., Quraishi, M. N., Bak, A., Green, C. A., Moore, D. J., et al. (2024). The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridioides difficile* infection and other potential indications: second

edition of joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *J. Hosp. Infect*. 148, 189–219. doi: 10.1016/j.jhin.2024.03.001

Napiórkowska-Baran, K., Biliński, J., Pujanek, M., Hałakuc, P., Pietryga, A., Szymczak, B., et al. (2024). Fecal microbiota transplantation in a patient with chronic diarrhea and primary and secondary immunodeficiency (common variable immunodeficiency and splenectomy). *Front. Cell. Infect. Microbiol.* 14:1456672. doi: 10.3389/fcimb.2024.1456672

Newman, T. M., Shively, C. A., Register, T. C., Appt, S. E., Yadav, H., Colwell, R. R., et al. (2021). Diet, obesity, and the gut microbiome as determinants modulating metabolic outcomes in a non-human primate model. *Microbiome* 9:100. doi: 10.1186/s40168-021-01069-y

Ng, S. C., Xu, Z., Mak, J., Yang, K., Liu, Q., Zuo, T., et al. (2022). Microbiota engraftment after faecal microbiota transplantation in obese subjects with type 2 diabetes: a 24-week, double-blind, randomised controlled trial. *Gut* 71, 716–723. doi: 10.1136/gutjnl-2020-323617

Nguyen, L. H., Örtqvist, A. K., Cao, Y., Simon, T. G., Roelstraete, B., Song, M., et al. (2020). Antibiotic use and the development of inflammatory bowel disease: a national case-control study in Sweden. *Lancet Gastroenterol. Hepatol.* 5, 986–995. doi: 10.1016/S2468-1253(20)30267-3

Ning, L., Zhou, Y. L., Sun, H., Zhang, Y., Shen, C., Wang, Z., et al. (2023). Microbiome and metabolome features in inflammatory bowel disease via multi-omics integration analyses across cohorts. *Nat. Commun.* 14:7135. doi: 10.1038/s41467-023-42788-0

Nishida, A., Imaeda, H., Ohno, M., Inatomi, O., Bamba, S., Sugimoto, M., et al. (2017). Efficacy and safety of single fecal microbiota transplantation for Japanese patients with mild to moderately active ulcerative colitis. *J. Gastroenterol.* 52, 476–482. doi: 10.1007/s00535-016-1271-4

Nooij, S., Ducarmon, Q. R., Laros, J., Zwittink, R. D., Norman, J. M., Smits, W. K., et al. (2021). Fecal microbiota transplantation influences procarcinogenic escherichia coli in recipient recurrent *Clostridioides difficile* patients. *Gastroenterology* 161, 1218–1228.e5. doi: 10.1053/j.gastro.2021.06.009

Nusbaum, D. J., Sun, F., Ren, J., Zhu, Z., Ramsy, N., Pervolarakis, N., et al. (2018). Gut microbial and metabolomic profiles after fecal microbiota transplantation in pediatric ulcerative colitis patients. *FEMS Microbiol. Ecol.* 94:fiy133. doi: 10.1093/femsec/fiy133

Paramsothy, S., Nielsen, S., Kamm, M. A., Deshpande, N. P., Faith, J. J., Clemente, J. C., et al. (2019). Specific bacteria and metabolites associated with response to fecal microbiota transplantation in patients with ulcerative colitis. *Gastroenterology* 156, 1440–1454.e2. doi: 10.1053/j.gastro.2018.12.001

Paramsothy, S., Paramsothy, R., Rubin, D. T., Kamm, M. A., Kaakoush, N. O., Mitchell, H. M., et al. (2017). Faecal microbiota transplantation for inflammatory bowel disease: a systematic review and meta-analysis. *J. Crohn's Colitis* 11, 1180–1199. doi: 10.1093/ecco-jcc/jjx063

Park, H., Laffin, M. R., Jovel, J., Millan, B., Hyun, J. E., Hotte, N., et al. (2019). The success of fecal microbial transplantation in *Clostridium difficile* infection correlates with bacteriophage relative abundance in the donor: a retrospective cohort study. *Gut Microbes* 10, 676–687. doi: 10.1080/19490976.2019.1586037

Parker, A., Romano, S., Ansorge, R., Aboelnour, A., Le Gall, G., Savva, G. M., et al. (2022). Fecal microbiota transfer between young and aged mice reverses hallmarks of the aging gut, eye, and brain. *Microbiome* 10:68. doi: 10.1186/s40168-022-01243-w

Patron, R. L., Hartmann, C. A., Allen, S., Griesbach, C. L., Kosiorek, H. E., DiBaise, J. K., et al. (2017). Vancomycin taper and risk of failure of fecal microbiota transplantation in patients with recurrent *Clostridium difficile* infection. *Clin. Infect. Dis*. 65, 1214–1217. doi: 10.1093/cid/cix511

Pavanello, A., Martins, I. P., Tófolo, L. P., Previate, C., Matiusso, C., Francisco, F. A., et al. (2022). Fecal microbiota transplantation during lactation programs the metabolism of adult wistar rats in a sex-specific way. *Arch. Med. Res*. 53, 492–500. doi: 10.1016/j.arcmed.2022.06.007

Peery, A. F., Kelly, C. R., Kao, D., Vaughn, B. P., Lebwohl, B., Singh, S., et al. (2024). AGA clinical practice guideline on fecal microbiota-based therapies for select gastrointestinal diseases. *Gastroenterology* 166, 409–434. doi: 10.1053/j.gastro.2024.01.008

Pérez-Cobas, A. E., Moya, A., Gosalbes, M. J., and Latorre, A. (2015). Colonization resistance of the gut microbiota against *Clostridium difficile*. *Antibiotics* 4, 337–357. doi: 10.3390/antibiotics4030337

Pinto, S., Šajbenová, D., Benincà, E., Nooij, S., Terveer, E. M., Keller, J. J., et al. (2024). Dynamics of gut microbiota after fecal microbiota transplantation in ulcerative colitis: success linked to control of prevotellaceae. *J. Crohn's Colitis*. doi: 10.1093/ecco-jcc/jjae137

Podlesny, D., Durdevic, M., Paramsothy, S., Kaakoush, N. O., Högenauer, C., Gorkiewicz, G., et al. (2022). Identification of clinical and ecological determinants of strain engraftment after fecal microbiota transplantation using metagenomics. *Cell Rep Med*. 3:100711. doi: 10.1016/j.xcrm.2022.100711

Proença, I. M., Allegretti, J. R., Bernardo, W. M., de Moura, D., Ponte Neto, A. M., Matsubayashi, C. O., et al. (2020). Fecal microbiota transplantation improves metabolic syndrome parameters: systematic review with meta-analysis based on randomized clinical trials. *Nutr. Res.* 83, 1–14. doi: 10.1016/j.nutres.2020.06.018

Ramos, R. J., Zhu, C., Joseph, D. F., Thaker, S., Lacomb, J. F., Markarian, K., et al. (2022). Metagenomic and bile acid metabolomic analysis of fecal microbiota transplantation for recurrent Clostridiodes difficile and/or inflammatory bowel diseases. *Med. Res. Arch.* 10:10.18103/mra.v10i10.3318. doi: 10.18103/mra.v10i10.3318

Rees, N. P., Shaheen, W., Quince, C., Tselepis, C., Horniblow, R. D., Sharma, N., et al. (2022). Systematic review of donor and recipient predictive biomarkers of response to faecal microbiota transplantation in patients with ulcerative colitis. *EBioMedicine* 81:104088. doi: 10.1016/j.ebiom.2022.104088

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 341:1241214. doi: 10.1126/science.1241214

Rinott, E., Youngster, I., Yaskolka Meir, A., Tsaban, G., Zelicha, H., Kaplan, A., et al. (2021). Effects of diet-modulated autologous fecal microbiota transplantation on weight regain. *Gastroenterology* 160, 158–173.e10. doi: 10.1053/j.gastro.2020.08.041

Rodig, N. M., Weatherly, M., Kaplan, A. L., Ballal, S. A., Elisofon, S. A., Daly, K. P., et al. (2023). Fecal microbiota transplant in pediatric solid organ transplant recipients. *Transplantation* 107, 2073–2077. doi: 10.1097/TP.0000000000004656

Rossen, N. G., Fuentes, S., van der Spek, M. J., Tijssen, J. G., Hartman, J. H., Duflou, A., et al. (2015). Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis. *Gastroenterology* 149, 110–118.e4. doi: 10.1053/j.gastro.2015.03.045

Ruan, W., Engevik, M. A., Spinler, J. K., and Versalovic, J. (2020). Healthy human gastrointestinal microbiome: composition and function after a decade of exploration. *Dig. Dis. Sci*. 65, 695–705. doi: 10.1007/s10620-020-06118-4

Schmidt, T., Li, S. S., Maistrenko, O. M., Akanni, W., Coelho, L. P., Dolai, S., et al. (2022). Drivers and determinants of strain dynamics following fecal microbiota transplantation. *Nat. Med*. 28, 1902–1912. doi: 10.1038/s41591-022-01913-0

Seekatz, A. M., Aas, J., Gessert, C. E., Rubin, T. A., Saman, D. M., Bakken, J. S., et al. (2014). Recovery of the gut microbiome following fecal microbiota transplantation. *MBio* 5, e00893–e00814. doi: 10.1128/mBio.00893-14

Sehgal, K., Yadav, D., Saha, S., Mara, K., Grover, M., and Khanna, S. (2024). Sex-Discordant Fecal microbiota transplantation for *Clostridioides difficile* may increase risk of postinfection irritable bowel syndrome. *Gastroenterology* 166, 704–706.e2. doi: 10.1053/j.gastro.2023.11.295

Serrano-Villar, S., Talavera-Rodríguez, A., Gosalbes, M. J., Madrid, N., Pérez-Molina, J. A., Elliott, R. J., et al. (2021). Fecal microbiota transplantation in HIV: a pilot placebo-controlled study. *Nat. Commun.* 12:1139. doi: 10.1038/s41467-021-21472-1

Settanni, C. R., Ianiro, G., Bibb,ò, S., Cammarota, G., and Gasbarrini, A. (2021). Gut microbiota alteration and modulation in psychiatric disorders: current evidence on fecal microbiota transplantation. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 109:110258. doi: 10.1016/j.pnpbp.2021.110258

Sheng, L., Jena, P. K., Hu, Y., and Wan, Y. Y. (2021). Age-specific microbiota in altering host inflammatory and metabolic signaling as well as metabolome based on the sex. *Hepatobiliary Surg. Nutr.* 10, 31–48. doi: 10.21037/hbsn-20-671

Shogbesan, O., Poudel, D. R., Victor, S., Jehangir, A., Fadahunsi, O., Shogbesan, G., et al. (2018). A systematic review of the efficacy and safety of fecal microbiota transplant for *Clostridium difficile* infection in immunocompromised patients. *Can J Gastroenterol Hepatol* 2018, 1394379. doi: 10.1155/2018/1394379

Shtossel, O., Turjeman, S., Riumin, A., Goldberg, M. R., Elizur, A., Bekor, Y., et al. (2023). Recipient-independent, high-accuracy FMT-response prediction and optimization in mice and humans. *Microbiome* 11:181. doi: 10.1186/s40168-023-01623-w

Shukla, R., Ghoshal, U., Dhole, T. N., and Ghoshal, U. C. (2015). Fecal microbiota in patients with irritable bowel syndrome compared with healthy controls using real-time polymerase chain reaction: an evidence of dysbiosis. *Dig. Dis. Sci*. 60, 2953–2962. doi: 10.1007/s10620-015-3607-y

Singh, P., Alm, E. J., Kelley, J. M., Cheng, V., Smith, M., Kassam, Z., et al. (2022). Effect of antibiotic pretreatment on bacterial engraftment after Fecal Microbiota Transplant (FMT) in IBS-D. *Gut Microbes* 14:2020067. doi: 10.1080/19490976.2021.2020067

Smillie, C. S., Sauk, J., Gevers, D., Friedman, J., Sung, J., Youngster, I., et al. (2018). Strain tracking reveals the determinants of bacterial engraftment in the human gut following fecal microbiota transplantation. *Cell Host Microbe* 23, 229–240.e5. doi: 10.1016/j.chom.2018.01.003

Soldi, S., Vasileiadis, S., Lohner, S., Uggeri, F., Puglisi, E., Molinari, P., et al. (2019). Prebiotic supplementation over a cold season and during antibiotic treatment specifically modulates the gut microbiota composition of 3-6 year-old children. *Benef. Microbes* 10, 253–263. doi: 10.3920/BM2018.0116

Song, Y. N., Yang, D. Y., Veldhuyzen van Zanten, S., Wong, K., McArthur, E., Song, C. Z., et al. (2022). Fecal microbiota transplantation for severe or fulminant *clostridioides difficile* infection: systematic review and meta-analysis. *J. Can. Assoc. Gastroenterol.* 5, e1–e11. doi: 10.1093/jcag/gwab023

Sood, A., Singh, A., Mahajan, R., Midha, V., Kaur, K., Singh, D., et al. (2020). Clinical Predictors of response to faecal microbiota transplantation in patients with active ulcerative colitis. *J. Crohn's Colitis*. doi: 10.1093/ecco-jcc/jjaa163

Spindelboeck, W., Schulz, E., Uhl, B., Kashofer, K., Aigelsreiter, A., Zinke-Cerwenka, W., et al. (2017). Repeated fecal microbiota transplantations attenuate diarrhea and lead to sustained changes in the fecal microbiota in acute, refractory gastrointestinal graft-versus-host-disease. *Haematologica* 102, e210–e213. doi: 10.3324/haematol.2016.154351

Staley, C., Kaiser, T., Vaughn, B. P., Graiziger, C. T., Hamilton, M. J., Rehman, T. U., et al. (2018). Predicting recurrence of *Clostridium difficile* infection following encapsulated fecal microbiota transplantation. *Microbiome* 6, 166. doi: 10.1186/s40168-018-0549-6

Stripling, J., Kumar, R., Baddley, J. W., Nellore, A., Dixon, P., Howard, D., et al. (2015). Loss of Vancomycin-resistant enterococcus fecal dominance in an organ transplant patient with *Clostridium difficile* colitis after fecal microbiota transplant. Open Forum Infect Dis 2, ofv078. doi: 10.1093/ofid/ofv078

Sulaiman, J. E., Thompson, J., Qian, Y., Vivas, E. I., Diener, C., Gibbons, S. M., et al. (2024). Elucidating human gut microbiota interactions that robustly inhibit diverse *Clostridioides difficile* strains across different nutrient landscapes. *Nat. Commun.* 15:7416. doi: 10.1038/s41467-024-51062-w

Tanaka, T., Talegawkar, S. A., Jin, Y., Candia, J., Tian, Q., Moaddel, R., et al. (2022). Metabolomic profile of different dietary patterns and their association with frailty index in community-dwelling older men and women. *Nutrients* 14:2237. doi: 10.3390/nu14112237

Tang, L. L., Feng, W. Z., Cheng, J. J., and Gong, Y. N. (2020). Clinical remission of ulcerative colitis after different modes of faecal microbiota transplantation: a meta-analysis. *Int. J. Colorectal Dis.* 35, 1025–1034. doi: 10.1007/s00384-020-03599-7

Tunali, V., Arslan, N. Ç., Ermiş, B. H., Derviş Hakim, G., Gündogdu, A., Hora, M., et al. (2024). A multicenter randomized controlled trial of microbiome-based artificial intelligence-assisted personalized diet vs low-fermentable oligosaccharides, disaccharides, monosaccharides, and polyols diet: a novel approach for the management of irritable bowel syndrome. *Am. J. Gastroenterol.* 119, 1901–1912. doi: 10.14309/ajg.0000000000002862

Urtecho, G., Moody, T., Huang, Y., Sheth, R. U., Richardson, M., Descamps, H. C., et al. (2024). Spatiotemporal dynamics during niche remodeling by super-colonizing microbiota in the mammalian gut. *Cell Syst* 15, 1002–1017.e4. doi: 10.1016/j.cels.2024.10.007

Vendrik, K., Terveer, E. M., Kuijper, E. J., Nooij, S., Boeije-Koppenol, E., Sanders, I., et al. (2021). Periodic screening of donor faeces with a quarantine period to prevent transmission of multidrug-resistant organisms during faecal microbiota transplantation: a retrospective cohort study. *Lancet Infect. Dis.* 21, 711–721. doi: 10.1016/S1473-3099(20)30473-4

Vich Vila, A., Zhang, J., Liu, M., Faber, K. N., and Weersma, R. K. (2024). Untargeted faecal metabolomics for the discovery of biomarkers and treatment targets for inflammatory bowel diseases. *Gut* 73, 1909–1920. doi: 10.1136/gutjnl-2023-329969

Wang, J., Zhong, Y., Zhu, H., Mahgoub, O. K., Jian, Z., Gu, L., et al. (2022). Different gender-derived gut microbiota influence stroke outcomes by mitigating inflammation. *J. Neuroinflammat.* 19, 245. doi: 10.1186/s12974-022-02606-8

Wang, J. J., Wang, J., Pang, X. Y., Zhao, L. P., Tian, L., and Wang, X. P. (2016). Sex differences in colonization of gut microbiota from a man with short-term vegetarian and inulin-supplemented diet in germ-free mice. *Sci. Rep.* 6:36137. doi: 10.1038/srep36137

Wang, T., Shi, Z., Ren, H., Xu, M., Lu, J., Yang, F., et al. (2024). Divergent age-associated and metabolism-associated gut microbiome signatures modulate cardiovascular disease risk. *Nat. Med.* 30, 1722–1731. doi: 10.1038/s41591-024-03038-y

Wang, Y., Wiesnoski, D. H., Helmink, B. A., Gopalakrishnan, V., Choi, K., DuPont, H. L., et al. (2018). Fecal microbiota transplantation for refractory immune checkpoint inhibitor-associated colitis. *Nat. Med.* 24, 1804–1808. doi: 10.1038/s41591-018-0238-9

Wang, Y., Zhang, S., Borody, T. J., and Zhang, F. (2022). Encyclopedia of fecal microbiota transplantation: a review of effectiveness in the treatment of 85 diseases. *Chin. Med. J.* 135, 1927–1939. doi: 10.1097/CM9.0000000000002339

Watane, A., Cavuoto, K. M., Rojas, M., Dermer, H., Day, J. O., Banerjee, S., et al. (2022). Fecal microbial transplant in individuals with immune-mediated dry eye. *Am. J. Ophthalmol.* 233, 90–100. doi: 10.1016/j.ajo.2021.06.022

Wei, S., Bahl, M. I., Baunwall, S., Dahlerup, J. F., Hvas, C. L., and Licht, T. R. (2022). Gut microbiota differs between treatment outcomes early after fecal microbiota transplantation against recurrent *Clostridioides difficile* infection. *Gut Microbes* 14:2084306. doi: 10.1080/19490976.2022.2084306

Wei, Y., Gong, J., Zhu, W., Tian, H., Ding, C., Gu, L., et al. (2016). Pectin enhances the effect of fecal microbiota transplantation in ulcerative colitis by delaying the loss of diversity of gut flora. *BMC Microbiol.* 16:255. doi: 10.1186/s12866-016-0869-2

Weingarden, A. R., Chen, C., Bobr, A., Yao, D., Lu, Y., Nelson, V. M., et al. (2014). Microbiota transplantation restores normal fecal bile acid composition in recurrent *Clostridium difficile* infection. *Am. J. Physiol. Gastrointest. Liver Physiol.* 306, G310–319. doi: 10.1152/ajpgi.00282.2013

Wilson, B. C., Vatanen, T., Cutfield, W. S., and O'Sullivan, J. M. (2019). The super-donor phenomenon in fecal microbiota transplantation. *Front. Cell. Infect. Microbiol.* 9:2. doi: 10.3389/fcimb.2019.00002

Wilson, B. C., Vatanen, T., Jayasinghe, T. N., Leong, K., Derraik, J., Albert, B. B., et al. (2021). Strain engraftment competition and functional augmentation in a multi-donor fecal microbiota transplantation trial for obesity. *Microbiome* 9:107. doi: 10.1186/s40168-021-01060-7

Witjes, J. J., Smits, L. P., Pekmez, C. T., Prodan, A., Meijnikman, A. S., Troelstra, M. A., et al. (2020). Donor fecal microbiota transplantation alters gut microbiota and metabolites in obese individuals with Steatohepatitis. *Hepatol Commun* 4, 1578–1590. doi: 10.1002/hep4.1601

Woodworth, M. H., Conrad, R. E., Haldopoulos, M., Pouch, S. M., Babiker, A., Mehta, A. K., et al. (2023). Fecal microbiota transplantation promotes reduction of antimicrobial resistance by strain replacement. *Sci. Transl. Med.* 15:eabo2750. doi: 10.1126/scitranslmed.abo2750

Wu, Q., Boonma, P., Badu, S., Yalcinkaya, N., So, S. Y., Garey, K. W., et al. (2023). Donor-recipient specificity and age-dependency in fecal microbiota therapy and probiotic resolution of gastrointestinal symptoms. *NPJ Biofilms Microbiom.* 9:54. doi: 10.1038/s41522-023-00421-4

Wu, X., Li, P., Wang, W., Xu, J., Ai, R., Wen, Q., et al. (2023). The underlying changes in serum metabolic profiles and efficacy prediction in patients with extensive ulcerative colitis undergoing fecal microbiota transplantation. *Nutrients* 15:3340. doi: 10.3390/nu15153340

Xiao, Y., Angulo, M. T., Lao, S., Weiss, S. T., and Liu, Y. Y. (2020). An ecological framework to understand the efficacy of fecal microbiota transplantation. *Nat. Commun.* 11:3329. doi: 10.1038/s41467-020-17180-x

Yakout, A., Bi, Y., and Harris, D. M. (2024). *Clostridioides difficile*: a concise review of best practices and updates. *J. Prim. Care Community Health* 15:21501319241249645. doi: 10.1177/21501319241249645

Yang, H., Wu, X., Li, X., Zang, W., Zhou, Z., Zhou, Y., et al. (2024). A commensal protozoan attenuates *Clostridioides difficile* pathogenesis in mice via arginine-ornithine metabolism and host intestinal immune response. *Nat. Commun.* 15:2842. doi: 10.1038/s41467-024-47075-0

Yang, Y., Zheng, X., Wang, Y., Tan, X., Zou, H., Feng, S., et al. (2022). Human fecal microbiota transplantation reduces the susceptibility to dextran sulfate sodium-induced germ-free mouse colitis. *Front. Immunol.* 13:836542. doi: 10.3389/fimmu.2022.836542

Yau, Y. K., Su, Q., Xu, Z., Tang, W., Ching, J., Mak, J., et al. (2023). Randomised clinical trial: Faecal microbiota transplantation for irritable bowel syndrome with diarrhoea. *Aliment. Pharmacol. Ther.* 58, 795–804. doi: 10.1111/apt.17703

Yurkovetskiy, L., Burrows, M., Khan, A. A., Graham, L., Volchkov, P., Becker, L., et al. (2013). Gender bias in autoimmunity is influenced by microbiota. *Immunity* 39, 400–412. doi: 10.1016/j.immuni.2013.08.013

Zellmer, C., Sater, M., Huntley, M. H., Osman, M., Olesen, S. W., and Ramakrishna, B. (2021). Shiga toxin-producing escherichia coli transmission via fecal microbiota transplant. *Clin. Infect. Dis.* 72, e876–e880. doi: 10.1093/cid/ciaa1486

Zhang, F., Zuo, T., Yeoh, Y. K., Cheng, F., Liu, Q., Tang, W., et al. (2021). Longitudinal dynamics of gut bacteriome, mycobiome and virome after fecal microbiota transplantation in graft-versus-host disease. *Nat. Commun.* 12:65. doi: 10.1038/s41467-020-20240-x

Zhang, S., Lu, G., Wang, W., Li, Q., Wang, R., Zhang, Z., et al. (2024). A predictive machine-learning model for clinical decision-making in washed microbiota transplantation on ulcerative colitis. *Comput. Struct. Biotechnol. J.* 24, 583–592. doi: 10.1016/j.csbj.2024.08.021

Zhang, X., Luo, X., Tian, L., Yue, P., Li, M., Liu, K., et al. (2023). The gut microbiome dysbiosis and regulation by fecal microbiota transplantation: umbrella review. *Front. Microbiol.* 14:1286429. doi: 10.3389/fmicb.2023.1286429

Zhang, Y., Liu, Q., Yu, Y., Wang, M., Wen, C., and He, Z. (2020). Early and short-term interventions in the gut microbiota affects lupus severity, progression, and treatment in MRL/lpr mice. *Front. Microbiol.* 11:628. doi: 10.3389/fmicb.2020.00628

Zhang, Y., Wang, S., Wang, H., Cao, M., Wang, M., Zhang, B., et al. (2024). Efficacy of donor-recipient-matched faecal microbiota transplantation in patients with IBS-D: a single-centre, randomized, double-blind placebo-controlled study. *Digestion* 105, 457–467. doi: 10.1159/000540420

Zhang, Y., Zhu, H., Du, S., Wang, H., Li, H., Wang, M., et al. (2023). Medium-chain and long-chain fatty acids are associated with diarrheal predominant irritable bowel syndrome revealed by DESI-MSI. *J. Gastroenterol.* 58, 1124–1133. doi: 10.1007/s00535-023-02030-6

Zhang, Z., Mocanu, V., Deehan, E. C., Hotte, N., Zhu, Y., Wei, S., et al. (2024). Recipient microbiome-related features predicting metabolic improvement following fecal microbiota transplantation in adults with severe obesity and metabolic syndrome: a secondary analysis of a phase 2 clinical trial. *Gut Microbes* 16:2345134. doi: 10.1080/19490976.2024.2345134

Zhong, Y., Cao, J., Deng, Z., Ma, Y., Liu, J., and Wang, H. (2021). Effect of fiber and fecal microbiota transplantation donor on recipient mice gut microbiota. *Front. Microbiol.* 12:757372. doi: 10.3389/fmicb.2021.757372

Zhu, J., Yin, J., Chen, J., Hu, M., Lu, W., Wang, H., et al. (2024). Integrative analysis with microbial modelling and machine learning uncovers potential alleviators for ulcerative colitis. *Gut Microbes* 16:2336877. doi: 10.1080/19490976.2024.2336877

Zhu, X., Huang, X., Hu, M., Sun, R., Li, J., Wang, H., et al. (2024). A specific enterotype derived from gut microbiome of older individuals enables favorable responses to immune checkpoint blockade therapy. *Cell Host Microbe* 32, 489–505.e5. doi: 10.1016/j.chom.2024.03.002

Zoll, J., Read, M. N., Heywood, S. E., Estevez, E., Marshall, J., Kammoun, H. L., et al. (2020). Fecal microbiota transplantation from high caloric-fed donors alters glucose metabolism in recipient mice, independently of adiposity or exercise status. *Am. J. Physiol. Endocrinol. Metab*. 319, E203–E216. doi: 10.1152/ajpendo.00037.2020

Zou, M., Jie, Z., Cui, B., Wang, H., Feng, Q., Zou, Y., et al. (2020). Fecal microbiota transplantation results in bacterial strain displacement in patients with inflammatory bowel diseases. *FEBS Open Bio.* 10, 41–55. doi: 10.1002/2211-5463.12744

Zuo, T., Wong, S. H., Cheung, C. P., Lam, K., Lui, R., Cheung, K., et al. (2018b). Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in *Clostridium difficile* infection. *Nat. Commun.* 9:3663. doi: 10.1038/s41467-018-06103-6

Zuo, T., Wong, S. H., Lam, K., Lui, R., Cheung, K., Tang, W., et al. (2018a). Bacteriophage transfer during faecal microbiota transplantation in *Clostridium difficile* infection is associated with treatment outcome. *Gut* 67, 634–643. doi: 10.1136/gutjnl-2017-313952

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 11 April 2025
DOI 10.3389/fcimb.2025.1535666

Check for updates

OPEN ACCESS

EDITED BY
Nazarii Kobyliak,
Bogomolets National Medical University, Ukraine

REVIEWED BY
Keith Fraser,
Rensselaer Polytechnic Institute, United States
Longhao Chen,
Zhejiang Chinese Medical University, China

*CORRESPONDENCE
Hongwei Gao
gaohongwei202210@163.com
Jing Zhao
jingzhao@sdu.edu.cn

†These authors have contributed equally to this work

RECEIVED 14 January 2025
ACCEPTED 28 March 2025
PUBLISHED 11 April 2025

CITATION
Ma P, Wang R, Chen H, Zheng J, Yang W, Meng B, Liu Y, Lu Y, Zhao J and Gao H (2025) Fecal microbiota transplantation alleviates lipopolysaccharide-induced osteoporosis by modulating gut microbiota and long non-coding RNA *TUG1* expression. *Front. Cell. Infect. Microbiol.* 15:1535666. doi: 10.3389/fcimb.2025.1535666

COPYRIGHT
© 2025 Ma, Wang, Chen, Zheng, Yang, Meng, Liu, Lu, Zhao and Gao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbiota transplantation alleviates lipopolysaccharide-induced osteoporosis by modulating gut microbiota and long non-coding RNA *TUG1* expression

Pengcheng Ma[1], Ruoyi Wang[1], Huizhi Chen[1], Jiachun Zheng[1], Weijie Yang[1], Bo Meng[1], Yifan Liu[1], Yao Lu[2], Jing Zhao[2*†] and Hongwei Gao[1,3*†]

[1]Shandong Public Health Clinical Center, Shandong University, Jinan, China, [2]Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Sciences, Shandong University, Qingdao, China, [3]School of Mechanical Engineering, Shandong University, Jinan, China

Purpose: To study whether fecal microbiota transplantation (FMT) can alleviate lipopolysaccharide (LPS)-induced osteoporosis (OP) by regulating the composition and abundance of gut microbiota and the expression level of long non-coding RNA (lncRNA) *TUG1*.

Methods: Twenty C57BL/6 mice were selected. Two mice were randomly designated as fecal donors, while the remaining mice were randomly divided into control group, LPS group, and LPS + FMT group. Each group consisted of 6 mice. The mice in the LPS and LPS + FMT groups were intraperitoneally injected with LPS to establish the OP model, and the mice in the LPS + FMT group were treated with donor feces by gavage. Micro-CT was used to scan the femur specimens of mice, and the bone structural parameters of the control and LPS groups were compared to verify the effectiveness of the OP model. HE staining was used to compare the microstructure of femurs in the 3 groups. 16S rRNA gene sequencing was used to analyze the composition and abundance of gut microbiota in mice. Immunofluorescence staining was used to compare the expression levels of Runt-related transcription factor 2 (RUNX2) in the femur of the 3 groups. Real-time quantitative reverse transcription PCR (qRT-PCR) was used to compare the expression levels of lncRNA *TUG1* in the intestines and serum of mice in the 3 groups.

Results: Micro-CT showed that compared with the control group, the mice in the LPS group had more bone loss. The bone mineral density, trabecular number, and trabecular thickness of the control group was higher, and the trabecular separation was smaller. The models were validated effectively. HE staining showed that compared with the control group, the bone trabeculae in the LPS group were thinner and sparse, while that in the LPS + FMT group were dense and clear. The 16s rRNA sequencing showed that the abundance of Bacteroides and Lactobacillus in LPS+FMT group was significantly higher than that in LPS group. Immunofluorescence staining showed that the RUNX2 level in the control

group and LPS + FMT group was similar, and both were higher than that in the LPS group. The qRT-PCR results showed that the *TUG1* mRNA level in the control group and LPS + FMT group was similar and significantly higher than that in the LPS group.

Conclusion: FMT can enhance osteoblast levels and improve bone structure by modulating the abundance of gut microbiota in OP mice (such as increasing Bacteroides and Lactobacillus populations) and promoting the expression of lncRNA *TUG1*, thereby alleviating LPS-induced OP.

KEYWORDS

fecal microbiota transplantation, osteoporosis, gut microbiota, lncRNA, lipopolysaccharides

1 Introduction

Osteoporosis (OP) is a metabolic disease characterized by osteopenia and destruction of bone microstructure, which can increase the risk of fracture. The pain and deformity caused by OP seriously reduce the quality of patients' survival (Anish and Nair, 2024). More than 150 million people are diagnosed with OP every year, and the high incidence has made it a global public health problem that needs to be solved urgently (Zhang et al., 2021). The pathogenesis of OP is imbalance of bone metabolism, and drugs to restore the homeostasis of bone formation and resorption are the main means of treating OP. However, long-term use of anti-OP drugs may cause side effects such as muscle pain and renal insufficiency, resulting in poor patient compliance (Reid and Billington, 2022). Currently, finding safer and more effective strategies for the prevention and treatment of OP has become a research hotspot.

Gut microbiota is the microbial community inhabiting the human gastrointestinal tract, which plays an important role in regulating bone development and biomechanics of bone tissue (Guss et al., 2017; Orwoll et al., 2022). Gut microbiota can delay the progression of OP by regulating the immune system and reducing the production of tumor necrosis factor. Gut microbiota can also indirectly regulate bone homeostasis through endocrine bone signaling factors such as estrogen and parathyroid hormone. In addition, metabolites of gut microbiota, such as short-chain fatty acids and 5-hydroxytryptamine, can affect the number and activity of osteoblasts and osteoclasts (Hao et al., 2024). The phylum of gut microbiota includes Firmicutes, Bacteroidetes, etc., and bacteria from different phyla have different effects on OP (Marchesi et al., 2016). It has been noted that bone mass loss was significantly improved in OP patients after oral administration of Lactobacillus (Li et al., 2022). Another study suggested that Enterobacteriales could aggravate OP by regulating amino acid metabolism (Wang S. et al., 2022). Therefore, it is necessary to explore new targets in the complex mechanisms by which the gut microbiota affects OP.

The composition and abundance of gut microbiota are affected by many factors. Such as long-term use of antibiotics can induce dysbiosis of gut microbiota and decrease the bending strength of bone. Consumption of certain prebiotics is beneficial for increasing the population of beneficial bacteria in the gut, enhancing the secretion of short-chain fatty acids (SCFAs), and improving the content and density of minerals in bone (Cronin et al., 2016). Fecal microbiota transplantation (FMT) represents a novel "organ transplant" technique that can reestablish a normal gut microbial ecology. Through various mechanisms such as correcting microbial dysbiosis, repairing gut barrier functions, and modulating immune responses, FMT participates in the regulation of bone metabolism (Zhang et al., 2022b). Although clinical evidence is still lacking to prove that FMT can be used to treat patients with OP, animal studies have confirmed the efficacy of FMT in treating OP. Ma's study demonstrated that transplanting feces from young rats to elderly OP rats can alter their gut microbiota composition, improve gut barrier functions, and ultimately reduce bone loss to alleviate OP in elderly rats (Ma et al., 2021). However, it remains to be investigated whether this phenomenon applies to lipopolysaccharide (LPS)-induced OP, and how the species and quantity of gut microbiota change in LPS-induced OP animal models treated with FMT.

Long non-coding RNA (lncRNA) is a kind of RNA that is not involved in protein coding and has the ability to regulate a variety of physiological and pathological processes (Jathar et al., 2017). For example, lncRNA *TUG1* can promote bone formation through various ways, including binding to Lin28a to promote the osteogenic differentiation of periodontal stem cells, and acting on the Wnt/β-catenin signaling pathway to enhance the proliferation of osteoblasts (He et al., 2018; Liu et al., 2019). The level of lncRNA *TUG1* is regulated by a variety of factors, such as the expression of lncRNA *TUG1* can be affected by the content of taurine, which is a metabolite of some gut bacteria (Young et al., 2005). Therefore, whether FMT can regulate OP by affecting the expression of lncRNA *TUG1* remains to be determined. The aim of this study

is to establish the mice model of LPS-induced OP and reshape the gut microbiota by FMT. The changes of gut microbiota and the level of lncRNA *TUG1* were observed to explore whether FMT could alleviate LPS-induced OP by regulating the gut microbiota and the expression of lncRNA *TUG1*, and provide a basis for the biological treatment of OP.

2 Materials and methods

2.1 Selection and handling of experimental animals

The study followed the principles for the use of experimental animals in the Animal Center of Shandong University and was approved by the Ethics Committee of Shandong University. Twenty 6-week-old C57BL/6 mice with similar body weights were selected. The experimental animals were all of the same strain of healthy mice, free from any diseases, including gastrointestinal ailments, ensuring a healthy digestive system function and a good gut microbial ecology. Two mice were randomly selected as fecal donors, with their gut microbial health was verified. The remaining mice were randomly divided into control, LPS, and LPS+FMT groups, with 6 mice in each group. Since estrogen levels can affect bone metabolism by inhibiting Th17 cell differentiation through estrogen receptor pathways and reducing the production of tumor necrosis factor-β receptor activators, thereby suppressing osteoclast function, and estrogen deficiency can trigger CD4+ T-cell mediated immune responses, producing pre-osteoclast factors, activating osteoclasts, and enhancing bone resorption (Jia et al., 2021; Yu et al., 2021). To minimize the impact of sex hormone level differences on the study outcomes and to avoid bias from using a single gender, the gender ratio in each group was set at 1:1.

Due to the lack of standardized guidelines for FMT, this study draws on the comprehensive review by Bokoliya et al. regarding the operational procedures and precautions for mouse FMT. We followed the FMT principles covered in the review, including, but not limited to, timing fecal collection based on the circadian rhythms of the gut microbiota, establishing the environmental conditions required for fecal processing and storage, and determining the appropriate routes, doses, and frequencies for FMT. Established practices from previous research provide guidance for this study (Bokoliya et al., 2021). All mice were housed in a clean environment with a temperature of (22 ± 1) °C and humidity of (50 ± 5) %, with free access to food and water, under a 12-hour light/dark cycle. Mice in both the LPS and LPS + FMT groups received an intraperitoneal injection of LPS solution at a dose of 10 mg/kg to establish OP models. In contrast, the control group received an equal volume of saline. Injections were administered every other day for a total duration of 10 days (Wang et al., 2020). After this period, fresh feces from the donor mice were collected under sterile conditions. The feces (100 mg) were dissolved in 1 mL of sterile saline, vortexed for 10 seconds, and centrifuged at 2000g for 10 minutes at 4°C to remove any residues. The supernatant was diluted and administered via gavage to the mice in the LPS + FMT group (200 μL/mouse), while the other 2 groups received saline via gavage for 7 days (Yang et al., 2018). Following this treatment, feces and venous blood samples from the mice were collected for further analysis. Subsequently, the mice were euthanized by cervical dislocation, and their right femurs and intestinal tissues were preserved.

2.2 Validation of mouse OP model

The femurs were immersed in a 4% paraformaldehyde solution for 24 hours and then scanned using micro-CT with a resolution of 10 μm to create 3-dimensional (3D) reconstructed images of the distal femur. The region between 2% and 7% proximal to the growth plate was selected as the region of interest. Bone mineral density (BMD), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular separation (Tb. Sp) were recorded. The effectiveness of the OP model was validated by comparing CT images and bone structural parameters between the control and LPS groups.

2.3 HE staining of mouse femur sections

The decalcified femurs were embedded in paraffin and sectioned. The sections were immersed in hematoxylin staining solution for 3 minutes, rinsed with water, then differentiated in 1% hydrochloric acid alcohol for several seconds before being rinsed again. After the water returned to blue for 3 minutes, the sections were stained with eosin for 3 minutes and rinsed with water again. The sections were dehydrated with gradient ethanol and transparent with xylene, then sealed with neutral resin. The bone tissue staining was observed and compared among the 3 groups.

2.4 Analysis of gut microbiota composition and abundance

DNA was extracted from mouse feces using the QIAamp DNA Kit. The bacterial 16S rRNA V3-V4 region was amplified through polymerase chain reaction (PCR) using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplification conditions were as follows: a predenaturation at 95°C for 3 minutes, followed by 27 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, stably extension at 72°C for 10 minutes. The samples were stored at 4°C. The amplified products were isolated, and purified in a purification Kit. Quantification of the products was performed using the Quant-iT PicoGreen dsDNA Assay Kit.

Equal concentrations of purified quantitative DNA were mixed to construct a library, which was sequenced using the Illumina HiSeq platform. The gut microbiota data were analyzed using QIIME (v2.0.0), creating bar plots of species distribution at the

phylum, class, order, family, and genus levels. The α diversity analysis was conducted using Shannon index, Simpson index, and Chao1 index. Additionally, principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) were performed. The Chao1 index measures microbial abundance, estimating the total number of microbial taxa in a sample. Both the Shannon and Simpson indices assess microbial diversity within the samples and are positively correlated with diversity. The difference lies in that the Shannon index focuses on quantifying the number of microbial species, whereas the Simpson index emphasizes the evenness of species distribution. PCoA and NMDS are different methods for analyzing β diversity; in PCoA plots, the greater the distance between samples, the greater the intergroup differences in microbial composition. NMDS focuses on reflecting the differences in ranking sample distances between groups, with greater distances indicating larger differences.

2.5 Immunofluorescence staining of mouse femur sections

Femur sections were blocked with a 5% BSA solution at room temperature for 30 minutes. The primary antibody (dilution ratio of 1:100) was added and incubated overnight at 4°C. After recovering the primary antibody, the sections were washed 3 times with 0.1M PBS buffer for 5 minutes each. A labeled fluorescent secondary antibody (dilution ratio of 1:200) was then added and incubated in the dark at room temperature for 60 minutes. Following this step, the secondary antibody was discarded, and the sections were washed 3 times with PBS buffer for 10 minutes each. DAPI was added to stain the nuclei in the dark for 10 minutes, followed by 3 washes with PBS for 5 minutes each. Finally, neutral resin was used to mount the sections. The levels of Runt-related transcription factor 2 (RUNX2), a specific marker of type I osteoblasts, were compared among the 3 groups.

2.6 Measurement of lncRNA *TUG1* levels in mouse gut and blood

Mouse intestinal tissue was ground into a powder in liquid nitrogen. For every 100 mg of powder, 1 mL of Trizol was added, mixed thoroughly, and allowed to stand for 10 minutes. Mouse venous blood was collected, allowed to stand at 4°C for 30 minutes, then centrifuged at room temperature at 3000 rpm for 15 minutes, and the upper layer of serum was left. Trizol was added to this serum and mixed before allowing it to stand for another 5 minutes.

Chloroform equal to 1/5 of the Trizol volume was added, shaken for 15 seconds, and left at room temperature for an additional 10 minutes. The mixture was then centrifuged at 4°C at 13000 rpm for 10 minutes; isopropanol equal to the supernatant volume was added after discarding the lower phase. Following another centrifugation step at 4°C at 12000 rpm for 10 minutes, the supernatant was discarded. Each tube received 75% ethanol to wash the precipitate before centrifuging again under similar conditions. The precipitate was dried at room temperature for 10 minutes before being dissolved in 30 μL DEPC water. RNA concentration was measured using a micro-spectrophotometer and stored at -80°C.

For reverse transcription, a reaction system of 20 μL was prepared on ice: containing 4 μL of 5×FastKing-RT SuperMix, total RNA (2 μg), and RNase-Free ddH2O to reach a total volume of 20 μL. This mixture was centrifuged after thorough mixing and subjected to a reverse transcription program: incubating at 42°C for 15 minutes followed by heating to 95°C for 3 minutes; cDNA was stored at -20°C.

A real-time quantitative reverse transcription PCR (qRT-PCR) reaction system of 15 μL was prepared: comprising 7.5 μL SYBR Green premix, cDNA template (1.5 μL), forward and reverse primers (0.75 μL each), and ddH2O (4.5 μL). Reaction conditions included predenaturation at 95°C for 10 minutes; denaturation at 95°C for 10 seconds; annealing at 60°C for 30 seconds; extension at 72°C for 30 seconds; followed by termination after completing a total of 40 cycles. Relative expression levels of lncRNA *TUG1* were calculated using the $2^{-\Delta\Delta CT}$.

2.7 Statistical methods

Each experimental procedure was repeated 3 times to obtain results analyzed using SPSS version 26.0. The Shapiro-Wilk test assessed whether experimental data followed a normal distribution; Brown-Forsythe test evaluated homogeneity of variances. Normally distributed data with equal variances were expressed as mean ± standard deviation ($\bar{x} \pm S$), with independent samples *t*-test was used for inter-group comparison, and paired *t*-test was used for intra-group comparison. Graphs were generated using GraphPad Prism version 8 software. *P*-value <0.05 (*) and <0.01 (**) were considered statistically significant.

3 Results

3.1 Validation of mouse OP model

Micro-CT results showed that compared with the control group, the mice in the LPS group had reduced cancellous bone and thinner cortical bone in the femur (Figure 1). The analysis of bone structural parameters showed that the BMD, Tb. N, and Tb. Th of the femur of the mice in the LPS group were smaller than those of the control group, while Tb. Sp was larger than that of the control group, and the differences were all statistically significant (Figure 2). The validation results showed that the mouse OP models established in this study were effective.

3.2 Comparative of HE staining of femoral sections

The results of HE staining showed that compared with the control group, the bone trabeculae in the LPS group were

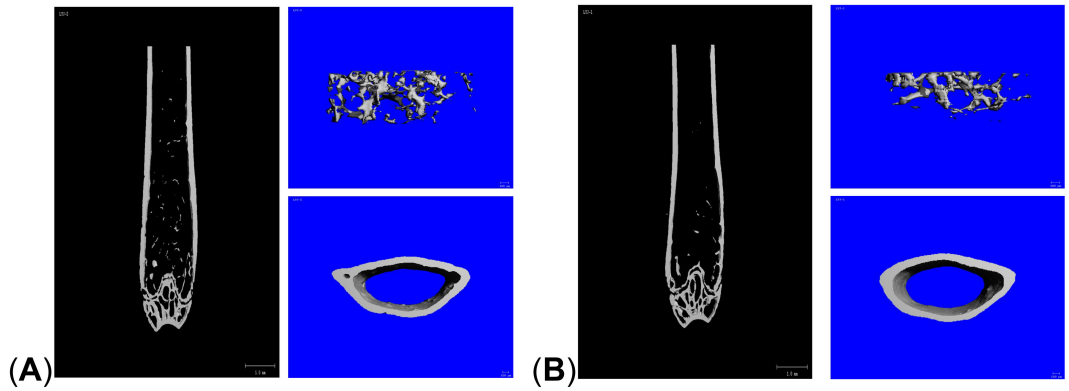


FIGURE 1
Micro-CT reconstructed images of the distal femur. **(A)** control group; **(B)** LPS group.

significantly thinner and more widely spaced, and there were large areas of trabecular bone loss. The density of adipocytes increased, showing the pathological characteristics of OP. However, in the LPS + FMT group, the thinning and sparsity of bone trabeculation was significantly improved, and the bone tissue structure was clear, which was basically restored to the same level as the control group, indicating that FMT could alleviate LPS-induced OP (Figure 3).

3.3 Analysis of gut microbiota composition and abundance

In terms of α diversity, there was a difference in the Chao1 index between the control and LPS groups ($P<0.05$), whereas the Chao1 index returned to normal levels after FMT treatment. It suggests that LPS can affect the abundance of gut microbiota in

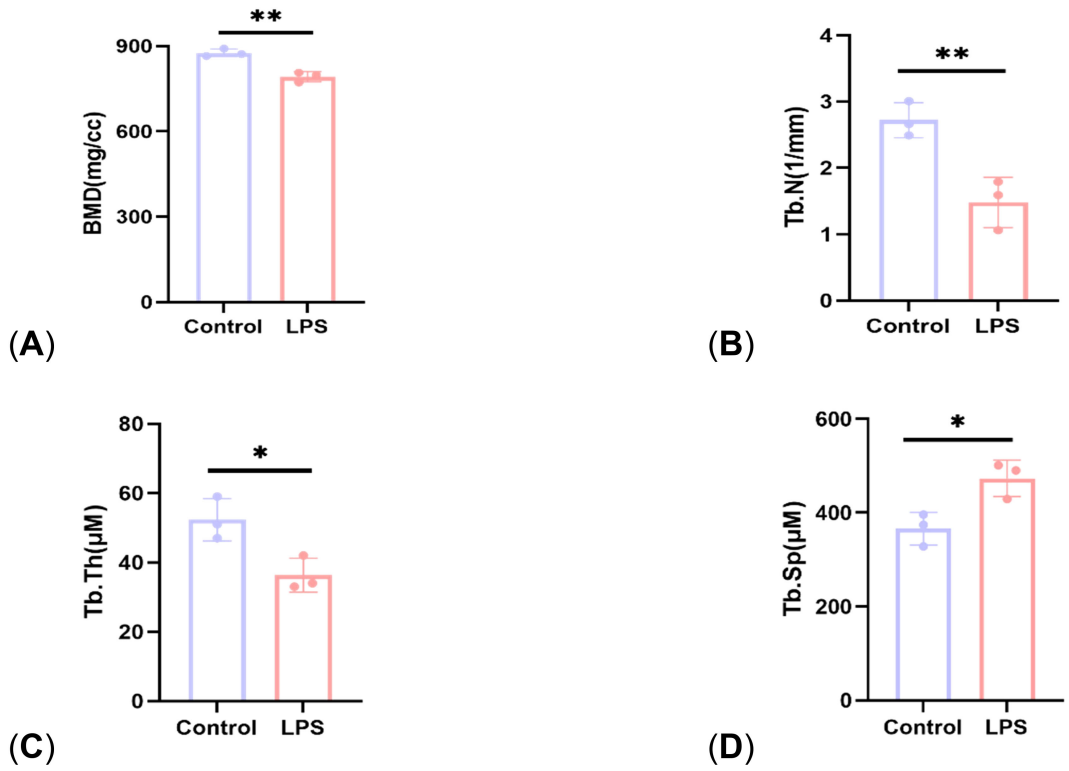


FIGURE 2
Comparison of bone structural parameters between control group and LPS group. **(A)** BMD; **(B)** Tb. N; **(C)** Tb. Th; **(D)** Tb. Sp. P<0.05 (*), P<0.01 (**).

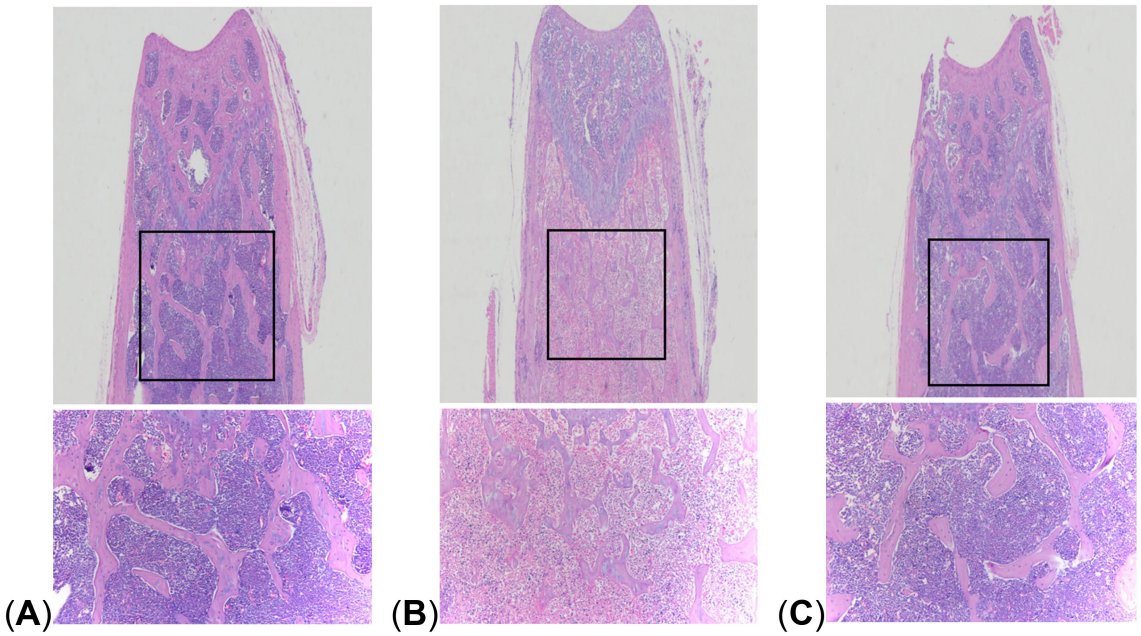


FIGURE 3
Comparison of HE staining. **(A)** control group; **(B)** LPS group; **(C)** LPS + FMT group.

mice, causing the absolute number of gut microbiota to decrease, and FMT can eliminate this effect. There were no statistically significant differences in the Shannon and Simpson indices among the 3 groups, indicating that neither LPS nor FMT treatments had a significant impact on microbial diversity or the evenness of microbial distribution (Figure 4). In terms of β diversity, the results of both PCoA and NMDS showed that the same color points representing the samples within the group were close, that is, the composition of gut microbiota within the group was clustered with small differences. However, the different color points representing the different groups were far, indicating that the composition and ranking of gut microbiota between the groups were significantly different (Figure 5).

The relative abundance of the top 10 bacterial groups was analyzed. At the phylum level, the Bacteroidetes and Firmicutes were significantly different among the groups. At the class level, the abundance of Bacteroidia and Bacilli decreased in the LPS group. At the order level, the abundance of Muribaculaceae was reduced in the LPS group. All these changes returned to normal after the FMT treatment. At the family level, the abundances of Bacteroidaceae and Lactobacillaceae in the LPS+FMT group were higher than in the LPS group, while Ruminococcaceae showed higher abundance in the LPS group. Notably, at the genus level, the abundances of Bacteroides and Lactobacillus were significantly higher in the LPS +FMT group compared to the LPS group (Figure 6). These results suggest that FMT could modulate the balance of gut microbiota, such as increasing the levels of Bacteroides and Lactobacillus.

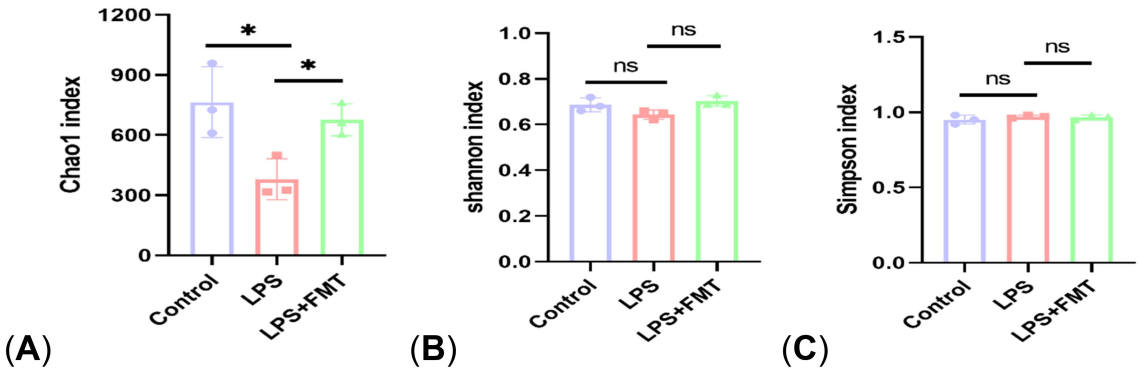


FIGURE 4
The α diversity analysis. **(A)** Chao 1 index; **(B)** Shannon index; **(C)** Simpson index. P<0.05 (*), P<0.01 (**), ns: no significance.

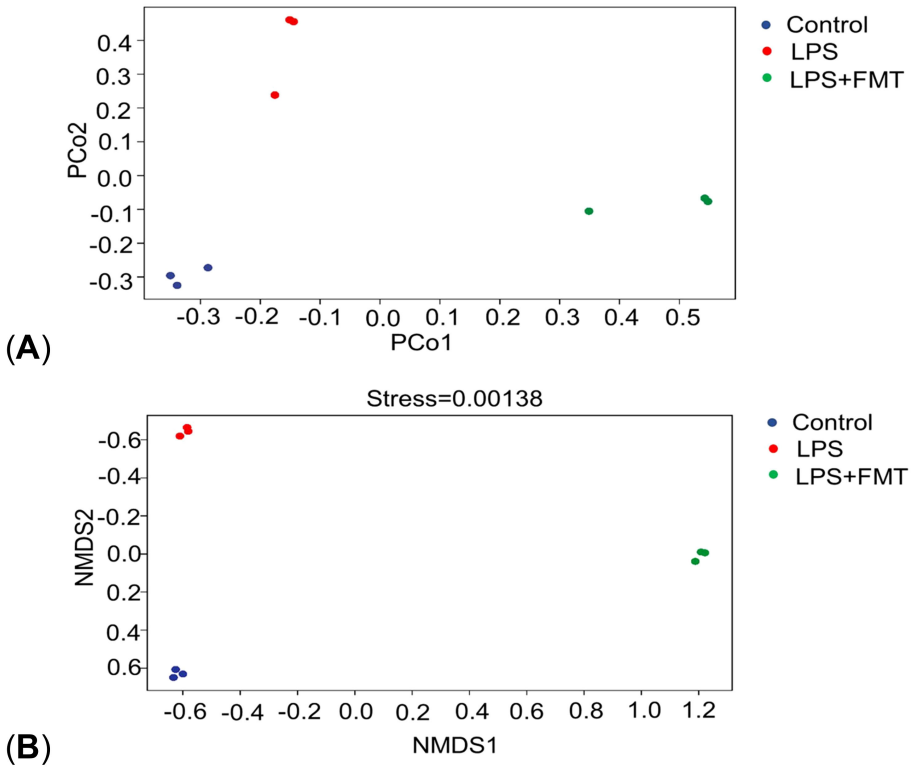


FIGURE 5
Results of PCoA and NMDS. **(A)** PCoA; **(B)** NMDS.

3.4 Comparative of immunofluorescence staining of femoral sections

Osteoblasts are directly involved in bone formation and their number is negatively correlated with the severity of OP. In the study, immunohistochemical staining for its specific marker RUNX2 was performed. The results showed that the expression of RUNX2 in the LPS group was significantly lower than that in the control group ($P < 0.01$), while the expression of RUNX2 in the LPS + FMT group was significantly increased (Figure 7). These results indicated that FMT could alleviate LPS-induced OP by increasing the number of osteoblasts.

3.5 Determination of lncRNA *TUG1* levels in mouse gut and blood

Bacteroides and Lactobacillus can regulate taurine production, and taurine can promote the expression of lncRNA *TUG1*, which regulates bone metabolism. To explore the mechanism by which FMT increases the number of osteoblasts, the expression levels of lncRNA *TUG1* in mouse gut and blood were examined in the study. The results showed that the *TUG1* mRNA levels in the LPS group were significantly lower than those in the control group, while the *TUG1* mRNA levels in the LPS + FMT group were significantly increased (Figure 8). These results suggest that FMT may alleviate OP by promoting the expression of lncRNA *TUG1* to restore the number of osteoblasts in OP mice.

4 Discussion

As the aging population intensifies, the number of OP patients is surging, imposing a significant economic burden on society (Gao et al., 2023). The results of this animal experiment indicate that FMT can enhance osteoblast levels and improve bone structure by modulating the abundance of gut microbiota in OP mice (such as increasing Bacteroides and Lactobacillus populations) and promoting the expression of lncRNA *TUG1*, thereby alleviating LPS-induced OP.

LPS is a common inducer of inflammatory OP. LPS activates specific pattern recognition receptors (PRRs) that detect microbial and infectious agents, triggering inflammatory responses, such as the LPS-TLR4/NF-κβ pathway, which produce inflammatory cytokines that impact the RANKL/RANK/OPG pathway leading to bone loss (Aquino-Martinez et al., 2020; de Vos et al., 2022). LPS also accelerates apoptosis in osteoblasts by increasing the expression of non-coding RNAs such as circular RNA circAtp9b and lncRNA TMC3-AS1 (Chen and Dai, 2022; Feng et al., 2022). Furthermore, LPS can promote oxidative stress damage by binding to the extracellular vesicles of harmful bacteria, compromising gut barrier functions (Chen S. et al., 2023). LPS-mediated

10.3389/fcimb.2025.1535666

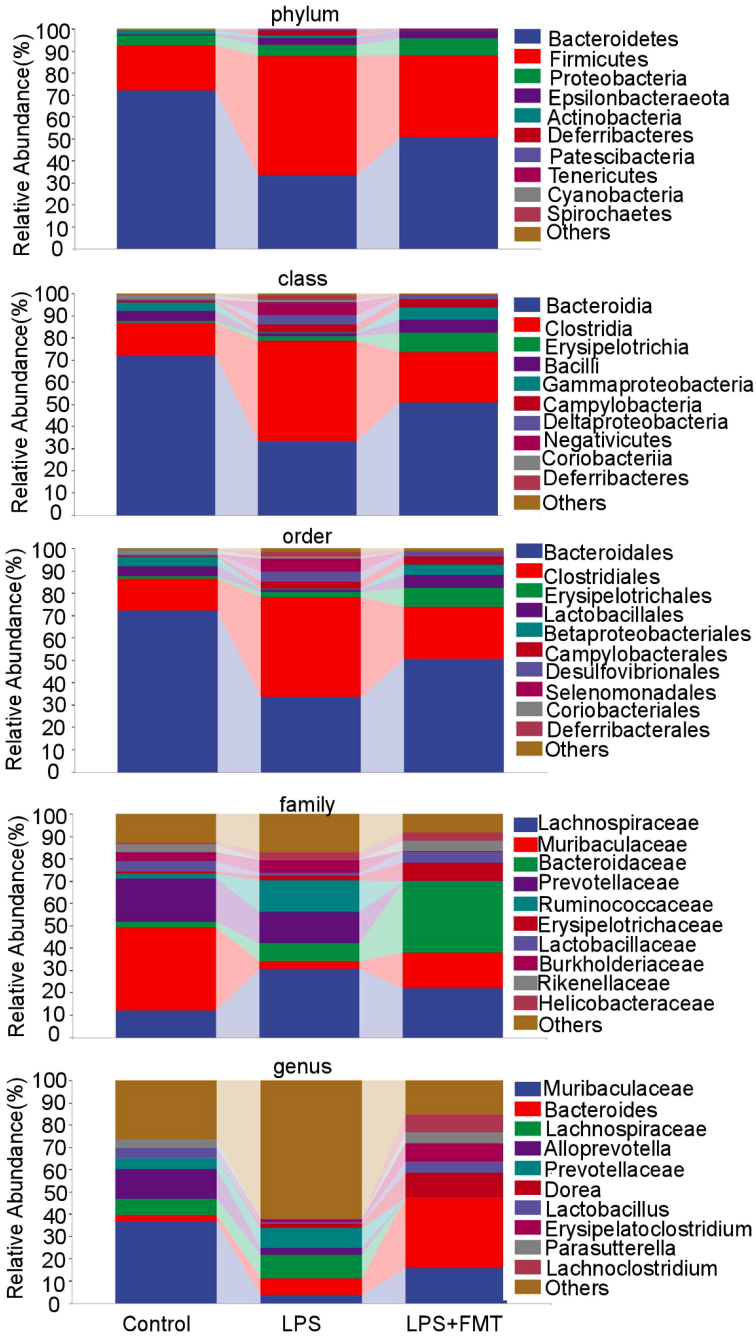


FIGURE 6
Analysis of gut microbiota composition at different taxonomic levels.

inflammation also affects the gut microbiota, increasing the abundance of pathogenic bacteria such as Enterococcus spp. and Enterobacteriaceae (including Escherichia coli and Salmonella typhimurium). Oxidative stress inhibits microbiota protein synthesis, thereby reducing the metabolism of beneficial microbes to SCFAs and other substances (Kroon et al., 2025). The metabolic products of harmful bacteria enter the bloodstream through the compromised gut barrier, activating cellular immune responses, while the beneficial metabolic products like SCFAs, which are conducive to bone formation, decrease, ultimately leading to the onset of OP (Safdari et al., 2016; Candelli et al., 2021).

Traditional anti-OP medications often face issues like numerous side effects and prolonged treatment times, leading to poor patient compliance. Consequently, emerging biological therapies such as FMT are being considered to address these problems. The gut microbiota plays a crucial role in physiological and immune functions; any abnormal changes in its diversity and quantity can trigger adverse outcomes like bone metabolism disorders (Tyagi et al., 2018). FMT

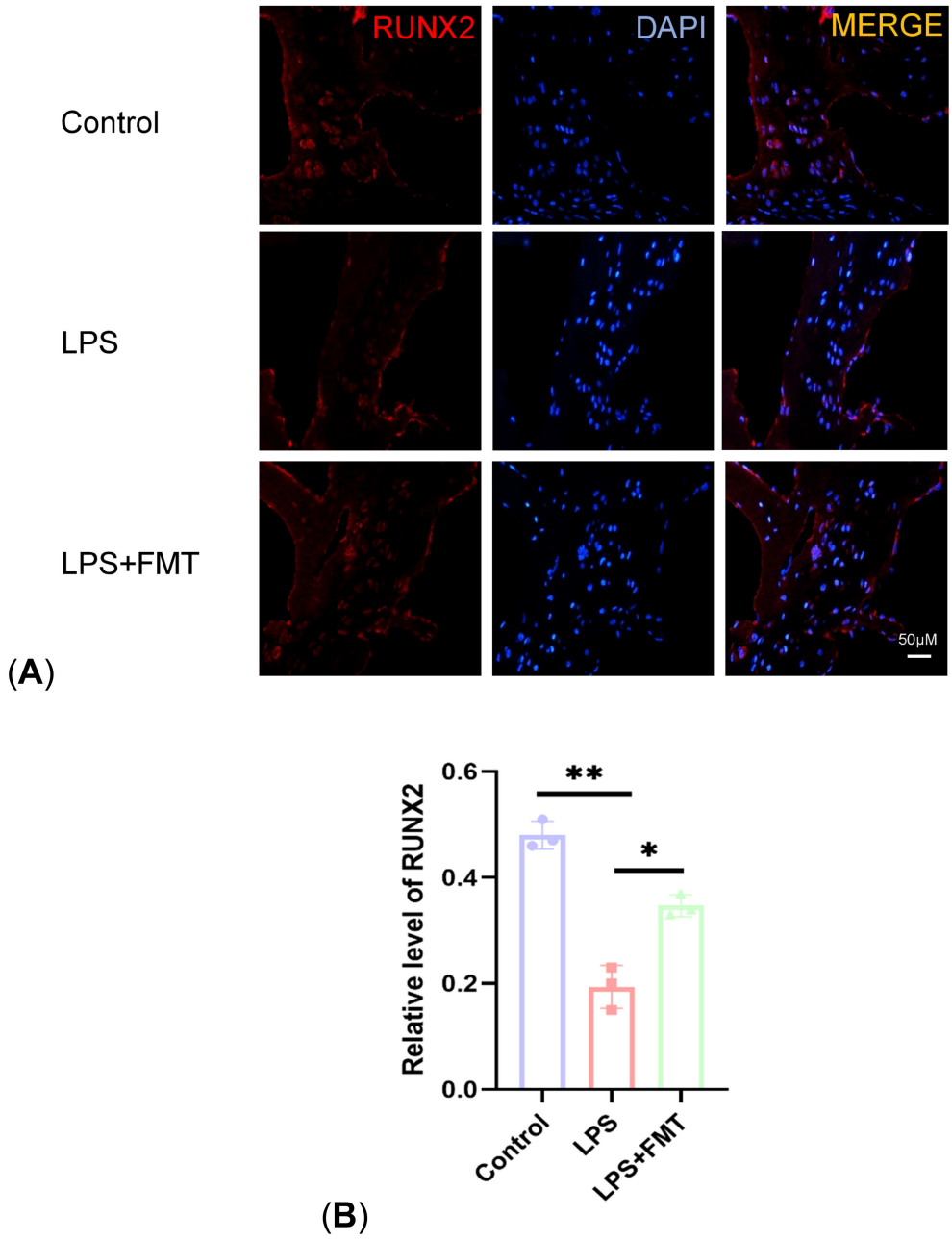


FIGURE 7
Comparison of RUNX2 expression levels. **(A)** Results of immunofluorescence staining; **(B)** Statistical plot of RUNX2 levels. P<0.05 (*), P<0.01 (**).

allows for the transfer of healthy donor gut microbiota into recipients to restore normal gut microecology. Compared to administering single probiotics, FMT has a longer-lasting effect on regulating gut microbiota and effectively controls the proliferation of harmful bacteria (Grehan et al., 2010). Currently, FMT has shown promising results in treating gastrointestinal diseases, but its efficacy and mechanisms in treating OP are still in preliminary exploration (Kim et al., 2024). This research findings indicate that FMT positively impacts alleviating LPS-induced OP. Previous studies have shown that after transplanting feces from juvenile rats, intestinal microbial structure improved in aged OP rats, with reduced bone loss (Ma et al., 2021). Another study demonstrated that FMT could enhance the expression of tight junction proteins between intestinal epithelial cells and inhibit the release of cytokines that promote osteoclastogenesis, thereby alleviating estrogen deficiency-induced OP (Zhang et al., 2022a). These pieces of evidence suggest that FMT has certain therapeutic effects in alleviating both primary and secondary OP.

The gut microbiota exhibits variations in species and abundance under different conditions such as age, diet, and disease. For instance, in the gut microbiota of aged OP rats, the abundance of the family Lachnospiraceae is higher, and this abundance decreases after the transplantation of feces from young rats (Ma et al., 2021).

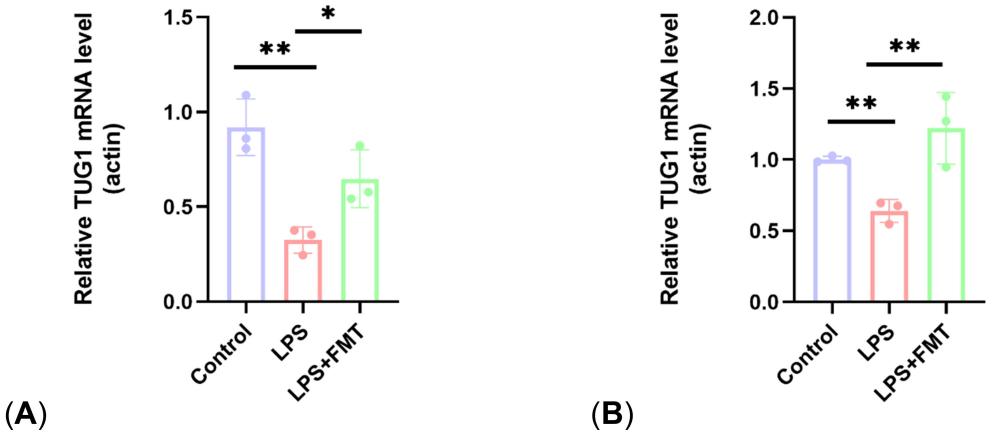


FIGURE 8
Comparison of lncRNA *TUG1* expression levels. **(A)** Expression levels of lncRNA *TUG1* in gut; **(B)** Expression levels of lncRNA *TUG1* in blood. P<0.05 (*), P<0.01 (**).

This study found that the abundance of Bacteroides and Lactobacillus in the gut microbiota of LPS-mediated OP mice was significantly increased after fecal transplantation from age-matched healthy mice. The gut microbiota can influence bone formation and resorption through its metabolic products or by regulating the host's metabolism, immunity, and endocrine functions; this connection is referred to as the gut-bone axis. Different gut bacteria play distinct roles in this gut-bone axis, making it necessary to clarify the relationship between specific gut bacteria and bone metabolism. Research by Chen indicated that Lactobacillus rhamnosus can reduce the expression of pro-inflammatory cytokines and nuclear factor κB while increasing the expression of the anti-inflammatory cytokine interleukin-10, thereby improving bone quality (Chen YW. et al., 2023). Another study found that Akkermansia muciniphila can promote the synthesis of polyamines such as spermine and spermidine, which increase bone trabecular volume and density, thus enhancing the biomechanical strength of bones in adult female and young male mice (Chevalier et al., 2020). On the other hand, some gut bacteria can promote the onset of OP. A large sample study indicated that the abundance of Clostridiales bacterium DTU089 is negatively correlated with tibial bone density (Okoro et al., 2023). This study also found that the abundance of Ruminococcaceae is significantly higher in the LPS group than in other groups, possibly related to the LPS-mediated inflammatory response. Ruminococcaceae UCG004 has been shown to reduce bone mass and increase the risk of OP (Zeng et al., 2024). Clarifying the relationship between specific bacteria in the gut microbiota and OP can help deepen our understanding of their mechanisms and promote the development of biological agents for treating OP. The results of this study demonstrate that FMT can increase the numbers of Bacteroides and Lactobacillus. Additionally, after FMT, the abundance of Muribaculaceae is also restored, which can promote osteogenesis by producing SCFAs and regulating T-cell differentiation. These beneficial bacteria play a positive role in mitigating LPS-mediated OP.

LncRNAs are regulatory non-coding RNAs longer than 200 nucleotides that play a crucial role in the osteogenic differentiation of various stem cells. LncRNA *AC092155* can promote the osteogenic differentiation of adipose stem cells through the miR-*143-3p*/STMN1 axis (Sun et al., 2021). LncRNA *KCNMA1-AS1* can enhance the osteogenic differentiation of human bone marrow mesenchymal stem cells by activating the SMAD9 signaling pathway (Mai et al., 2023). Additionally, lncRNAs regulate certain bone metabolism markers and cytokines involved in bone metabolism. For instance, lncRNA *SNHG1* can inhibit the expression of osteoprotegerin by affecting its methylation status, thereby promoting bone resorption (Yu et al., 2022). LncRNA *CASC11* can drive the development of postmenopausal osteoporosis by upregulating tumor necrosis factor-α levels (Yu et al., 2019). Research has shown that Bacteroides and Lactobacillus can promote the methylation of lncRNA *MEG3* to inhibit osteoclastogenesis, indicating that gut microbiota can regulate bone metabolism by influencing lncRNA expression (Huang et al., 2022). In this study, FMT not only restored the abundance of Bacteroides and Lactobacillus but also elevated the levels of lncRNA *TUG1*. This may be related to certain metabolites produced by Bacteroides and Lactobacillus (such as taurine) that promote lncRNA *TUG1* expression. Bacteroides and Lactobacillus produce bile salt hydrolase (BSH), which hydrolyzes the C-24 N-acyl bond between taurine and bile acids, breaking down taurocholate-a compound of bile acids and taurine entering the intestine via bile-into free bile acids and taurine (Cai et al., 2022). Taurine, a cysteine derivative, can bind to glycine-receptor α2 and GABA(A) receptors, releasing signals that upregulate the level of lncRNA *TUG1* (Young et al., 2005). LncRNA *TUG1* can facilitate the differentiation of bone marrow mesenchymal stem cells into osteoblasts and inhibit their apoptosis by targeting the miRNA-*34a*/FGFR1 pathway (Wang et al., 2021a). These studies illustrate the potential for Bacteroides and Lactobacillus to increase bodily taurine levels and subsequently upregulate lncRNA *TUG1*. These theories may support the results of our study, which showed that

fecal transplantation could alleviate LPS-mediated OP by regulating the expression of lncRNA *TUG1*. The study also found that the restoration levels of lncRNA *TUG1* in the blood post-FMT were higher than in the gut, which may be related to the different ability of lncRNA *TUG1* expression in different tissue cells. A study found that *TUG1* levels are higher in the central nervous system than in other tissues (Subhramanyam and Hu, 2017). Another study found that *TUG1* was mainly distributed in the cytoplasm and nucleus, so the distribution of *TUG1* in different cellular structures was different (Liu et al., 2020). However, the reasons for these differences still need further investigation.

Research indicates that Lactobacillus can drive RNA m6A modification mediated by METTL3 through its production of lactic acid, thereby upregulating lncRNA *TUG1* (Xiong et al., 2022; Xi et al., 2024). Bacteroides can downregulate the TLR4/NF-κβ signaling pathway through its production of capsular polysaccharide A, reversing LPS-mediated inflammatory responses (Wang et al., 2021b). These findings reveal potential mechanisms by which Lactobacillus and Bacteroides influence LPS-mediated OP through other metabolic products, with more mechanisms yet to be explored.

In addition to affecting the gut microbiota and levels of lncRNA *TUG1*, FMT can influence bone metabolism by regulating gut barrier functions and the levels of inflammatory factors. FMT increases the expression of ZO-1 and Occludin to restore gut barrier function and inhibits the release of TNF-α and IL-1 β to suppress osteoclast formation, all of which are beneficial for alleviating OP (Zhang et al., 2022a). Moreover, transplanting feces from elderly OP rats to young rats compromised the intestinal barrier of the young (Wang N. et al., 2022). Transplanting feces from postmenopausal OP women to germ-free mice treated with antibiotics elevated levels of TNF-α and IL-17A in the mice (Chen et al., 2024). These studies highlight the roles of gut barrier integrity and systemic inflammatory status in FMT.

Previous animal studies on FMT for the treatment of OP mostly used elderly animal models or animal models with estrogen deficiency after ovariectomy. In this study, LPS was used to establish animal models of inflammation-mediated OP, and the positive role of FMT in the treatment of LPS-mediated OP and the possible mechanism were investigated. This study also found that the number of Bacteroides and Lactobacillus in the intestine of OP mice increased after FMT treatment, and identifying these potentially beneficial bacteria can provide reference for other treatment methods such as intake of probiotics. This study is the first to focus on the changes in the level of LncRNA *TUG1* during FMT treatment of OP, and lncRNA *TUG1* has been shown to promote the proliferation of osteoblasts and inhibit their apoptosis. The results of this study can provide new ideas for the mechanism of FMT treatment of OP.

However, there are certain limitations in this study. The study focused solely on the changes in the gut microbiota and lncRNA *TUG1* levels following FMT, but did not evaluate the effects of changes in gut barrier function and inflammatory factor levels on bone metabolism. Furthermore, it did not analyze the functional aspects of the gut microbiota through metabolomics. This study noted an increase in the abundance of Bacteroides and Lactobacillus post-FMT and highlighted the elevated levels of lncRNA *TUG1*. Building on previous studies on taurine metabolism and efficacy, it outlined a positive pathway involving Bacteroides/Lactobacillus-taurine-lncRNA *TUG1* (Young et al., 2005; Cai et al., 2022). Future studies will consider incorporating Phylogenetic Investigation of Communities by Reconstruction of Unobserved States or metabolomics analyses to provide more comprehensive evidence for the mechanisms by which FMT alleviates OP. Additionally, the functional assays for lncRNA *TUG1* need to be improved, such as clarifying whether knocking down lncRNA *TUG1* can reverse the effects of FMT. These issues will be addressed in further research.

Currently, FMT faces numerous challenges in treating human OP. Inappropriate donor selection can not only fail to treat OP but also increase the presence of harmful components in the gut microbiota or even lead to cross-infection. Donor screening is difficult and costly; one study showed that only about 10% of potential donors passed the screening due to reasons such as medication use, parasitic infections, and Helicobacter pylori infections, with the entire process costing 64.112 euros (Bénard et al., 2022). The selection of recipients is equally important; causes of OP include aging, inflammation, and estrogen deficiency, and it is unclear which subtypes benefit most from FMT. It is also uncertain whether FMT could cause adverse reactions in patients with other diseases or those taking other medications (Zhang et al., 2022b). Future research should seek solutions to these issues, further standardize the selection of fecal donors and recipients, and the transplantation routes, explore the appropriate number of transplantations, and weigh the pros and cons of combination therapies. For treating different subtypes of OP, personalized microbiome intervention strategies should be a direction for future research, such as personalized enrichment treatments with beneficial gut bacteria. Additionally, establishing a large-scale microbial library based on standard healthy donor gut microbiota could help reduce the risks and costs associated with FMT. As research continues to deepen, it is anticipated that fecal transplantation therapy for OP will bring more benefits to patients and society.

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

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

Author contributions

PM: Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. RW: Data curation, Formal Analysis, Software, Validation, Visualization, Writing – review & editing. HC: Data curation, Formal Analysis, Methodology, Validation, Writing – review & editing. JCZ: Data curation, Formal Analysis, Methodology, Validation, Writing – review & editing. WY: Data curation, Formal Analysis, Methodology, Validation, Writing – review & editing. BM: Data curation, Formal Analysis, Methodology, Validation, Writing – review & editing. YFL: Data curation, Formal Analysis, Methodology, Validation, Writing – review & editing. YL: Data curation, Formal Analysis, Methodology, Validation, Writing – review & editing. JZ: Conceptualization, Supervision, Validation, Writing – review & editing. HG: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article.

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 author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Anish, R. J., and Nair, A. (2024). Osteoporosis management-current and future perspectives - A systemic review. *J. Orthop* 53, 101–113. doi: 10.1016/j.jor.2024.03.002

Aquino-Martinez, R., Rowsey, J. L., Fraser, D. G., Eckhardt, B. A., Khosla, S., Farr, J. N., et al. (2020). LPS-induced premature osteocyte senescence: Implications in inflammatory alveolar bone loss and periodontal disease pathogenesis. *Bone* 132, 115220. doi: 10.1016/j.bone.2019.115220

Bénard, M. V., de Bruijn, C. M. A., Fenneman, A. C., Wortelboer, K., Zeevenhoven, J., Rethans, B., et al. (2022). Challenges and costs of donor screening for fecal microbiota transplantations. *PloS One* 17, e0276323. doi: 10.1371/journal.pone.0276323

Bokoliya, S. C., Dorsett, Y., Panier, H., and Zhou, Y. (2021). Procedures for fecal microbiota transplantation in murine microbiome studies. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.711055

Cai, J., Sun, L., and Gonzalez, F. J. (2022). Gut microbiota-derived bile acids in intestinal immunity, inflammation, and tumorigenesis. *Cell Host Microbe* 30, 289–300. doi: 10.1016/j.chom.2022.02.004

Candelli, M., Franza, L., Pignataro, G., Ojetti, V., Covino, M., Piccioni, A., et al. (2021). Interaction between lipopolysaccharide and gut microbiota in inflammatory bowel diseases. *Int. J. Mol. Sci.* 22, 6242. doi: 10.3390/ijms22126242

Chen, S., and Dai, M. (2022). Lipopolysaccharide-Induced lncRNA TMC3-AS1 is Highly Expressed in Osteoporosis and Promotes Osteoblast Apoptosis by Suppressing the Formation of Mature miR-708. *Int. J. Gen. Med.* 15, 3345–3352. doi: 10.2147/IJGM.S350081

Chen, Y. W., Lee, M. L., Chiang, C. Y., and Fu, E. (2023). Effects of systemic Bifidobacterium longum and Lactobacillus rhamnosus probiotics on the ligature-induced periodontitis in rat. *J. Dent. Sci.* 18, 1477–1485. doi: 10.1016/j.jds.2023.04.013

Chen, S., Lei, Q., Zou, X., and Ma, D. (2023). The role and mechanisms of gram-negative bacterial outer membrane vesicles in inflammatory diseases. *Front. Immunol.* 14. doi: 10.3389/fimmu.2023.1157813

Chen, T., Wang, N., Hao, Y., and Fu, L. (2024). Fecal microbiota transplantation from postmenopausal osteoporosis human donors accelerated bone mass loss in mice. *Front. Cell Infect. Microbiol.* 14. doi: 10.3389/fcimb.2024.1488017

Chevalier, C., Kieser, S., Çolakoğlu, M., Hadadi, N., Brun, J., Rigo, D., et al. (2020). Warmth prevents bone loss through the gut microbiota. *Cell Metab.* 32, 575–590.e7. doi: 10.1016/j.cmet.2020.08.012

Cronin, B. E., Allsopp, P. J., Slevin, M. M., Magee, P. J., Livingstone, M. B., Strain, J. J., et al. (2016). Effects of supplementation with a calcium-rich marine-derived multi-mineral supplement and short-chain fructo-oligosaccharides on serum lipids in postmenopausal women. *Br. J. Nutr.* 115, 658–665. doi: 10.1017/S0007114515004948

de Vos, W. M., Tilg, H., Van Hul, M., and Cani, P. D. (2022). Gut microbiome and health: mechanistic insights. *Gut* 71, 1020–1032. doi: 10.1136/gutjnl-2021-326789

Feng, Y., Ding, L., and Li, L. (2022). LPS-inducible circAtp9b is highly expressed in osteoporosis and promotes the apoptosis of osteoblasts by reducing the formation of mature miR-17-92a. *J. Orthop Surg. Res.* 17, 193. doi: 10.1186/s13018-022-03072-x

Gao, Y., Chen, N., Fu, Z., and Zhang, Q. (2023). Progress of wnt signaling pathway in osteoporosis. *Biomolecules* 13, 483. doi: 10.3390/biom13030483

Grehan, M. J., Borody, T. J., Leis, S. M., Campbell, J., Mitchell, H., and Wettstein, A. (2010). Durable alteration of the colonic microbiota by the administration of donor fecal flora. *J. Clin. Gastroenterol.* 44, 551–561. doi: 10.1097/MCG.0b013e3181e5d06b

Guss, J. D., Horsfield, M. W., Fontenele, F. F., Sandoval, T. N., Luna, M., Apoorva, F., et al. (2017). Alterations to the gut microbiome impair bone strength and tissue material properties. *J. Bone Miner Res.* 32, 1343–1353. doi: 10.1002/jbmr.3114

Hao, L., Yan, Y., Huang, G., and Li, H. (2024). From gut to bone: deciphering the impact of gut microbiota on osteoporosis pathogenesis and management. *Front. Cell Infect. Microbiol.* 14. doi: 10.3389/fcimb.2024.1416739

He, Q., Yang, S., Gu, X., Li, M., Wang, C., and Wei, F. (2018). Long noncoding RNA TUG1 facilitates osteogenic differentiation of periodontal ligament stem cells via interacting with Lin28A. *Cell Death Dis.* 9, 455. doi: 10.1038/s41419-018-0484-2

Huang, F., Wong, P., Li, J., Lv, Z., Xu, L., Zhu, G., et al. (2022). Osteoimmunology: The correlation between osteoclasts and the Th17/Treg balance in osteoporosis. *J. Cell Mol. Med.* 26, 3591–3597. doi: 10.1111/jcmm.17399

Jathar, S., Kumar, V., Srivastava, J., and Tripathi, V. (2017). Technological developments in lncRNA biology. *Adv. Exp. Med. Biol.* 1008, 283–323. doi: 10.1007/978-981-10-5203-3_10

Jia, L., Tu, Y., Jia, X., Du, Q., Zheng, X., Yuan, Q., et al. (2021). Probiotics ameliorate alveolar bone loss by regulating gut microbiota. *Cell Prolif* 54, e13075. doi: 10.1111/cpr.13075

Kim, D. Y., Lee, S. Y., Lee, J. Y., Whon, T. W., Lee, J. Y., Jeon, C. O., et al. (2024). Gut microbiome therapy: fecal microbiota transplantation vs live biotherapeutic products. *Gut Microbes* 16, 2412376. doi: 10.1080/19490976.2024.2412376

Kroon, S., Malcic, D., Weidert, L., Bircher, L., Boldt, L., Christen, P., et al. (2025). Sublethal systemic LPS in mice enables gut-luminal pathogens to bloom through oxygen species-mediated microbiota inhibition. *Nat. Commun.* 16, 2760. doi: 10.1038/s41467-025-57979-0

Li, P., Ji, B., Luo, H., Sundh, D., Lorentzon, M., and Nielsen, J. (2022). One-year supplementation with Lactobacillus reuteri ATCC PTA 6475 counteracts a degradation of gut microbiota in older women with low bone mineral density. *NPJ Biofilms Microbiomes* 8, 84. doi: 10.1038/s41522-022-00348-2

Liu, S., Qiu, J., He, W., Geng, C., He, G., Liu, C., et al. (2020). TUG1 long non-coding RNA enlists the USF1 transcription factor to overexpress ROMO1 leading to hepatocellular carcinoma growth and metastasis. *MedComm* 1, 386–399. doi: 10.1002/mco2.38

Liu, S. C., Sun, Q. Z., Qiao, X. F., Li, X. G., Yang, J. H., Wang, T. Q., et al. (2019). LncRNA TUG1 influences osteoblast proliferation and differentiation through the Wnt/β-catenin signaling pathway. *Eur. Rev. Med. Pharmacol. Sci.* 23, 4584–4590. doi: 10.26355/eurrev_201906_18035

Ma, S., Wang, N., Zhang, P., Wu, W., and Fu, L. (2021). Fecal microbiota transplantation mitigates bone loss by improving gut microbiome composition and gut barrier function in aged rats. *PeerJ* 9, e12293. doi: 10.7717/peerj.12293

Mai, Z., Liu, J., Jiang, X., Gu, W., Wang, W., Li, S., et al. (2023). Long noncoding RNA KCNMA1-AS1 promotes osteogenic differentiation of human bone marrow mesenchymal stem cells by activating the SMAD9 signaling pathway. *Biol. Direct* 18, 81. doi: 10.1186/s13062-023-00425-2

Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D., Hirschfield, G. M., Hold, G., et al. (2016). The gut microbiota and host health: a new clinical frontier. *Gut* 65, 330–339. doi: 10.1136/gutjnl-2015-309990

Okoro, P. C., Orwoll, E. S., Huttenhower, C., Morgan, X., Kuntz, T. M., McIver, L. J., et al. (2023). A two-cohort study on the association between the gut microbiota and bone density, microarchitecture, and strength. *Front. Endocrinol. (Lausanne)* 14. doi: 10.3389/fendo.2023.1237727

Orwoll, E. S., Parimi, N., Wiedrick, J., Lapidus, J., Napoli, N., Wilkinson, J. E., et al. (2022). Analysis of the associations between the human fecal microbiome and bone density, structure, and strength: the osteoporotic fractures in men (MrOS) cohort. *J. Bone Miner Res.* 37, 597–607. doi: 10.1002/jbmr.4518

Reid, I. R., and Billington, E. O. (2022). Drug therapy for osteoporosis in older adults. *Lancet* 399, 1080–1092. doi: 10.1016/S0140-6736(21)02646-5

Safdari, B. K., Sia, T. C., Wattchow, D. A., and Smid, S. D. (2016). Effects of pro-inflammatory cytokines, lipopolysaccharide and COX-2 mediators on human colonic neuromuscular function and epithelial permeability. *Cytokine* 83, 231–238. doi: 10.1016/j.cyto.2016.04.017

Subhramanyam, C. S., and Hu, Q. (2017). Non-coding RNA in brain development and disorder. *Curr. Med. Chem.* 24, 1983–1997. doi: 10.2174/0929867324666170124151436

Sun, J., Zhang, F., Luo, X., Shi, G., Li, F., Zheng, B., et al. (2021). Long noncoding RNA AC092155 facilitates osteogenic differentiation of adipose-derived stem cells through the miR-143-3p/STMN1 axis. *J. Gene Med.* 23, e3363. doi: 10.1002/jgm.3363

Tyagi, A. M., Yu, M., Darby, T. M., Vaccaro, C., Li, J. Y., Owens, J. A., et al. (2018). The microbial metabolite butyrate stimulates bone formation via T regulatory cell-mediated regulation of WNT10B expression. *Immunity* 49, 1116–1131.e7. doi: 10.1016/j.immuni.2018.10.013

Wang, X., He, J., Wang, H., Zhao, D., Geng, B., Wang, S., et al. (2021a). Fluid shear stress regulates osteoblast proliferation and apoptosis via the lncRNA TUG1/miR-34a/FGFR1 axis. *J. Cell Mol. Med.* 25, 8734–8747. doi: 10.1111/jcmm.16829

Wang, N., Ma, S., and Fu, L. (2022). Gut microbiota dysbiosis as one cause of osteoporosis by impairing intestinal barrier function. *Calcif Tissue Int.* 110, 225–235. doi: 10.1007/s00223-021-00911-7

Wang, S., Wang, S., Wang, X., Xu, Y., Zhang, X., Han, Y., et al. (2022). Effects of icariin on modulating gut microbiota and regulating metabolite alterations to prevent bone loss in ovariectomized rat model. *Front. Endocrinol. (Lausanne)* 13. doi: 10.3389/fendo.2022.874849

Wang, X., Ye, C., Xun, T., Mo, L., Tong, Y., Ni, W., et al. (2021b). Bacteroides fragilis polysaccharide A ameliorates abnormal voriconazole metabolism accompanied with the inhibition of TLR4/NF-κB pathway. *Front. Pharmacol.* 12. doi: 10.3389/fphar.2021.663325

Wang, J., Zhang, Y., Xu, X., Jin, W., Jing, C., Leng, X., et al. (2020). ASP2-1, a polysaccharide from Acorus tatarinowii Schott, inhibits osteoclastogenesis via modulation of NFATc1 and attenuates LPS-induced bone loss in mice. *Int. J. Biol. Macromol* 165, 2219–2230. doi: 10.1016/j.ijbiomac.2020.10.077

Xi, Q., Yang, G., He, X., Zhuang, H., Li, L., Lin, B., et al. (2024). M6A-mediated upregulation of lncRNA TUG1 in liver cancer cells regulates the antitumor response of CD8+ T cells and phagocytosis of macrophages. *Adv. Sci. (Weinh)* 11, e2400695. doi: 10.1002/advs.202400695

Xiong, J., He, J., Zhu, J., Pan, J., Liao, W., Ye, H., et al. (2022). Lactylation-driven METTL3-mediated RNA m6A modification promotes immunosuppression of tumor-infiltrating myeloid cells. *Mol. Cell* 82, 1660–1677.e10. doi: 10.1016/j.molcel.2022.02.033

Yang, H., Xiang, Y., Robinson, K., Wang, J., Zhang, G., Zhao, J., et al. (2018). Gut microbiota is a major contributor to adiposity in pigs. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.03045

Young, T. L., Matsuda, T., and Cepko, C. L. (2005). The noncoding RNA taurine upregulated gene 1 is required for differentiation of the murine retina. *Curr. Biol.* 15, 501–512. doi: 10.1016/j.cub.2005.02.027

Yu, M., Pal, S., Paterson, C. W., Li, J. Y., Tyagi, A. M., Adams, J., et al. (2021). Ovariectomy induces bone loss via microbial-dependent trafficking of intestinal TNF+ T cells and Th17 cells. *J. Clin. Invest.* 131, e143137. doi: 10.1172/JCI143137

Yu, X., Song, M. S., Rong, P. Z., Chen, X. J., Shi, L., Wang, C. H., et al. (2022). LncRNA SNHG1 modulates adipogenic differentiation of BMSCs by promoting DNMT1 mediated Opg hypermethylation via interacting with PTBP1. *J. Cell Mol. Med.* 26, 60–74. doi: 10.1111/jcmm.16982

Yu, H., Zhou, W., Yan, W., Xu, Z., Xie, Y., and Zhang, P. (2019). LncRNA CASC11 is upregulated in postmenopausal osteoporosis and is correlated with TNF-α. *Clin. Interv Aging* 14, 1663–1669. doi: 10.2147/CIA.S205796

Zeng, H. Q., Li, G., Zhou, K. X., Li, A. D., Liu, W., and Zhang, Y. (2024). Causal link between gut microbiota and osteoporosis analyzed via Mendelian randomization. *Eur. Rev. Med. Pharmacol. Sci.* 28, 542–555. doi: 10.26355/eurrev_202401_35052

Zhang, Y. W., Cao, M. M., Li, Y. J., Lu, P. P., Dai, G. C., Zhang, M., et al. (2022a). Fecal microbiota transplantation ameliorates bone loss in mice with ovariectomy-induced osteoporosis via modulating gut microbiota and metabolic function. *J. Orthop Translat* 37, 46–60. doi: 10.1016/j.jot.2022.08.003

Zhang, Y. W., Cao, M. M., Li, Y. J., Zhang, R. L., Wu, M. T., Yu, Q., et al. (2022b). Fecal microbiota transplantation as a promising treatment option for osteoporosis. *J. Bone Miner Metab.* 40, 874–889. doi: 10.1007/s00774-022-01375-x

Zhang, Y. W., Lu, P. P., Li, Y. J., Dai, G. C., Cao, M. M., Xie, T., et al. (2021). Low dietary choline intake is associated with the risk of osteoporosis in elderly individuals: a population-based study. *Food Funct.* 12, 6442–6451. doi: 10.1039/d1fo00825k

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 07 May 2025
DOI 10.3389/fmicb.2025.1589902

Check for updates

OPEN ACCESS

EDITED BY
Ludovico Abenavoli,
Magna Græcia University, Italy

REVIEWED BY
Lei Li,
Second Military Medical University, China
Giuseppe Guido Maria Scarlata,
University Magna Graecia of Catanzaro, Italy

*CORRESPONDENCE
Jie Men
✉ menjie2020@126.com

†These authors have contributed equally to this work and share first authorship

RECEIVED 10 March 2025
ACCEPTED 11 April 2025
PUBLISHED 07 May 2025

CITATION
Men J, Cui C, Li H, Li Z, Zhang Y, Liu Z, Wang Q, Liu P, Zou S, Yu Z, Zhang Y, Wu S, Zhu G, Wang P and Huang X (2025) Cold water swimming reshapes gut microbiome to improve high-fat diet-induced obesity. *Front. Microbiol.* 16:1589902. doi: 10.3389/fmicb.2025.1589902

COPYRIGHT
© 2025 Men, Cui, Li, Li, Zhang, Liu, Wang, Liu, Zou, Yu, Zhang, Wu, Zhu, Wang and Huang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Cold water swimming reshapes gut microbiome to improve high-fat diet-induced obesity

Jie Men[1,2*†], Chenglong Cui[1†], Hao Li[1†], Zhaowei Li[1], Yu Zhang[1], Zhiyu Liu[1], Qi Wang[1], Penghong Liu[3], Shuangling Zou[4], Zhengyang Yu[1], Yuxi Zhang[1], Simin Wu[1], Guoyu Zhu[1], Pengbo Wang[1] and Xiaoli Huang[5]

[1]Department of Clinical Medicine, Fenyang College of Shanxi Medical University, Fenyang, China, [2]Department of Basic Medical Sciences, Key Discipline of Fenyang College of Shanxi Medical University (Physiology), Fenyang, China, [3]Department of Psychiatry, First Hospital of Shanxi Medical University, Taiyuan, China, [4]Center for Translational Medicine, Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, West China Second University Hospital, Sichuan University, Chengdu, Sichuan, China, [5]Longhua County Traditional Chinese Medicine Hospital, Chengde, China

Hypothermia and swimming have been shown to alleviate high-fat diet (HFD)-induced obesity, with effects linked to the gut microbiota (GM). However, whether the effects of cold water swimming (CWS) on GM can be effectively transferred through fecal microbiota transplantation (FMT) has not been investigated. This study established mice models of obesity, CWS and FMT to investigate the mechanism by which CWS reshapes GM to improve HFD-induced obesity. Additionally, we analyzed the relationship between obesity phenotypes, GM composition, gene expression and CWS. The study found that HFD induced obesity phenotypes and GM dysbiosis in mice, while CWS produced opposite effects. The FMT results confirmed that CWS effectively alleviated HFD-induced lipid accumulation, metabolic disorders, and chronic inflammatory responses, which are associated with increased GM diversity, enrichment of beneficial bacteria, and the repair of intestinal barrier damage. Furthermore, these beneficial effects can be effectively transferred via FMT. The evidence from this study suggests that GM plays a critical role in the anti-obesity effects of CWS, with intestinal barrier repair emerging as a potential therapeutic target. This also provides scientific evidence for the feasibility of FMT as a strategy to combat obesity.

KEYWORDS

cold water swimming, gut microbiome, fecal microbiota transplantation, obesity, aerobic exercise

Introduction

The global estimate for high body mass index (BMI $\geq$ 25kg/m^2) indicates that ~2.63 billion individuals will be affected in 2023 and this number is projected to exceed 4 billion by 2035 (World obesity atlas, 2024; Nomura et al., 2024). High BMI is associated with the early onset of various non-communicable diseases, including type 2 diabetes, cardiovascular disease (CVD), cancer, and stroke (Piché et al., 2020). Obesity further exacerbates these risks. Currently, the primary approaches for treating obesity are medications, surgical treatment, physical activity, and dietary management (the significant role of the Mediterranean diet in obesity prevention and management should not be overlooked) (Dominguez et al., 2023). However, long-term use of medicine is often accompanied by adverse gastrointestinal reactions (Rubino et al., 2021; Sodhi et al., 2023), hypertension, and mental disorders (Gudzune and Kushner, 2024)

and may even lead to significant reductions in heart weight and cardiomyocyte area (Martens et al., 2024). Surgical interventions carry risks of postoperative and metabolic complications (e.g., gastroesophageal reflux and severe nutrient deficiencies) as well as the need for re-operation (Perdomo et al., 2023). Notably, pharmacological and surgical interventions primarily address the obesity phenotype but do not positively impact obesity-induced declines in cardiopulmonary fitness, metabolic dysfunction, or mental disorders. Exercise, another effective intervention, is recommended by the American College of Cardiology (ACC) and the European Society of Cardiology (ESC). It should be emphasized that although exercise does not cause serious adverse effects or excessive disease burden, forms of exercise other than swimming may increase the risk of joint injuries in individuals due to high BMI (Roos and Arden, 2016).

Growing evidence indicates that gut microbiota (GM) is closely associated with diseases, including high-fat diet (HFD)-induced obesity (Perler et al., 2023). Meanwhile, GM is highly plastic and easily influenced by environmental factors, including diet and exercise. Differences in GM composition and function were observed between obese individuals and healthy subjects, and this finding was closely related to HFD-induced dyslipidemia and pro-inflammatory GM changes (Aron-Wisnewsky et al., 2021; Chambers et al., 2019). Clinical trials have shown that GM diversity and abundance of beneficial bacteria are significantly higher in individuals engaged in aerobic exercise than in obese individuals (Leong et al., 2020). Animal studies also support these findings, attributing the positive changes to GM remodeling (Dohnalova et al., 2022). Recent studies have found that low-temperature exposure induces dramatic changes in GM diversity, and composition, leading to weight loss (Ziętak et al., 2016), which is achieved by promoting fat browning, increasing energy expenditure, and repairing intestinal barrier damage (Li et al., 2019). Interestingly, these changes can be effectively transferred through fecal microbiota transplantation (FMT) (Chevalier et al., 2015), thus offering a novel option for obesity treatment.

In recent years, FMT has gradually attracted significant attention. Whether GM was transplanted from healthy mice to HFD-induced obese mice or from obesity-improved mice to healthy mice, the donor GM phenotype was consistently replicated (Barcena et al., 2019; Shtossel et al., 2023), emphasizing the causal relationship between FMT-mediated GM regulation and disease improvement. Moreover, although there is a correlation between GM donors and acceptors (Porcari et al., 2023), higher GM diversity and abundance in the donor are associated with an increased success rate of setting values when recipient conditions remain constant (Kump et al., 2018). Hence, the success of FMT engraftment is more influenced by the donor. Both exercise and hypothermia significantly enhanced GM diversity and abundance while demonstrating unique advantages in energy consumption. However, little is known about how to reshape GM based on Cold water swimming (CWS) to improve the mechanism of obesity.

To elucidate the relationship between CWS and HFD-induced iGM imbalance and to determine whether GM composition and changes are associated with the anti-obesity effects induced by long-term CWS. This study transplanted GM from mice subjected to long-term CWS into obese mice, validating GM as a key target for the an-ti-obesity effects of CWS, which also found that FMT effectively transferred the anti-obesity effects induced by CWS, including significant increases in GM diversity and abundance, as well as the repair of intestinal barrier damage and metabolic dysfunction. These findings lay the foundation for GM-targeted interventions to improve or treat obesity.

Materials and methods

Experimental animals and protocol

Experimental animals

This study used 46 SPF-grade male C57BL/6J mice aged 4 weeks, weighing 12–14 g, purchased from the Animal Research Center of Shanxi Medical University [SCXK (Shanxi) 2019-0004]. The laboratory conditions included a temperature of (25 ± 2°C), relative humidity (50 ± 5%), and a 12-h light-dark cycle. All mice were housed in open cages within a flexible membrane isolator with filtered air ventilation. Free access to food and water was provided, and after 1 week of acclimatization, the mice were randomly divided into the HFD group ($n = 12$), normal-fat diet (NFD) group ($n = 12$), CWS group ($n = 12$), and FMT group ($n = 10$). All animal experiments were approved by the Ethics Committee of Shanxi Medical University, Ethical Review Number: 2023020.

Experimental protocol

The NFD and CWS groups received standard chow (13.5% of calories from fat), while the HFD group was fed HFD (60% of calories from fat), provided by Book Yu Biological Technology (Shanghai) Co., Ltd., License No.: Su Feeding Permit (2019) 01008, and continued for 18 weeks. The CWS protocol was based on Da's study (Da Silva et al., 2020), with an adaptive CWS protocol (2 weeks): 3 days/week, 10 min/day, each session increased by 5 min; the temperature was 37°C, each reduction was 3°C; formal exercise protocol (16 weeks): 20°C, 30 min/day, 3 days/week, for a total of 16 weeks.

Fecal microbiota transplantation

The FMT procedure was modified by Wang et al. (2020) and performed after the construction of the HFD and CWS models. Fresh feces from the donor (CWS group) were collected, homogenized in sterile phosphate buffer saline (PBS), and centrifuged at 4000 r/min for 5 min. The supernatant was aliquoted and stored at −80°C for further use. After 1 week of antibiotic pretreatment, the FMT was performed on the recipient (HFD group) with 200 μl per injection, three times per week until the end of the experiment. Feces from the FMT group were collected at the end of the study, snap-frozen in liquid nitrogen, and stored at −80°C for further analysis.

Biochemical analysis

After modeling, the mice were fasted for 5 h and injected intraperitoneally with 25% urethane at a dose of 0.5 ml/100 g

(20 g urethane in water, final volume 100 mL, 100 g mouse injected with 0.5 ml), and anesthesia was successfully achieved. Fresh blood samples were collected via cardiac puncture, centrifuged at 4000 r/min for 7 min, and the supernatant was used to measure serum biochemical parameters. According to the manufacturer's instructions, high-density lipoprotein (HDL), low-density lipoprotein (LDL), Total Cholesterol (TC), and Triglyceride (TG) were measured by colorimetric assays using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Abbkine. Serum Lipopolysaccharide (LPS) levels were measured using a solid-phase sandwich ELISA kit purchased from Bio Swamp Ltd., following the manufacturer's instructions.

Histological analysis

Tissue blocks (fat, liver, colon) from each mouse were collected, fixed in 4% para-formaldehyde solution for 36 h, and embedded in paraffin, followed by cutting into 4 μm slices. These slices were stained with Hematoxylin and Eosin (H&E) using standard techniques and then used for histopathological analysis. Observations were made under a light microscope to examine fat cell size in fat tissues, pathological changes in liver tissues the length of villi, and the number of goblet cells in the colon.

Real-time fluorescent quantitative PCR (qrt-PCR)

Total RNA was isolated from adipose tissue and liver tissue, and quantitative qRT-PCR for mRNA was performed by adding a fluorescent substance to the qRT-PCR reaction system. The fluorescent signal was detected in real-time through binding to amplification products. The experimental procedure was as follows: First, RNA extraction was carried out, followed by reverse transcription, and then relative quantitative analysis of mRNA was performed. Information on tissue genes and the housekeeping gene GAPDH can be found in Table 1. Total RNA was isolated from the right lateral lobe of the mouse liver and adipose tissue, and qRT-PCR was performed as previously described. Based on the results of the aforementioned bioinformatics analysis, genes Uncoupling protein 1 (UCP-1), Peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC-1α), Protein kinase AMP-activated catalytic subunit alpha 1 (PRKAA1), and tumor necrosis factor-α (TNF-α) were selected for validation. The sample size was increased, and qRT-PCR technology was used to detect the expression levels of these genes in the NFD, HFD, CWS, and FMT groups. Their expression levels were measured. Total RNA was extracted using Buffer RLA, Buffer RW3, Buffer RW, and proteinase K. One microgram of total RNA from each sample was reverse transcribed using a reverse transcription system. Real-time amplification was performed using Master Mix and Primers. The initial stage was set at 95°C for 30 s, followed by a two-step program of 95°C for 10 s and 60°C for 30 s, cycled 40 times, and finally a three-step program of 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. The relative expression of genes was evaluated using the 2^(−ΔΔCT) method.

TABLE 1 Gene organization and the reference gene GAPDH information.

ID	Primer name	Sequence, 5′→ 3′	Base number
UCP-1	UCP-1 F	AGGCTTCCAGTACCATTAGGT	21
	UCP-1 R	CTGAGTGAGGCAAAGCTGATTT	22
PGC-1α	PGC-1α F	TATGGAGTGACATAGAGTGTGCT	23
	PGC-1α R	CCACTTCAATCCACCCAGAAAG	22
PRKAA1	PRKAA1 F	GTCAAAGCCGACCCAATGATA	21
	PRKAA1 R	CGTACACGCAAATAATAGGGGTT	23
TNF-α	TNF-α F	GACGTGGAACTGGCAGAAGAG	21
	TNF-α R	TTGGTGGTTTGTGAGTGTGAG	21
GAPDH	GAPDH F	GTGTTCCTACCCCCAATGTGT	21
	GAPDH R	ATTGTCATACCAGGAAATGAGCTT	24

Gut microbiota analysis

DNA extraction from samples

The total genomic DNA from GM was extracted according to the E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, U.S.), following the manufacturer's protocol. The quality of the extracted genomic DNA was checked using 1% agarose gel electrophoresis, and DNA concentration and purity were determined using NanoDrop2000 (Thermo Scientific, USA).

PCR amplification and sequencing library construction

Using the extracted DNA as a template, PCR was performed with the barcode-labeled forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V3-V4 variable regions of the 16S rRNA gene. Each sample was amplified in triplicates. After recovery and purification of the PCR products from the same sample, quantification was performed. The purified PCR products were then used to build sequencing libraries using the NEXTFLEX Rapid DNA-Seq Kit, and the original data was uploaded to the National Center for Biotechnology Information (NCBI) database under the sequence number PRJNA1203435.

Sequence data analysis

Quality control was performed on the paired-end raw sequencing sequences using fastp (Chen et al., 2018) (version 0.19.6). The FLASH (Magoc and Salzberg, 2011) (version 1.2.11) software was used for sequence merging. The sequencing analysis was based on the DADA2 (Bolyen et al., 2019) plugin in the

Qiime2 pipeline (Callahan et al., 2016), where optimized sequences were denoised. The denoised sequences obtained from DADA2 were Amplicon Sequence Variants (ASVs). Mitochondrial and chloroplast sequences were removed from all samples. All samples were rarefied to equal sequencing depth, followed by taxonomic analysis at the ASVs level using a Naive Bayes classifier. Functional prediction analysis of the 16S rRNA sequences was performed using PICRUSt2 (Douglas et al., 2020) (version 2.2.0).

Statistical analysis

Statistical analysis and plotting were performed using GraphPad Prism 10. If data met a normal distribution, means ± standard deviation were used to represent the data, and one-way analysis of variance (ANOVA) was used for intergroup comparisons. Non-normally distributed data were expressed as median (IQR), and group comparisons were performed using the Kruskal-Wallis test. Statistical significance was set at $P < 0.05$

All sequencing data analyses were conducted on the MegBio Cloud Platform, with the following details: Alpha diversity was calculated using the Mothur (Schloss, 2020) software, including Shannon and Chao indices and the Wilcoxon rank sum test was used for intergroup differences in Alpha diversity analysis. The similarity of GM structure among samples was tested using Principal co-ordinates analysis (PCoA) based on the Bray-Curtis distance algorithm and PERMANOVA non-parametric test was used to analyze significant differences in GM structure between sample groups. Linear discriminant analysis Effect Size (LEfSe) analysis (Segata et al., 2011) [Linear discriminant analysis (LDA) > 4, $P < 0.05$] was used to identify significant differences in bacterial taxa from phylum to genus level between groups. Distance-based redundancy analysis (db-RDA) was used to analyze the effects of CWS on GM structural changes. Linear regression analysis was used to assess the impact of CWS on GM alpha diversity indices in db-RDA. Species were selected for correlation network analysis based on Spearman correlation ($|r| > 0.6$, $P < 0.05$).

Results

CWS improves HFD-induced obesity phenotypes

To evaluate the effect of CWS-based FMT on HFD-induced obesity, 10-week HFD-induced obesity and CWS mice models were established. Compared with the NFD group, mice in the CWS, and HFD groups showed significantly higher body weight ($P < 0.01$), consistent with changes in body size, with the HFD group exhibiting the most pronounced differences (Figure 1A). The total fat mass, visceral fat, and mice anatomy exhibited trends similar to those of body weight (Figures 1B, D, I). Notably, compared with the NFD group, the body weight-to-total fat ratio was significantly higher in the CWS and HFD groups, which can be attributed to weight gain induced by the HFD. However, the CWS group accumulated more fat, resulting in the highest body weight-to-total fat ratio. This suggested two mechanisms: on one hand, it shows that the compensation mechanism can obtain more energy from the physical objects consumed (Worthmann et al., 2017), and on the other hand, adaptive thermogenesis during CWS, which increased weight and fat mass in response to the low-temperature environment (Aldiss et al., 2022). To clarify the effects of CWS on mice fat, we analyzed fat types. Compared with the NFD group, epididymal white adipose tissue (eWAT) weight significantly increased in both the CWS and HFD groups ($P < 0.001$, Figure 1F). Inguinal white adipose tissue (iWAT) and brown adipose tissue (BAT) weights were significantly higher in the CWS group compared with the NFD group ($P < 0.001$, Figures 1E, G), indicating that CWS induces thermogenic regulation of energy homeostasis, leading to fat browning, a result consistent with previous studies (Cani and Van Hul, 2024). These findings suggested significant differences in obesity phenotypes between CWS and HFD models, but whether CWS-based FMT can improve HFD-induced obesity phenotypes remains unclear. Thus, after 8 weeks of CWS and HFD modeling, an FMT model was established. Remarkably, FMT replicated the CWS-improved obesity phenotype induced by HFD ($P < 0.001$, Figures 1A–G), suggesting that the GM altered by CWS held potential for obesity improvement.

CWS improves HFD-induced metabolic disorders, browning pro-cesses, intestinal barrier function and gene expression

Long-term CWS-induced adaptive changes positively affected dyslipidemia, inflammatory responses, fat browning, and gene expression. These changes could be replicated in recipients via FMT. To verify this possibility, we analyzed key lipid and inflammatory markers. Compared with the NFD group, HFD-induced mice exhibited dyslipidemia, with significantly elevated LDL, TC, and TG levels ($P < 0.001$, Figures 2B–D), reduced HDL levels ($P < 0.001$, Figure 2A), and increased LPS levels ($P < 0.001$, Figure 2E). The CWS group showed trends opposite to those of the HFD group, and the FMT group mirrored the CWS group's patterns. Combined with obesity phenotype data (Figure 1), these results demonstrated that HFD-induced weight gain was strongly correlated with dyslipidemia and inflammation, consistent with previous studies (Huang et al., 2021). To better understand the browning process of fat after CWS, pathological sections and H&E staining of adipose tissues and liver were conducted, and the H&E results were as expected. Compared with the NFD group, the HFD group exhibited significant expansion of eWAT cells and lipid accumulation in the liver (Figure 2F), whereas the CWS and FMT groups showed opposing results. BAT and iWAT contain higher mitochondrial density than eWAT, resulting in more efficient thermogenesis and metabolic benefits (Becher et al., 2021), consistent with the findings of this study. Compared with the NFD and HFD groups, the CWS group significantly inhibited adipocyte expansion and induced eWAT browning and BAT increases, as evidenced by differences in adipocyte size, number, and density (Figure 2F). These findings indicated that eWAT browning and BAT increases were more pronounced under dual stimuli of low-temperature exposure and exercise, which were significantly associated with improved HDL, LDL, TC, TG, and LPS levels. Notably, similar changes were observed in the FMT

10.3389/fmicb.2025.1589902

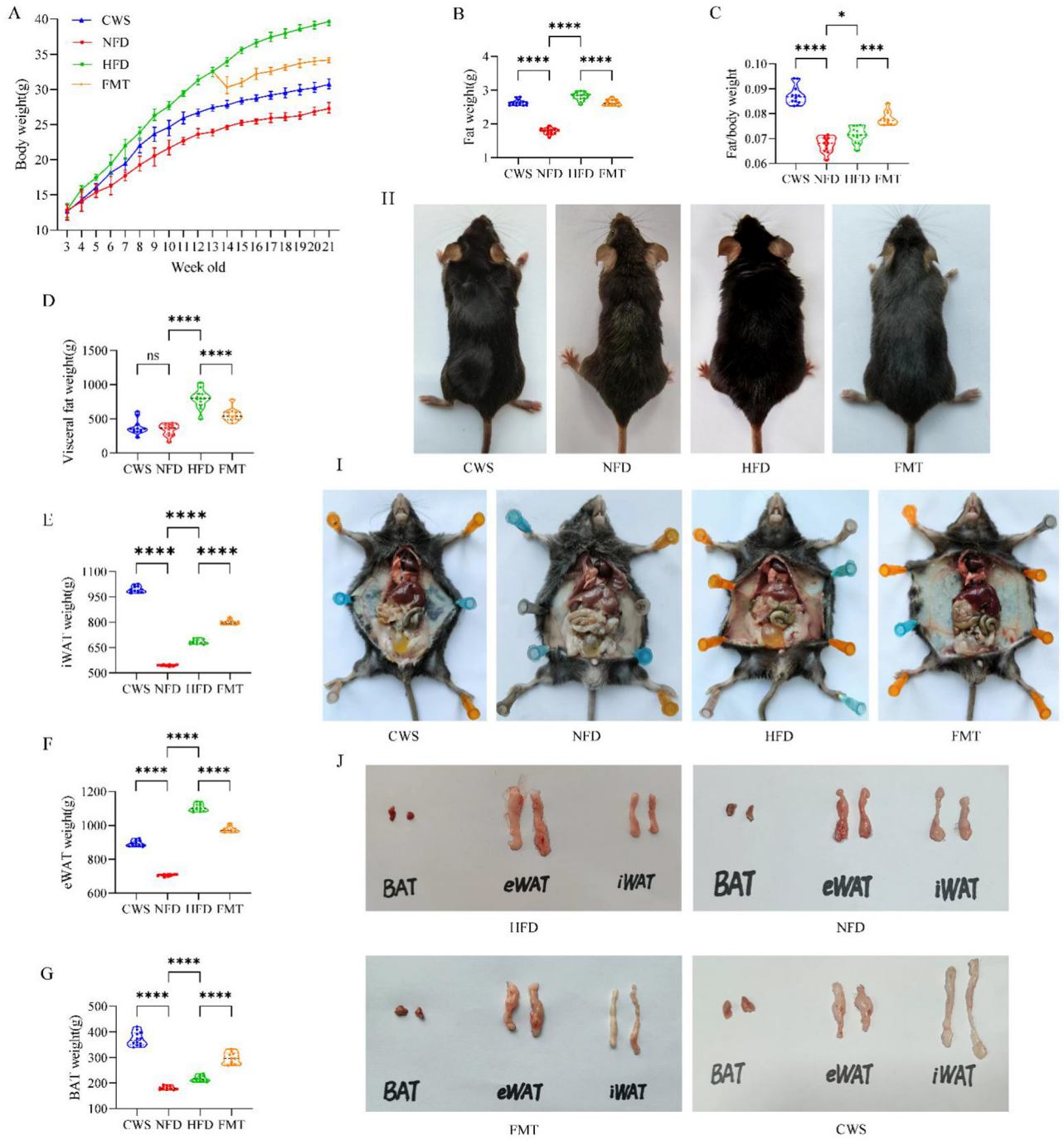


FIGURE 1
CWS improves the obese phenotype induced by HFD. **(A)** Changes in mice body weight during the experiment; **(B)** Total fat mass; **(C)** Fat/weight ratio; **(D)** Visceral fat; **(E)** iWAT; **(F)** eWAT; **(G)** BAT; **(H)** Mice morphology image; **(I)** Visceral fat image in the abdominal cavity; **(J)** Morphological image of fat types. Data are presented as mean ± SD; ns, no significant difference; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$.

group, indicating that the GM phenotype reshaped by CWS could be effectively transferred via FMT.

HFD-induced dyslipidemia and inflammatory responses disrupted intestinal integrity and the intestinal barrier. Similarly, this study found that HFD-induced obesity resulted in a significant reduction in the number of goblet cells in the colon, along with decreased intestinal wall thickness and villus height, compared with the NFD group (Figure 2F). Dyslipidemia and increased LPS levels further supported evidence of intestinal integrity and barrier damage. Each intestinal cell contained up to 1,000 microvilli, structures that expanded the absorptive surface area by 25-fold. In contrast, the CWS group exhibited a marked increase in goblet cell numbers, intestinal wall thickness, and villus height (Figure 2F). These findings suggest that cold exposure enhanced intestinal

10.3389/fmicb.2025.1589902

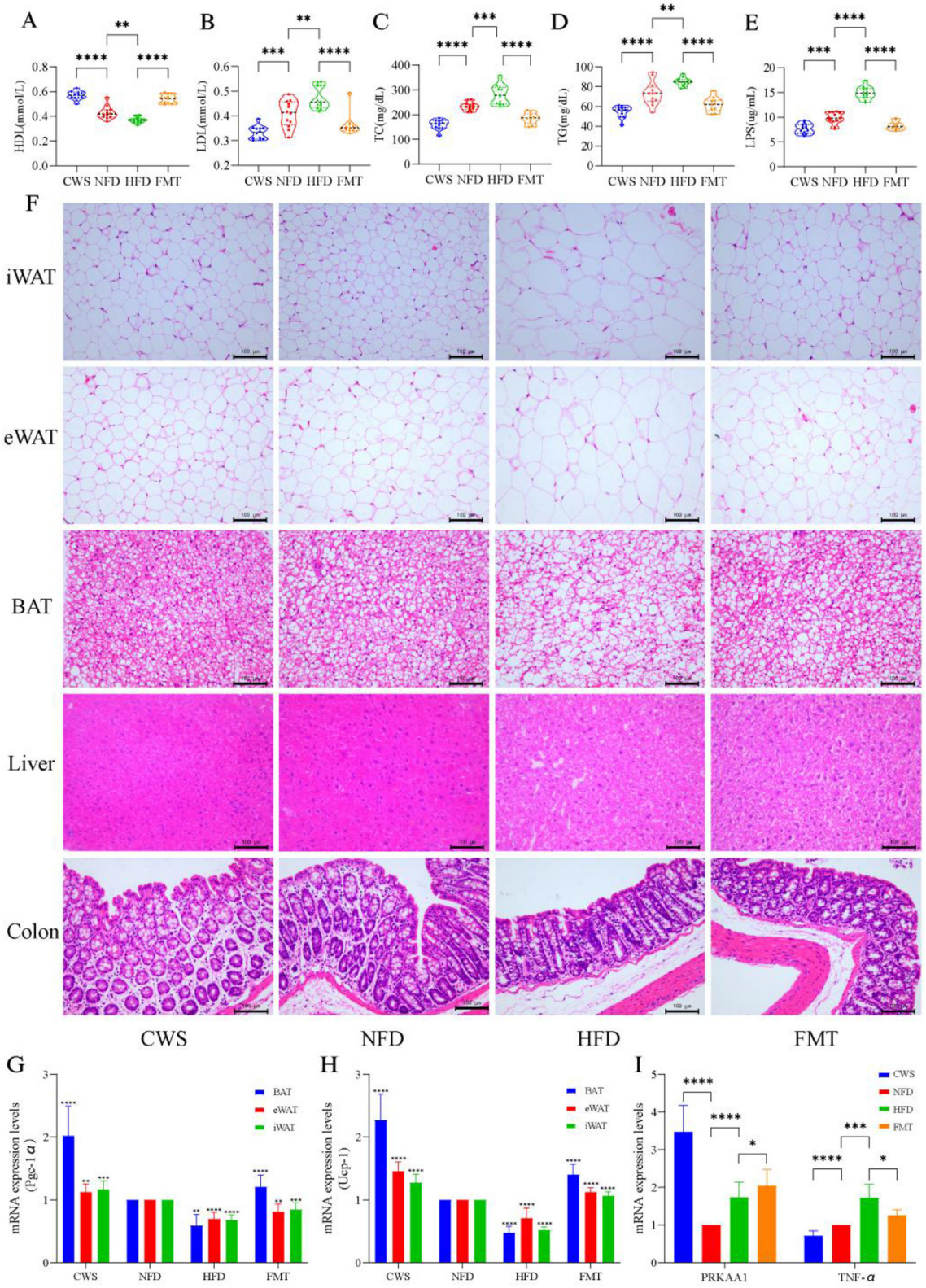


FIGURE 2
CWS improves HFD induced metabolic disturbances, browning processes, gut barrier function, and gene expression. **(A–E)** Impact on serum biochemical indicators: **(A)** HDL levels; **(B)** LDL levels; **(C)** TC levels; **(D)** TG levels; **(E)** LPS levels; **(F)** Representative images of fat and liver from H&E staining; **(G)** PGC-1α expression levels; **(H)** UCP-1 expression levels; **(I)** PRKAA1 and TNF-α mRNA abundance was determined by qRT-PCR analysis expression levels. Data are presented as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$.

absorptive capacity through these structural adaptations, and FMT effectively replicates these beneficial changes.

Low-temperature exposure activated UCP-1-mediated thermogenesis in BAT to counteract obesity. This study found that CWS significantly increased the expression of PGC-1α in various types of adipose tissue, with the highest increase observed in BAT ($P < 0.001$, Figure 2G). Similarly, the expression of UCP-1, which is regulated by PGC-1α, followed a similar trend (Figure 2H). This result aligned with fat browning induced by CWS and was linked to enhanced mitochondrial biogenesis and improved metabolism due to low-temperature exposure (Caesar et al., 2015). Another critical pathway involved in metabolism and inflammation was AMP-activated protein kinase (AMPK). The deletion of its PRKAA1 subunit can induce obesity and hepatic steatosis (Yang et al., 2022). Our findings revealed a significant increase in PRKAA1 expression in the CWS group compared with the NFD group ($P < 0.001$, Figure 2I), with similar changes observed in the FMT group. This indicates that CWS improves metabolism by inducing PRKAA1 expression, consistent with previous studies (Mulligan et al., 2007). Additionally, compared with the NFD group, the CWS group showed significantly inhibited TNF-α expression in the liver ($P < 0.001$, Figure 2I), whereas the HFD group exhibited a significant upregulation ($P < 0.01$, Figure 2I). After FMT, TNF-α levels were significantly reduced. In summary, long-term CWS ameliorated HFD-induced obesity by enhancing metabolism, promoting fat browning, increasing intestinal absorption, and modulating gene expression. These benefits were transferable through FMT.

CWS alters gut microbiota diversity and community structure

Obesity is negatively correlated with GM diversity, and a decrease in GM diversity is consistent with previous studies observed in HFD mice (Wastyk et al., 2021). However, it remains unclear whether CWS and FMT can affect the GM diversity induced by HFD obesity. Fecal samples from the four mice groups were collected, and 16S rRNA gene sequencing was used to obtain 2,323,439 optimized sequences with 975,132,110 bases and an average sequence length of 420 bp. Alpha diversity results showed significant differences between groups; compared to the NFD group, the HFD group showed significant reductions in Chao, Simpson, and Shannon indices, while the CWS group showed significant increases ($P < 0.05$, Figures 3A–C). In comparison to the HFD group, the FMT group also showed significant increases. Venn diagrams (Figures 3D, E) and Chao index rarefaction curves (Figure 3F) further confirmed that HFD significantly reduced GM diversity, while CWS significantly increased GM diversity, and FMT could replicate the result of increasing GM diversity in the CWS group. Based on the Bray-Curtis distance, PCoA analysis visualized the differences in GM structure among the four groups (Figure 3G). PC1 and PC2 explained 16.03% and 9.22% of the variation, respectively, and the PCoA plot showed different clustering patterns: the HFD group clustered in the top left quadrant, the NFD group in the bottom left quadrant, while the CWS and FMT groups were clustered along the right axis. This indicates significant differences in the GM community structure among the HFD, NFD, CWS, and FMT groups, and the similarity of the GM community structure between the CWS and FMT groups further confirms the feasibility and efficacy of FMT. These results suggest that HFD significantly altered the GM community structure, while long-term CWS or FMT effectively prevents drastic changes in the GM community structure.

To further investigate the specific changes in GM, the relative abundance of GM at the phylum level was analyzed among different groups. Firmicutes and Bacteroidota were the dominant phyla, accounting for about 80% of the total sequences (Figures 3H, I). Compared to the NFD group, there was a significant 7.13% increase in Firmicutes abundance in the HFD group ($P < 0.05$, Figures 3H, I, K), consistent with previous studies (Zhang et al., 2020). However, CWS did not reverse this change. Compared to the NFD group, long-term CWS significantly reduced Bacteroidota abundance by 19.98%, while the FMT group showed a reduction of 8.11%. Similar trends were observed when comparing CWS and FMT to the HFD group (reductions of 19.02% and 7.15%, respectively). The consistent trend between the CWS and FMT groups suggests a relationship between long-term CWS and FMT. The ratio of Bacteroidota to Firmicutes (B/F ratio) was correlated with obesity; compared to the NFD group, the HFD group had a significantly lower B/F ratio ($P < 0.05$, Figure 3J). However, the trend in the CWS and FMT groups was inconsistent with the hypothesis, suggesting that the discrepancy might have been attributed to CWS and requires further verification.

CWS alters GM composition

The obese phenotype induced by HFD is closely related to GM. In HFD mice, we observed increased lipid accumulation, metabolic endotoxemia (elevated blood lipid and inflammation), disrupted gut barrier integrity, and altered GM diversity and gene expression. These findings indicated that the composition of GM induced by HFD may have changed. To assess the effects of CWS, FMT, and HFD, we analyzed microbial composition at the phylum and genus levels. The LEfSe results indicated significant differences among bacterial taxonomic groups induced by CWS, HFD, and FMT, including phyla such as Firmicutes, Bacteroidota, Proteobacteria, Actinobacteria, Patescibacteria, and Verrucomicrobiota (Figure 4A). The LDA bar graph at the phylum-genus level showed 72 dominant genera, where CWS accounted for 38.89% (28 genera), HFD for 34.72% (25 genera), FMT for 18.05% (13 genera), and NFD for 8.33% (6 genera) (Figure 4B).

Further analysis at the genus level revealed significant differences among the dominant genera between groups (Figure 4C). Using the testing method for intergroup differences on the top 10 genera by relative abundance, we identified significant differences in genera between HFD and NFD groups, HFD and FMT groups, and NFD and CWS groups. Based on the community abundance data, a pairwise test method was used to analyze the top 10 genera with significant differences ($P < 0.05$). Harmful genera such as *Ileibacterium* and *Coriobacteriaceae_UCG-002* are significantly increased in the HFD group compared to the NFD group (Figure 4D). In the CWS group, beneficial genera such as *Lachnospiraceae_NK4A136_group*, *Prevotellaceae_NK3B31_group*,

10.3389/fmicb.2025.1589902

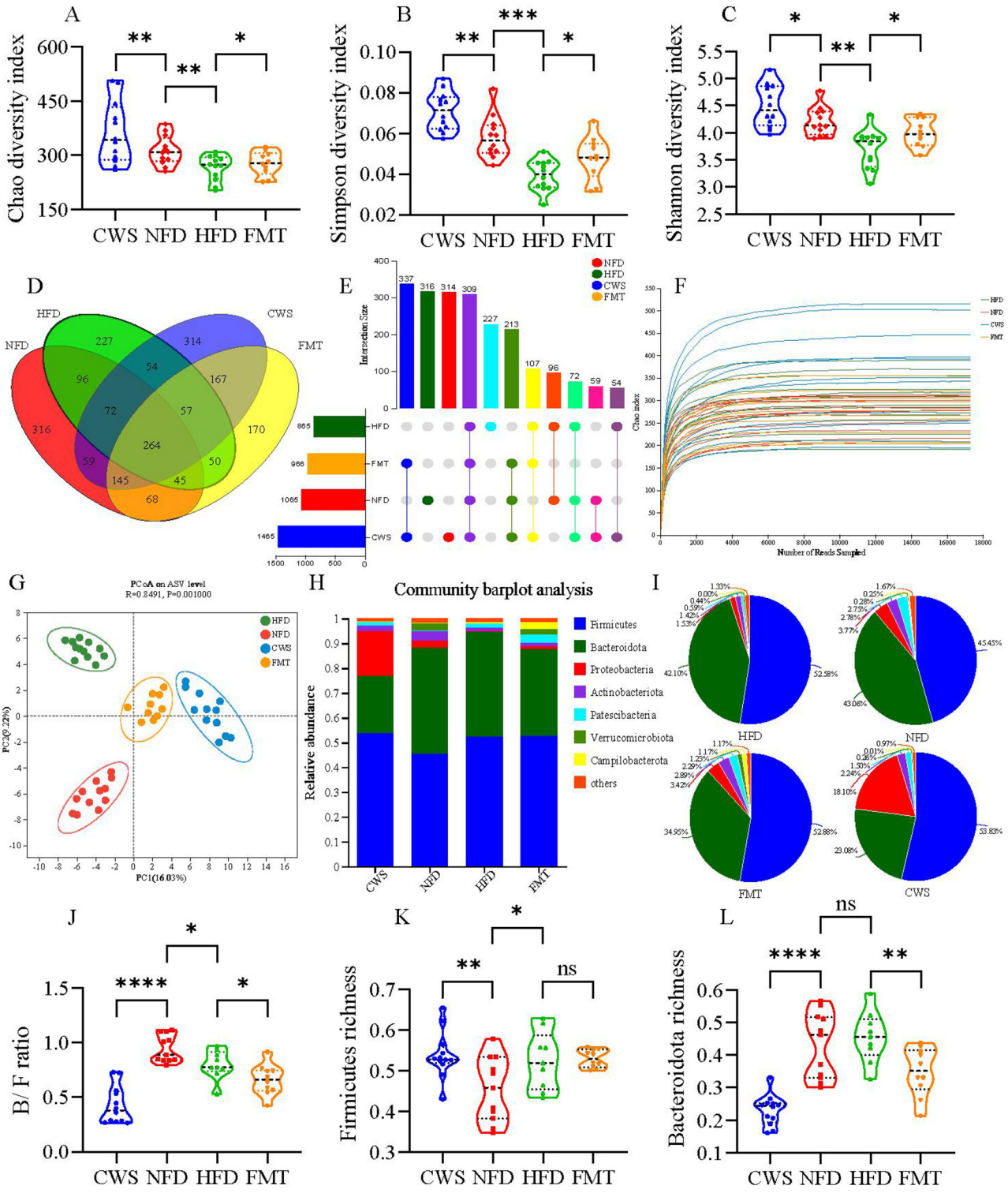


FIGURE 3
CWS alters GM diversity and community structure. **(A)** Chao index in α-diversity analysis; **(B)** Simpson index in α-diversity analysis; **(C)** Shannon index in α-diversity analysis; **(D)** Venn diagram of ASV levels; **(E)** UpSet plot of ASV levels; **(F)** Species rarefaction curve at Chao index level; **(G)** PCoA analysis of GM based on Bray_Curtis distance in feces. Principal components (PCs) 1 and 2 explained 16.03 and 9.22% of the variance, respectively; **(H)** Taxonomic composition of GM at the phylum level; **(I)** Pie chart of GM community at the phylum level; **(J)** B/F ratio; **(K, L)** Relative abundance of GM at the phylum level; **(K)** Firmicutes; **(L)** Bacteroidetes. Data are presented as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$.

and *Sporosarcina* were significantly increased compared to the NFD group (Figure 4F). The changes in the relative abundance of these inflammation-related genera were consistent with changes in serum LPS levels. It is worth noting that in obese mice induced by a HFD, the abundance of beneficial bacteria such as *g_norank_f_norank_o_Clostridia_UCG-014*, *Lactobacillus*,

10.3389/fmicb.2025.1589902

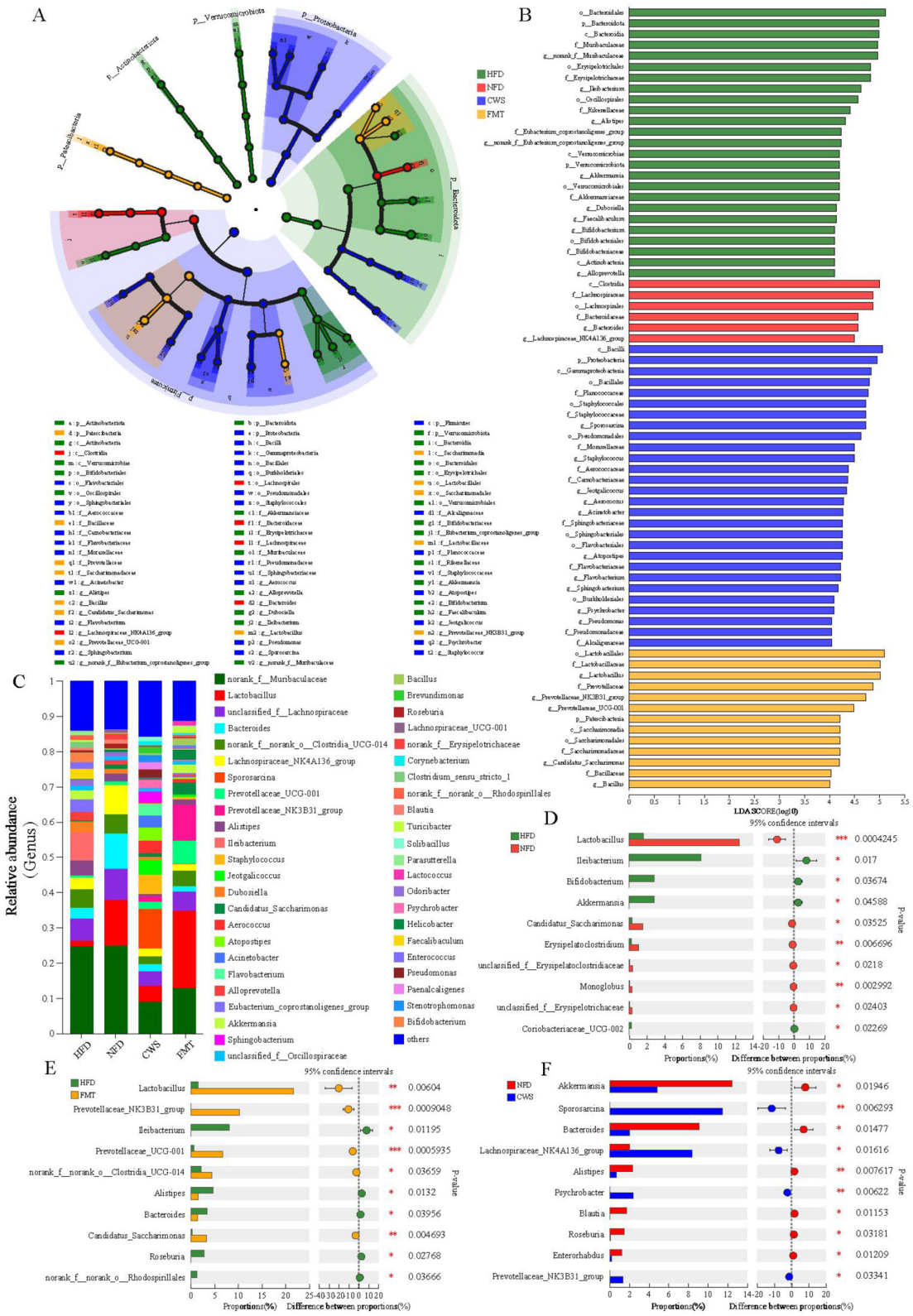


FIGURE 4
CWS alters the composition of GM. **(A)** LEfSe analysis of intergroup GM at the phylum-genus level Cladogram; **(B)** LDA at the phylum-genus level. (LDA >3 was considered to be the differential characteristic taxon); **(C)** Composition of GM at the genus level; **(D–F)** Dominant genera with significant differences between groups; **(D)** Comparison of HFD and NFD groups; **(E)** Comparison of HFD and FMT groups; **(F)** Comparison of CWS and NFD groups. Data are presented as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

10.3389/fmicb.2025.1589902

Prevotellaceae_NK3B31_group, *Prevotellaceae_UCG-001*, and *Candidatus_Saccharimonas* significantly increased after FMT (Figure 4E). In summary, these results indicate that the differential changes in dominant microbial communities are consistent with the hypothesis of this study, that long-term regular CWS can reshape the GM composition in HFD-induced obese mice, and that this reshaping can be effectively transferred through FMT.

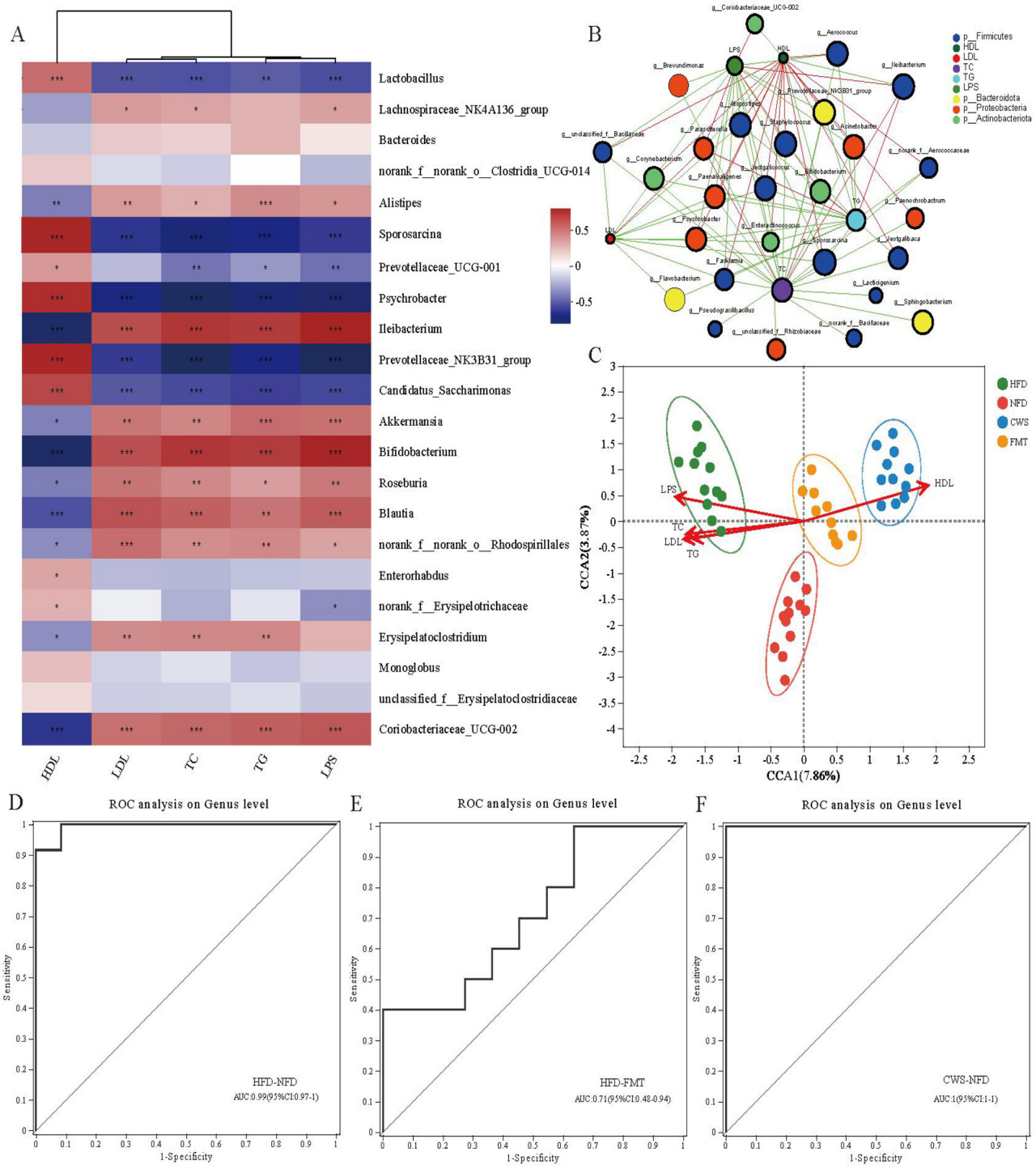


FIGURE 5
The changes in GM induced by CWS and HFD are correlated with metabolic indicators. **(A)** Correlation analysis of the top 22 dominant genera with metabolic indi-cators (red indicates positive correlation, blue indicates negative correlation); **(B)** Bipartite network plot of genera and metabolic indicators; **(C)** Ordination plot of species by RDA/CCA. CCA1 and 2 explained 7.86 and 3.87% of the variance, respectively.; **(D)** HFD and NFD related. Area Under Curve (AUC:0.99); **(E)** HFD and FMT related. (AUC:0.71); **(F)** CWS and NFD related. (AUC:1). ROC; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

CWS and HFD-induced GM changes correlate with metabolic indicators

At the genus level, a total of 22 bacterial genera with the highest abundance were selected, and a correlation matrix was generated using the Spearman correlation coefficient (Figure 5A). The potential relationship between GM changes and metabolic indicators caused by cryogenic swimming and HFD was clarified through correlation analysis. The results showed that most key bacterial abundances were highly correlated with metabolic indicators. Beneficial bacteria affected by CWS maintain intestinal integrity, increase Short-chain fatty acids (SCFAs) and improve blood lipids and inflammation, such as *Prevotellaceae_UCG-001*, *Prevotellaceae_NK3B31_group*, *Candidatus_Saccharimonas*, *Sporosarcina*, *Lactobacillus* and *Psychrobacter*, whose abundance is positively correlated with HDL, while LDL, TC, TG and LPS are negatively correlated. On the contrary, the abundance of harmful bacteria affected by HFD, *Alistipes*, *Ileibacterium*, and *Erysipelatoclostridium*, was significantly negatively correlated with HDL while positively correlated with LDL, TC, TG, and LPS. The results of the two-factor correlation network analysis between GM and metabolic indicators were consistent with the correlation analysis (Figure 5B). Meanwhile, TC and TG showed more bacterial genus correlations, followed by LPS, HDL, and LDL.

To further analyze the relationship between metabolic indicators and GM across different groups, Redundancy analysis/Canonical correspondence analysis (RDA/CCA) analysis was conducted (Figure 5C). The results showed that the sample distributions of each group were concentrated and significantly different. The microbiota functions of the CWS and FMT groups were similar, which was consistent with the PCoA results. The GM in the HFD group is highly correlated with TC, TG, LDL, and LPS, directly affecting the composition and function of GM in the HFD group. In contrast, the GM in the CWS and FMT groups is highly correlated with HDL, similarly affecting the composition and function of GM in the CWS and FMT groups. This difference is related to low temperature and HFD and is also consistent with the research hypothesis that CWS reshapes HFD-induced GM. In order to reveal the interrelationship between sensitivity and specificity among different groups, as well as the biomarkers at the genus level between different groups, a receiver operating characteristic (ROC) curve was constructed. The ROC curve analysis showed AUCs of 99%, 71%, and 100% respectively (Figures 5D–F). These results indicate that the changes in GM caused by CWS and HFD are highly correlated with metabolic indicators. The changes in GM in the FMT group are similar to those in the CWS group, providing further evidence to support the improvement of obesity by FMT.

Discussion

Research indicates that weight loss in obese mice is associated with CWS-remodeled GM enhancing energy expenditure, but how CWS remodels GM in HFD-induced obese mice to ameliorate obesity, and whether this effect can be effectively transmitted through FMT remain unclear. This study systematically reveals for the first time that CWS alleviates HFD-induced obesity through multiple mechanisms, with evidence including the following aspects. First, a novel pattern of CWS remodeling GM to coordinate energy homeostasis. CWS activates the AMPK/SIRT1/PGC-1α signaling pathway to induce adipose browning, significantly improving energy metabolism in HFD mice, which synergizes with enhanced GM diversity and specific enrichment of anti-inflammatory and metabolism-regulating bacterial genera. Second, this study provides the first evidence that CWS induces increased intestinal barrier integrity and absorptive area in mice. CWS reduces LPS levels while significantly enriching intestinal barrier-repairing bacterial genera, markedly enhancing gut barrier function. Third, CWS-remodeled GM can be effectively transferred through FMT. Recipients post-FMT show high similarity in GM structure with donors, with a concurrent recapitulation of obesity phenotype improvement and intestinal barrier restoration.

Increasing evidence suggests that low-temperature exposure alleviates HFD-induced obesity and is associated with the reshaping of the GM (Worthmann et al., 2017; Ziętak et al., 2016). The results of our study showed that CWS, while increasing energy expenditure and reducing lipid accumulation, also induced significant changes in GM. GM diversity reflects the co-evolution between microbial communities and the host, which is consistent with the decrease in GM diversity caused by HFD and the increase due to CWS. Our study further revealed that CWS increases the proportions of the phyla Firmicutes and Bacteroidota. Overall, these results suggest that adaptive mechanisms in the host play a role in improving metabolism and controlling body weight. Both low-temperature exposure (Chevalier et al., 2015) and exercise (Cani et al., 2019) can modulate GM to facilitate fat browning (Pereira et al., 2022).

Mechanistically, CWS induces adipose browning and increases energy expenditure by activating UCP-1 and PGC-1α expression in BAT, ultimately reducing lipid accumulation (Liu et al., 2019). Through dual stimulation (cold exposure and exercise), CWS activated AMPK through dual stimulation (cold exposure + exercise) and catalyzed upregulation of subunit PRKAA1 expression (Figure 2I), which further promotes SIRT1 activity and leads to significant upregulation of PGC-1α, the core regulator of mitochondrial biosynthesis. This drove eWAT browning and BAT activation, manifested as a marked increase in iWAT and BAT mass (Figures 1E, G). Additionally, PGC-1α promoted mitochondrial thermogenesis and energy expenditure by elevating UCP-1 transcriptional levels, thereby reducing lipid accumulation (Figure 1F). These findings demonstrate that CWS likely promotes adipose browning via the AMPK/SIRT1/PGC-1α pathway, attributable to both exercise and cold exposure. This mechanism shares commonalities with metabolic improvements observed in cold exposure studies (Chevalier et al., 2015), but this study is the first to demonstrate that cold-water swimming synergizes with exercise adaptation through the AMPK/SIRT1/PGC-1α axis, achieving more pronounced adipose browning and metabolic benefits. Notably, FMT successfully transfers these adaptive changes. The data further support FMT as an effective strategy for ameliorating obesity.

HFD-induced GM imbalance led to a pro-inflammatory environment that accelerates metabolic disorders (Cai et al., 2024), while also disrupting intestinal barrier function and

increasing intestinal permeability (Tulkens et al., 2020). These changes accelerate the release of LPS into the blood, inducing systemic chronic inflammation. A growing body of evidence suggests that impaired intestinal barrier function was associated with obesity (Beisner et al., 2021; Zhang et al., 2020). This study found that in HFD mice, CWS significantly reduced the levels of some bacteria associated with LPS and obesity, including *Ileibacterium*, *Coriobacteriaceae_UCG-002*, *Akkermansia,* and *Bifidobacterium*. On the contrary, the levels of beneficial bacteria that resist LPS and obesity were significantly increased, including *Lachnospiraceae_NK4A136_group*, *Prevotellaceae_NK3B31_group*, *Sporosarcina*, etc. This ultimately led to a decrease in LPS levels in the CWS group and FMT group. Correlation analysis showed that HFD-induced bacterial spectrum was positively correlated with LPS, while CWS could significantly inhibit the increase in LPS. An increase in the abundance of *Alistipes*, *Ileibacterium,* and *Erysipelatoclostridium* was observed in the HFD group, but a decrease in relative abundance in the CWS and FMT groups. LPS stimulated macrophages to produce TNF-α, which could further activate macrophages, forming a vicious two-way cycle, thereby exacerbating the inflammatory response (He et al., 2012). In this study, it was found that elevated levels of LPS are associated with a significant upregulation of TNF-α mRNA expression levels and were related to HFD induction, while CWS significantly improved this vicious cycle. The changes in intestinal barrier and permeability are the main reasons for the increased levels of LPS in the blood. Evidence of intestinal damage was observed in the HFD group, including a significant reduction in intestinal wall thickness, villus height, and the number of goblet cells, as well as a marked decrease in intestinal absorptive area. Conversely, the CWS group showed the opposite results. Interestingly, when the GM after CWS intervention was transplanted into obese mice, similar changes to the CWS group were observed. These changes helped the host adapt to low-temperature exposure and assisted in gut injury repair, consistent with previous research (Chevalier et al., 2015). Reports have indicated that low-temperature exposure increases LPS and TNF-a levels (Luo et al., 2019), and decreases GM diversity and beneficial bacterial abundance, which is inconsistent with this study. These studies mainly focus on single or short-term low-temperature exposure, whereas this study is based on long-term low-temperature exposure and includes swimming adaptation. Moreover, the subjects of this study are obese mice induced by HFD. The results of short-term low-temperature exposure suggest that changes in the GM are related to temperature and not related to the GM changes caused by obesity (Ziętak et al., 2016). This difference can also be further explained by the coordination of energy homeostasis in response to low-temperature exposure, which includes fat browning, energy intake, and environmental adaptation.

GM can be effectively transferred through FMT to treat diseases induced by GM imbalance due to HFD, including metabolic syndrome and obesity. An effective FMT setting is the key and prerequisite for remodeling host GM. On the one hand, it is influenced by the donor. Studies have shown that the success rate of receptor setting is related to the donor GM, and the difference ranges from 0 to 90% (Wang et al., 2024). This variability is primarily affected by the composition of the donor GM, including GM diversity and beneficial bacterial abundance (Porcari et al., 2023). A non-random cohort study found that an increase in the donor's GM abundance was correlated with the clinical success of FMT (Kump et al., 2018). Another systematic review of 25 studies evaluating FMT indicated that donor GM diversity is a predictive factor for treatment outcomes (Rees et al., 2022). On the other hand, the recipient's GM tolerance is another crucial factor for the success of FMT, with lower initial GM diversity in subjects associated with improved outcomes (Kootte et al., 2017). Population cohort studies further demonstrated the bidirectional relationship between donors and recipients (Shtossel et al., 2023), emphasizing that donor selection should be a priority for FMT. This study selected GM after low-temperature exposure and exercise as the donor, which had a positive impact on the recipient. To further validate the hypothesis of this study that gut barrier and GM reshaping are effective strategies for improving HFD-induced obesity, FMT was performed. The experimental results of this study support this hypothesis with evidence, including the suppression of dyslipidemia and LPS. Since dyslipidemia and LPS have a mutually vicious cycle relationship and form the pathological basis of obesity, their improvement is crucial for improving obesity. In the FMT group, improvements in dyslipidemia and inflammation were observed, which were consistent with the CWS group, indicating that GM from CWS successfully colonized in obese mice through FMT. Second, the reduction in LPS-induced gut barrier damage. The involvement of the gut barrier in various gastrointestinal diseases is significant (Di Tommaso et al., 2021), coupled with adverse events related to FMT, occurring only in subjects with gut barrier dysfunction (Marcella et al., 2021). Although LPS leakage and gut barrier damage were observed in the HFD-induced obese mice, no gut damage was found after FMT. Instead, evidence of gut barrier repair was found, including decreased LPS levels, increased goblet cell numbers, and increased villus height in the FMT group. Third, significant changes in GM composition were observed in the FMT group, particularly an increase in GM diversity and beneficial bacterial abundance. A growing body of evidence suggests that exercise improves colonic antioxidant (Almasi et al., 2023) and anti-inflammatory (Cook et al., 2016) status, thereby enhancing the function of the intestinal epithelium, repairing intestinal barrier damage, and reshaping GM to improve obesity (Shao et al., 2022). Convincing evidence from FMT indicates that reshaping of the intestinal barrier and GM are alternative targets for CWS to improve HFD-induced obesity.

GM dysbiosis is an independent risk factor for obesity. Although we confirmed that obesity alleviation is linked to CWS-remodeled GM and FMT, our study has certain limitations. First, experiments were conducted only in a single mouse strain, and differences in responses across genetic backgrounds were not evaluated. Second, the functional roles of specific microbiota (e.g., the *Lachnospiraceae NK4A136* and *Prevotellaceae NK3B31* groups) remain unvalidated due to the lack of monobacterial transplantation or metabolomic analyses. Third, molecular mechanisms rely solely on correlational data, and direct causal relationships require further verification using pathway inhibitors or gene knockout approaches. Fourth, the adaptability and safety of long-term FMT therapy require further assessment. Future research should fully leverage multi-omics approaches

(e.g., metagenomics, metabolomics) to realize their pivotal role in diagnostic applications (Marascio et al., 2023). Additionally, experiments in models with diverse genetic backgrounds are required to clarify the functions of specific microbiota and molecular mechanisms, as well as to evaluate the safety of FMT. Furthermore, whether lifestyle interventions (e.g., the Mediterranean diet) and CWS exhibit synergistic/additive effects warrant further exploration, which would provide critical insights for clinical translation.

In summary, this study found that HFD-induced obese mice had specific GM features, including a reduction in GM diversity and an increased abundance of bacteria associated with pro-inflammatory and metabolic disorders. Conversely, CWS provided key evidence for improving HFD-induced obesity, and these positive effects were effectively transferred through FMT. This study shows that CWS improves lipid metabolism, and inflammatory responses by reshaping GM, thereby improving HFD-induced obesity, and that FMT may become an attractive treatment strategy for improving obesity.

Data availability statement

All data generated or analyzed during this study are included in this published article and the original data was uploaded to the NCBI database under the sequence number PRJNA1203435. All other raw data generated in this study are available upon request from the corresponding author.

Ethics statement

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

Author contributions

JM: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. CC: Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization. HL: Investigation, Resources, Software, Writing – review & editing. ZLi: Investigation, Resources, Validation, Writing – review & editing. YZ: Formal analysis, Investigation, Resources, Validation, Writing – review & editing. ZLiu: Validation, Writing – review & editing. QW: Project administration, Validation, Writing – review & editing. PL: Project administration, Validation, Writing – review & editing. SZ: Validation, Writing – review & editing. ZY: Validation, Writing – review & editing. YZ: Validation, Writing – review & editing. SW: Validation, Writing – review & editing. GZ: Validation, Writing – review & editing. PW: Validation, Writing – review & editing. XH: Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by the National Natural Science Foundation of China (82201691); the Shanxi Provincial Key Laboratory Cultivation Base for Metabolic and Cardiovascular Disease Research, jointly established by the Ministry of Science and Technology (TSGJ001); the Science and Technology Innovation Program of the Education Department of Shanxi Province (2023L472); the Shanxi Province University Students Innovation and Entrepreneurship Training Program (S20241011401032); the Key R&D Project in the Field of Social Development of Lvliang City Science and Technology Plan (2023SHFZ48); and the Talent Introduction Start-up Fund of Fenyang College, Shanxi Medical University (2022A01).

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 author(s) declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Aldiss, P., Lewis, J. E., Lupini, I., Bloor, I., Chavoshinejad, R., Boocock, D. J., et al. (2022). Cold exposure drives weight gain and adiposity following chronic suppression of brown adipose tissue. *Int. J. Mol. Sci.* 23:1869. doi: 10.3390/ijms23031869

Almasi, N., Torok, S., Al-Awar, A., Veszelka, M., Kiraly, L., Borzsei, D., et al. (2023). Voluntary exercise-mediated protection in TNBS-induced rat colitis: the involvement of NETosis and prdx antioxidants. *Antioxidants* 12:1531. doi: 10.3390/antiox12081531

Aron-Wisnewsky, J., Warmbrunn, M. V., Nieuwdorp, M., and Clément, K. (2021). Metabolism and metabolic disorders and the microbiome: the intestinal microbiota associated with obesity, lipid metabolism, and metabolic health—pathophysiology and therapeutic strategies. *Gastroenterology* 160, 573–599. doi: 10.1053/j.gastro.2020.10.057

Barcena, C., Valdes-Mas, R., Mayoral, P., Garabaya, C., Durand, S., Rodriguez, F., et al. (2019). Healthspan and lifespan extension by fecal microbiota transplantation

into progeroid mice. *Nat. Med.* 25, 1234–1242. doi: 10.1038/s41591-019-0504-5

Becher, T., Palanisamy, S., Kramer, D. J., Eljalby, M., Marx, S. J., Wibmer, A. G., et al. (2021). Brown adipose tissue is associated with cardiometabolic health. *Nat. Med.* 27, 58–65. doi: 10.1038/s41591-020-1126-7

Beisner, J., Filipe, R. L., Kaden-Volynets, V., Stolzer, I., Gunther, C., and Bischoff, S. C. (2021). Prebiotic inulin and sodium butyrate attenuate obesity-induced intestinal barrier dysfunction by induction of antimicrobial peptides. *Front. Immunol.* 12:678360. doi: 10.3389/fimmu.2021.678360

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37:1091. doi: 10.1038/s41587-019-0209-9

Caesar, R., Tremaroli, V., Kovatcheva-Datchary, P., Cani, P. D., and Bäckhed, F. (2015). Crosstalk between gut microbiota and dietary lipids aggravates WAT inflammation through TLR signaling. *Cell Metab.* 22, 658–668. doi: 10.1016/j.cmet.2015.07.026

Cai, J., Li, C., Liu, S., Tan, M., Sun, Y., Sun, X., et al. (2024). Angiogenin-mediated tsRNAs control inflammation and metabolic disorder by regulating NLRP3 inflammasome. *Cell Death Differ.* 31, 1057–1069. doi: 10.1038/s41418-024-01311-8

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cani, P. D., and Van Hul, M. (2024). Gut microbiota in overweight and obesity: crosstalk with adipose tissue. *Nat. Rev. Gastroenterol. Hepatol.* 21, 164–183. doi: 10.1038/s41575-023-00867-z

Cani, P. D., Van Hul, M., Lefort, C., Depommier, C., Rastelli, M., and Everard, A. (2019). Microbial regulation of organismal energy homeostasis. *Nat Metab* 1, 34–46. doi: 10.1038/s42255-018-0017-4

Chambers, E. S., Byrne, C. S., Morrison, D. J., Murphy, K. G., Preston, T., Tedford, C., et al. (2019). Dietary supplementation with inulin-propionate ester or inulin improves insulin sensitivity in adults with overweight and obesity with distinct effects on the gut microbiota, plasma metabolome and systemic inflammatory responses: a randomised cross-over trial. *Gut* 68, 1430–1438. doi: 10.1136/gutjnl-2019-318424

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

Chevalier, C., Stojanović, O., Colin, D. J., Suarez-Zamorano, N., Tarallo, V., and Veyrat-Durebex, C., et al. (2015). Gut microbiota orchestrates energy homeostasis during cold. *Cell.* 163, 1360–1374. doi: 10.1016/j.cell.2015.11.004

Cook, M. D., Allen, J. M., Pence, B. D., Wallig, M. A., Gaskins, H. R., White, B. A., et al. (2016). Exercise and gut immune function: evidence of alterations in colon immune cell homeostasis and microbiome characteristics with exercise training. *Immunol. Cell Biol.* 94, 158–163. doi: 10.1038/icb.2015.108

Da Silva, J. T., Cella, P. S., Testa, M. T. D. J., Perandini, L. A., Festuccia, W. T., Deminice, R., et al. (2020). Mild-cold water swimming does not exacerbate white adipose tissue browning and brown adipose tissue activation in mice. *J. Physiol. Biochem.* 76, 663–672. doi: 10.1007/s13105-020-00771-z

Di Tommaso, N., Gasbarrini, A., and Ponziani, F. R. (2021). Intestinal barrier in human health and disease. *Int. J. Environ. Res. Public Health* 18: 12836. doi: 10.3390/ijerph182312836

Dohnalova, L., Lundgren, P., Carty, J., Goldstein, N., Wenski, S.L., Nanudorn, P., et al. (2022). A microbiome-dependent gut-brain pathway regulates motivation for exercise. *Nature* 612, 739–747. doi: 10.1038/s41586-022-05525-z

Dominguez, L. J., Veronese, N., Di Bella, G., Cusumano, C., Parisi, A., and Tagliaferri, F., et al. (2023). Mediterranean diet in the management and prevention of obesity. *Exp. Gerontol.* 174:112121. doi: 10.1016/j.exger.2023.112121

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., et al. (2020). PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* 38, 685–688. doi: 10.1038/s41587-020-0548-6

Gudzune, K. A., and Kushner, R. F. (2024). Medications for obesity: a review. *JAMA* 332, 571–584. doi: 10.1001/jama.2024.10816

He, X., Shu, J., Xu, L., Lu, C., and Lu, A. (2012). Inhibitory effect of astragalus polysaccharides on lipopolysaccharide-induced TNF-a and IL-1beta production in THP-1 cells. *Molecules* 17, 3155–3164. doi: 10.3390/molecules17033155

Huang, T., Xu, T., Wang, Y., Zhou, Y., Yu, D., Wang, Z., et al. (2021). Cannabidiol inhibits human glioma by induction of lethal mitophagy through activating TRPV4. *Autophagy* 17, 3592–3606. doi: 10.1080/15548627.2021.1885203

Kootte, R. S., Levin, E., Salojarvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., et al. (2017). Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. *Cell Metab.* 26, 611–619. doi: 10.1016/j.cmet.2017.09.008

Kump, P., Wurm, P., Gröchenig, H. P., Wenzl, H., Petritsch, W., Halwachs, B., et al. (2018). The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of faecal microbiota transplantation in therapy refractory ulcerative colitis. *Aliment. Pharmacol. Ther.* 47, 67–77. doi: 10.1111/apt.14387

Leong, K. S. W., Jayasinghe, T. N., Wilson, B. C., Derraik, J. G. B., Albert, B. B., Chiavaroli, V., et al. (2020). Effects of fecal microbiome transfer in adolescents with obesity: the gut bugs randomized controlled trial. *JAMA Netw Open* 3:e2030415. doi: 10.1001/jamanetworkopen.2020.30415

Li, B., Li, L., Li, M., Lam, S. M., Wang, G., Wu, Y., et al. (2019). Microbiota depletion impairs thermogenesis of brown adipose tissue and browning of white adipose tissue. *Cell Rep.* 26, 2720–2737. doi: 10.1016/j.celrep.2019.02.015

Liu, J., Wang, Y., and Lin, L. (2019). Small molecules for fat combustion: targeting obesity. *Acta Pharm. Sin. B.* 9, 220–236. doi: 10.1016/j.apsb.2018.09.007

Luo, B., Shi, H., Zhang, K., Wei, Q., Niu, J., Wang, J., et al. (2019). Cold stress provokes lung injury in rats co-exposed to fine particulate matter and lipopolysaccharide. *Ecotoxicol. Environ. Saf.* 168, 9–16. doi: 10.1016/j.ecoenv.2018.10.064

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

Marascio, N., Scarlata, G. G. M., Romeo, F., Cicino, C., Trecarichi, E. M., and Quirino, A., et al. (2023). The role of gut microbiota in the clinical outcome of septic patients: state of the art and future perspectives. *Int. J. Mol. Sci.* 24, 9307. doi: 10.3390/ijms24119307

Marcella, C., Cui, B., Kelly, C. R., Ianiro, G., Cammarota, G., and Zhang, F. (2021). Systematic review: the global incidence of faecal microbiota transplantation-related adverse events from 2000 to 2020. *Aliment. Pharmacol. Ther.* 53, 33–42. doi: 10.1111/apt.16148

Martens, M. D., Abuetabh, Y., Schmidt, M. A., Zolondek, M. C. P., Silver, H. L., Levasseur, J. L., et al. (2024). Semaglutide reduces cardiomyocyte size and cardiac mass in lean and obese mice. *JACC Basic Transl. Sci.* 9, 1429–1431. doi: 10.1016/j.jacbts.2024.07.006

Mulligan, J. D., Gonzalez, A. A., Stewart, A. M., Carey, H. V., and Saupe, K. W. (2007). Upregulation of AMPK during cold exposure occurs via distinct mechanisms in brown and white adipose tissue of the mouse. *J. Physiol.* 580, 677–684. doi: 10.1113/jphysiol.2007.128652

Nomura, K., Kinoshita, S., Mizusaki, N., Senga, Y., Sasaki, T., Kitamura, T., et al. (2024). Adaptive gene expression of alternative splicing variants of PGC-1alpha regulates whole-body energy metabolism. *Mol Metab* 86:101968. doi: 10.1016/j.molmet.2024.101968

Perdomo, C. M., Cohen, R. V., Sumithran, P., Clement, K., and Fruhbeck, G. (2023). Contemporary medical, device, and surgical therapies for obesity in adults. *Lancet* 401, 1116–1130. doi: 10.1016/S0140-6736(22)02403-5

Pereira, R. O., Olvera, A.C., Marti, A., Fang, S., White, J. R., Westphal, M., et al. (2022). OPA1 regulates lipid metabolism and cold-induced browning of white adipose tissue in mice. *Diabetes* 71, 2572–2583. doi: 10.2337/db22-0450

Perler, B. K., Friedman, E. S., and Wu, G. D. (2023). The role of the gut microbiota in the relationship between diet and human health. *Annu. Rev. Physiol.* 85, 449–468. doi: 10.1146/annurev-physiol-031522-092054

Piché, M., Tchernof, A., and Després, J. (2020). Obesity phenotypes, diabetes, and cardiovascular diseases. *Circ. Res.* 126, 1477–1500. doi: 10.1161/CIRCRESAHA.120.316101

Porcari, S., Benech, N., Valles-Colomer, M., Segata, N., Gasbarrini, A., Cammarota, G., et al. (2023). Key determinants of success in fecal microbiota transplantation: from microbiome to clinic. *Cell Host Microbe* 31, 712–733. doi: 10.1016/j.chom.2023.03.020

Rees, N. P., Shaheen, W., Quince, C., Tselepis, C., Horniblow, R. D., Sharma, N., et al. (2022). Systematic review of donor and recipient predictive biomarkers of response to faecal microbiota transplantation in patients with ulcerative colitis. *EBioMedicine* 81:104088. doi: 10.1016/j.ebiom.2022.104088

Roos, E. M., and Arden, N. K. (2016). Strategies for the prevention of knee osteoarthritis. *Nat. Rev. Rheumatol.* 12, 92–101. doi: 10.1038/nrrheum.2015.135

Rubino, D., Abrahamsson, N., Davies, M., Hesse, D., Greenway, F. L., Jensen, C., et al. (2021). Effect of continued weekly subcutaneous semaglutide vs. placebo on weight loss maintenance in adults with overweight or obesity: the STEP 4 randomized clinical trial. *JAMA* 325, 1414–1425. doi: 10.1001/jama.2021.3224

Schloss, P. D. (2020). Reintroducing mothur: 10 years later. *Appl. Environ. Microbiol.* 86, e02343–19. doi: 10.1128/AEM.02343-19

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

Shao, C., Li, Y., Chen, J., Zheng, L., Chen, W., Peng, Q., et al. (2022). Physical exercise repairs obstructive jaundice-induced damage to intestinal mucosal barrier function via h2s-mediated regulation of the HMGB1/toll like receptors 4/nuclear factor kappa b pathway. *Front. Physiol.* 12:732780. doi: 10.3389/fphys.2021.732780

Shtossel, O., Turjeman, S., Riumin, A., Goldberg, M. R., Elizur, A., Bekor, Y., et al. (2023). Recipient-independent, high-accuracy FMT-response prediction and optimization in mice and humans. *Microbiome* 11:181. doi: 10.1186/s40168-023-01623-w

Sodhi, M., Rezaeianzadeh, R., Kezouh, A., and Etminan, M. (2023). Risk of gastrointestinal adverse events associated with glucagon-like peptide-1 receptor agonists for weight loss. *JAMA* 330, 1795–1797. doi: 10.1001/jama.2023.19574

Tulkens, J., Vergauwen, G., Van Deun, J., Geeurickx, E., Dhondt, B., Lippens, L., et al. (2020). Increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction. *Gut* 69, 191–193. doi: 10.1136/gutjnl-2018-317726

Wang, P., Gao, J., Ke, W., Wang, J., Li, D., Liu, R., et al. (2020). Resveratrol reduces obesity in high-fat diet-fed mice via modulating the structure and metabolic function of the gut microbiota. *Free Radic. Biol. Med.* 156, 83–98. doi: 10.1016/j.freeradbiomed.2020.04.013

Wang, Z., Wu, Y., Li, X., Ji, X., and Liu, W. (2024). The gut microbiota facilitate their host tolerance to extreme temperatures. *BMC Microbiol.* 24:131. doi: 10.1186/s12866-024-03277-6

Wastyk, H. C., Fragiadakis, G. K., Perelman, D., Dahan, D., Merrill, B. D., Yu, F. B., et al. (2021). Gut-microbiota-targeted diets modulate human immune status. *Cell* 184, 4137–4153. doi: 10.1016/j.cell.2021.06.019

World obesity atlas (2024). *World Obesity Federation. World obesity atlas, 2024. London: World Obesity Federation, 2024.* Available online at: https://data.worldobesity.org/publications/?cat=22 (accessed April 25, 2025).

Worthmann, A., John, C., Rühlemann, M.C., Baguhl, M., Heinsen, F., Schaltenberg, N., et al. (2017). Cold-induced conversion of cholesterol to bile acids in mice shapes the gut microbiome and promotes adaptive thermogenesis. *Nat. Med.* 23, 839–849. doi: 10.1038/nm.4357

Yang, Q., Ma, Q., Xu, J., Liu, Z., Mao, X., Zhou, Y., et al. (2022). Endothelial AMPKα1/PRKAA1 exacerbates inflammation in HFD-fed mice. *Br. J. Pharmacol.* 179, 1661–1678. doi: 10.1111/bph.15742

Zhang, X., Chen, J., Yi, K., Peng, L., Xie, J., Gou, X., et al. (2020). Phlorizin ameliorates obesity-associated endotoxemia and insulin resistance in high-fat diet-fed mice by targeting the gut microbiota and intestinal barrier integrity. *Gut Microbes* 12, 1–18. doi: 10.1080/19490976.2020.1842990

Ziętak, M., Kovatcheva-Datchary, P., Markiewicz, L. H., Ståhlman, M., Kozak, L. P., and Bäckhed, F. (2016). Altered microbiota contributes to reduced diet-induced obesity upon cold exposure. *Cell Metab.* 23, 1216–1223. doi: 10.1016/j.cmet.2016.05.001

10.3389/fmicb.2025.1589902

Glossary

HDF, High-fat diet; GM, Gut microbiota; FMT, Fecal microbiota transplantation; CWS, Cold water swimming; BMI, Body mass index; CVD, Cardiovascular disease; ACC, American College of Cardiology; ESC, European Society of Cardiology; NFD, Normal fat diet; PBS, Phosphate buffer saline; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; TC, Total Cholesterol; TG, Triglyceride; LPS, Lipopolysaccharide; ELISA, Enzyme-linked immunosorbent assay; H&E, Hematoxylin and Eosin; qRT-PCR, Real-Time Fluorescent Quantitative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UCP-1, Uncoupling protein 1; PGC-1α, Peroxisome proliferator-activated receptor γ coactivator 1-alpha; PRKAA1, Protein kinase AMP-activated catalytic subunit alpha 1; TNF-α, Tumor necrosis factor-α; NCBI, National Center for Biotechnology Information; ASVs, Amplicon Sequence Variants; PCoA, Principal co-ordinates analysis; LEfSe, Linear discriminant analysis Effect Size; db-RDA, Distance-based redundancy analysis; eWAT, Epididymal white adipose tissue; iWAT, Inguinal white adipose tissue; BAT, Brown adipose tissue; AMPK, AMP-activated protein kinase; LDA, Linear discriminant analysis; B/F ratio, The ratio of Bacteroidota to Firmicutes; SCFAs, Short-chain fatty acids; RDA/CCA, Redundancy analysis/Canonical correspondence analysis; ROC, Receiver operating characteristic.

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 09 May 2025
DOI 10.3389/fmicb.2025.1589704

Check for updates

OPEN ACCESS

EDITED BY
Ludovico Abenavoli,
Magna Græcia University, Italy

REVIEWED BY
Sindhuja Koneru,
Henry Ford Health System, United States
Sara Ellegaard Paaske,
Aarhus University Hospital, Denmark

*CORRESPONDENCE
Sepideh Pakpour
✉ sepideh.pakpour@ubc.ca

RECEIVED 10 March 2025
ACCEPTED 22 April 2025
PUBLISHED 09 May 2025

CITATION
Karimianghadim R, Satokari R, Yeo S, Arkkila P, Kao D and Pakpour S (2025) Prolonged effect of antibiotic therapy on the gut microbiota composition, functionality, and antibiotic resistance genes' profiles in healthy stool donors.
Front. Microbiol. 16:1589704.
doi: 10.3389/fmicb.2025.1589704

COPYRIGHT
© 2025 Karimianghadim, Satokari, Yeo, Arkkila, Kao and Pakpour. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Prolonged effect of antibiotic therapy on the gut microbiota composition, functionality, and antibiotic resistance genes' profiles in healthy stool donors

Ramin Karimianghadim[1], Reetta Satokari[2], Sam Yeo[1], Perttu Arkkila[3], Dina Kao[4] and Sepideh Pakpour[1]*

[1]School of Engineering, University of British Columbia, Kelowna, BC, Canada, [2]Human Microbiome Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland, [3]Department of Gastroenterology, Helsinki University Hospital and Helsinki University, Helsinki, Finland, [4]Division of Gastroenterology, Department of Medicine, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada

Introduction: Fecal microbiota transplantation (FMT) is highly effective in preventing *Clostridioides difficile* recurrence by restoring gut microbiota composition and function. However, the impact of recent antibiotic use, a key exclusion criterion for stool donors, on gut microbiota recovery is poorly understood.

Methods: We investigated microbial recovery dynamics following antibiotic use in three long-term stool donors from Canada and Finland. Using longitudinal stool sampling, metagenomic sequencing, and qPCR, we assessed changes in bacterial diversity, community composition, microbial functions, the gut phageome, and the risk of transmitting antibiotic-resistant genes (ARGs).

Results: Antibiotics caused lasting disruption to bacterial communities, significantly reducing important taxa like *Bifidobacterium bifidum*, *Blautia wexlerae*, *Akkermansia muciniphila*, *Eubacterium* sp. *CAG 180*, and *Eubacterium hallii*, with effects persisting for months. Functional analyses revealed alterations in housekeeping genes critical for energy production and biosynthesis, with no direct links to key health-related pathways. Antibiotics also disrupted viral populations, decreasing diversity and increasing *crAssphage* abundance, reflecting disrupted host-bacteriophage dynamics. No significant increase in clinically important ARGs was detected.

Discussion: These findings highlight the unpredictable and complex recovery of gut microbiota post-antibiotics. Individualized suspension periods in donor programs, guided by metagenomic analyses, are recommended to optimize FMT outcomes in various indications by considering antibiotic spectrum, duration, and host-specific factors.

KEYWORDS

antibiotic treatment, fecal microbiota transplant, gut microbiome, antibiotic resistant genes, FMT donors

1 Introduction

Fecal Microbiota Transplantation (FMT) is highly effective in managing recurrent *Clostridioides difficile* infection (rCDI), achieving success rates of 80 to 90%, and is recommended by multiple practice guidelines (Van Nood, 2013; McDonald et al., 2018; Ng et al., 2020; Cammarota et al., 2019; Haifer et al., 2020). FMT has also shown promise in

several other dysbiosis-associated conditions, including inflammatory bowel disease (IBD) (Paramsothy et al., 2017; Fischer et al., 2016; Moayyedi et al., 2015), metabolic syndrome (Mocanu et al., 2021), irritable bowel syndrome (Holvoet et al., 2021), multidrug-resistant bacteria de-colonization (Battipaglia et al., 2019; Bilinski et al., 2017), and immune checkpoint inhibitor-associated colitis (Wang et al., 2018). Presently, there are approximately 572 registered clinical trials worldwide (WHO, 2023), exploring FMT's therapeutic potential across these diverse conditions (ClinicalTrials.gov, 2023).

Although the mechanisms of action underpinning the therapeutic efficacy of FMT remains incompletely understood, bacterial engraftment is thought to be crucial (Podlesny et al., 2022). While pathogen-free donor stool is essential, specific microbiota composition may be less critical given high success rates (Van Nood, 2013; Cammarota et al., 2015; Kassam et al., 2013). However, for conditions with complex pathophysiology, the therapeutic efficacy of FMT may be closely tied to the diversity, specific microbial taxa, and functionalities provided by the donor microbiota (Paramsothy et al., 2017).

Antibiotics disrupt the gut microbiota by reducing microbial diversity, eliminating individual taxa, with changes that can persist for months or even years. Moreover, recovery is not solely about microbial diversity; functional stability is critical, given that specific microbial activities are governed at the strain level. While diversity may rebound relatively quickly, certain beneficial strains and their associated functions may take much longer to fully recover (Dethlefsen and Relman, 2011; Lozupone et al., 2012; Palleja et al., 2018). Another significant concern with antibiotic exposure in FMT donors is the enrichment of antibiotic-resistant microbes and increased risk for transmission of antibiotic-resistant genes (ARGs) to the recipient's microbiota (Strati et al., 2021), posing long-term health risks if resistance genes transfer to pathogenic strains. The persistence of ARGs in the donor microbiota can occur even after apparent recovery of microbial diversity.

Balancing safety and efficacy remains particularly challenging in non-rCDI cases, where donor selection can substantially influence therapeutic success (Liptak et al., 2021). While the efficacy of FMT for rCDI is well-established, rare but severe complications, such as transmission of multi-drug resistant organism resulting in death has been reported, emphasizing the importance of meticulous donor screening to avoid transmitting pathogens or ARGs, particularly in immunocompromised individuals (Food and Drug Administration, 2020, 2022, 2023; van Lingen, 2023).

Current guidelines recommend a three-month suspension period post-antibiotic use (Kelly et al., 2016; Keller et al., 2021; Mullish et al., 2018). However, this three-month threshold is not strongly evidence-based and stems from limited studies. This is concerning, as key bacterial taxa essential for various physiological functions may take significantly longer to return to baseline levels (Dethlefsen and Relman, 2011; Palleja et al., 2018; Raymond et al., 2016), highlighting a gap in donor requalification practices (Dethlefsen and Relman, 2011; Jernberg et al., 2007; Korpela et al., 2016; Ng et al., 2019). Notably, a recent study demonstrated that antibiotic use by donors within 3–12 months prior to donation significantly decreased FMT effectiveness (Grosen et al., 2025). Therefore, determining an optimal antibiotic-free interval is essential to ensure that the donor microbiota has adequately recovered in both diversity and function to support FMT efficacy across a range of clinical applications. This study aims to investigate the effects of antibiotic exposure on donor microbiota composition, functional stability, and the persistence of ARGs using advanced high-throughput metagenomic sequencing. By clarifying microbiome recovery dynamics in real-life examples of fecal donors who received antibiotic therapy and were subsequently quarantined from donating, this research seeks to improve donor screening practices, and inform evidence-based guidelines that enhance both the safety and therapeutic efficacy of FMT.

2 Materials and methods

2.1 Sample collection and DNA extraction

Stool samples were collected longitudinally from three individuals before and after antibiotic treatments. Ethics approval for the use of donor samples in this study was obtained (refer to the Ethics Approval section for details). Donor 1 (Alberta, Canada) provided 34 samples over a 31-month period between June 25, 2019, and January 15, 2022, during which he underwent a 3-month Trimethoprim treatment. Donor 2 (Helsinki, Finland) provided 19 samples over a 19-month period between May 29, 2012, and December 19, 2014, and was exposed to a one-week *Helicobacter pylori* (*H. pylori*) eradication therapy (amoxicillin 500 mg four times a day, metronidazole 400 mg three times a day, and lansoprazole 30 mg twice a day). Lastly, Donor 3 (Helsinki, Finland) provided 8 samples over a 13-month period between May 29, 2012, and June 1, 2013, and underwent two antibiotic treatments: a one-week amoxicillin regimen (500 mg three times per day) followed by a one-week cefalexin regimen (500 mg twice per day). The timeline of all three donors' samples is depicted in Figure 1A. All stool samples were aliquoted and stored at −80° C within 4 h of stool collection. The donors were quarantined from acting as FMT donors for at least 3 months after the antibiotic exposure.

Microbial DNA from stool samples was extracted using the FastDNA Spin Kit for Feces (MP Biomedicals, Solon, Ohio) for whole genome shotgun sequencing. Metagenome libraries were constructed with the Nextera XT protocol (developed by Illumina) and sequenced on an Illumina MiSeq platform using a paired-end 300 cycle protocol.

2.2 Sequencing and bioinformatics

The preprocessing of metagenomic sequencing data aimed to ensure high-quality data for downstream analysis. The KneadData[1] pipeline (v0.10) was used to preprocess raw reads, employing Trimmomatic (v0.39), Bowtie2 (v2.3.5.1), and custom scripts to remove low-quality reads, primers, and sequencing adapters, and host contamination from the input sequencing data. Adjustments to default settings included SLIDINGWINDOW:4:25, HEADCROP:10, and MINLEN:77. Post-processing, an average of 25 million reads per sample was obtained, with a read length of 131 base pairs and a quality score above 35, providing sufficient sequencing depth.

The bacterial community composition was analyzed using MetaPhlan3 (v3.0.14) and Kraken2 (v2.1.2), and their performances were compared (Beghini et al., 2021; Wood et al., 2019). MetaPhlan3 was run with the "-t rel_ab_w_read_stats" parameter, mapping

1 https://huttenhower.sph.harvard.edu/kneaddata

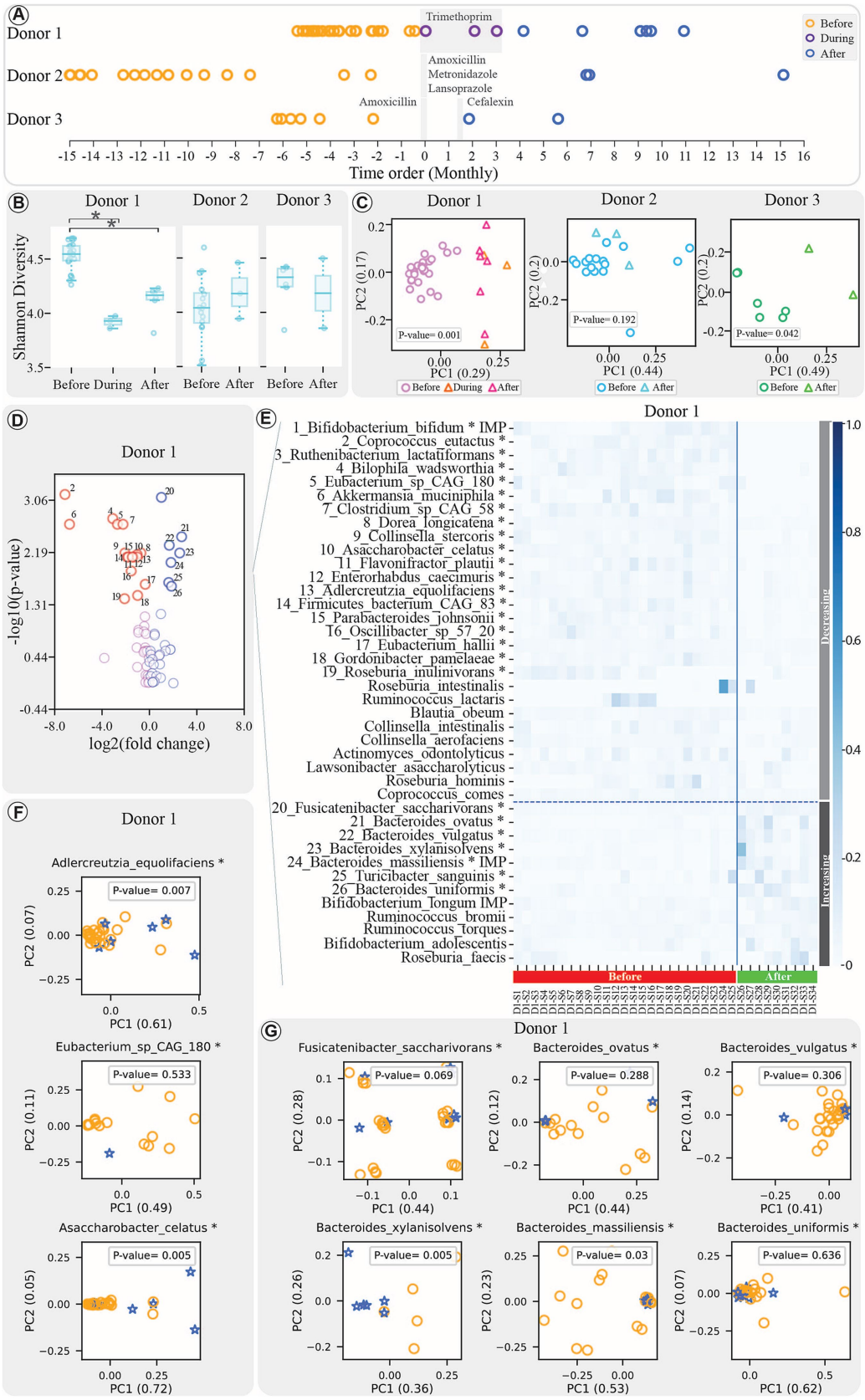


FIGURE 1
(A) Donor samples before, during (for Donor 1), and after treatment are represented by the orange, purple, and blue colors, respectively. The x-axis reflects a one-month time interval, with donor sampling dates rescaled to facilitate direct comparisons. All timelines are standardised so that antibiotic

(Continued)

FIGURE 1 (Continued)
therapy begins at month 0 for each donor, despite their collection at various time periods. The shaded gray regions represent the duration of the specific treatment applied to each donor, with the type of antibiotics indicated in the figure for each donor. **(B)** Boxplots comparing the Shannon diversity index of samples' bacteriome profile before, during (for Donor 1), and after treatment. (*indicates significant change with FDR-adjusted *p*-value < 0.05). **(C)** PCoA plots comparing beta diversity of samples before, during (for Donor 1), and after treatment for each donor, with *p*-values displayed within the figure. **(D)** Volcano plot displaying bacterial species' response to treatment. The x-axis shows $\log_2$ change in feature abundances before vs. after treatment; the y-axis shows $-\log_{10}$ of the adjusted *p*-value. Significant changes (FDR-adjusted *p*-value < 0.05, Mann–Whitney) are indicated in the figure. **(E)** Heatmap illustrating dynamics of the top 40 bacteria (sorted by raw *p*-values), with horizontal dashed line separating decreasing and increasing bacteria, and solid vertical line distinguishing before and after treatment samples (*indicates significant change with FDR-adjusted *p*-value < 0.05, Mann–Whitney test). The samples are presented in chronological order in this figure. **(F,G)** PCA plots of strain communities with significant decrease **(F)** or increase **(G)** post-treatment, using samples meeting the StrainPhlan pipeline's minimum thresholds (details in Methods). Circles and stars denote pre- and post-treatment samples, respectively.

quality-controlled reads to a marker gene database (v30[2]) with Bowtie2. Post-processing involved removing low-abundance taxa (<0.01%), singletons, doubletons, and species found in fewer than half of the samples, resulting in 72 distinct bacterial species. Kraken2 was run with a standard database (March 13, 2022) and specific parameters (-kmer-len: 35, -minimizer-len: 31, and -minimizer-spaces: 7). The read count profile for each sample was normalized by dividing each value by the total sequence count in that sample. Post-processing involved filtering singletons, doubletons, and bacteria below 1, 0.1, or 0.01% abundance, followed by the exclusion of species present in fewer than half of the samples.

Microbial strain analysis focused on characterizing strain-level variations in bacterial species. The StrainPhlAn pipeline was used in this regard (v4.0.3; Truong et al., 2017). Samples with at least 20 markers were retained (−sample_with_n_markers), and markers present in at least 50% of the samples were kept (−marker_in_n_samples). StrainPhlAn then categorized these samples as distinct strains based on the distance between strain sequences. This distance was used to create a distance matrix for PCoA plots, implemented with the Scikit-bio[3] Python library (v0.5.6). Strain analysis was conducted only on species that showed significant changes in the study.

For virome analysis, the same Kraken2 pipeline and database settings as used for bacteriome analysis were applied. Kraken2 output was filtered to include only viruses, and post-processing normalized read counts per sample by dividing by the total number of sequences. Singletons, doubletons, viruses with abundances below 0.01%, and viruses found in fewer than half of the samples were removed. After processing, 14 distinct viral species remained, accounting for nearly 96% of the viral reads. It is important to note that our virome analysis exclusively detects DNA viruses, as RNA viruses would require complementary RNA-specific extraction and sequencing methods.

CrAssphage abundance was assessed using two approaches: relative abundance from metagenomics and absolute abundance via qPCR. For relative abundance, *crAssphage* levels were extracted from Kraken2 virome analysis outputs. For absolute abundance of *crAssphage*, qPCR assays were conducted using a 126 bp gBlock gene fragment (crAssphage CPQ_056 amplicon) as a standard (Stachler et al., 2017). Calibration curves were created with 10-fold dilutions from 10 ng/μL to 10^{-7} ng/μL of the gBlock. qPCR was performed in triplicate with Itaq™ Universal Probes Supermix, primers, and probe, using the CFX Opus 96 instrument. DNA concentrations were converted to genomic copies/μL, as described in (Stachler et al., 2017), which provides additional details of the qPCR assays. Also, detailed information regarding primer sequences and qPCR cycling conditions is provided in Supplementary Table S1.

The functional potential of bacterial communities was assessed using the Humann3 pipeline (v3.6) by profiling the pathway abundances in samples (Beghini et al., 2021). Default parameters were applied, and pathway abundances were normalized using the TSS approach with the "--units relab" option for relative abundances. The post-processing steps were applied by removing singletons, doubletons, pathways below 0.01% abundance, and those present in fewer than half of the samples. This resulted in a final set of 286 unique pathways.

To investigate the ARGs profiles of samples, the Comprehensive Antibiotic Resistance Database (CARD, v3.1.4) and its Resistance Gene Identifier (RGI, v5.2.1) program were used (Alcock et al., 2023). The "bwt" option for metagenomic short reads and Bowtie2 for alignment were employed. Read counts for mapped reads were analyzed at the gene level, and sample tables were merged using custom Python scripts. The obtained abundance table was subsequently subjected to a post-processing step to remove singletons, doubletons, and ARGs with abundances below 0.1%. The final table contained 121 unique ARGs.

2.3 Statistical and data analysis

For majority of the data analysis, Python (v3.9) packages and scripts were used, with visualization done by the Matplotlib package (v3.7.1; Hunter, 2007).

Stacked bar plots were created with Matplotlib (v3.7.1). Samples were rescaled by dividing abundances by the total sum per sample, and features (bacteria/ARGs) were sorted by their average values. Only a subset of highly abundant features was colored and shown in the legend.

Alpha diversity was assessed using the Shannon metric from Scikit-bio library (v0.5.6) and visualized with boxplots from Matplotlib library (v3.7.1). Statistical significance was determined using the Mann–Whitney U test from SciPy library (v1.7.3) with a significance threshold set at a *p*-value < 0.05 (Virtanen et al., 2020). For Donor 1, which includes three groups (before, during, and after antibiotic exposure), the Kruskal–Wallis test from the SciPy library was first applied to assess overall differences. This was followed by pairwise Mann–Whitney U tests for *post-hoc* analysis, with FDR

2 mpa_v30_CHOCOPhlAn_201901.

3 http://scikit-bio.org/

correction applied to account for multiple comparisons. Beta diversity was analyzed with PCoA plots based on Bray-Curtis distances using the Scikit-learn library (Pedregosa et al., 2011). The comparison between the groups is performed using the PERMANOVA test from the Scikit-bio library, with a significance threshold set at a p-value < 0.05. For Donor 1, if the PERMANOVA test yielded a significant p-value, a *post-hoc* analysis (pairwise PERMANOVA with FDR correction) was conducted.

If no significant differences were detected between the during and after antibiotic exposure samples in Donor 1, these samples were combined and considered as after antibiotic exposure samples to increase the sample size for subsequent analyses.

For correlation network analysis, Spearman correlations between bacterial species and ARGs were calculated using SciPy (version 1.7.3). The correlation analysis included ARGs and only the significantly changed bacteria as well as bacteria important for FMT engraftment (Supplementary Table S2). Only positive correlations with FDR-adjusted p-values < 0.05 and R-values > 0.6 were retained. Networks were created with NetworkX (version 2.6.3) and visualized with Gephi (version 0.9.6), where node size reflects the significance of the change after treatment (Mann–Whitney U test, $p < 0.05$) and edge thickness indicates correlation strength. Green links represent correlations unique to post-treatment samples, which did not exist in before treatment samples.

For constructing the phylogenetic tree via the ETE Python library (v3.1.3; Huerta-Cepas et al., 2010), the union of all bacterial species (from all the samples) identified by either Kraken2 or MetaPhlan3, utilizing various filtering thresholds, is employed. The tree, annotated with metadata on species identification methods, was visualized using the Interactive Tree of Life (iTOL; v6.8; Letunic and Bork, 2019).

3 Results

3.1 Assessing gut microbial diversity and composition in donors before and after antibiotic treatment

In our investigation of gut microbiota, we aimed to understand how antibiotic exposure influences the composition and diversity of the gut bacteriome in donor samples. To ensure accurate bacterial species profiling, we chose the MetaPhlan3 pipeline over Kraken2 for its conservative approach to bacterial species identification. Supplementary Table S3 shows the number of unique species identified at various thresholds, with species coverage illustrated in Supplementary Figure S1.

Since the baseline gut microbiota may determine its ability to recover following perturbation, we compared the bacteriomes of donors before antibiotic treatment. Alpha diversity analysis revealed significant differences between Donor 1 and Donors 2 and 3 (Mann–Whitney U test with FDR correction, adjusted p-values < 0.0001 and < 0.01, respectively), while no significant difference was observed between Donors 2 and 3 (Supplementary Figure S2A). Beta-diversity analysis confirmed that each donor had a distinct microbial community, with significant differences across all donors (Pairwise PERMANOVA test with FDR correction, adjusted p-value < 0.01; Supplementary Figure S2B). Furthermore, bacterial composition at the phylum level (Supplementary Figure S2C) showed Firmicutes as the predominant phylum in all donors. Actinobacteria was the second most abundant in Donor 2, while Bacteroidetes was the second most abundant phylum in Donors 1 and 3. At the species level (Supplementary Figure S2D), *Faecalibacterium prausnitzii* dominated in Donors 1 and 3, while *Bifidobacterium adolescentis* and *Bifidobacterium longum* were prevalent in Donor 2.

Following antibiotic treatment, we observed significant changes in the microbial communities. Donor 1 showed a significant difference in alpha diversity across the before, during, and after antibiotic exposure groups (Kruskal–Wallis test, $p < 0.0001$). *Post-hoc* analysis in this donor revealed a significant decrease in alpha diversity in both the during and after treatment samples compared to before treatment group (Mann–Whitney U test with FDR correction, adjusted p-value < 0.001). Also, Donor 3 showed a non-significant decrease (Mann–Whitney U test, p-value = 0.42). In contrast, Donor 2, sampled 7–15 months post-treatment, exhibited a slight, non-significant increase in diversity (Mann–Whitney U test, p-value = 0.86; Figure 1B). Beta diversity analysis revealed significant compositional changes in Donors 1 and 3 (PERMANOVA test, p-value < 0.05), but not in Donor 2, whose microbial composition remained stable (PERMANOVA test, p-value = 0.208; Figure 1C). Furthermore, *post-hoc* analysis of Donor 1 samples revealed that the bacteriome composition of the during and after treatment samples was significantly different from the before treatment group (PERMANOVA test with FDR correction, adjusted p-value < 0.005), while no significant difference was observed between the during and after treatment communities (PERMANOVA test with FDR correction, adjusted p-value = 0.86).

A closer look at specific bacterial species showed that Donor 1 experienced significant shifts in 26 taxa, with 19 decreasing and 7 increasing (Mann–Whitney U test with FDR correction, adjusted p-value < 0.05; Figures 1D,E). Donor 2 exhibited fewer changes: *Blautia wexlerae* showing a significant decrease, and six taxa—including *Bacteroides fragilis* and *Bacteroides vulgatus*— showing significant increases (Mann–Whitney U test with FDR correction, adjusted p-value < 0.05; Supplementary Figures S3A,B). Donor 3's bacterial landscape remained stable, with no significant changes post-treatment (Supplementary Figures S3D,E).

To gain a more detailed understanding, strain-level analysis was conducted using the StrainPhlAn pipeline. In Donor 1, although some species showed significant changes, the strain populations within these species remained relatively stable. For instance, among the bacteria with decreasing abundance, eg. *Eubacterium* sp. *CAG 180*, and among those with increasing abundance, eg. *Fusicatenibacter saccharivorans*, *Bacteroides ovatus*, *Bacteroides vulgatus*, and *Bacteroides uniformis,* have not shown significant strain population changes (Figures 1F,G, respectively). In Donor 2, strains of *Eubacterium sp_CAG 180* and *Bacteroides vulgatus* remained stable, while *Blautia obeum* exhibited significant strain changes post-treatment (PERMANOVA test, p-value < 0.05; Supplementary Figure S3C). Strain analysis was not performed for Donor 3 due to the lack of significant findings at the species level.

3.2 Phageome composition and *crAssphage* abundance in donor samples before and after antibiotic exposure

To comprehend how antibiotic exposure influences the composition and diversity of the gut virome in donor samples,

10.3389/fmicb.2025.1589704

we examined the viral species composition of the donor samples. Overall, 14 viral species were identified belonging to the phylum Uroviricota. The viral species composition of Donors samples is illustrated using the stacked bar graphs in Figure 2A. Notably, the samples from Donor 3 exhibited a different virome composition compared to the other donors. In Donor 1 and Donor 2, the most abundant virus is "*uncultured_crassphage*." However, it is intriguing that this virus was hardly detectable in Donor 3. Additionally, when examining the impact of antibiotic treatment on post-treatment sample diversity, a decrease in the alpha diversity of viruses was observed in all donors, as shown in Figure 2B. This decrease reached statistical significance only in Donor 2 (Mann–Whitney U test, p-value < 0.01), while no significant differences were observed between groups in Donor 1 (Kruskal–Wallis test, $p = 0.085$) or Donor 3 (Mann–Whitney U test, $p = 0.285$).

Since *CrAssphage* is known as a dominant phage in the human gut microbiota, it was taken into further investigation. The results demonstrated that total *crAssphage* abundance increased following antibiotic treatment, with statistical significance observed in Donor 1 and Donor 2 (Mann–Whitney U test, p-value < 0.05; Figure 2C). The findings were validated by qPCR to obtain absolute abundance measurements for *crAssphage*. The qPCR results supported the

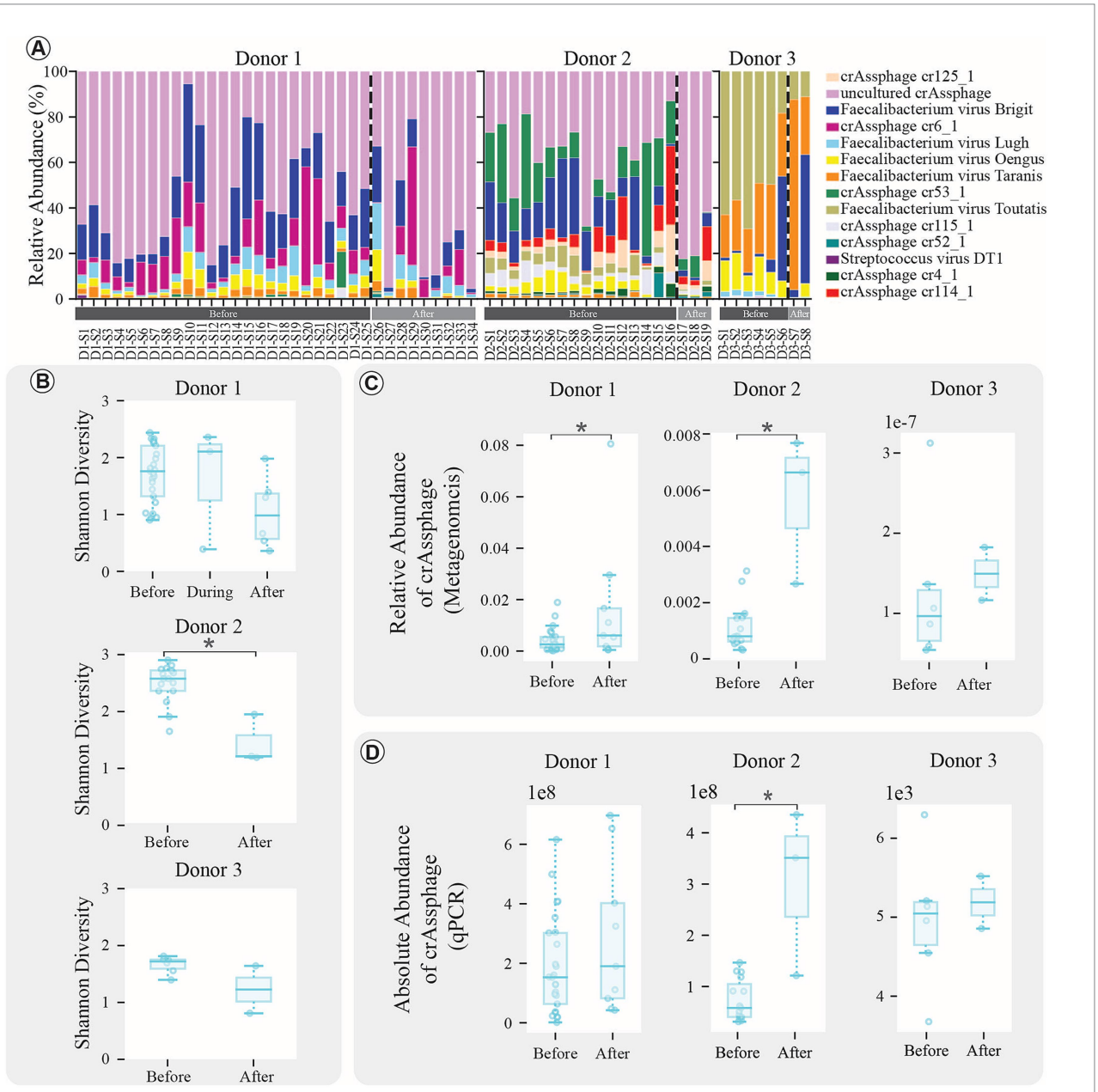


FIGURE 2
Virome composition analysis results. **(A)** Stacked bar plots depicting virome compositions at the species level. Before, during (for Donor 1), and after treatment samples are differentiated by a dashed line and the samples are presented in chronological order. **(B)** Boxplots comparing the Shannon diversity index of samples before, during (for Donor 1), and after treatment. (*indicates significant change with p-value < 0.05). **(C)** Boxplots comparing the relative abundance of *crAssphage* using the metagenomics data from Kraken2 pipeline (*indicates significant change with p-value < 0.05). **(D)** Boxplots comparing the absolute abundance of *crAssphage* using the qPCR approach data (*indicates significant change with p-value < 0.05).

increase in *crAssphage* abundance after antibiotic therapy, reaching statistical significance in Donor 2 (Mann–Whitney U test, p-value < 0.05; Figure 2D). Standard curves for each donor showed high reliability with R-squared values near 1, indicating robust qPCR assays across all donors, further supporting the validity of these findings (Supplementary Figure S4).

Beyond the antibiotic impact on the gut virome, we explored potential correlations between *crAssphage* abundance, bacterial species, and ARGs. We hypothesized that *crAssphage* abundance might be linked to alterations in the bacterial community and ARGs. The analysis, conducted using qPCR results to avoid autocorrelation issues from metagenomics data, did not reveal any significant correlations between *crAssphage* abundance and specific bacterial species or ARGs (Spearman correlation, p-value < 0.05, R-value < 0.6).

3.3 Functional changes in the gut microbiome post-antibiotic treatment

To explore how antibiotic exposure affects the functional genes within the donor microbiota, we analyzed changes in pathway abundance profiles. Our analysis revealed that only Donor 1 experienced significant changes in both alpha and beta diversity during and after treatment compared to pre-treatment phase (Figures 3A,B; Kruskal–Wallis test, p-value < 0.05; Mann–Whitney U test and PERMANOVA test, FDR-adjusted p-value < 0.05). In contrast, Donors 2 and 3 showed no substantial alterations (Figure 3C). Moreover, in Donor 1, no significant difference was observed between during and after treatment groups (Mann–Whitney U test, FDR-adjusted p-value = 0.262).

In Donor 1, 43 pathways were significantly altered after antibiotic exposure, 29 decreased and 14 increased (Supplementary Table S4). These pathways span 13 unique super classes, with the most frequently decreased ones including "Amino Acid Biosynthesis" (8 times), "Nucleoside and Nucleotide Biosynthesis" (6 times), and "Cofactor, Carrier, and Vitamin Biosynthesis" (4 times). Among the increasing groups, "Secondary Metabolite Biosynthesis" appeared most frequently (3 times). Within these categories, certain pathways exhibited significant decreases and increases following treatment, encompassing a variety of metabolic processes, biosynthesis pathways, and degradation processes (Mann–Whitney U test with FDR correction method, adjusted p-values < 0.05; Figure 3D; Supplementary Table S4).

The pathway analysis revealed that bacterial contributions to these pathways come not only from significantly altered species but also from those without significant changes, contributing at least 5% to certain pathways (Supplementary Figures S5, S6). For example, the "O-antigen building blocks biosynthesis" pathway (OANTIGEN-PWY) involves *Bifidobacterium adolescentis* and *Collinsella aerofaciens*, while the "thiamine diphosphate biosynthesis III" pathway (THISYNARA-PWY) is influenced by both significantly altered and stable bacteria, such as *Bacteroides uniformis*, *Ruminococcus torques*, *Faecalibacterium prausnitzii*, and *Roseburia faecis*.

A significantly increased bacterium, *Bacteroides vulgatus*, has contributed to both the reduction and increase of several key pathways: PWY-7977, PWY-1042, PWY-6703, and RIBOSYN-PWY among the decreased pathways, and PWY66-429, RHAMCAT-PWY, and PWY-5121 among the increased pathways (Supplementary Figures S5, S6). *Bifidobacterium bifidum*, significantly decreased post-treatment, contributed to the reduced "glycogen degradation I" pathway (GLYCOCAT-PWY; Supplementary Figure S5). These findings highlight the complex interplay between bacterial species—both significantly altered and stable—and their collective influence on pathway dynamics.

3.4 Diversity and dynamics of ARGs and their species associations post-antibiotic treatment

The profiles of ARGs were analyzed to determine whether antibiotic exposure leads to changes in the abundance and prevalence of ARGs within the donor microbiota, assessing potential increased risks of transfer in FMT. The results revealed that while overall alpha diversity of ARGs showed no significant changes across donors (Kruskal–Wallis test and Mann–Whitney U test, p-value > 0.05; Figure 4A), beta diversity revealed distinct patterns (Figure 4B). Donor 1, in particular, exhibited a significant shift in ARG community composition post-treatment (PERMANOVA test, FDR-adjusted p-value < 0.01), while no significant difference was observed between the during and after treatment groups (Mann–Whitney U test, FDR-adjusted p-value = 0.090). The full names of the significantly changed ARGs for Donor 1 are listed in Supplementary Table S5.

As the composition of post-treatment samples in Donor 1 significantly differed from the pre-treatment samples, the dynamics of ARG abundance in this donor were further investigated. The post-treatment ARG abundance for this donor revealed significant increases and decreases in several ARGs, visualized in a volcano plot and heatmap (Figures 4C,D; Mann–Whitney U test with FDR correction, adjusted p-value < 0.05).

Association mapping identified several noteworthy correlations between ARGs and specific microbial taxa (Figure 4E). For example, the Mgen_23S_MULT gene showed a significant positive association with *Bacteroides massiliensis* (Spearman correlation with FDR correction, adjusted p-value < 0.05, R-value > 0.6). Similarly, *Bifidobacterium longum*, was positively correlated with carA (adjusted p-value < 0.01, R-value > 0.6). Also, *Bacteroides vulgatus* (currently *Phocaeicola vulgatus*), exhibited new associations with ARGs such as Bbur_16S_GEN and Bhyo_23S_TYL (adjusted p-values < 0.01 and < 0.05, respectively). Other positive correlations are shown in Figure 4E.

4 Discussion

While the efficacy of FMT at preventing CDI recurrence is well established, the duration of donor suspension following a course of antibiotic is under-investigated. Our analysis highlights differences in recovery in three long term stool donors in microbial diversity, functionality, phage abundance and diversity, as well as ARG profiles. The most notable disruptions were seen in Donor 1, who underwent a longer course of antibiotics and showed significant changes in microbial diversity, composition, functionality, and ARG profiles that persisted even 8 months post-treatment. In contrast, Donor 2 exhibited relative compositional stability between 7 and 15 months after treatment. These findings suggest that in cases of prolonged

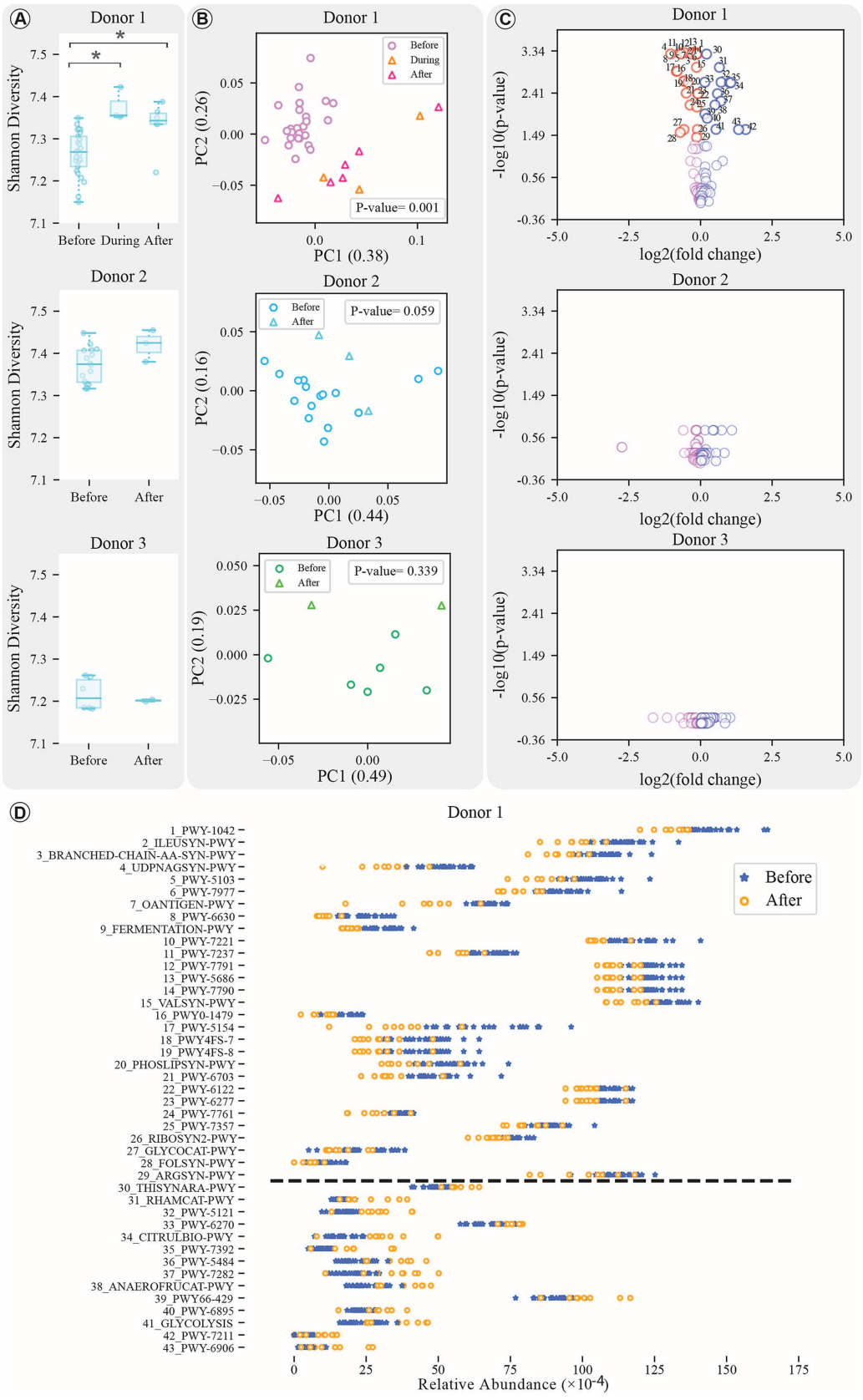


FIGURE 3
Pathway analysis results. **(A)** Boxplots comparing the Shannon diversity index of samples before, during (for Donor 1), and after treatment for all donors (*indicates significant change with p-value < 0.05). **(B)** PCoA plots comparing beta diversity of samples before, during (for Donor 1), and after treatment

(Continued)

FIGURE 3 (Continued)
for each donor, with *p*-values displayed within the figures. **(C)** Volcano plot of pathways' response to treatment. The x-axis shows $\log_2$ change in feature abundances before vs. after treatment; the y-axis shows $-\log_{10}$ of the adjusted *p*-value. Only the top 100 pathways contributing to PCA components are shown. Significant changes (FDR-adjusted *p*-value < 0.05, Mann–Whitney) are indicated in the figure. **(D)** Dynamics of the significantly changed pathways for Donor 1 (increased and decreasing pathways are separated using a dashed line).

antibiotic exposure, microbiota recovery may take up to 8 months or longer. Conversely, for shorter antibiotic courses, an 8-month window may represent a reasonable timeframe for microbiota recovery and donor requalification.

Studies have shown that several engrafted bacteria during FMT treatment, especially from the Bacteroidetes and Actinobacteria phyla, are closely linked to beneficial functions like SCFA and bile acid metabolism (listed in Supplementary Table S2) Ojima et al., 2020; Morrison and Preston, 2016; Harris et al., 2018; Smith et al., 2022; Smillie et al., 2018; Kootte et al., 2017; Ianiro et al., 2022). Our study revealed that antibiotic exposure leads to reduction or near complete disappearance of specific SCFA producers like *Bifidobacterium bifidum*, *Roseburia inulinivorans*, *Eubacterium hallii*, and *Akkermansia muciniphila*. In one donor, this disruption extended beyond microbiota composition, affecting metabolic pathways, including the biosynthesis of essential amino acids like L-arginine and L-methionine, which are particularly underrepresented in IBD patients (Hellmann et al., 2020; Li et al., 2023). We also observed alterations in the mixed acid fermentation pathway, which is associated with acetate production (Förster and Gescher, 2014). Even when the abundance of some bacteria remains relatively unchanged, shifts in their strain communities, as seen with *Bacteroides massiliensis* and *Bacteroides xylanisolvens*, may still impact their functionality. These strain-level shifts underscore the complexity of microbial recovery following antibiotic exposure, as even subtle variations in strain composition can lead to significant changes in functional capacity (Koo et al., 2019). For example, bacteria such as *Bacteroides vulgatus*, which increased post-treatment, contributed to both reductions and increases in key pathways, reflecting the duality of their functional impacts on the microbiome. Conversely, the depletion of *Bifidobacterium bifidum*, critical for glycogen degradation, underscores the risks posed by the loss of beneficial strains. Moreover, our findings indicate that functional contributions stem not only from bacterial species with altered strains but also from those whose strains remained stable in abundance. For example, pathways such as thiamine biosynthesis and O-antigen building blocks biosynthesis were influenced by bacteria like *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, and *Roseburia faecis*, which remained relatively stable in their abundances post-antibiotic treatment. However, despite their stable population levels, changes in the surrounding microbial community likely altered their relative functional contributions. Such dynamics suggest that stable taxa can adapt their metabolic outputs in response to environmental changes, including those induced by antibiotic exposure.

We observed reduced viral diversity in donor samples following antibiotic exposure, further underscoring the disruption of the gut microbial ecosystem. Viruses, particularly bacteriophages, play an integral role in shaping bacterial fitness and maintaining gut homeostasis (Lam et al., 2022; Norman et al., 2015). Our findings demonstrated a decrease in alpha diversity of viruses in all donors post-antibiotic treatment, with a particularly pronounced reduction observed in Donor 2. Such reductions may reflect a diminished ability of the virome to regulate bacterial populations. Notably, the sustained rise in *crAssphage* abundance post-antibiotic treatment is of particular interest. *CrAssphage* is a dominant bacteriophage in the human gut microbiota, and studies have shown its association with stable and healthy microbiome in balanced proportions (Remesh and Viswanathan, 2024). However, in our study, its significant increase in Donor 1 and Donor 2 post-antibiotic treatment may also reflect potential disruption of microbial balance. *CrAssphage* relies on specific host bacteria for replication, and its sustained high levels, even 8 months or more after antibiotic exposure, indicate that recovery of the virome to a pre-treatment state may be incomplete. This persistence suggests a shift in host-bacteriophage dynamics, potentially driven by reduced bacterial diversity or altered ecological niches created by antibiotic disruption. Moreover, disproportionate *crAssphage* abundance has been linked to various diseases such as inflammatory bowel disease, obesity, metabolic syndrome, and colorectal cancer (Norman et al., 2015; Gogokhia et al., 2019; Cervantes-Echeverría et al., 2023). Viral components may contribute to FMT efficacy (Zuo et al., 2018; Fujimoto et al., 2021), but the clinical implications of virome/phageome changes in FMT donors remain unclear. It should be noted that the virome analysis in this study is limited to DNA. As a result, RNA viruses were not captured, which likely contributed to the low number of detected viral species. Future studies incorporating RNA sequencing methodologies, such as metatranscriptomics, would be necessary to provide a more comprehensive characterization of the gut virome.

One critical aspect of FMT safety is the transmission of antibiotic-resistant organisms (van Lingen, 2023; Food and Drug Administration, 2020; DeFilipp et al., 2019). These concerns underscore the broader challenge of antimicrobial resistance, where ARGs, capable of horizontal transfer, not only persist but actively reshape microbial ecosystems (Sabtu et al., 2015; Murray, 2022; Centers for Disease Control and Prevention, 2019). Our findings suggest that shifts in ARG diversity reflect the lasting impact of antibiotic exposure (Jernberg et al., 2007; Jakobsson et al., 2010; Xu et al., 2020), potentially altering the competitive dynamics of gut bacteria like *Bacteroides massiliensis* and *Bifidobacterium longum,* which were positively correlated with ARGs. While these correlations hint at possible ARG acquisition, they do not necessarily imply that these bacteria have become antibiotic-resistant. Fortunately, these changes did not involve the most hazardous ARGs, classified as "Rank I ARGs: current threats" (Zhang et al., 2021). Nevertheless, how these ARGs interacts with a dysbiotic microbial population remains incompletely understood.

Our study has several strengths. First, we performed longitudinal sampling of three long term stool donors with high efficacy in preventing rCDI. Second, we undertook deep shotgun metagenomics sequencing which enabled us to address many questions. Our study also has several limitations, including a small number of donors, different antibiotic regimens and durations for each donor, and varying durations of follow up. We did not capture success rates of recipients

10.3389/fmicb.2025.1589704

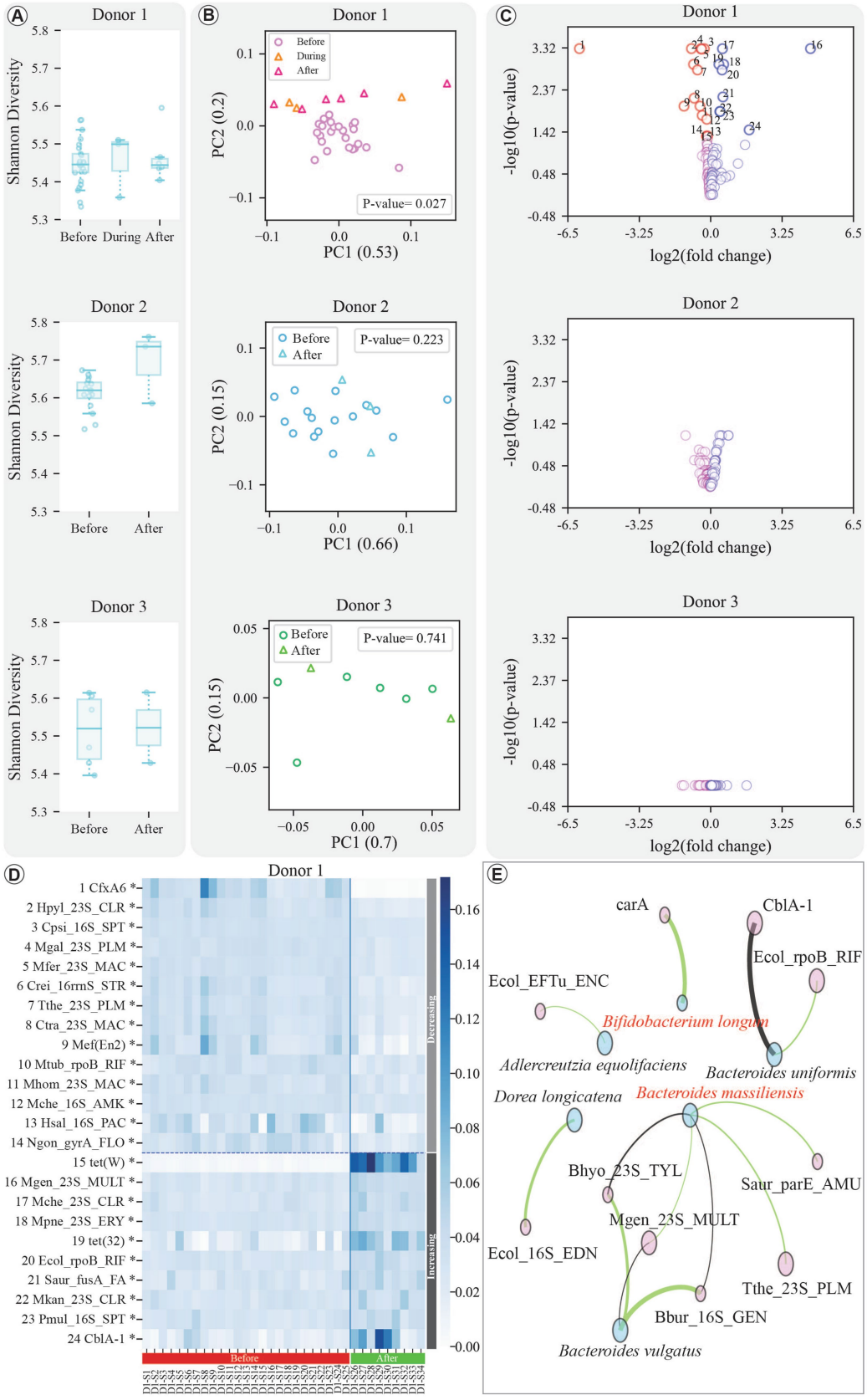


FIGURE 4
Results of ARG analysis. **(A)** Comparative Boxplots illustrating the Shannon diversity index of samples before, during (for Donor 1), and after treatment across all donors. **(B)** PCoA (Principal Coordinates Analysis) plots depicting the beta diversity of samples before, during (for Donor 1), and after

(Continued)

FIGURE 4 (Continued)
treatment for each, with *p*-values displayed within the figures. **(C)** Volcano plot of ARGs' response to treatment. The x-axis shows $\log_2$ change in feature abundances before vs. after treatment; the y-axis shows $-\log_{10}$ of the adjusted *p*-value. Significant changes (FDR-adjusted *p*-value < 0.05, Mann–Whitney) are indicated in the figure. **(D)** Heatmap displaying the dynamics of significantly altered ARGs for Donor 1. The heatmap differentiates between increased and decreased pathways using a horizontal dashed line, and before-and-after treatment samples are separated by a vertical solid line. The samples are presented in chronological order in this figure. **(E)** Correlation network illustrating positive and significant links among significantly changed bacterial species (as well as the crucial bacteria for FMT engraftment, highlighted in red text) and ARGs. The network considers post-treatment samples and employs correlation *p*-value and R-value thresholds of 0.05 and 0.6, respectively, using the Spearman correlation method (details in Methods). Node size corresponds to the magnitude of change after treatment, with larger nodes indicating significantly changed bacteria or ARGs after treatment.

once these donors came out of quarantine in order to examine how these changes may impact efficacy. Although we profiled ARGs, we did not examine antibiotic resistant organisms in these stool donors.

In conclusion, while our sample size is limited, we found no increased risk of potential ARGs dissemination from donors to recipients after antibiotic exposure of donors, as assessed by the donor's ARG profiles. The alterations in ARG profiles were minimal and do not raise major safety concerns for FMT. However, the impact of antibiotic exposure on donor microbiota was more pronounced than anticipated, with lasting and complex changes, particularly in key bacterial species and their functionality. These changes could potentially affect the efficacy of FMT, especially in indications beyond rCDI, and suggest that donor microbiota performance post-antibiotic treatment may be unpredictable. Therefore, donor suspension periods may need to be individualized, taking into account the spectrum of antibiotics, treatment duration, and other host-specific factors. Future studies should consider a broader range of antibiotics, varying treatment durations, and more frequent sampling, alongside additional host-specific factors like diet, lifestyle, and the use of additional medications like Proton Pump Inhibitors (PPIs) or probiotics. This comprehensive approach will help better understand the long-term effects of antibiotics on the gut microbiome and inform stool banks which is evidence-based.

Data availability statement

The metagenomic sequencing data from this study has been processed and deposited at NCBI, accessible under project ID PRJNA1202717, https://www.ncbi.nlm.nih.gov/bioproject/1202717. The custom scripts used for data analysis in this study are available in a GitHub repository, https://github.com/ramin-karimian/fmt_pj.

Ethics statement

The studies involving humans were approved by the University of Alberta Research Ethics Board (Pro0089012) for Donor 1; the Helsinki University Hospital Ethics Committee, Helsinki, Finland (HUS124/13/03/01/11 and HUS/1405/2020) for Donors 2 and 3. 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

RK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. RS: Funding acquisition, Investigation, Supervision, Validation, Writing – review & editing, Conceptualization, Project administration. SY: Investigation, Writing – review & editing, Methodology, Data curation. PA: Investigation, Writing – review & editing. DK: Funding acquisition, Investigation, Validation, Writing – review & editing, Conceptualization, Methodology, Project administration. SP: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing, Resources.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. We like to acknowledge Natural Sciences and Engineering Research Council of Canada (NSERC, grant GR008435), University of Alberta Hospital Foundation (grant ZI418), and The Sigrid Juselius Foundation, Finland, for their financial support of this project.

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 author(s) declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1589704/full#supplementary-material

References

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., et al. (2023). CARD 2023: expanded curation, support for machine learning, and resistome prediction at the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 51, D690–D699. doi: 10.1093/nar/gkac920

Battipaglia, G., Malard, F., Rubio, M. T., Ruggeri, A., Mamez, A. C., Brissot, E., et al. (2019). Fecal microbiota transplantation before or after allogeneic hematopoietic transplantation in patients with hematologic malignancies carrying multidrug-resistance bacteria. *Haematologica* 104, 1682–1688. doi: 10.3324/haematol.2018.198549

Beghini, F., McIver, L. J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., et al. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *eLife* 10:e65088. doi: 10.7554/eLife.65088

Bilinski, J., Grzesiowski, P., Sorensen, N., Madry, K., Muszynski, J., Robak, K., et al. (2017). Fecal microbiota transplantation in patients with blood disorders inhibits gut colonization with antibiotic-resistant bacteria: results of a prospective, single-center study. *Clin. Infect. Dis.* 65, 364–370. doi: 10.1093/cid/cix252

Cammarota, G., Ianiro, G., Kelly, C. R., Mullish, B. H., Allegretti, J. R., Kassam, Z., et al. (2019). International consensus conference on stool banking for faecal microbiota transplantation in clinical practice. *Gut* 68, 2111–2121. doi: 10.1136/gutjnl-2019-319548

Cammarota, G., Masucci, L., Ianiro, G., Bibbò, S., Dinoi, G., Costamagna, G., et al. (2015). Randomised clinical trial: faecal microbiota transplantation by colonoscopy vs. vancomycin for the treatment of recurrent *Clostridium difficile* infection. *Aliment. Pharmacol. Ther.* 41, 835–843. doi: 10.1111/apt.13144

Centers for Disease Control and Prevention (2019). Antibiotic resistance threats in the United States. Department of Health and Human Services. Available at: https://www.cdc.gov/antimicrobial-resistance/data-research/threats/index.html (Accessed November 07, 2024).

Cervantes-Echeverría, M., Gallardo-Becerra, L., Cornejo-Granados, F., and Ochoa-Leyva, A. (2023). The two-faced role of crAssphage subfamilies in obesity and metabolic syndrome: between good and evil. *Gen. Dent.* 14:139. doi: 10.3390/genes14010139

ClinicalTrials.gov. (2023). Search results for "fecal microbiota transplantation" with active, recruiting, and not yet recruiting studies. Available online at: https://clinicaltrials.gov/search?intr=Fecal%20Microbiota%20Transplantation&aggFilters=status:act%20rec%20not (Accessed November 13, 2024).

DeFilipp, Z., Bloom, P. P., Torres Soto, M., Mansour, M. K., Sater, M. R. A., Huntley, M. H., et al. (2019). Drug-resistant *E. coli* bacteremia transmitted by fecal microbiota transplant. *N. Engl. J. Med.* 381, 2043–2050. doi: 10.1056/NEJMoa1910437

Dethlefsen, L., and Relman, D. A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci.* 108, 4554–4561. doi: 10.1073/pnas.1000087107

Food and Drug Administration (2020). Fecal microbiota for transplantation: Safety alert - risk of serious adverse events likely due to transmission of pathogenic organisms. U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Biologics Evaluation and Research: Silver Spring, Maryland, USA. Available at: https://www.fda.gov/safety/medical-product-safety-information/fecal-microbiota-transplantation-safety-alert-risk-serious-adverse-events-likely-due-transmission (Accessed November 07, 2024).

Food and Drug Administration (2022). Enforcement policy regarding investigational new drug requirements for use of fecal microbiota for transplantation to treat Clostridium difficile infection not responsive to standard therapies. Center for Biologics Evaluation and Research, Food and Drug Administration: Silver Spring, Maryland, USA. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/enforcement-policy-regarding-investigational-new-drug-requirements-use-fecal-microbiota (Accessed November 07, 2024).

Food and Drug Administration (2023). FDA approves first orally administered fecal microbiota product for the prevention of recurrence of clostridioides difficile Infection. Center for Biologics Evaluation and Research, Food and Drug Administration: Silver Spring, Maryland, USA. Available at: https://www.fda.gov/news-events/press-announcements/fda-approves-first-orally-administered-fecal-microbiota-product-prevention-recurrence-clostridioides (Accessed November 07, 2024).

Fischer, M., Kao, D., Kelly, C., Kuchipudi, A., Jafri, S. M., Blumenkehl, M., et al. (2016). Fecal microbiota transplantation is safe and efficacious for recurrent or refractory *Clostridium difficile* infection in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* 22, 2402–2409. doi: 10.1097/MIB.0000000000000908

Förster, A. H., and Gescher, J. (2014). Metabolic engineering of *Escherichia coli* for production of mixed-acid fermentation end products. *Front. Bioeng. Biotechnol.* 2:16. doi: 10.3389/fbioe.2014.00016

Fujimoto, K., Kimura, Y., Allegretti, J. R., Yamamoto, M., Zhang, Y. Z., Katayama, K., et al. (2021). Functional restoration of bacteriomes and viromes by fecal microbiota transplantation. *Gastroenterology* 160, 2089–2102.e12. doi: 10.1053/j.gastro.2021.02.013

Gogokhia, L., Buhrke, K., Bell, R., Hoffman, B., Brown, D. G., Hanke-Gogokhia, C., et al. (2019). Expansion of bacteriophages is linked to aggravated intestinal inflammation and colitis. *Cell Host Microbe* 25, 285–299.e8. doi: 10.1016/j.chom.2019.01.008

Grosen, A. K., Mikkelsen, S., Hindhede, L. A., Paaske, S. E., Baunwall, S. M. D., Hansen, M. M., et al. (2025). Effects of clinical donor characteristics on the success of faecal microbiota transplantation for patients in Denmark with Clostridioides difficile infection: a single-centre, prospective cohort study. *Lancet Microbe*. doi: 10.1016/j.lanmic.2024.101034

Haifer, C., Kelly, C. R., Paramsothy, S., Andresen, D., Papanicolas, L. E., McKew, G. L., et al. (2020). Australian consensus statements for the regulation, production and use of faecal microbiota transplantation in clinical practice. *Gut* 69, 801–810. doi: 10.1136/gutjnl-2019-320260

Harris, S. C., Devendran, S., Méndez-García, C., Mythen, S. M., Wright, C. L., Fields, C. J., et al. (2018). Bile acid oxidation by *Eggerthella lenta* strains C592 and DSM 2243T. *Gut Microbes* 9, 523–539. doi: 10.1080/19490976.2018.1458180

Hellmann, J., Andersen, H., Fei, L., Linn, A., Bezold, R., Lake, K., et al. (2020). Microbial shifts and shorter time to bowel resection surgery associated with *C. difficile* in pediatric Crohn's disease. *Inflamm. Bowel Dis.* 26, 1212–1221. doi: 10.1093/ibd/izz263

Holvoet, T., Joossens, M., Vázquez-Castellanos, J. F., Christiaens, E., Heyerick, L., Boelens, J., et al. (2021). Fecal microbiota transplantation reduces symptoms in some patients with irritable bowel syndrome with predominant abdominal bloating: short-and long-term results from a placebo-controlled randomized trial. *Gastroenterology* 160, 145–157.e8. doi: 10.1053/j.gastro.2020.07.013

Huerta-Cepas, J., Dopazo, J., and Gabaldón, T. (2010). ETE: a python environment for tree exploration. *BMC bioinformatics*, 11, 1–7. doi: 10.1186/1471-2105-11-24

Hunter, J. D. (2007). Matplotlib: a 2D graphics environment. *Comput. Sci. Eng.* 9, 90–95. doi: 10.1109/MCSE.2007.55

Ianiro, G., Punčochář, M., Karcher, N., Porcari, S., Armanini, F., Asnicar, F., et al. (2022). Variability of strain engraftment and predictability of microbiome composition after fecal microbiota transplantation across different diseases. *Nat. Med.* 28, 1913–1923. doi: 10.1038/s41591-022-01964-3

Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjölund-Karlsson, M., Jansson, J. K., and Engstrand, L. (2010). Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* 5:e9836. doi: 10.1371/journal.pone.0009836

Jernberg, C., Löfmark, S., Edlund, C., and Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J.* 1, 56–66. doi: 10.1038/ismej.2007.3

Kassam, Z., Lee, C. H., Yuan, Y., and Hunt, R. H. (2013). Fecal microbiota transplantation for *Clostridium difficile* infection: systematic review and meta-analysis. *Am. J. Gastroenterol.* 108, 500–508. doi: 10.1038/ajg.2013.59

Keller, J. J., Ooijevaar, R. E., Hvas, C. L., Terveer, E. M., Lieberknecht, S. C., Högenauer, C., et al. (2021). A standardised model for stool banking for faecal microbiota transplantation: a consensus report from a multidisciplinary UEG working group. *United European Gastroenterol J* 9, 229–247. doi: 10.1177/2050640620967898

Kelly, C. R., Khoruts, A., Staley, C., Sadowsky, M. J., Abd, M., Alani, M., et al. (2016). Effect of fecal microbiota transplantation on recurrence in multiply recurrent *Clostridium difficile* infection: a randomized trial. *Ann. Intern. Med.* 165, 609–616. doi: 10.7326/M16-0271

Koo, H., Hakim, J. A., Crossman, D. K., Kumar, R., Lefkowitz, E. J., and Morrow, C. D. (2019). Individualized recovery of gut microbial strains post antibiotics. *NPJ Biofilms Microbiomes* 5:30. doi: 10.1038/s41522-019-0103-8

Kootte, R. S., Levin, E., Salojärvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., et al. (2017). Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. *Cell Metab.* 26, 611–619.e6. doi: 10.1016/j.cmet.2017.09.008

Korpela, K., Salonen, A., Virta, L. J., Kekkonen, R. A., Forslund, K., Bork, P., et al. (2016). Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. *Nat. Commun.* 7:10410. doi: 10.1038/ncomms10410

Lam, S., Bai, X., Shkoporov, A. N., Park, H., Wu, X., Lan, P., et al. (2022). Roles of the gut virome and mycobiome in faecal microbiota transplantation. The lancet. *Gastroenterol. Hepatol.* 7, 472–484. doi: 10.1016/S2468-1253(21)00303-4

Letunic, I., and Bork, P. (2019). Interactive tree of life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res.* 47, W256–W259. doi: 10.1093/nar/gkz239

Li, J.-Y., Guo, Y. C., Zhou, H. F., Yue, T. T., Wang, F. X., Sun, F., et al. (2023). Arginine metabolism regulates the pathogenesis of inflammatory bowel disease. *Nutr. Rev.* 81, 578–586. doi: 10.1093/nutrit/nuac070

Liptak, R., Gromova, B., and Gardlik, R. (2021). Fecal microbiota transplantation as a tool for therapeutic modulation of non-gastrointestinal disorders. *Front. Med.* 8:665520. doi: 10.3389/fmed.2021.665520

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

McDonald, L. C., Gerding, D. N., Johnson, S., Bakken, J. S., Carroll, K. C., Coffin, S. E., et al. (2018). Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin. Infect. Dis.* 66, e1–e48. doi: 10.1093/cid/cix1085

Moayyedi, P., Surette, M. G., Kim, P. T., Libertucci, J., Wolfe, M., Onischi, C., et al. (2015). Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology* 149, 102–109.e6. doi: 10.1053/j.gastro.2015.04.001

Mocanu, V., Zhang, Z., Deehan, E. C., Kao, D. H., Hotte, N., Karmali, S., et al. (2021). Fecal microbial transplantation and fiber supplementation in patients with severe obesity and metabolic syndrome: a randomized double-blind, placebo-controlled phase 2 trial. *Nat. Med.* 27, 1272–1279. doi: 10.1038/s41591-021-01399-2

Morrison, D. J., and Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7, 189–200. doi: 10.1080/19490976.2015.1134082

Mullish, B. H., Quraishi, M. N., Segal, J. P., McCune, V. L., Baxter, M., Marsden, G. L., et al. (2018). The use of faecal microbiota transplant as treatment for recurrent or refractory *Clostridium difficile* infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. *Gut* 67, 1920–1941. doi: 10.1136/gutjnl-2018-316818

Murray, C. J. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399, 629–655. doi: 10.1016/S0140-6736(21)02724-0

Ng, K. M., Aranda-Díaz, A., Tropini, C., Frankel, M. R., van Treuren, W., O'Loughlin, C. T., et al. (2019). Recovery of the gut microbiota after antibiotics depends on host diet, community context, and environmental reservoirs. *Cell Host Microbe* 26, 650–665.e4. doi: 10.1016/j.chom.2019.10.011

Ng, S. C., Kamm, M. A., Yeoh, Y. K., Chan, P. K. S., Zuo, T., Tang, W., et al. (2020). Scientific frontiers in faecal microbiota transplantation: joint document of Asia-Pacific Association of Gastroenterology (APAGE) and Asia-Pacific Society for Digestive Endoscopy (APSDE). *Gut* 69, 83–91. doi: 10.1136/gutjnl-2019-319407

Norman, J. M., Handley, S. A., Baldridge, M. T., Droit, L., Liu, C. Y., Keller, B. C., et al. (2015). Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell* 160, 447–460. doi: 10.1016/j.cell.2015.01.002

Ojima, M. N., Gotoh, A., Takada, H., Odamaki, T., Xiao, J. Z., Katoh, T., et al. (2020). *Bifidobacterium bifidum* suppresses gut inflammation caused by repeated antibiotic disturbance without recovering gut microbiome diversity in mice. *Front. Microbiol.* 11:1349. doi: 10.3389/fmicb.2020.01349

Palleja, A., Mikkelsen, K. H., Forslund, S. K., Kashani, A., Allin, K. H., Nielsen, T., et al. (2018). Recovery of gut microbiota of healthy adults following antibiotic exposure. *Nat. Microbiol.* 3, 1255–1265. doi: 10.1038/s41564-018-0257-9

Paramsothy, S., Kamm, M. A., Kaakoush, N. O., Walsh, A. J., van den Bogaerde, J., Samuel, D., et al. (2017). Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet* 389, 1218–1228. doi: 10.1016/S0140-6736(17)30182-4

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., et al. (2011). Scikit-learn: machine learning in Python. *J. Mach. Learn. Res.* 12, 2825–2830. doi: 10.5555/1953048.2078195

Podlesny, D., Durdevic, M., Paramsothy, S., Kaakoush, N. O., Högenauer, C., Gorkiewicz, G., et al. (2022). Identification of clinical and ecological determinants of strain engraftment after fecal microbiota transplantation using metagenomics. *Cell Reports Medicine*, 3. doi: 10.1016/j.xcrm.2022.100711

Raymond, F., Ouameur, A. A., Déraspe, M., Iqbal, N., Gingras, H., Dridi, B., et al. (2016). The initial state of the human gut microbiome determines its reshaping by antibiotics. *ISME J.* 10, 707–720. doi: 10.1038/ismej.2015.148

Remesh, A. T., and Viswanathan, R. (2024). CrAss-like phages: from discovery in human fecal metagenome to application as a microbial source tracking marker. *Food Environ. Virol.* 16, 121–135. doi: 10.1007/s12560-024-09584-5

Sabtu, N., Enoch, D., and Brown, N. (2015). Antibiotic resistance: what, why, where, when and how? *Br. Med. Bull.* 116:ldv041. doi: 10.1093/bmb/ldv041

Smillie, C. S., Sauk, J., Gevers, D., Friedman, J., Sung, J., Youngster, I., et al. (2018). Strain tracking reveals the determinants of bacterial engraftment in the human gut following fecal microbiota transplantation. *Cell Host Microbe* 23, 229–240.e5. doi: 10.1016/j.chom.2018.01.003

Smith, B. J., Piceno, Y., Zydek, M., Zhang, B., Syriani, L. A., Terdiman, J. P., et al. (2022). Strain-resolved analysis in a randomized trial of antibiotic pretreatment and maintenance dose delivery mode with fecal microbiota transplant for ulcerative colitis. *Sci. Rep.* 12:5517. doi: 10.1038/s41598-022-09307-5

Stachler, E., Kelty, C., Sivaganesan, M., Li, X., Bibby, K., and Shanks, O. C. (2017). Quantitative CrAssphage PCR assays for human fecal pollution measurement. *Environ. Sci. Technol.* 51, 9146–9154. doi: 10.1021/acs.est.7b02703

Strati, F., Pujolassos, M., Burrello, C., Giuffrè, M. R., Lattanzi, G., Caprioli, F., et al. (2021). Antibiotic-associated dysbiosis affects the ability of the gut microbiota to control intestinal inflammation upon fecal microbiota transplantation in experimental colitis models. *Microbiome* 9, 1–15. doi: 10.1186/s40168-020-00991-x

Truong, D. T., Tett, A., Pasolli, E., Huttenhower, C., and Segata, N. (2017). Microbial strain-level population structure and genetic diversity from metagenomes. *Genome Res.* 27, 626–638. doi: 10.1101/gr.216242.116

van Lingen, E. (2023). Short-and long-term follow-up after fecal microbiota transplantation as treatment for recurrent Clostridioides difficile infection in patients with inflammatory bowel disease. *Ther. Adv. Gastroenterol.* 16:17562848231156285. doi: 10.1177/17562848231156285

Van Nood, E. (2013). Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *N. Engl. J. Med.* 368, 407–415. doi: 10.1056/NEJMoa1205037

Virtanen, P., Gommers, R., Oliphant, T. E., Haberland, M., Reddy, T., Cournapeau, D., et al. (2020). SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* 17, 261–272. doi: 10.1038/s41592-019-0686-2

Wang, Y., Wiesnoski, D. H., Helmink, B. A., Gopalakrishnan, V., Choi, K., DuPont, H. L., et al. (2018). Fecal microbiota transplantation for refractory immune checkpoint inhibitor-associated colitis. *Nat. Med.* 24, 1804–1808. doi: 10.1038/s41591-018-0238-9

WHO. World Health Organization: International clinical trials registry platform (ICTRP). (2023); Available online at: https://trialsearch.who.int/ (Accessed November 07, 2024).

Wood, D. E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with kraken 2. *Genome Biol.* 20, 1–13. doi: 10.1186/s13059-019-1891-0

Xu, L., Surathu, A., Raplee, I., Chockalingam, A., Stewart, S., Walker, L., et al. (2020). The effect of antibiotics on the gut microbiome: a metagenomics analysis of microbial shift and gut antibiotic resistance in antibiotic treated mice. *BMC Genomics* 21, 1–18. doi: 10.1186/s12864-020-6665-2

Zhang, A.-N., Gaston, J. M., Dai, C. L., Zhao, S., Poyet, M., Groussin, M., et al. (2021). An omics-based framework for assessing the health risk of antimicrobial resistance genes. *Nat. Commun.* 12:4765. doi: 10.1038/s41467-021-25096-3

Zuo, T., Wong, S. H., Lam, K., Lui, R., Cheung, K., Tang, W., et al. (2018). Bacteriophage transfer during faecal microbiota transplantation in *Clostridium difficile* infection is associated with treatment outcome. *Gut* 67, 634–643. doi: 10.1136/gutjnl-2017-313952

frontiers | Frontiers in Microbiomes

TYPE Systematic Review
PUBLISHED 16 May 2025
DOI 10.3389/frmbi.2025.1553243

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Christina Tsigalou,
Democritus University of Thrace, Greece
Elias Toubi,
Technion Israel Institute of Technology, Israel

*CORRESPONDENCE
Mohammad Adawi
adawimo1802@gmail.com

RECEIVED 30 December 2024
ACCEPTED 22 April 2025
PUBLISHED 16 May 2025

CITATION
Adawi M (2025) The role of gut microbiota in autoimmune disease progression and therapy: a comprehensive synthesis. *Front. Microbiomes* 4:1553243. doi: 10.3389/frmbi.2025.1553243

COPYRIGHT
© 2025 Adawi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The role of gut microbiota in autoimmune disease progression and therapy: a comprehensive synthesis

Mohammad Adawi*

Laniado MC, Ariel University, General Health Services, Natanya, Israel

Autoimmune diseases arise from the immune system's dysregulated attack on the body's own tissues, influenced by a complex interplay of genetics, environment, and the microbiome. This comprehensive review and meta-analysis examines the dynamic relationship between gut microbiota and autoimmune diseases, highlighting their role in disease onset, progression, and potential therapeutic interventions. Emerging evidence underscores the bidirectional interactions between microbiota and immune pathways, particularly through mechanisms like mucosal immune modulation and regulatory T-cell activity. Microbiota dysbiosis, characterized by altered diversity and function, is consistently associated with autoimmune conditions such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes. The review identifies critical microbiota-driven factors, including antigenic mimicry and inflammatory signaling pathways that disrupt immune tolerance and exacerbate autoimmunity. Meta-analysis findings reveal a consistent reduction in microbial diversity across autoimmune diseases, emphasizing the role of specific taxa and their metabolites in influencing disease severity and immune responses. Therapeutic strategies, such as probiotics, prebiotics, and microbiome-targeted interventions, offer promising avenues to restore microbiome balance and mitigate autoimmune inflammation. Despite significant advances, challenges in methodology, limited longitudinal studies, and heterogeneity in results highlight the need for standardized research protocols and larger, well-controlled clinical trials. Future studies should prioritize personalized approaches to microbiome modulation, integrating dietary, genetic, and environmental factors to improve disease management and prevention. This work consolidates current knowledge, providing a framework for future research and clinical applications in the field of microbiome-autoimmune interactions.

KEYWORDS

gut microbiome, autoimmune diseases, dysbiosis, immune regulation, microbiome-targeted therapy

1 Introduction

Autoimmune diseases are conditions in which the immune system, which typically fights off foreign invaders, turns its destructive power against the body's own cells or tissues. This aberrant response can result from the interplay between genetics, environment, and gut microbiota. The gut microbiota are the microorganisms living under homeostasis in our digestive tracts, including bacteria, viruses, fungi, and protozoa. Exposing the gut to foreign environmental antigens should result in either an inactivating mucosal immune response or immune tolerance. However, a disruption to the gut microbiome or impaired mucosal barrier can allow entering antigens to interface with the mucosal immune system in a pro-inflammatory way. Over time, this dysfunction can trigger a systemic immune response, driving pathogenic immune cells and autoimmune disease. We, therefore, undertook a comprehensive review explore the potential relationships between the gut microbiome and various autoimmune diseases. We found experimental evidence that the probable relationships become apparent through modulation of microbiota communities or their individual members, which has led to a demonstrable impact on autoimmunity in both human and rodent gut models of disease and is, therefore, now a widely accepted concept. A class of T-helper 17 cells was identified as the mucosal pathogen at the forefront of this mechanism. This cell population is positively correlated with disease severity. We further discussed the potential functional roles of vaccine-induced responses to the microbiome in autoimmune disease abrogation and treatment. As such, the gut microbiome has potential diagnostic significance to predict disease progression (Belkaid and Hand, 2014; De Luca and Shoenfeld, 2018).

1.1 Background and rationale

Autoimmune and autoinflammatory diseases (ADs and AIDs) represent a significant health burden. The microbiome, which encompasses the microbiota, the host genome, and environmental factors, creates an environment that influences immune responses. ADs and AIDs are linked to the microbiome through factors such as the gut-brain axis and the determination of predisposition. With the increasing interest in the microbiome, much research has focused on the pathology of each individual disease, yet this has yielded controversial and inconsistent results. Integrating each disease from the perspective of genetics and clinical phenotypes is necessary, systematic review can help to illustrate the changes of the microbiome in the different paths and phenomena within human microbial diseases. The mechanisms of the relationship between the microbiome and autoimmunity can also be demonstrated through the literature. We collect up-to-date related research and select the available studies by filtering criteria and searching on reputable global articles. We illustrate the features and species affecting microbial diversity in ADs through alpha and beta diversity analyses and create a pipeline that can demonstrate the relationship among diseases (Zhang et al., 2023).

1.2 Scope and objectives

The review and original analysis herein undertaken help provide a connection between autoimmune diseases and antibiotic use, as well as the digestion of specific immune-modulating dietary components, such as fibers and sweeteners. The primary objective of the review was to develop a comprehensive overview of all current mapping studies on the microbiome of autoimmune diseases in order to establish relationships among them and find common trends that will ultimately be able to explain the current results trend for both the microbiome's impact on autoimmune diseases and autoimmune disease effects on the microbiome. A second perspective of the work is to provide a mapping framework for all future studies on this topic and develop a way of tracking research on this subject. The third objective of the work is to show that disparities exist between studies and that more publications on microbiome and autoimmunity disease research are needed to provide sufficient information to develop a deeper understanding of microbiome contributions to autoimmune disease development and progression. Lastly, it is important to identify the haphazard use of the rational manipulation of the microbiome that can help prevent or cure new and even existing illnesses. To achieve this result, this review evaluates the significant methodical disparities among microbiome studies and how they affect the results of the study and how the relative exiguity of strategic treatment recommendations can be adopted from up-to-date research findings. Furthermore, we explore pertinent proof gathered in the progression of some of the most common ailments, demonstrating that other microbiome manipulation techniques are corroborated and that significant scientific trials are missing. To fulfill these aims, we entice studies from a broad range of sectors and, focused on current knowledge, employ an interdisciplinary approach to merge all relevant sources. Cutbacks in internal microbiome multifaceted diversity are associated with serious diseases, including autoimmunity. In this assessment, we have previously expanded work to benefit the public; particularly, approaches concerned with autoimmune diseases incorporate joint information (Wang et al., 2024).

2 Understanding the microbiome

While some scientists and laypersons have known about the existence of commensal bacteria in the human gut and on the skin for decades, many breakthrough discoveries in the understanding of how these species contribute to host biology and environmental ecology have occurred within the last 20 to 30 years, enabled by innovations in bioinformatics and supported by the rapidly decreasing costs of whole-genome shotgun sequencing. These species, individually termed microbiota, interact with the host and environmental pathogens to provide a number of essential functions that the host lacks, including metabolism of complex carbohydrates, fermentation of otherwise indigestible substrates, and metabolism of plant polysaccharides, which together produce short-chain fatty acids, such as butyrate, acetate, and propionate,

that can contribute upward of 10% of a human's total caloric intake. The bacterial residents of the gastrointestinal lumen also play critical roles in resistance to exogenous pathogens by facilitating the development of the host's mucosal immunity. Animal models suggest that gut commensal bacteria play an important role in modulating a variety of autoimmune responses, including neurological autoimmunity, thyroiditis, and gastritis. Indeed, the presence of many of these species in the infant gut influences early development of the immune system and etiological risk for autoimmune diseases. Moreover, maintenance of a healthy colonic microbiome influences the chronic inflammation and disease progression homeostasis in patients with many autoimmune and autoinflammatory diseases. Homeostatic autoreactivity in these organs is shaped directly by the presence of elevated pro-inflammatory metabolites, especially in the gut and oral microbiome. High levels of systemic lipopolysaccharides and autoantigens are, consequently, prone to breach the normally protective gut lumen barrier, leading to chronic inflammation and breakdown in oral tolerance that can contribute to the genesis and persistence of autoimmune disease (Berg et al., 2020).

2.1 Definition and composition

The gastrointestinal microbiome, also termed microbiota, is typically described as a distinct unit of bacterial communities. In addition to prokaryotic bacteria, the biliary microbiome also consists of fungal and eukaryotic components connected to liver diseases. Nevertheless, the gut microbiome is composed of thousands of different taxa, including bacteria, viruses, and fungi, incorporated in a specific environment of host-fed, whole habitat bacterial complexes. Moreover, metatranscriptomics and metaproteomics have indicated that functional performance is not fairly mirrored by every taxonomic inventory. Human feces can reach bacterial particle concentrations as high as 10^11 or 10^12 per gram of feces, leading to the duplication of over 10 million genes in human intestinal communities. Therefore, setting apart person-to-person genetic diversity, the coding project DNA contained by indigenous human microorganisms far surpasses the predictable gene copies found in the genome of humans alone. Indeed, the microbial gene count might be as much as 150 times as heavy as the related host genome (Gosalbes et al., 2011).

2.2 Role in human health

The development of the human immune system is influenced by both genetic and environmental factors. In recent years, scientific interest has been actively developing, linking the state of the human immune system with environmental influences, including microbiota and its components. Accumulated evidence suggests that the microbiota contributes to the education of the immune system. The shaping of immune-competent cells of the systemic immune system and the balance between the processes of tolerance and immune activation are not completely formed, with the education of the child's immune system including the synthesis of cytokines that form the direction of the immune response closely linked to the presence of an intact microbiota. The first experimental evidence indicating the influence of the microbiota on human health was discovered in studies evaluating the development and functionality of the immune system. These studies concluded that the colonization of children with 'good' microorganisms after birth is important for both the development of resistance to infections and the prevention of inflammatory conditions. Studies assessing groups of monozygotic and dizygotic twins showed a greater similarity between monozygotes associated with a more similar structure of the microbiota and greater variability in the expression of genes associated with immunity in dizygotes. In other words, the development of an individual is characterized by the personality of immunity, which is related to the unique individual structure of the microbiota. More recent studies have described a complex relationship between microbes, immunity, and disease. The result of this balance between the two is the state of the host immune responses, with the microbiota influencing a variety of different aspects. It determines our defenses against infections and modulates our immune responses to commensal or harmful microbes, influences the development of immune tolerance at epithelial and systemic levels, shapes the profiles of different effector elements, both innate and acquired, and influences the function of various hematopoietic cells in secondary lymphoid organs and barriers, such as intestine and lung (Ahn and Hayes, 2021).

3 Autoimmunity: mechanisms and implications

Autoimmune diseases are an increasingly prevalent group of non-communicable disorders driven by autoimmunity, which is defined as an adaptive immune response that damages its host. Autoimmunity is a complex disorder characterized by the presence of circulating autoantibodies and autoreactive T cells against self-antigens, combined with an autoimmune response against specific organs and tissues of the body. Autoimmune diseases have a strong genetic basis; this is supported by classical twin studies, sibling studies, and segregation analyses of familial risk. However, genes do not act in isolation; non-genetic factors related to the environment, and especially the gut microbiota, provide further potential insights into the development of these diseases. The term microbiota encompasses all the genetic material within a microbiome, including bacteria, archaea, fungi, protozoa, and viruses, alongside their cohabiting commensal and sentinel immune cell partners. The microbiota exerts a protective influence on autoimmunity by promoting immune regulation, although it can also trigger a pro-inflammatory immune response, thus favoring the progression of autoimmune responses and the development of inflammatory and autoimmune diseases. The exact nature of the microbiome's influence on autoimmunity is likely to be multifactorial. The implications of the cross talk between the microbiome and autoimmunity are widespread and prompt us to consider how to

best maintain a harmonious relationship with the microbiome to promote health and well-being throughout our lives. Data from humanized mice showed that the ability to develop immune tolerance is lost in response to colonization and the interaction with environmental bacteria—a process described as the hygiene deficit. In these mice, the development of autoimmune diseases such as type 1 diabetes or other inflammatory diseases became faster than in specific pathogen-free animals. In sum, certain environmental bacteria or other microorganisms might protect the host from the development of chronic mucosal diseases by acting as natural adjuvants or immunostimulants that induce a tonic volumetric homeostasis of the immune system (Miller, 2022).

3.1 Definition and examples

The concept of the "microbiome" refers to all microbes, their genetic material, or their products within a specific compartment or in the organism. The most studied microbiome is the gut microbiome, but there are other microbiomes such as the lung, skin, and oral microbiomes. Like human genetic data, the microbiome is incredibly diverse and variable. There is great variability in microbiome composition between individuals and body sites within individuals. The microbiome is influenced by host genetics, the local environment, health status, and external interventions such as antibiotics, immunosuppressants, and anti-inflammatories (Hou et al., 2022).

3.2 Pathogenesis and triggers

The Gut Barrier and Autoimmunity Mechanisms that are responsible for the development of autoimmune diseases often feature damage to bodily tissues due to T-cell mediated auto aggression. In the case of type 1 diabetes, for example, the major antigen is expressed on the islet beta-cell surface. Once infiltrated, the immune system can differentiate these cells as pathologically changed and launch an immune response against them. The natural history of such disease development has a number of clear steps. First, the immune system of susceptible individuals is sensitized to self-antigens due to exposure to various triggers. Second, antigen-specific, autoreactive T cells escape thymic negative selection and are activated by environmental triggers, moving the disease into the clinically silent phase. Finally, the autoimmune reaction that leads to disease manifests and progresses. The triggers that initiate autoantigen self-activation in order to form clonal proliferating immune cells and break self-tolerance have historically been divided into interplaying genetic and environmental factors. The disease itself or a genetic predisposition to some pathologies could sometimes be introduced into a healthy body by the transplantation of hematopoietic stem cells carrying apoptotic bodies formed in the blood of diseased subjects. This process has been called disorder transfer through blood stem cell transplantation. These data, therefore, strongly support the hypothesis that immune cells committed to auto aggression can enter a quiescent state, and this trait could be inherited by daughter cell generations. After that, disruption in peripheral control mechanisms could provoke disease onset. Despite the fact that among autoimmune diseases, there are both candidates for thymic presentation and ectopic lymphoid organs, there is no clear proof of the general role of the thymus in controlling autoimmune reactions (Burrack et al., 2017).

4 The interplay between microbiome and autoimmunity

The relationship between the gastrointestinal microbiome and autoimmunity may be bidirectional in nature. Studies to date have indeed shown that the human microbiome is influenced by genetic, nutritional, and environmental factors, including alterations affected by medications. The impact of diet and environmental exposure, including living with pets and geographical location, on the human microbiota and on the immune system in health and disease has been well characterized. In turn, commensal microbes are critical for the development and maintenance of the host immune system by enhancing innate pattern recognition receptor expression, maintaining the recruitment of immunosuppressive cells such as regulatory T cells, and protecting the host against infection. Children and adults with autoimmune diseases are at risk for expression of the disease phenotype given the potential susceptibility of their microbiota. In animal models, it has been demonstrated that the gut microbiota can direct not only the frequency, function, and phenotypic characteristics of conventional Tregs in multiple mouse strains but can also support the differentiation of pTregs and the programming of pTregs. Tregs are present in the thymus of germ-free mice, suggesting that the gut microbiota is required for their generation in the thymus rather than their circulation from the thymus into the periphery. Given the influence of the microbiome on Tregs, the possibility that segmented filamentous bacteria or other luminal microfloral clusters may survive or migrate extra-intestinally to cause an untoward Treg response in a specific genetic host remains a tantalizing possibility. Thus, given the well-characterized importance of Tregs in controlling T-cell-dependent autoimmunity, commensal bacteria have been hypothesized to likewise modulate autoimmune diseases. It is also important to consider that these concepts may be subject to alternative explanations (De Luca and Shoenfeld, 2018; Hou et al., 2022).

4.1 Direct interactions

Several direct interactions have been described between the microbiome and the host immune system when a trigger like a pathogen infects the host. For example, lactate produced by Lactobacillus has immunosuppressive effects during growth. Microbe ligands also influence APCs upon TCR triggering and promote Th activation. Components of viruses, bacteria, fungi, and protozoans can directly lead to cross-reactive responses with

peptides of self-antigens, increasing autoimmunity. This leads to an equilibrium in the immune system where innate and adaptive immune responses against commensals are accompanied by an induction of regulatory mechanisms that avoid immunopathology induction.

In the classical concept of a commensal lymphoid trigger, microbes induce Th17 and regulatory T cell responses that influence inflammation and homeostasis in parallel with T permeating before reported inflammation initiation. Microbes have epigenetic effects on genes that regulate the immune response, such as TLR and NOD2. Some bacteria can also produce SCFAs that can act to modulate signals in the immune system, which has important effects on inflammation and host adaptation. It is important to point out that Eubacterium limosum is capable of regulating Th/T CD4+ T ratios by expanding regulatory T cells. The species also produces a key ligand for the TCR and it is able to induce Th17 and CD4+ responses that contribute to autoimmunity if this colitogenic bacterium colonizes the gut late in life (Wiertsema et al., 2021).

4.2 Indirect effects

Recent evidence suggests that both gut dysbiosis and a disrupted oral microbiota are key players involved in the etiology of autoimmune diseases. Since the microbiome is capable of modifying both the innate and adaptive immune systems, indirect effects of the microbiome on autoimmune diseases are often observed. Therefore, the microbiome indirectly affects host immune response homeostasis through the mucosal immune system (Shaheen et al., 2022).

5 Methodology and literature search strategy

A literature extraction was conducted on various databases for peer-reviewed conditions employing thyroid, connective tissue and intestines. A focused search in journals of immunology, immunotherapy, microbiome, autoimmunity, inflammation, and allergy was conducted, respectively (Shi et al., 2017). A comprehensive systematic literature search was conducted in the MEDLINE database using a Medical Subject Headings (MeSH) string combined with the Boolean operators "AND" and "OR." The search terms for the first concept were as follows: "autoimmunity," "autoimmune disease," "autoimmune disorder," and "self-reactive response" combined using "OR." As for the second concept, the exploded MeSH term "bacteria" was used in combination with synonyms for bacteria, which were combined with the Boolean operator "OR" as follows: "acetobacter," "Acetobacteraceae," "Aerococcus," "Bacillaceae," "bacillus," "corynebacterium," and "Escherichia coli." The third concept was set as for the second but using "AND" as follows: "lactic acid bacteria," "Lactobacillaceae," "Lactobacillus," "probiotics," "bifidobacteria," and "bifidi." Moreover, for the final concept, the exploded MeSH term "inflammatory response" was used. As for further concept combinations, we used, as explained above, "AND" or "OR." We used the following major inclusion criteria (1): clinical trials (2) preclinical studies. The exclusion criteria were: (1) reviews (2) research involving prokaryotes (3) no relation to autoimmunity (4) no specific interest in intestinal flora (5) no relationship with probiotics (6) gene not belonging to the prokaryote (7) commentaries.

5.1 Inclusion and exclusion criteria

All studies met the following inclusion criteria: 1) studies had to be original research articles; review papers, case reports, or *in vitro* studies were initially excluded; 2) studies were required to report human primary outcomes related to the microbiome in autoimmune diseases; and 3) the studies had to use high-throughput sequencing to evaluate the composition and/or function of the microbiome. We conducted the literature search regardless of linguistic limitations. After initial exclusions and the subsequent review of the full text, we then evaluated whether the study contained baseline information about the autoimmune disease that was analyzed. Using this approach, the following diseases were selected: RA, T1D, G1D, SLE, and IBD. Then, we evaluated whether the autoimmune disease in question was diagnosed, and thereafter the progression of the effects on the microbiome was analyzed. In this step, studies that did not contain the following information were excluded: genus, family, or species levels; and relative abundance, absolute abundance from differences between the groups, or richness equal to or greater than 50% of the cuts through the taxonomic identification of bacteria (Ventelä et al., 2023).

5.2 Key findings from literature review

Based on the findings of this study, several novel aspects about gut microbiota dysbiosis associated with autoimmunity and inflammatory diseases were discussed. Based on the collated data, we suggest that autoimmune conditions share common features such as enrichment of specific taxa and genes but not groups of taxa. We posit that despite the need for sample size expansion and study verifications, the possible universal method for amelioration of autoimmunity could involve modification of the gut environment and promotion of gut health. To achieve such results, we foresee fundamental influences such as diet, living environment, hygienic conditions, and oral use of gut health-promoting probiotics, and lifestyle changes (McLean et al., 2014; Shaheen et al., 2022).

5.3 Systematic review results

The majority of the exponents in both datasets reported a decrease in microbiome diversity across all major immune-mediated diseases examined. The estimated diversity of

microbiota across all immune-mediated diseases increases by 0.36 when absolute taxonomic richness is considered. Consistently, disease was associated with a 0.11 decrease, furthermore showing little heterogeneity. While no clear conclusions may be drawn from a funnel plot, the medium number of studies did not directly support any risk of a large number of experiments reporting smaller effects. Thus, careful interpretation of all results is appropriate; in particular, certain contributory factors regarding the intrinsic implications of the effect may need amendments to current standards in studies of immune-related diseases (Wang et al., 2023).

6 Implications for clinical practice

There are significant clinical implications for the study of the microbiome in relation to autoimmunity. Solutions to diseases like MS, lupus, and rheumatoid arthritis, diseases with a "common thread" for disease onset to understand and control, have thus far been elusive. Treatments such as T-cell therapy or stem cell therapy may be available in some countries, but have significant potential for serious side effects, including mortality. Probiotic therapy presents a paradigm shift in how we may treat diseases such as MS, lupus, and rheumatoid arthritis. These treatments have been described as being active, healthy, and inexpensive and positively contribute to a person's health. There are well-documented outcomes in reducing infections, helping with obesity, and treating inflammatory bowel disease, all without significant negative side effects. In the context of autoimmunity, there is unique power in the potential ability to correct the problem at its very earliest cellular stages, prior to activation of innate and adaptive immune responses. Finally, the growing scientific support for the microbiome's role in autoimmunity reinforces the notion that probiotic use can be rational to recommend, and that such recommendations can be embodied by guidelines that a physician can integrate into clinical practice (Bogović Crnčić et al., 2024).

6.1 Potential therapeutic strategies

Targeted gastrointestinal manipulation is an intriguing area. Dietary interventions can be an attractive strategy for these microbiome-targeted therapies, including prebiotics, probiotics, fecal microbiota transplantation, and/or synbiotics, as well as the incorporation of novel non-digestible carbohydrates, polyunsaturated fatty acids, and a variety of other nutrition-based intakes referred to as postbiotics and metabiotics. It is also well known that bacteria in co-culture tend to be more effective; this has paved the way for a viable combination and greater efficacy in therapeutics. Postbiotics such as short-chain fatty acids, melatonin, and indole derivatives can mitigate inflammation. To this end, a more comprehensive analysis has shown that the adjuvant therapy of melatonin in addition to traditional antidepressant therapy appears to be useful in improving depressive symptoms. Small molecules from certain bacteria reverse microbiota changes that contribute to obesity and metabolic disorders, suggesting that these molecules may be useful to mitigate the risk of developing depression in patients with obesity. Banana flakes mixed with lupin have shown a promising effect in regulating the gut microbiome and inflammation in animal studies. Gut microbiome-targeted therapeutics aim to correct the dysbiosis in the mucosa and restore the network of bacterial targeting and crosstalk between multiple immune-influencing cells, both innate and adaptive that dictate gut tolerance or enteric inflammation. Hence, such a therapy could represent a promising approach to prevent and/or control dysbiosis-induced systemic inflammation, which is considered to contribute to autoimmune and autoimmune-related conditions. Collectively, a large body of animal and human studies has suggested that microbial bioproducts can harness enteric immune system development and function and beneficially clear systemic inflammation, which can ultimately drive systemic autoimmune responses (Cresci et al., 2020).

6.2 Future research directions

Autoimmune diseases constitute a significant public health burden, particularly in Western societies. Information regarding genes, dietary and lifestyle factors, and the immune phenotype that are linked to the microbiome can provide a synthesis of the triggering mechanisms. Future research studies focusing on both set points of the immune phenotype and long-term probiotic administration are needed to gain a full understanding of the potential therapeutic interventions related to the human microbiome (Kim et al., 2023).

7 Conclusion and summary

Currently, studies focusing on the role of the microbiome in autoimmunity are predominantly described using animal models. Different methodologies that lack sufficient validity result in unsystematic data analysis. The diminished sample size utilization leads to a scattering of data distribution and robustness. We are aware of the limitations of human studies, including the potential risk of bias. Animal model studies can sometimes provide controlled measures of disease induction and resolution. The role of microbiome influences on autoimmunity is more elusive than for infectious diseases. At this stage, it is not possible to predict the accrual necessary to definitively show clinical benefits of specific microbiome manipulations. Basic and clinical experiments should be incorporated into evaluating impacts on the microbiome in the context of autoimmune states. Additional robust studies and more meta-analyses are required before the underlying pathological mechanisms of microbiome influence on autoimmunity are better elucidated. Such studies are still opening new avenues and have the potential to decrease inflammation through manipulation of the microbiome and may avert or delay the onset of autoimmunity. In

summary, our analyses suggest that the role of the microbiome in autoimmunity is credible, and current evidence supports the development of strategies to modulate the variability in gut microbiota for the management of autoimmune diseases.

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.

Author contributions

MA: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

Conflict of interest

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

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Ahn, J., and Hayes, R. B. (2021). Environmental influences on the human microbiome and implications for noncommunicable disease. *Annu. Rev. Public Health* 42, 277–292. doi: 10.1146/annurev-publhealth-012420-105020

Belkaid, Y., and Hand, T. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Berg, G., Rybakova, D., Schelkle, B., Schloter, M., et al. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8, 103. doi: 10.1086/s40168-020-00875-0

Bogović Crnčić, T., Ćurko-Cofek, B., and Batičić, L. (2024). Navigating the neuro-immunomodulation frontier: pioneering approaches and promising horizons—A comprehensive review. *Int. J. Mol. Sci.* 25, 9695.

Burrack, A. L., Martinov, T., and Fife, B. T. (2017). T cell-mediated beta cell destruction: autoimmunity and alloimmunity in the context of type 1 diabetes. *Front. Endocrinol. (Lausanne)* 8, 343. doi: 10.3389/fendo.2017.00343

Cresci, G. A. M., Lampe, J. W., and Gibson, G. (2020). Targeted approaches for *in situ* gut microbiome manipulation. *JPEN J. Parenter Enteral Nutr.* 44, 581–588. doi: 10.1002/jpen.v44.4

De Luca, F., and Shoenfeld, Y. (2018). The microbiome in autoimmune diseases. *Clin. Exp. Immunol.* 195, 74–85. doi: 10.1111/cei.13158

Gosalbes, M. J., Durbán, A., Pignatelli, M., Abellan, J. J., Jiménez-Hernández, N., Pérez-Cobas, A. E., et al. (2011). Metatranscriptomic approach to analyze the functional human gut microbiota. *PLoS One* 6, e17447. doi: 10.1371/journal.pone.0017447

Hou, K., Wu, Z. X., Chen, X. Y., Wang, J. Q., Zhang, D., Xiao, C., et al. (2022). Microbiota in health and diseases. *Signal Transduction Targeted Ther.* 7, 135. doi: 10.1038/s41392-022-00974-4

Kim, C. S., Jung, M. H., Choi, E. Y., and Shin, D. M. (2023). Probiotic supplementation has sex-dependent effects on immune responses in association with the gut microbiota in community-dwelling older adults: a randomized, double-blind, placebo-controlled, multicenter trial. *Nutr. Res. Pract.* 17, 883–898. doi: 10.4162/nrp.2023.17.5.883

McLean, M. H., Dieguez, D. Jr, Miller, L. M., and Young, H. A. (2014). Does the microbiota play a role in the pathogenesis of autoimmune diseases? *Gut* 64, 332–341. doi: 10.1136/gutjnl-2014-308514

Miller, F. W. (2022). The increasing prevalence of autoimmunity and autoimmune diseases: an urgent call to action for improved understanding, diagnosis, treatment and prevention. *Curr. Opin. Immunol.* 80, 102266. doi: 10.1016/j.coi.2022.102266

Shaheen, W. A., Quraishi, M. N., and Iqbal, T. H. (2022). Gut microbiome and autoimmune disorders. *Rev. Clin. Exp. Immunol.* 209, 161–174. doi: 10.1093/cei/uxac057

Shi, N., Li, N., Duan, X., and Niu, H. (2017). Interaction between the gut microbiome and mucosal immune system. *Mil Med. Res.* 14. doi: 10.1186/s40779-017-0122-9

Ventelä, J., Korja, M., Auvinen, A., Lohi, O., and Nikkilä, A. (2023). Dual direction associations between common autoimmune diseases and leukemia among children and young adults: A systematic review. *Cancer Epidemiol.* 86, 102411. doi: 10.1016/j.canep.2023.102411

Wang, T., Sternes, P. R., Guo, X. K., Zhao, H., Xu, C., and Xu, H. (2023). Autoimmune diseases exhibit shared alterations in the gut microbiota. *Rheumatol. (Oxford)* 63, 856–865. doi: 10.1093/rheumatology/kead364

Wang, X., Yuan, W., Yang C, Wang, Z., Zhang, J., Xu, D., Sun, X., et al. (2024). Emerging role of gut microbiota in autoimmune diseases. *Front. Immunol.* 63 (3), 856-865. doi: 10.3389/fimmu.2024.1365554

Wiertsema, S. P., van Bergenhenegouwen, J., Garssen, J., and Knippels, L. M. J. (2021). The interplay between the gut microbiome and the immune system in the context of infectious diseases throughout life and the role of nutrition in optimizing treatment strategies. *Nutrients* 13, 886. doi: 10.3390/nu13030886

Zhang, Y., Peng, Y., and Xia, X. (2023). Autoimmune diseases and gut microbiota: a bibliometric and visual analysis from 2004 to 2022. *Clin. Exp. Med.* 23, 2813–2827. doi: 10.1007/s10238-023-01028-x

frontiers | Frontiers in Microbiology

TYPE Systematic Review
PUBLISHED 31 July 2025
DOI 10.3389/fmicb.2025.1604571

Check for updates

OPEN ACCESS

EDITED BY
Giuseppe Guido Maria Scarlata,
University "Magna Graecia" of Catanzaro, Italy

REVIEWED BY
Najla A. Obaid,
Umm Al-Qura University, Saudi Arabia
Yomna Ali,
Alexandria University, Egypt
Yan Xu,
Emergency General Hospital, China

*CORRESPONDENCE
Sheng Zhang
aloof3737@126.com

†These authors have contributed equally to this work

RECEIVED 02 April 2025
ACCEPTED 23 June 2025
PUBLISHED 31 July 2025

CITATION
Wang K, Gao C, Zhu L, Chen M, Tong YX and Zhang S (2025) Fecal microbiota transplantation for chronic constipation: a systematic review and meta-analysis of clinical efficacy, safety, and microbial dynamics.
Front. Microbiol. 16:1604571.
doi: 10.3389/fmicb.2025.1604571

COPYRIGHT
© 2025 Wang, Gao, Zhu, Chen, Tong and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbiota transplantation for chronic constipation: a systematic review and meta-analysis of clinical efficacy, safety, and microbial dynamics

Ke Wang[1], Chun Gao[2†], Li Zhu[2†], Min Chen[2], Yi Xin Tong[2] and Sheng Zhang[2*]

[1]The Second Department of Surgery, Jiyuan Hospital of Traditional Chinese Medicine, Jiyuan, China, [2]Department of Gastrointestinal Surgery, Tongji Medical College, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China

Background: Chronic constipation, a prevalent gastrointestinal disorder with limited treatment efficacy in refractory cases, has prompted exploration of fecal microbiota transplantation (FMT) as a novel therapeutic strategy. This systematic review and meta-analysis evaluate the efficacy, safety, and gut microbial dynamics of FMT in adults with chronic constipation.

Methods: We systematically searched PubMed, Embase, Web of Science, and Cochrane Library up to January 2025, identifying 1,072 records. Nine studies (n = 245 patients) met inclusion criteria for qualitative synthesis, with eight contributing to meta-analysis. Outcomes included constipation remission and improvement, stool metrics, quality of life, and microbiota changes. Random-effects models analyzed pooled remission rates, mean differences (MDs), and heterogeneity (I^2 statistics).

Results: Fecal microbiota transplantation achieved a 50.7% pooled remission rate (95% CI: 38.7%–62.7%) and 64.8% improvement rate (95% CI: 51.4%–76.3%). Significant improvements were observed in stool consistency (MD = 1.32, 95% CI: 1.05–1.35), quality of life (GIQLI MD = 32.19, 95% CI: 17.15–47.23), and symptom severity (Wexner MD = −4.83, 95% CI: −7.15–2.51). Post-FMT microbiota analyses revealed enrichment of beneficial taxa (Bifidobacterium, Prevotella; Firmicutesacteroidetes) and suppression of pro-inflammatory Enterobacteriaceae. Transient gastrointestinal adverse events (e.g., bloating: 17.3%) resolved spontaneously, with no severe complications reported.

Conclusion: Fecal microbiota transplantation demonstrates clinically meaningful symptom relief and gut microbiota remodeling in chronic constipation, supported by favorable short-term safety. While heterogeneity in protocols and limited RCT data warrant caution, these findings advocate standardized FMT frameworks and confirmatory trials to optimize therapeutics for refractory constipation.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42025643634.

KEYWORDS

fecal microbiota transplantation, chronic constipation, gut microbiome, microbiota remodeling, refractory constipation

Introduction

Chronic constipation, a prevalent functional gastrointestinal disorder affecting 10%–15% of adults globally, imposes substantial burdens on quality of life and healthcare systems. Its prevalence escalates with age, disproportionately impacting women (female-to-male ratio ∼2:1), and manifests as heterogeneous subtypes: slow-transit, outlet obstruction, or a combination thereof—as defined by Rome IV criteria. First-line therapies, including dietary fiber, osmotic laxatives, and prokinetic agents, fail to achieve sustained relief in 30%–40% of patients, underscoring an urgent need for mechanistically grounded interventions targeting underlying pathophysiology (Bharucha and Wald, 2019; Brenner et al., 2022; Drossman and Hasler, 2016; Harris and Chang, 2022; Wald, 2016).

Recent advances have delineated gut microbial dysbiosis as a pivotal contributor to chronic constipation. Comparative metagenomic analyses reveal consistent depletion of short-chain fatty acid (SCFA)-producing taxa (e.g., *Faecalibacterium prausnitzii*) and enrichment of methanogens (e.g., *Methanobrevibacter smithii*) in constipated individuals, disrupting colonic motility through impaired serotonin signaling, altered enteric nervous system activity, and methane-induced slowing of transit (Attaluri et al., 2010; Banaszak et al., 2023; Goya-Jorge et al., 2023; Huang et al., 2018). Germ-free rodent models transplanted with microbiota from constipated donors recapitulate delayed gut motility, while probiotic supplementation partially restores peristalsis, directly implicating microbial communities in disease pathogenesis (Ge et al., 2017; Peng et al., 2023; Roy and Dhaneshwar, 2023).

Fecal microbiota transplantation (FMT), which reconstitutes gut ecological balance through engraftment of donor-derived microbiota, has demonstrated transformative success in recurrent *Clostridioides difficile* infection (cure rates > 90%) and moderate efficacy in ulcerative colitis (Paramsothy et al., 2017; Walter and Shanahan, 2023). In functional gastrointestinal disorders, emerging evidence highlights FMT's potential to modulate gut-brain axis signaling. A 2020 randomized controlled trial (RCT) reported significant improvements in stool frequency and abdominal pain in irritable bowel syndrome patients receiving FMT, mediated by restored microbial diversity and enhanced SCFA production (El-Salhy et al., 2020). Preliminary studies in chronic constipation suggest FMT may ameliorate symptoms by upregulating serotonin biosynthesis, reducing methane production, and reinforcing intestinal barrier integrity, yet current evidence remains limited to small, non-randomized cohorts (Li et al., 2024; Liu et al., 2021; Singh et al., 2022; Wang et al., 2024).

This systematic review and meta-analysis synthesize existing clinical data to rigorously evaluate FMT's efficacy in adult chronic constipation, quantifying its effects on core endpoints (stool frequency, consistency) and patient-reported outcomes. By integrating microbial compositional changes with clinical responses, we aim to define actionable patterns linking microbiota remodeling to therapeutic success. Our findings will address critical gaps in evidence, inform future RCT design, and advance translational strategies leveraging the gut microbiome as a therapeutic target for chronic constipation.

Materials and methods

Protocol and registration

In this study, all procedures were conducted in accordance with the guidelines specified in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2020 (PRISMA-2020) protocol (Page et al., 2021). The methodological approaches were consistent with the standards outlined in the Cochrane Handbook. (Higgins and Thomas, 2024) Additionally, this study has been registered with the International Prospective Register of Systematic Reviews, with the registration number PROSPERO: CRD42025643634.

Search strategy and study selection

Up to January 2025, we conducted searches across four electronic databases: PubMed, Cochrane Library, Scopus, and Web of Science. We conducted our search using the following query to identify relevant studies on fecal microbiota transplantation: ("Fecal Microbiota Transplant*" OR FMT OR "Fecal Microbiota Transplant*" OR "microbiota transplant*" OR "microbiota transfer" OR "microbiome transfer" OR "microbiome transplant*" OR "feces transplant*" OR "Fecal Transplant*" OR "Fecal Transfer" OR "stool Transplant*" OR "stool Transfer" OR "stool therapy" OR "Donor Feces" OR "donor fecal" OR "donor stool" OR "fecal flora transplant*" OR "microflora transplant*" OR "Fecal therapy" OR "fecal bacteriotherapy" OR "feces bacteriotherapy" OR "rectal bacteriotherapy" OR "fecal flora bacteriotherapy" OR "fecal reconstitution" OR "flora reconstitution" OR "microbiome reconstitution" OR "feces reconstitution") AND ("chronic constipation" OR "slow transit constipation" OR "constipation"). We only include studies in English in our search. Furthermore, we reviewed the reference lists of the included studies to identify additional relevant citations.

Titles and abstracts were independently screened by two reviewers, C.G. and M.C., to identify studies meeting the inclusion criteria and exclude irrelevant ones. Duplicate records were systematically removed, and reference lists of selected studies were manually searched to capture additional eligible studies. Discrepancies during the selection process were resolved through consultation with a third reviewer, Y.X.T., ensuring methodological rigor and decision consistency.

Inclusion and exclusion criteria

Studies were considered for full review if they met the following criteria: single-arm or two-arm studies, retrospective studies, prospective studies, and randomized controlled trials, focusing on the effects of FMT on constipation. The population included adults with chronic constipation or slow transit constipation. Studies were excluded if they were abstracts, letters to the editor, conference proceedings, review articles, case reports, comments, or book chapters. Studies with insufficient data to summarize were also Additionally, a manual search of reference lists was conducted to identify potentially relevant articles for further study

inclusion. Exclusion criteria comprised: (1) review articles, case reports, letters, comments, or book chapters; (2) studies with insufficient data to extract predefined efficacy endpoints (e.g., missing stool frequency metrics or symptom scores); (3) non-English publications.

Bowel movement measurements and outcomes

The bowel movement measurement was evaluated by CSBM (complete weekly spontaneous bowel movements), CTT (colonic transit time), and BSFS (Bristol Stool Form Scale). Other evaluations included PAC-SYM (Patient Assessment of Constipation-Symptoms); PAC-QOL (Patient Assessment of Constipation Quality of Life); GIQLI (Gastrointestinal Quality-of-Life Index); SACS (Self-assessment of constipation symptoms). The primary outcome assessed in this systematic review was clinical remission rate, defined as the proportion of patients who had an average of three or more spontaneous complete bowel movements per week during the observation period. The secondary outcome was clinical improvement rate, defined as proportion of patients who were in remission or had an average increase of one or more CSBMs/week compared with baseline. Furthermore, the implementation of fecal microbial profiling during both pre- and post-transplantation phases was systematically recorded and critically appraised across all eligible studies in this systematic review.

Data extraction and quality assessment

In this systematic review, data extraction was independently conducted by two reviewers using a pre-specified data extraction form. Key variables included first author, publication year, trial design, sample size, participant characteristics (e.g., constipation type), FMT intervention details (e.g., administration route), outcome measures (e.g., bowel movement frequency, symptom scores), and primary results (summarized in Table 1). Outcome data from intention-to-treat or per-protocol analyses were prioritized to ensure alignment with original study conclusions. To enhance reliability, the dual-reviewer system was implemented for cross-verification, with any discrepancies re-examined through iterative discussions until consensus was achieved. Additionally, the inclusion or omission of pre- and post-FMT fecal microbiome analysis in each study was explicitly documented to assess microbial dynamics in relation to clinical outcomes.

Study quality was assessed using domain-specific tools: the Cochrane Risk of Bias 2.0 (RoB 2) tool for randomized trials and the Newcastle-Ottawa Scale (NOS) for observational studies. Two reviewers independently evaluated selection bias, performance bias, detection bias, attrition bias, and reporting bias for RCTs using RoB 2's signaling questions. Observational studies were scored across NOS domains (selection, comparability, outcome) (Sterne et al., 2019; Wells et al., 2014). Discrepancies were resolved through consensus. The NOS evaluates studies based on eight criteria, encompassing three key domains: the selection of study participants, the comparability of the study's design and analysis, and the assessment of study outcomes. Additionally, the level of certainty of the evidence presented in each study was assessed utilizing the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology (Guyatt et al., 2011)

Statistical analysis and publication bias

A single-arm meta-analysis was conducted to estimate the pooled response rate of fecal microbiota transplantation (FMT) in chronic constipation. Proportions of responders (defined by study-specific criteria) were synthesized using a generalized linear mixed model (GLMM) with random effects, which accounts for between-study heterogeneity and stabilizes variance in sparse data through logit transformation. The GLMM framework was selected for its robustness in handling overdispersed binomial data and its alignment with recommendations for meta-analyses of proportions. Interstudy heterogeneity was quantified using the Cochran's Q statistic (significance threshold $p < 0.10$) and the I^2 statistic, interpreted as follows: $I^2 < 25\%$ (low), 25%–50% (moderate), 50%–75% (substantial), and $> 75\%$ (considerable). To assess the robustness of pooled estimates, sensitivity analyses were performed by iteratively excluding individual studies. Potential sources of heterogeneity were qualitatively explored through examination of methodological variations in donor selection criteria, fecal processing protocols, and follow-up duration across studies.

A prediction interval (95% PI) was calculated to estimate the potential range of response rates in future studies. The analysis was performed using the metaprop function in the R package meta (v6.5-0). Forest plots were generated to visualize individual study proportions (with 95% confidence intervals), the pooled estimate, and prediction intervals. Weights assigned to each study were derived from the inverse variance method under the random-effects assumption. All statistical code was executed in R 4.3.1, with reproducibility ensured through script-based workflows. The GLMM models included a logit link function and restricted maximum likelihood (REML) estimation for variance components. Due to the absence of randomized controlled trials in the included studies, formal risk of bias assessment was not applicable.

Results

Search results and characteristics of included studies

The systematic search identified a total of 1,072 citations from electronic databases and manual sources, with 377 duplicates removed, yielding 695 unique records for initial screening. After title and abstract review, 52 potentially relevant articles were selected for full-text evaluation. Following rigorous assessment against predefined inclusion criteria (e.g., study design, population, intervention), 43 articles were excluded due to irrelevance to the research question, insufficient data, or protocol overlaps.

TABLE 1 Characteristics of studies included in systematic review.

References	Year		Patients' characteristics	Study design	Sample size	Study endpoints	Follow-up and outcomes
Ge et al., 2016	2016	China	Slow transit constipation	Retrospective, Single-arm FMT group: via nasojejunal tube	21	CSBM, stool consistency (BSFS), GIQLI, PAC-SYM, CTT	12 weeks Remission rate: 42.9% Improvement rate: 66.7% Adverse event
Tian et al., 2016	2016	China	Slow transit constipation	Retrospective, Single-arm FMT group: via nasojejunal tube	24	CSBM, BSFS, Wexner score, GIQLI, CTT	12 weeks Remission rate: 37.5% Improvement rate: 50%
Tian et al., 2017)	2017	China	Slow transit constipation	RCT, FMT (via nasojejunal tube) vs. conventional therapy	49 (25/24)	CSBM, CTT, BSFS, Wexner score	12 weeks Remission rate: 36% vs 13.3% Improvement rate: 52% vs. 20% Adverse event
Ding et al., 2018	2018	China	Slow transit constipation	Propective, Single-arm FMT group: via nasojejunal tube	52	CSBM, BSFS, PAC-SYM, PAC-QOL	24 weeks Remission rate: 32.7% Improvement rate: 44.2% Adverse event
Tian et al., 2020	2020	China	Chronic functional constipation	Propective, Single-arm FMT group: via nasojejunal tube	34	Wexner score, SACS, BSFS, PAC-QOL, SDS, SAS	12 weeks Remission rate: 73.5% Improvement rate: 88.2% Adverse event
Zhang et al., 2021	2021	China	Refractory constipation	Retrospective, Single-arm FMT group: via nasojejunal tube	18	CSBM, stool consistency (BSFS), Wexner score, PAC-SYM, PAC-QOL	4 weeks Remission rate: 77.8% Microbime and metabolite change
Xie et al., 2021	2021	China	Slow transit constipation	Retrospective, Single-arm FMT group: via nasojejunal tube	8	CSBM, Wexner score, GIQLI, HAMD	6 weeks Remission rate: 62.5% Improvement rate: 75% Microbime and metabolite change Adverse event
Yang et al., 2021	2021	China	Mixed constipation	Retrospective, double-arm FMT group vs. Biofeedback group	85 (45/40)	CSBM, BSFS, PAC-SYM, GIQLI	48 weeks Mean CSBM: 3.61/week vs. 2.15/week (1 month); 3.25/week vs. 2.01/week (6 months); 2.71/week vs. 1.98/week (12 months) Microbime and metabolite change Adverse event
Wu et al., 2023	2023	China	Refractory functional constipation	Retrospective, Single-arm FMT group: colonscopy or nasojejunal tube	63	CSBM, Wexner score, BSFS score	24 weeks Remission rate: 50% Improvement rate: 73% Adverse event

FMT, fecal microbiome transplantation; CSBM, complete weekly spontaneous bowel movements; CTT, colonic transit time; BSFS, Bristol Stool Form Scale; PAC-SYM, Patient Assessment of Constipation-Symptoms; SBMs, spontaneous bowel movements; PAC-QOL, Patient Assessment of Constipation Quality of Life, GIQLI, Gastrointestinal Quality-of-Life Index; SACS, Self-assessment of constipation symptoms; SDS, Zung's self-rating depression scale; SAS, Zung's self-rating anxiety scale; HAMD, Hamilton Depression Scale.

10.3389/fmicb.2025.1604571

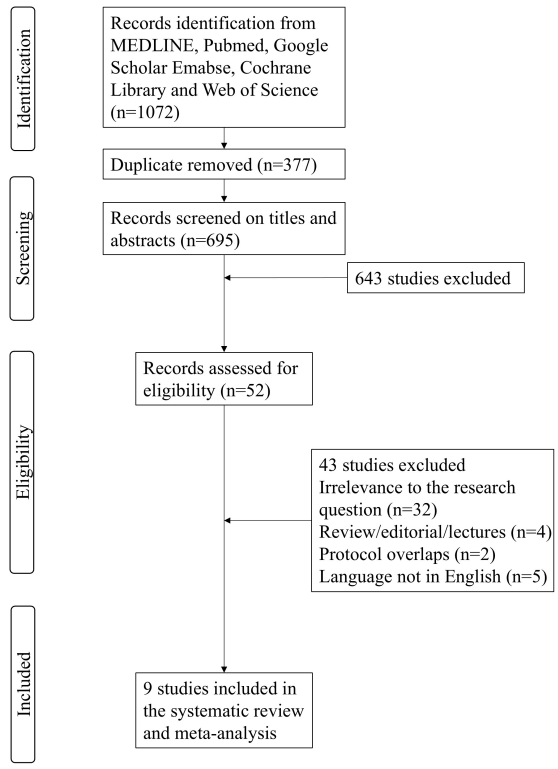


FIGURE 1
PRISMA-2020 flow diagram of search and inclusion strategy.

Ultimately, nine studies met all eligibility requirements and were included in the qualitative synthesis (Ding et al., 2018; Ge et al., 2016; Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021; Yang et al., 2021; Zhang et al., 2021). Of these, eight studies provided extractable outcome data for quantitative meta-analysis. The selection process adhered to PRISMA guidelines, with discrepancies resolved through consensus discussions among reviewers (Figure 1).

Table 1 summarized the characteristics of nine studies included in the meta-analysis. The sample size of included studies ranges from 8 to 85. Majority studies were single-arm studies evaluating FMT on chronic constipation (Ding et al., 2018; Ge et al., 2016; Tian et al., 2016; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021; Zhang et al., 2021). One study compared FMT and biofeedback therapy on constipation (Yang et al., 2021). One RCT compared FMT and conventional therapy on constipation (Tian et al., 2017). The follow-up period ranges from 4 to 48 weeks. Four studies analyzed gut microbiota composition pre- and post-FMT (Tian et al., 2020; Xie et al., 2021; Yang et al., 2021; Zhang et al., 2021). Table 2 presents the microbiota composition data of included studies. Two studies also performed the metabolite analysis pre- and post FMT therapy (Xie et al., 2021; Zhang et al., 2021).

Among the included studies, eight studies had available data to analyze the remission rate of FMT on constipation (Ding et al., 2018; Ge et al., 2016; Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021; Zhang et al., 2021). Seven studies had available data to analyze the improvement rate of FMT on constipation (Ding et al., 2018; Ge et al., 2016; Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021), seven studies (Ge et al., 2016; Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Yang et al., 2021; Zhang et al., 2021), three studies (Ge et al., 2016; Tian et al., 2016; Tian et al., 2017), six studies (Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021; Zhang et al., 2021), four studies (Ge et al., 2016; Tian et al., 2016; Xie et al., 2021; Yang et al., 2021), four studies (Ding et al., 2018; Ge et al., 2016; Yang et al., 2021; Zhang et al., 2021), three studies [(Ding et al., 2018; Tian et al., 2020; Zhang et al., 2021) had data on stool consistency, colonic transit time, Wexner score, GIQLI, PAC-SYM and PAC-QOL respectively.

Pooled efficacy of FMT in the treatment of chronic constipation

As shown in Figure 2, the pooled efficacy of FMT on remission rate of constipation was 50.7% (95%CI 38.7%–62.7%) from eight studies (245 patients, I^2 = 68%). The pooled efficacy of FMT on improvement rate of constipation was 64.8% (95% 51.4%–76.3%) from seven studies (237 patients, I^2 = 72%), indicating that FMT may improve bowel movement in patients with chronic constipation. Meta-analysis also revealed that FMT improved stool consistency and patients' quality of life, with pooled increase mean difference (MD) of 1.32 points (95% CI: 1.05–1.35, 230 patients, I^2 = 88%) and 32.19 points (95%CI: 17.15–47.23, 98 patients, I^2 = 98%) for BSFS and GIQLI score respectively. FMT also significantly relief the constipation related symptoms and improve quality of life, as shown in the pooled MD as −4.83 points (95% CI: −7.15 to 2.51, 172 patients, I^2 = 97%), −3.56 points (95% CI: −9.63 to 2.51, 136 patients, I^2 = 96.4%), −23.2 points (95% CI: −46.5 to 0.03, 104 patients, I^2 = 99%) for Wexner constipation scale, PAC-SYM and PAC-QOL, respectively. In addition, colonic transit time was also significantly decreased after FMT treatment, as shown in the pooled decrease of MD as −20.3 h (95% CI: −28.5 to 12.0 h, I^2 = 94%) (70 patients) from three studies (Figures 3A–F). The summary of the meta-analysis was present in Table 3.

Dynamics of gut microbiome after FMT treatment

Four studies performed fecal microbiome testing based on 16S rDNA Gene analysis before and after FMT of the included patients. All four studies revealed that the composition of fecal microbiota in the post-FMT patients significantly differed from baseline in the phylum-level and genus-level abundances. Alpha and Beta diversity analysis also revealed that gut microbiota diversity elevated in patients after FMT Treatment.

As summarized in Table 2, following FMT, the bacterial taxa demonstrating significant enrichment predominantly included Phylum Firmicutes (included *Coprococcus*, *Phascolarctobacterium*, *Acidaminococcaceae, Lactobacillus, Megamonas, Fusicatenibacter*); Phylum Bacteroidetes (included *Prevotella*, *Paraprevotella*, *Butyricimonas*); Phylum Actinobacteria (included *Bifidobacterium, Collinsella, Slackia*); Phylum Proteobacteria (included *Escherichia)* and Other Notable Taxa (included *Weissella and Allisonella*).

TABLE 2 Microbiota composition and metabolite data in included studies.

References	Microbiota measurement	Microbiota composition data ↑after FMT	Microbiota composition data ↓after FMT	Key beneficial bacteria	Key metabolite
Guyatt et al., 2011	N/A	N/A	N/A	N/A	N/A
Ge et al., 2016	N/A	N/A	N/A	N/A	N/A
Tian et al., 2016	N/A	N/A	N/A	N/A	N/A
Tian et al., 2017	N/A	N/A	N/A	N/A	N/A
Ding et al., 2018	Alpha and Beta diversity, LEfSe	*Prevotella*, *Megamonas*, *Acidaminococcaceae*, *Butyricimonas*	Enterobacteriaceae Bacteroides	*Prevotella*, *Megamonas*, *Acidaminococcus*, *Butyricimonas*	N/A
Tian et al., 2020	Alpha & Beta diversity, PCoA, LEfSe	*Paraprevotella*, *Weissella*, *Coprococcus*, *Phascolarctobacterium*, *Allisonella*, *Fusicatenibacter*, *Acidaminococcaceae*, *Leuconostocaceae*, *Clostridia*	Lachnoanaerobaculum, Anaerofilum, Neisseria	*Fusicatenibacter*, *Paraprevotella*	Butyric acid↑
Zhang et al., 2021	Alpha and Beta diversity, PCoA, LEfSe	Actinobacteria (*Bifidobacterium*), Proteobacteria (*Escherichia*), Firmicute (*Lactobacillus*)	Bacteroidetes (*Prevotell, Bacteroides*), Firmicute (*Roseburia, Blautia*)	*Lactobacillus*	N-Acetyl-Lglutamate, gamma-L-Glutamyl-L-glutamic acid, Glycerophosphocholine
Xie et al., 2021	Alpha and Beta diversity, PCoA,	*Prevotella*, *Bifidobacterium*, *Paraprevotella*, *Collinsella*, *Barnesiellaceae*, *Actinobacteria*, *Slackia*, *Adlercreutzia*, *Enterococcus*	Bacteroidaceae, Victivallaceae	*Prevotella*, *Bifidobacterium*, *Actinobacteria*	N/A
Yang et al., 2021	N/A	N/A	N/A	N/A	N/A

LEfSe, LDA Effect Size; PCoA, Co-ordinates Analysis.

fecal microbiota transplantation led to consistent reductions in bacterial taxa associated with pro-inflammatory or dysbiosis-related functions, categorized as follows: Phylum Proteobacteria (included *Enterobacteriaceae and Neisseria*); Phylum Bacteroidetes (included *Bacteroidaceae*); Phylum Firmicutes (included *Lachnoanaerobaculum, Anaerofilum, Roseburia* and *Blautia*); Phylum Lentisphaerae (*Victivallaceae*).

The critical bacterial taxa linked to the therapeutic effects of FMT on chronic constipation include phylogenetically and functionally distinct groups: Phylum Bacteroidetes (included *Prevotella, Paraprevotella and Butyricimonas*); Phylum Firmicutes (*Megamonas, Fusicatenibacter and Lactobacillus*); Phylum Actinobacteria (*Bifidobacterium and Actinobacteria*) and Family Acidaminococcaceae (*Acidaminococcus*) (Table 2).

Safety of FMT

The safety of FMT was rigorously evaluated across nine included studies, with seven explicitly documenting treatment-related adverse events (AEs) (Ding et al., 2018; Ge et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021; Yang et al., 2021). Critically, no severe AEs (e.g., systemic infections, hospitalization, or mortality) were reported in any studies. The majority of AEs were transient, mild-to-moderate gastrointestinal symptoms, including diarrhea (0.9%–9.5%), abdominal pain (0.9%–14.3%), flatulence (1.8%–17.3%), nausea (0.9%–6.7%), and vomiting (0%–9.5%), all of which resolved spontaneously without intervention. A subset of studies identified procedure-related discomfort, such as naso-intestinal tube irritation, that ceased immediately post-FMT. Mechanistically, these observations align with the transient microbial engraftment dynamics and procedural tolerability. Collectively, the absence of severe AEs and the self-limiting nature of reported symptoms underscore the favorable safety profile of FMT in functional constipation, supporting its feasibility for further clinical translation.

Quality assessment and heterogeneity analysis

The methodological rigor of included studies was evaluated using domain-specific tools. For Tian's study, RoB 2 tool revealed critical concerns: high risk in outcome measurement due to unblinded assessment (patients are unblined). Observational studies (n = 8) scored fair on the Newcastle-Ottawa Scale (NOS). Common limitations included insufficient adjustment for confounding variables (e.g., laxative use or not) and non-representative sampling of constipated populations. Detailed risk of bias assessments is provided in Supplementary Tables 1, 2.

We also performed sensitivity analyses and the results demonstrated that the pooled remission and improvement rates remained stable with sequential exclusion of individual studies, indicating the overall robustness of primary findings. Nevertheless, high heterogeneity persisted across all outcomes ($I^2 > 70\%$), likely attributable to inter-study variations in donor screening strategies

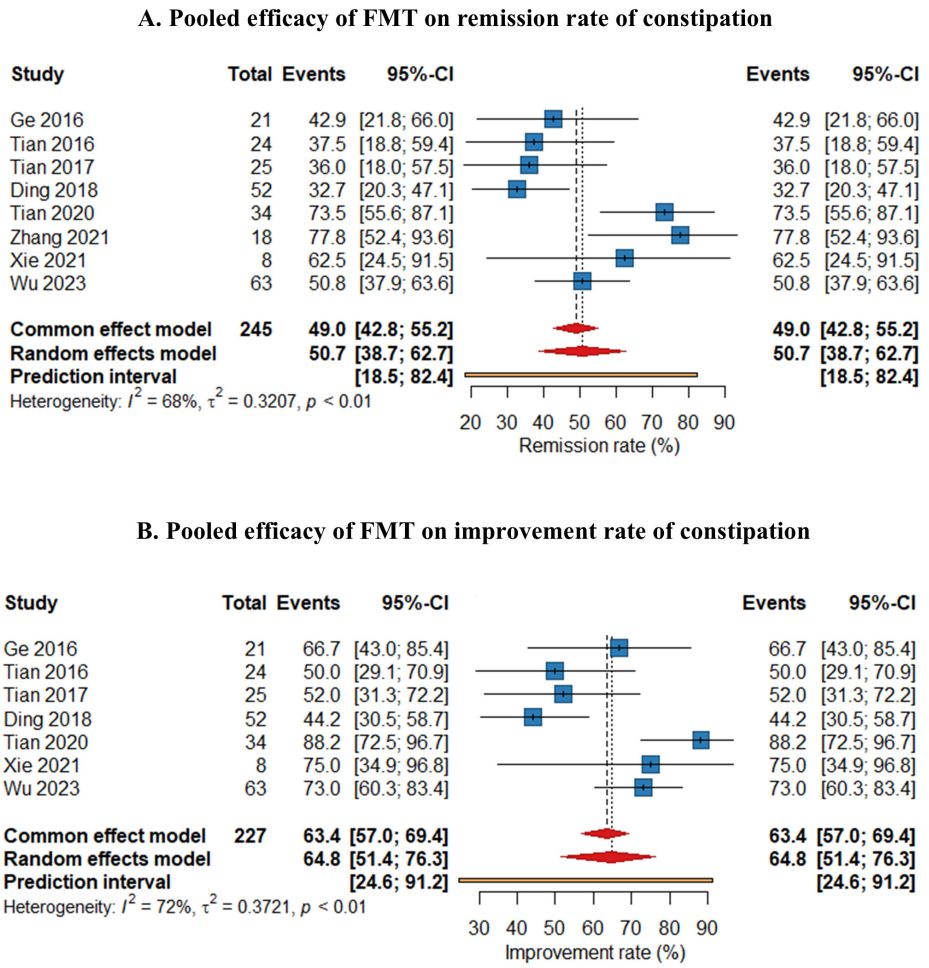


FIGURE 2
Forest plots of efficacy of fecal microbiota transplantation (FMT) on remission rate **(A)** and improvement rate **(B)** of constipation.

(e.g., inclusion/exclusion of constipated donors), fecal preparation methods (fresh vs. frozen), and differential follow-up periods.

Discussion

In this systematic review and meta-analysis, we demonstrate that fecal microbiota transplantation (FMT) holds significant potential to alleviate chronic constipation. Our synthesis of nine clinical studies reveals that FMT achieved complete symptom remission in 50.7% of patients and measurable improvement in 66.7%, with parallel enhancements in stool consistency, colonic transit time, and quality-of-life metrics. Mechanistically, four integrated microbiota analyses identified a post-FMT expansion of phylogenetically and functionally distinct taxa: *Prevotella and Paraprevotella* (Bacteroidetes)–polysaccharide degraders linked to mucosal glycoprotein metabolism; *Fusicatenibacter and Megamonas* (Firmicutes)–butyrate producers critical for enteric neuronal activation; *Bifidobacterium* (Actinobacteria)–a keystone genus modulating gut motility through acetate-mediated vagal signaling; and *Acidaminococcus* (Acidaminococcaceae), a glutamate-fermenting symbiont implicated in visceral hypersensitivity regulation. These taxonomic shifts align with preclinical models where microbial-derived metabolites (e.g., butyrate, acetate) enhance neuromuscular coordination and dampen proinflammatory cascades. Despite transient gastrointestinal symptoms in some of the recipients, the absence of severe adverse events underscores FMT's short-term safety. However, heterogeneity in donor selection, delivery routes, and remission criteria complicates cross-study comparisons, while the lack of randomized controlled trials and long-term data precludes causal inference.

The therapeutic landscape for chronic constipation reveals striking contrasts in efficacy across interventions. Conventional first-line therapies—osmotic laxatives like polyethylene glycol (PEG) and stimulants such as bisacodyl–demonstrate 30%–50% response rates in clinical trials, with high relapse rates upon discontinuation (Chassagne et al., 2017; Luthra et al., 2019; Nelson et al., 2017). Probiotics show modest symptom improvement (15%–25% over placebo) in meta-analyses, though strain-specific effects limit generalizability (Dimidi et al., 2020; Huang et al., 2025). Biofeedback therapy achieves 45%–55% sustained response in pelvic floor dysfunction subtypes yet requires specialized infrastructure (Chiarioni et al., 2006; Rao et al., 2010). Against this backdrop, FMT's 50.7% remission

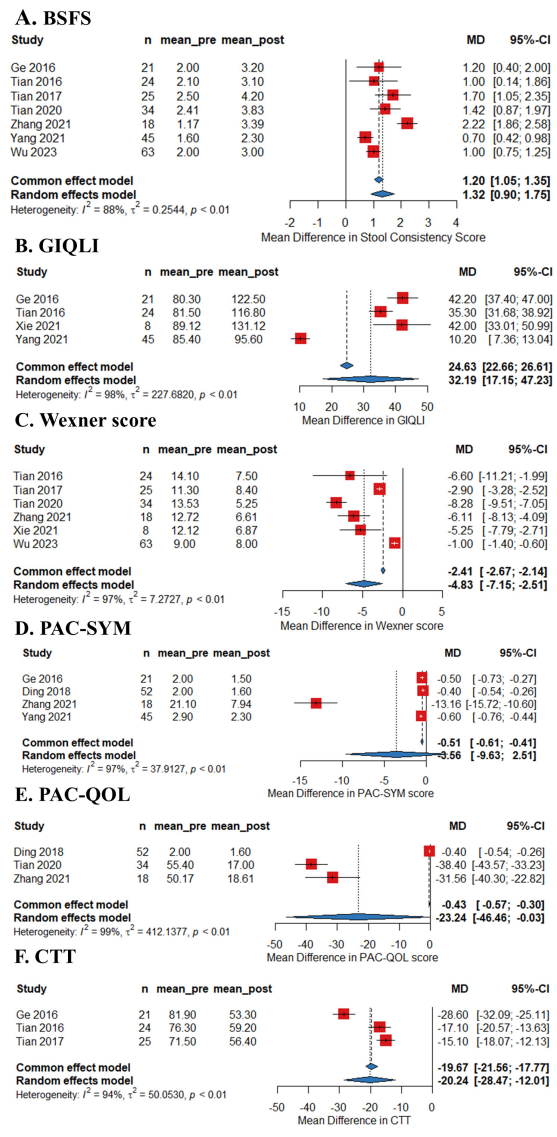


FIGURE 3
Forest plots of efficacy of fecal microbiota transplantation (FMT) on Bristol Stool Form Scale (BSFS) **(A)**, Gastrointestinal Quality-of-Life Index (GIQLI) **(B)**, Wexner score **(C)**, Patient Assessment of Constipation-Symptoms (PAC-SYM) **(D)**, and Patient Assessment of Constipation Quality of Life (PQC-QOL) **(E)** and colonic transit time (CTT) **(F)** of constipation.

rate (95% CI: 38.7%–62.7%) and 66.7% improvement rate in refractory cases–derived from eight studies of 245 patients–suggests microbiome modulation outperforms these approaches in specific populations. Crucially, FMT concurrently elevates stool consistency (BSFS MD = 1.32) and gastrointestinal quality of life (GIQLI MD = 32.19), outcomes rarely achieved by monotherapies. Four studies (Tian et al., 2020; Xie et al., 2021; Yang et al., 2021; Zhang et al., 2021) trace these clinical gains to durable microbiota restructuring, while two (Xie et al., 2021; Zhang et al., 2021) implicate bile acid and short-chain fatty acid metabolic shifts–mechanistic dimensions absent in conventional interventions. This biosynthetic capacity positions FMT not merely as a symptomatic treatment, but as an ecosystem-level reset for dysbiotic constipation phenotypes. Variability in preparation methods (fresh vs. frozen), delivery routes (colonoscopic vs. oral), and dosing schedules obscures causal links between microbial signatures and clinical outcomes–a challenge mirrored across FMT applications. For FMT therapy in most clinical studies, currently no studies have directly compared the efficacy of different FMT delivery routes (e.g., colonoscopic vs. oral capsules). In inflammatory bowel disease (IBD), current evidence suggests no significant differences in clinical outcomes, such as mucosal healing rates, have been observed between fresh and frozen FMT preparations (Fehily et al., 2021). Among the 9 studies included in our analysis, all FMT interventions for constipation utilized nasoenteric tube administration. Consequently, there is a lack of evidence directly comparing the efficacy differences between various delivery routes (e.g., nasoenteric tube vs. oral or colonoscopic approaches). Current data remains insufficient to clarify which administration route holds a definitive advantage. Standardization must prioritize donor selection criteria and engraftment monitoring to isolate therapeutic strains and metabolites.

Across nine studies in this systematic review, systematic adverse (AE) tracking identified only transient gastrointestinal disturbances–diarrhea (0.9%–9.5%), abdominal pain (9%–14.3%), flatulence (1.8%–17.3%) –and minor procedure-linked effects (e.g., naso-intestinal irritation) that resolved without intervention (Ge et al., 2016; Tian et al., 2017; Wu et al., 2023; Xie et al., 2021; Yang et al., 2021). Rigorous donor screening protocols likely contributed to the absence of severe infections or systemic complications, reinforcing methodological priorities in microbial therapeutic development. Mechanistic parallels between these self- and transient post-FMT microbial engraftment dynamics invite deeper exploration of-microbial adaptability. This collective safety evidence, anchored in adverse resolution and procedural robustness, elevates FMT clinical feasibility. Nevertheless, targeted studies must now address granular risk-benefit stratification, including donor-recipient strain compatibility, to propel rational translation.

Post-FMT microbial profiling across four studies (Tian et al., 2020; Xie et al., 2021; Yang et al., 2021; Zhang et al., 2021) revealed a functional restructuring of gut communities, marked by enrichment of phylogenetically distinct taxa with motility-modulating and anti-inflammatory properties. Recipients exhibited increased abundance of *Prevotella* and *Paraprevotella* (Bacteroidetes)–mucin specialists linked to enhanced mucosal glycoprotein turnover (Glover et al., 2022) –and *Megamonas* and *Fusicatenibacter* (Firmicutes), which drive butyrate synthesis to stimulate enteric neurons (Cavaliere and Traina, 2023; Lanza et al., 2021). Concurrently, FMT suppressed pro-inflammatory *Enterobacteriaceae* (Proteobacteria) and *Bacteroidaceae*, taxa associated with gut barrier disruption and visceral hypersensitivity. Critically, *Bifidobacterium* (Actinobacteria) and *Acidaminococcus* (Acidaminococcaceae) emerged as keystone taxa, their post-FMT expansion correlating with acetate-mediated vagal signaling and glutamate metabolism, respectively (Mayer et al., 2015; Zhang et al., 2022). These shifts coincided with elevated alpha diversity and restored beta diversity patterns, suggesting FMT reestablishes ecological resilience in constipated microbiomes.

TABLE 3 Summary of findings and certainty of the evidence.

Outcomes	Measurement	Studies (participants)	Pooled efficacy of FMT (95% CI, 95% PI)	Heterogeneity (Q, *p*-value, I^2, %)	Certainty of the evidence (GRADE)*	Conclusion
Remission rate	CSBM	8 studies (245) (Ding et al., 2018; Ge et al., 2016; Guyatt et al., 2011; Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Yang et al., 2021; Zhang et al., 2021)	50.7% (38.7–62.7%) (18.5–82.4%)	Substantial (26, < 0.05, 68%)	Very low 1, 2, 3	Approximately half of patients achieved clinically completely symptom relief by FMT therapy
Improvement rate	CSBM	7 studies (237) (Ding et al., 2018; Ge et al., 2016; Guyatt et al., 2011; Tian et al., 2016; Tian et al., 2017; Yang et al., 2021; Zhang et al., 2021)	64.8% (51.4–76.3%) (24.6–91.2%)	Substantial (18.9, < 0.05, 72%)	Very low 1, 2, 3	64.8% of patients achieved clinical improvement of symptom relief by FMT therapy
Stool consistency	BSFS	7 studies (230) (Ding et al., 2018; Ge et al., 2016; Guyatt et al., 2011; Tian et al., 2016; Tian et al., 2020; Xie et al., 2021; Yang et al., 2021)	1.32 (1.05–1.35)	Considerable (32.1, < 0.05, 88%)	Very low, 1, 2, 4	FMT significantly improved stool consistency scores by a mean difference of 1.32 points.
Constipation related quality of life	GIQLI	4 studies (98) (Ge et al., 2016; Guyatt et al., 2011; Xie et al., 2021; Zhang et al., 2021)	32.19 points (17.15–47.23)	Considerable (96o3, < 0.05, 98%)	Very low 1, 2, 4, 5	The pooled MD of 32.19 points exceeds the minimally clinically important difference for GIQLI (typically 10–15 points), indicating substantial improvement in gastrointestinal quality of life post-FMT.
	Wexner score	6 studies (172) (Ding et al., 2018; Ge et al., 2016; Tian et al., 2016; Tian et al., 2020; Yang et al., 2021; Zhang et al., 2021)	−4.83 points (−7.15 to 2.51)	Considerable (38.7, < 0.05, 97%)	Very low 1, 2, 4, 5	FMT significantly reduced Wexner scores by 4.83 points, indicating improved constipation symptoms
	PAC-SYM	4 studies (136) (Guyatt et al., 2011; Tian et al., 2017; Tian et al., 2020; Xie et al., 2021)	−3.56 points (−9.63 to 2.51)	Considerable (83.1, < 0.05, 96.4%)	Very low 1, 2, 3, 5	The pooled MD of −3.56 points suggested a non-significant trend toward symptom improvement ($p = 0.37$) of FMT.
	PAC-QOL	3 studies (104) (Ding et al., 2018; Tian et al., 2017; Tian et al., 2020)	−23.2 points (−46.5 to 0.03)	Considerable (54.8, < 0.05, 99%)	Very low 1, 2, 4, 5	The pooled MD of −23.2 points suggested a statistically significant improvement in PAC-QOL scores post-FMT ($p = 0.002$).
Bowel movement	CTT	3 studies (70) (Ge et al., 2016; Guyatt et al., 2011; Tian et al., 2016)	−20.3 h (−28.5 to 12.0)	Considerable (50.1, < 0.05, 94%)	Very low 1, 2, 4, 5	FMT significantly reduced CTT by 20.3 h (95% CI: −28.5 to −12.0), demonstrating accelerated colonic transit.

*(1). Downgraded one point because of moderate or high risk of bias associated with study attrition, prognostic factor measurement, and statistical analysis. (2). Downgraded one point because of inconsistency due to substantial heterogeneity. (3). Downgraded one point because of publication bias. (4). Downgraded one point because of inconsistency of forest plot. (5). Downgraded one point because of imprecision. FMT, fecal microbiome transplantation; CSBM, complete weekly spontaneous bowel movements; CTT, colonic transit time; BSFS, Bristol Stool Form Scale; PAC-SYM, Patient Assessment of Constipation-Symptoms; SBMs: spontaneous bowel movements; PAC-QOL, Patient Assessment of Constipation Quality of Life, GIQLI, Gastrointestinal Quality-of-Life Index.

10.3389/fmicb.2025.1604571

The taxonomic and functional shifts above may align with a multi-hit mechanism for FMT efficacy: (1) butyrate-driven neuromuscular activation via Firmicutes (*Fusicatenibacter*) restores colonic motility (Lanza et al., 2021; Tixier et al., 2006); (2) acetate and GABA modulation by *Bifidobacterium* and *Acidaminococcus* dampens visceral pain signaling (Duranti et al., 2020; Pokusaeva et al., 2017); (3) mucosal barrier repair mediated by *Prevotella*-derived mucin degradation reduces endotoxin translocation (Wright et al., 2000; Yuan et al., 2022). These findings nominate *Prevotella* and *Bifidobacterium* abundance and *Enterobacteriaceae* suppression as candidate biomarkers for FMT responsiveness. Yet, causal validation requires integrating metagenomics, metabolomics, and host transcriptomics in longitudinal cohorts to disentangle microbial contributions from confounders like diet or transit time. Standardizing donor selection around these taxa–while prioritizing butyrogenic consortia–may optimize FMT's therapeutic precision.

Our synthesis integrates clinical and microbiome data across nine studies (Ding et al., 2018; Ge et al., 2016; Tian et al., 2016; Tian et al., 2017; Tian et al., 2020; Wu et al., 2023; Xie et al., 2021; Yang et al., 2021; Zhang et al., 2021), offering the first holistic assessment of FMT's dual role in alleviating chronic constipation and restructuring gut ecosystems. The substantial heterogeneity observed across clinical outcomes (I^2 = 72.9%–96.4%) reflects both methodological diversity and biological complexity in FMT interventions. While our synthesis adhered to PRISMA guidelines and employed random-effects models to account for variability, the interpretative power remains constrained by inherent limitations: (1) Protocol heterogeneity–divergent donor screening practices (e.g., only 33% of studies excluded constipated donors), fecal processing methods (fresh vs. frozen), and remission criteria; (2) Temporal discordance–follow-up durations ranging from 4 to 48 weeks, with diminishing response rates beyond 12 weeks suggesting transient engraftment; (3) Ecological compatibility gaps–unstandardized donor-recipient matching potentially compromising motility-regulating taxa (e.g., *Bifidobacterium*) survival. Despite these challenges, the convergence of multi-omics evidence (Xie et al., 2021; Zhang et al., 2021)—linking stool consistency improvements (MD = 0.94, 95% CI: 0.42–1.46) to *Bifidobacterium*-driven metabolite shifts—strengthens causal inference for FMT's pleiotropic mechanisms.

The lack of control groups in most studies precludes definitive attribution of symptom improvement to FMT itself, as placebo effects—which account for 30%–40% of response in functional gastrointestinal disorders—cannot be ruled out. While our pooled estimates suggest clinical potential, the open-label designs inherently conflate microbiota-mediated effects with natural disease fluctuation and non-specific therapeutic expectations. Furthermore, the Tian et al. (2017) RCT's active comparator design, while pragmatic for clinical practice, inherently conflates FMT efficacy with relative superiority over laxatives rather than absolute therapeutic mechanisms. In addition, publication bias likely inflates the observed 50.5% remission rate. Furthermore, microbiota meta-analyses were hampered by non-standardized reporting of diversity metrics (e.g., conflicting definitions of alpha diversity) and taxonomic classification (e.g., *Prevotell* labeled as beneficial or pathogenic across studies). Quality appraisal revealed methodological limitations in included RCTs. Tian's et al. (2017) exhibited high risk in outcome measurement due to unblinded design—a recognized challenge in FMT trials given the procedural distinctiveness. While observational studies demonstrated adequate selection criteria, the lack of adjustment for laxative use in 6/7 cohort studies may confound efficacy estimates.

The predominance of single-arm studies (8/9 trials) and inclusion of only one underpowered RCT markedly constrain causal inference. While our pooled analyses suggest therapeutic potential, the current evidence level parallels Phase II exploratory trials, necessitating validation through large-scale, double-blind RCTs with sham-FMT controls. Future research must prioritize adequately powered RCTs employing standardized protocols to isolate microbiota-specific effects from placebo responses. Such trials should integrate longitudinal multi-omics (e.g., metagenomics, metabolomics) to mechanistically dissect donor-recipient interactions—a prerequisite for advancing FMT from empirical practice to precision microbial therapeutics. This requires harmonized frameworks integrating three pillars: (1) rigorous donor screening through metagenomic profiling to exclude dysbiotic signatures (e.g., methanogen overgrowth); (2) longitudinal multi-omics tracking (> 24 weeks) to differentiate durable microbial engraftment from transient ecological shifts; and (3) mechanistic validation of microbiota-derived metabolites (e.g., butyrate) through parallel gnotobiotic models. Only through such coordinated efforts can we transform FMT from empirical practice into targeted ecosystem therapeutics, ensuring sustained clinical benefits beyond temporary microbiota perturbations.

Conclusion

This systematic review establishes fecal microbiota transplantation (FMT) as a mechanistically grounded therapy for chronic constipation, demonstrating clinically meaningful symptom remission (50.7%) coupled with functional restructuring of gut ecosystems—specifically enriching *Bifidobacterium* and butyrate-producing *Fusicatenibacter* while suppressing pro-inflammatory *Enterobacteriaceae*. These microbial shifts may correlate with restored gut-brain axis signaling through metabolites like butyrate and acetate, which enhance neuromuscular coordination and barrier integrity. While rodent models provide mechanistic hypotheses (e.g., butyrate's role in serotonin synthesis), the extrapolation to human pathophysiology remains provisional. Concurrent human host-response data—such as colonic serotonin receptor expression or fecal metabolomics—are critically needed to confirm causality.

In summary, current evidence remains provisional due to heterogeneous protocols, transient engraftment, and undersized cohorts. To transcend empirical application, future RCTs must prioritize standardized donor-recipient matching, longitudinal multi-omics profiling (> 24 weeks), and mechanistic validation of microbial metabolites in gnotobiotic models. Success in these areas will propel FMT into precision microbiome engineering—a paradigm that recalibrates gut ecology at the ecosystem level, addressing refractory constipation through sustained microbiota-host dialogue rather than symptomatic relief alone.

Data availability statement

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

Author contributions

KW: Writing – review and editing, Data curation, Resources. CG: Conceptualization, Writing – review and editing. LZ: Conceptualization, Methodology, Software, Writing – original draft. MC: Conceptualization, Data curation, Formal Analysis, Writing – review and editing. YT: Conceptualization, Methodology, Software, Validation, Writing – review and editing. SZ: Conceptualization, Data curation, Formal Analysis, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by the Natural Science Foundation of Hainan province, (No. 821MS0855).

Acknowledgments

We thank Cheng Chen for the English grammar correction of this manuscript.

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 Generative AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1604571/full#supplementary-material

References

Attaluri, A., Jackson, M., Valestin, J., and Rao, S. (2010). Methanogenic flora is associated with altered colonic transit but not stool characteristics in constipation without IBS. *Am. J. Gastroenterol.* 105, 1407–1411. doi: 10.1038/ajg.2009.655

Banaszak, M., Górna, I., Woźniak, D., Przysławski, J., and Drzymała-Czyż, S. (2023). Association between Gut Dysbiosis and the Occurrence of SIBO. *LIBO, SIFO and IMO. Microorganisms* 11:573. doi: 10.3390/microorganisms11030573

Bharucha, A., and Wald, A. (2019). Chronic constipation. *Mayo Clin. Proc.* 94, 2340–2357. doi: 10.1016/j.mayocp.2019.01.031

Brenner, D., Harris, L., Chang, C., Waldman, S., Poppers, D., Kassebaum-Ladewski, A., et al. (2022). Real-world treatment strategies to improve outcomes in patients with chronic idiopathic constipation and irritable bowel syndrome with constipation. *Am. J. Gastroenterol.* 117, S21–S26. doi: 10.14309/ajg.0000000000001709

Cavaliere, G., and Traina, G. (2023). Neuroinflammation in the brain and role of intestinal microbiota: An overview of the players. *J. Integr. Neurosci.* 22:148. doi: 10.31083/j.jin2206148

Chassagne, P., Ducrotte, P., Garnier, P., and Mathiex-Fortunet, H. (2017). Tolerance and long-term efficacy of polyethylene Glycol 4000 (Forlax$) compared to lactulose in elderly patients with chronic constipation. *J. Nutr. Health Aging* 21, 429–439. doi: 10.1007/s12603-016-0762-6

Chiarioni, G., Heymen, S., and Whitehead, W. (2006). Biofeedback therapy for dyssynergic defecation. *World J. Gastroenterol.* 12, 7069–7074. doi: 10.3748/wjg.v12.i44.7069

Dimidi, E., Mark Scott, S., and Whelan, K. (2020). Probiotics and constipation: Mechanisms of action, evidence for effectiveness and utilisation by patients and healthcare professionals. *Proc. Nutr. Soc.* 79, 147–157. doi: 10.1017/S0029665119000934

Ding, C., Fan, W., Gu, L., Tian, H., Ge, X., Gong, J., et al. (2018). Outcomes and prognostic factors of fecal microbiota transplantation in patients with slow transit constipation: Results from a prospective study with long-term follow-up. *Gastroenterol. Rep (Oxf).* 6, 101–107. doi: 10.1093/gastro/gox036

Drossman, D., and Hasler, W. (2016). Rome IV-functional GI disorders: Disorders of gut-brain interaction. *Gastroenterology* 150, 1257–1261. doi: 10.1053/j.gastro.2016.03.035

Duranti, S., Ruiz, L., Lugli, G., Tames, H., Milani, C., Mancabelli, L., et al. (2020). Bifidobacterium adolescentis as a key member of the human gut microbiota in the production of GABA. *Sci. Rep.* 10:14112. doi: 10.1038/s41598-020-70986-z

El-Salhy, M., Hatlebakk, J., Gilja, O., Bråthen Kristoffersen, A., and Hausken, T. (2020). Efficacy of faecal microbiota transplantation for patients with irritable bowel syndrome in a randomised, double-blind, placebo-controlled study. *Gut* 69, 859–867. doi: 10.1136/gutjnl-2019-319630

Fehily, S., Basnayake, C., Wright, E., and Kamm, M. (2021). Fecal microbiota transplantation therapy in Crohn's disease: Systematic review. *J. Gastroenterol. Hepatol.* 36, 2672–2686. doi: 10.1111/jgh.15598

Ge, X., Tian, H., Ding, C., Gu, L., Wei, Y., Gong, J., et al. (2016). Fecal microbiota transplantation in combination with soluble dietary fiber for treatment of slow transit constipation: A pilot study. *Arch. Med. Res.* 47, 236–242. doi: 10.1016/j.arcmed.2016.06.005

Ge, X., Zhao, W., Ding, C., Tian, H., Xu, L., Wang, H., et al. (2017). Potential role of fecal microbiota from patients with slow transit constipation in the regulation of gastrointestinal motility. *Sci. Rep.* 7:441. doi: 10.1038/s41598-017-00612-y

Glover, J., Ticer, T., and Engevik, M. (2022). Characterizing the mucin-degrading capacity of the human gut microbiota. *Sci. Rep.* 12:8456. doi: 10.1038/s41598-022-11819-z

Goya-Jorge, E., Bondue, P., Gonza, I., Laforêt, F., Antoine, C., Boutaleb, S., et al. (2023). Butyrogenic, bifidogenic and slight anti-inflammatory effects of a green kiwifruit powder (Kiwi FFG§) in a human gastrointestinal model simulating mild constipation. *Food Res. Int.* 173:113348. doi: 10.1016/j.foodres.2023.113348

Guyatt, G., Oxman, A., Akl, E., Kunz, R., Vist, G., Brozek, J., et al. (2011). GRADE guidelines: 1. Introduction-GRADE evidence profiles and summary of findings tables. *J. Clin. Epidemiol.* 64, 383–394. doi: 10.1016/j.jclinepi.2010.04.026

Harris, L., and Chang, C. (2022). Burden of constipation: Looking beyond bowel movements. *Am. J. Gastroenterol.* 117, S2–S5. doi: 10.14309/ajg.0000000000001708

Higgins, J., and Thomas, J. (2024). *Cochrane handbook for systematic reviews of interventions VERSION 6.5, 2024. cochrane*. Available online at: https://training.cochrane.org/handbook/current (accessed January, 2025).

Huang, L., Zhu, Q., Qu, X., and Qin, H. (2018). Microbial treatment in chronic constipation. *Sci. China Life Sci.* 61, 744–752. doi: 10.1007/s11427-017-9220-7

Huang, Y., Shi, J., Luo, X., Luo, S., Cheung, P., Corke, H., et al. (2025). How do probiotics alleviate constipation? A narrative review of mechanisms. *Crit. Rev. Biotechnol.* 45, 80–96. doi: 10.1080/07388551.2024.2336531

Lanza, M., Filippone, A., Ardizzone, A., Casili, G., Paterniti, I., Esposito, E., et al. (2021). SCFA treatment alleviates pathological signs of migraine and related intestinal alterations in a mouse model of NTG-induced migraine. *Cells* 10:2756. doi: 10.3390/cells10102756

Li, Y., Zhang, X., and Wang, Z. (2024). Microbiota treatment of functional constipation: Current status and future prospects. *World J. Hepatol.* 16, 776–783. doi: 10.4254/wjh.v16.i5.776

Liu, J., Gu, L., Zhang, M., Zhang, S., Wang, M., Long, Y., et al. (2021). The fecal microbiota transplantation: A remarkable clinical therapy for slow transit constipation in future. *Front. Cell. Infect. Microbiol.* 11:732474. doi: 10.3389/fcimb.2021.732474

Luthra, P., Camilleri, M., Burr, N., Quigley, E., Black, C., and Ford, A. (2019). Efficacy of drugs in chronic idiopathic constipation: A systematic review and network meta-analysis. *Lancet Gastroenterol. Hepatol.* 4, 831–844. doi: 10.1016/S2468-1253(19)30246-8

Mayer, E., Tillisch, K., and Gupta, A. (2015). Gut/brain axis and the microbiota. *J. Clin. Invest.* 125, 926–938. doi: 10.1172/JCI76304

Nelson, A., Camilleri, M., Chirapongsathorn, S., Vijayvargiya, P., Valentin, N., Shin, A., et al. (2017). Comparison of efficacy of pharmacological treatments for chronic idiopathic constipation: A systematic review and network meta-analysis. *Gut* 66, 1611–1622. doi: 10.1136/gutjnl-2016-311835

Page, M., McKenzie, J., Bossuyt, P., Boutron, I., Hoffmann, T., Mulrow, C., et al. (2021). The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ* 372:n71. doi: 10.1136/bmj.n71

Paramsothy, S., Kamm, M., Kaakoush, N., Walsh, A., van den Bogaerde, J., Samuel, D., et al. (2017). Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: A randomised placebo-controlled trial. *Lancet* 389, 1218–1228. doi: 10.1016/S0140-6736(17)30182-4

Peng, Y., Ma, Y., Luo, Z., Jiang, Y., Xu, Z., and Yu, R. (2023). Lactobacillus reuteri in digestive system diseases: Focus on clinical trials and mechanisms. *Front. Cell. Infect. Microbiol.* 13:1254198. doi: 10.3389/fcimb.2023.1254198

Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., et al. (2017). GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. *Neurogastroenterol. Motil.* 29:e12904. doi: 10.1111/nmo.12904

Rao, S., Valestin, J., Brown, C., Zimmerman, B., and Schulze, K. (2010). Long-term efficacy of biofeedback therapy for dyssynergic defecation: Randomized controlled trial. *Am. J. Gastroenterol.* 105, 890–896. doi: 10.1038/ajg.2010.53

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

Singh, R., Zogg, H., Ghoshal, U., and Ro, S. (2022). Current treatment options and therapeutic insights for gastrointestinal dysmotility and functional gastrointestinal disorders. *Front. Pharmacol.* 13:808195. doi: 10.3389/fphar.2022.808195

Sterne, J., Savoviæ, J., Page, M., Elbers, R., Blencowe, N., Boutron, I., et al. (2019). RoB 2: A revised tool for assessing risk of bias in randomised trials. *BMJ* 366:l4898. doi: 10.1136/bmj.l4898

Tian, H., Ding, C., Gong, J., Ge, X., McFarland, L., Gu, L., et al. (2016). Treatment of slow transit constipation with fecal microbiota transplantation: A pilot study. *J. Clin. Gastroenterol.* 50, 865–870. doi: 10.1097/MCG.0000000000000472

Tian, H., Ge, X., Nie, Y., Yang, L., Ding, C., McFarland, L., et al. (2017). Fecal microbiota transplantation in patients with slow-transit constipation: A randomized, clinical trial. *PLoS One* 12:e0171308. doi: 10.1371/journal.pone.0171308

Tian, Y., Zuo, L., Guo, Q., Li, J., Hu, Z., Zhao, K., et al. (2020). Potential role of fecal microbiota in patients with constipation. *Therap. Adv. Gastroenterol.* 13:1756284820968423. doi: 10.1177/1756284820968423

Tixier, E., Galmiche, J., and Neunlist, M. (2006). Acidity induces c-Fos expression in a subpopulation of human colonic submucosal neurons. *Neurosci. Lett.* 404, 23–27. doi: 10.1016/j.neulet.2006.05.058

Wald, A. (2016). Constipation: Advances in diagnosis and treatment. *JAMA* 315, 185–191. doi: 10.1001/jama.2015.16994

Walter, J., and Shanahan, F. (2023). Fecal microbiota-based treatment for recurrent Clostridioides difficile infection. *Cell* 186:1087. doi: 10.1016/j.cell.2023.02.034

Wang, L., Xu, Y., Li, L., Yang, B., Zhao, D., Ye, C., et al. (2024). The impact of small intestinal bacterial overgrowth on the efficacy of fecal microbiota transplantation in patients with chronic constipation. *mBio* 15:e0202324. doi: 10.1128/mbio.02023-24

Wells, G., Shea, B., O'Connell, D., Peterson, J., Welch, V., Losos, M., et al. (2014). *Newcastle-Ottawa quality assessment scale cohort studies*. Ottawa: University of Ottawa.

Wright, D., Rosendale, D., and Robertson, A. (2000). Prevotella enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin. *FEMS Microbiol. Lett.* 190, 73–79. doi: 10.1111/j.1574-6968.2000.tb09265.x

Wu, L., Yuan, Q., Wu, L., Hua-Xiang Xia, H., Zhong, M., Liu, T., et al. (2023). Efficacy of washed microbiota transplantation for therapeutic targets of refractory functional constipation and the influencing factors: A single-center, retrospective, 24-week follow-up study. *BMC Gastroenterol.* 23:291. doi: 10.1186/s12876-023-02929-7

Xie, L., Xu, C., Fan, Y., Li, Y., Wang, Y., Zhang, X., et al. (2021). Effect of fecal microbiota transplantation in patients with slow transit constipation and the relative mechanisms based on the protein digestion and absorption pathway. *J. Transl. Med.* 19:490. doi: 10.1186/s12967-021-03152-2

Yang, B., Tian, H., Ye, C., Lin, Z., Zhao, D., Ma, C., et al. (2021). The efficacy and safety of fecal microbiota transplantation combined with biofeedback for mixed constipation: A retrospective cohort study. *Front. Med (Lausanne).* 8:746990. doi: 10.3389/fmed.2021.746990

Yuan, H., Zhou, J., Li, N., Wu, X., Huang, S., and Park, S. (2022). Isolation and identification of mucin-degrading bacteria originated from human faeces and their potential probiotic efficacy according to host-microbiome enterotype. *J. Appl. Microbiol.* 133, 362–374. doi: 10.1111/jam.15560

Zhang, X., Li, N., Chen, Q., and Qin, H. (2021). Fecal microbiota transplantation modulates the gut flora favoring patients with functional constipation. *Front. Microbiol.* 12:700718. doi: 10.3389/fmicb.2021.700718

Zhang, Y., Fan, Q., Hou, Y., Zhang, X., Yin, Z., Cai, X., et al. (2022). *Bacteroides* species differentially modulate depression-like behavior via gut-brain metabolic signaling. *Brain Behav. Immun.* 102, 11–22. doi: 10.1016/j.bbi.2022.02.007

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 05 August 2025
DOI 10.3389/fmicb.2025.1599107

Check for updates

OPEN ACCESS

EDITED BY
Omneya Magdy Omar,
Alexandria University, Egypt

REVIEWED BY
Jingjie Du,
Cornell University, United States
Xiaofei Zhou,
Shanghai Zhongqiao Vocational Technical College, China

*CORRESPONDENCE
Chuanxing Xiao
xiaoxx@163.com
Zhenqiang Hong
594710961@qq.com
Bangzhou Zhang
geebzbz@163.com

RECEIVED 24 March 2025
ACCEPTED 18 July 2025
PUBLISHED 05 August 2025

CITATION
Yuan S, Jia W, Liu X, Liu R, Cao M, Wu Y, Li Y, Xu W, Xiao C, Hong Z and Zhang B (2025) Therapeutic effect of fecal microbiota transplantation on hyperuricemia mice by improving gut microbiota.
Front. Microbiol. 16:1599107.
doi: 10.3389/fmicb.2025.1599107

COPYRIGHT
© 2025 Yuan, Jia, Liu, Liu, Cao, Wu, Li, Xu, Xiao, Hong and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Therapeutic effect of fecal microbiota transplantation on hyperuricemia mice by improving gut microbiota

Songjian Yuan[1], Wenting Jia[1], Xiaomei Liu[1], Ruzhen Liu[2], Man Cao[2], Yuting Wu[2], Yuantao Li[2], Wei Xu[2], Chuanxing Xiao[1,2]*, Zhenqiang Hong[1]* and Bangzhou Zhang[1,2]*

[1]Fujian University of Traditional Chinese Medicine, Fuzhou, China, [2]Xiamen Treatgut Biotechnology Co. Ltd., Xiamen, China

Objective: The primary objective of this study was to assess the impact of fecal microbiota transplantation (FMT) on serum biochemical parameters, renal injury, and gut microbiota in hyperuricemia (HUA) mice.

Methods: Six-week-old male C57BL/6 J mice were given a high-purine diet and potassium oxonate injections to induce HUA, followed by a two-week FMT treatment. Regular body weight checks, serum biochemical analyses, and fecal sampling for 16S rRNA gene sequencing were conducted to evaluate the treatment's impact on gut microbiota.

Results: The model group showed significant increases in uric acid (UA), creatinine (Cr), blood urea nitrogen (BUN) levels, and increased xanthine oxidase (XOD) activity compared to controls ($p < 0.05$). FMT treatment effectively reduced these levels and XOD activity ($p < 0.05$). At the genus level, specific taxa like *Muribaculaceae* and *Prevotellaceae_UCG-001* were less abundant, while *Blautia* and *Ruminiclostridium_9* were more abundant in the model group. Following FMT, gut microbiota composition returned to near-normal levels, with significant differences from the model group ($p < 0.05$).

Conclusion: This study demonstrates that FMT holds therapeutic potential for HUA mice by reducing UA levels, alleviating renal damage, and restoring gut microbiota balance.

KEYWORDS

fecal microbiota transplantation, hyperuricemia, microbiome, renal injury, gut microbiota

1 Introduction

In recent years, there has been a notable rise in the incidence of hyperuricemia (HUA), positioning it as the fourth most prevalent chronic condition, following hyperglycaemia, hyperlipidaemia, and hypertension. Moreover, HUA is recognized as a significant risk factor for the onset of various diseases, such as diabetes, cardiovascular disorders, and atherosclerosis (Wang et al., 2023). Consequently, HUA has become a pressing public health issue (Chen et al., 2016). This condition is characterized by an imbalance in purine metabolism, primarily attributed to either excessive uric acid (UA) production or impaired UA metabolism (Xu et al., 2021). The accumulation of urate crystals can exacerbate the progression of gout and potentially result in chronic nephritis or renal failure. UA is predominantly synthesized

through the metabolism of purines in the liver, which can be categorized as either endogenous or exogenous purines. Exogenous purines are typically found in foods rich in protein, fat, purines, and fructose (Kim and Jun, 2022; Skoczyńska et al., 2020). Current pharmacological interventions for HUA generally involve xanthine oxidase (XOD) inhibitors and UA excretion agents, such as allopurinol, febuxostat, and benzbromarone (Mehmood et al., 2019; Yan et al., 2022). However, the prevalence of adverse effects associated with these medications, including liver and kidney damage, nausea, vomiting, dermatitis, and other negative reactions, is notably high (Tien et al., 2022). Moreover, prolonged administration of UA-lowering medications has been linked to a heightened risk of cardiovascular disease in individuals with HUA (Sawada et al., 2023). As a result, there is an urgent need for the development of safer and more effective therapeutic alternatives.

Several studies have identified the kidneys and intestines as the primary organs involved in the elimination of UA. Approximately 70% of UA is excreted via the kidneys, while the remaining 30% is eliminated through the intestines, facilitated by UA transporter proteins in the epithelial cells. Furthermore, the gut microbiota contributes to the degradation of UA within the intestinal tract (Wang et al., 2023). The colonization of gut microbiota in the human intestinal tract establishes a symbiotic relationship with the host, aiding in immune regulation (Su et al., 2022). Modifications in the composition of gut microbiota have been implicated as potential factors contributing to metabolic disorders, particularly HUA. Research has demonstrated a significant reduction in both the abundance and diversity of gut microbiota in HUA mice, along with alterations in the microbial structure compared to the control group (Wei et al., 2022). Specifically, HUA mice exhibited elevated levels of pathogenic and opportunistic bacteria and diminished levels of probiotic bacteria within their gut microbiota (Pan et al., 2020). The gut microbiota plays a crucial role in UA metabolism by facilitating the synthesis of purine-metabolizing enzymes, releasing inflammatory cytokines, and decreasing UA levels.

Fecal microbiota transplantation (FMT) involves the transfer of stool or frozen samples from a healthy donor to a recipient suffering from diseases associated with dysbiosis in the gut microbiota. The primary objective of FMT is to restore microbial balance and enhance microbial diversity in the recipient's gut by introducing beneficial microorganisms (Shao et al., 2023). Xie et al. demonstrated the efficacy of FMT in reducing UA levels, alleviating gout symptoms, and enhancing intestinal barrier function in patients experiencing acute and recurrent gout (Xie et al., 2022). In another study, Fu et al. reported that modulation of gut microbiota through antibiotic intervention could decrease serum blood urea nitrogen (BUN), creatinine (Cr), XOD, and UA levels, as well as ameliorate renal tubular necrosis in HUA geese on a high-cholesterol and purine (HCP) diet (Fu et al., 2024). Liu et al. observed that although antibiotic treatment reduced UA levels in HUA rats, fecal transplants from HUA rats to recipient rats resulted in increased UA levels in the latter (Liu et al., 2020). The above studies indicate that gut microbiota may play a potential role in the development of HUA, and modulating gut microbiota via FMT may be an effective strategy for managing HUA symptoms.

This study aims to evaluate the efficacy of FMT in treating HUA by modulating the gut microbiota of HUA mice induced by a high-purine diet and potassium oxonate. The gut microbiota composition and diversity in HUA mice were analyzed through 16S rRNA gene sequencing using the Illumina MiSeq platform. Additionally, this study seeks to investigate the mechanisms through which FMT reduces UA levels in HUA mice by examining the role of gut microbiota. The outcomes of this research will contribute to the development of preventive and therapeutic strategies for HUA and provide novel treatment options for affected individuals.

2 Materials and methods

2.1 Reagents and materials

Potassium oxonate (PO, with a purity of no less than 98%), allopurinol and carboxymethyl cellulose sodium (CMC) were procured from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Assay kits for UA, XOD, BUN, Cr were supplied by Nanjing JianCheng Bioengineering Institute (Nanjing, China).

2.2 Preparation of donor fecal transplant materials

Donor candidates were evaluated through a questionnaire and physical examination to determine their health status, which included assessments of medical history, medication history, family history, and potential risk of infectious diseases. Individuals who smoked or consumed alcohol were excluded from participation, as these factors can disturb the gut microbiota (He et al., 2021). To maintain the integrity of the donated fecal samples, donors were instructed to provide fresh samples in designated containers within a controlled clean room environment. The fecal microbiota was then extracted using an automated fecal microbiota extractor, TG-01 Extn, from Xiamen Treatgut Biotechnology Co., Ltd. (Xiamen, China). The sample was ultimately obtained from the Donor 1101. Fresh fecal specimens were subjected to centrifugation at 5,000 × g for 5 min to isolate a purified microbial fraction. The resultant bacterial cells were then resuspended in normal saline within a homogenizer bag, achieving a bacterial concentration of 1×10^9 CFU/mL. Subsequently, the suspension was aliquoted into sterile tubes and preserved at −80°C for future animal experimentation (Liu et al., 2023; Shaheen et al., 2025). All donor candidates participated voluntarily, signed an informed consent form, and the study was approved by the Ethics Committee of the Third Affiliated Hospital of Fujian University of Traditional Chinese Medicine (Approval No.: 2022-k1-037).

2.3 Animals experimental design

In previous experiments, we utilized a model of PO-induced acute HUA in mice and observed minimal pathological damage to the kidney, which aligns with findings from a similar acute HUA mouse model developed by Dhouibi et al. (2021). Consequently, the current study utilized a high-purine diet in conjunction with PO to establish a chronic HUA model in mice. C57BL/6 J mice (5–6 weeks old, weighing 20 ± 2 g) were obtained from Guangdong Pharmachem Biotechnology Co Ltd. (SCXK (GD) 2020-0054). Mice were acclimatized for 1 week prior to experimentation, during which they

had ad libitum access to food and water in a well-ventilated room maintained at an ambient temperature of 24°C ± 1°C, with humidity levels at 50% ± 10%, and a 12-h light/dark cycle (lights on from 07: 00 to 20: 00).

Thirty-two male C57BL/6 J mice were acclimatized and subsequently divided into four groups based on their initial body weights: control (CON) group, modelling (MOD) group, positive drug (POS) group, and FMT group. The CON group received a standard mouse diet, while the MOD group was provided with a high-purine diet, with all groups having unrestricted access to water. Following a 7-day feeding period, the CON group was administered an intraperitoneal injection of 0.5% CMC-Na solution, while the other groups received intraperitoneal injections of 250 mg/kg/day of PO for 7 days. After 2 weeks of modelling, the CON and MOD groups were given equal volumes of saline, the POS group received 5 mg/kg of allopurinol, and the FMT group was administered 10 μL/g of gut microbiota suspension (with a viable bacterial count of 1×10^9 CFU/mL) for 14 consecutive days. Individual body weights of the mice in each group were recorded every 3 days during the experiment, continuing until the conclusion of drug administration. All animal experiments conducted in this study were approved by the Laboratory Animal Ethics Committee of Fujian University of Traditional Chinese Medicine (Approval No.: FJTCM IACUC 2024201) (Figure 1).

2.4 Biochemical analysis

Mice were subjected to fasting and dehydration for 12 h on the 7th and 14th days of modelling, as well as on the 7th and 14th days of treatment. Blood samples were collected from the inner canthus of the mouse eye and placed in a blood collection tubes. Following mixing and centrifugation, the supernatant was transferred to EP tubes for biochemical assays utilizing kits from the Nanjing JianCheng Bioengineering Institute. The parameters measured included UA, BUN, Cr, and XOD activity.

2.5 Histological analysis

At the end of the treatment period, mice were euthanized via cervical dislocation, and the kidneys were promptly excised, weighed, and examined for alterations in color, texture, and size of the renal tissues. To observe the detailed histopathological changes, kidneys from the various groups were preserved in a 10% buffered formalin solution, subsequently embedded in paraffin, sectioned into 5–7 μm slices, stained with hematoxylin and eosin (H&E), and analyzed under a light microscope.

2.6 DNA extraction

Following 14 days of treatment, fresh stool samples were collected and stored at −80°C for gut microbiota DNA extraction. Genomic DNA from the fecal samples was extracted using the QIAamp Fast DNA Stool Mini kit (Qiagen, CA, United States). The concentration and purity of the isolated DNA were evaluated using spectrophotometry (Multiskan™ GO, Thermo Fisher Scientific, United States). Extracted DNA was purified with a DNA gel purification kit (Qiagen, Germany), and the quality of the DNA extracts was assessed through agarose (1.5%) gel electrophoresis in 1 × Tris-Acetate-EDTA buffer. The purified DNA was stored at −80°C for sequencing.

2.7 16S rRNA amplicon sequencing

The variable V4 region of the 16S rRNA gene was amplified using forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer 806R (5′-GGACTACNVGGGTWTCTAAT-3′) (Cao et al., 2022). Thirty cycles of PCR amplification were conducted (95°C for 3 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and finally, 72°C for 10 min) (Sun et al., 2022). Purification was achieved by adding 0.8 times the volume of magnetic beads (Vazyme VAHTS DNA Clean beads) to 25 μL of the PCR product. Sequencing libraries were prepared using Illumina's TruSeq Nano DNA LT library Prep Kit (Qiu et al., 2022). The quality of the library was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, China) and Agilent Bioanalyzer 2,100 system. Ultimately, the libraries were sequenced on an Illumina MiniSeq.

2.8 Bioinformatics and statistical analysis

Raw paired-end reads were assembled using FLASH. Primers were removed with Cutadapt, and the clean tags were generated by removing the lower reads using the Cutadapt (Caporaso et al., 2010). Chimera checking and operational taxonomic unit (OTU) clustering were performed on the clean tags using USEARCH, following the established pipeline. Specifically, all reads were demultiplexed into individual files, clustered at 97% similarity, then the chimera checking was performed using UCHIME in reference mode (Edgar, 2010). Representative sequences were generated, singletons were discarded, and a final OTU table was created. The representative OTU sequences were aligned against the SILVA_132_97_16S database for taxonomic classification via the RDP Classifier.

Subsequent analyses of alpha and beta diversity were conducted using R software. The Vegan package of R software was employed to calculate the alpha diversity index. Principal coordinate analysis (PcoA) and non-metric multidimensional scaling (NMDS) analyses of Bray-Curtis distances were utilized to reflect the beta diversity of the gut microbiota. R software was used to create two-dimensional plots for PCoA and NMDS. Intergroup differences in the gut microbiota composition were analyzed using linear discriminant analysis (LDA) effect sizes (LEfSe), which employed the non-parametric factors Kruskal-Wallis and rank tests to identify features with significant abundance differences between taxa and used LDA to assess the impact of each feature (Segata et al., 2011).

Data processing and statistical analyses were performed using GraphPad Prism version 9.0 and SPSS 27.0 statistical software. Comparisons of multiple sample means were made using the one-way ANOVA test, and pairwise comparisons were made using the LSD test. $p < 0.05$ was considered significant, and $p < 0.01$ was considered highly significant. In addition, all obtained data are expressed as the mean ± standard deviation (SD).

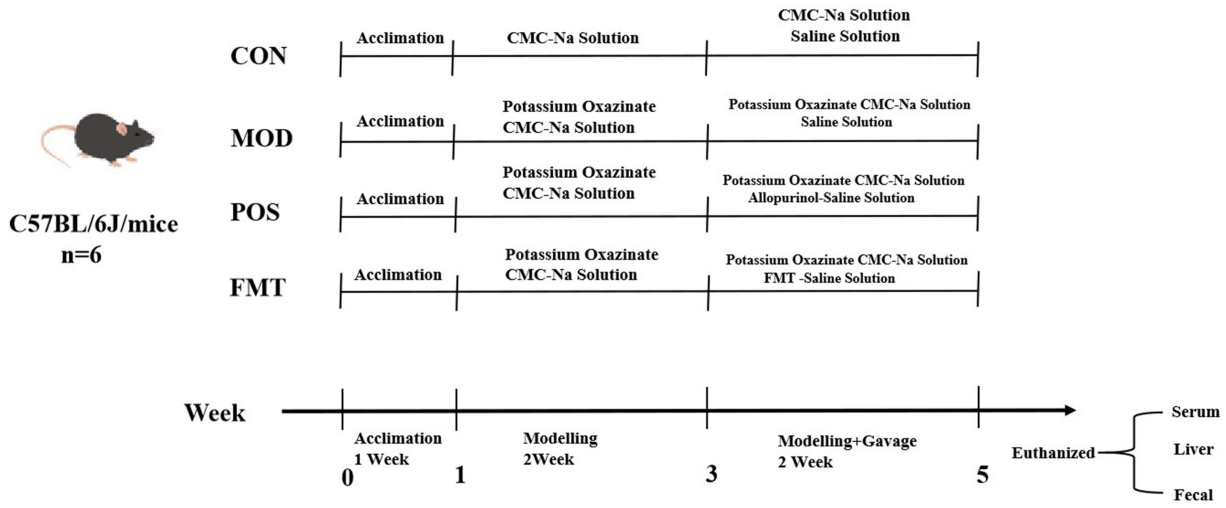


FIGURE 1
Experimental chart in the treatment of HUA mice.

3 Results

3.1 Effect of FMT on body weight in HUA mice

To assess the potential ameliorative effects of FMT on HUA, we established a HUA model in mice through a high-purine diet for the initial 7 days, followed by injections of PO for the next 7 days, culminating in a 14-day modelling period. Commencing on day 15, mice in the CON and MOD groups received saline via gavage, while the POS group was administered the drug allopurinol, and FMT group received a live bacterial preparation via gavage. Following the 14-day modelling period, the body weights of mice in the FMT, POS, and MOD groups were significantly lower than those in the CON group, exhibiting a consistent decline from the onset of modelling (Figure 2A). It is noteworthy that mice receiving FMT gained an average of 3.1 ± 0.6 g during the treatment period, compared to a 1.2 ± 0.4 g loss in the MOD group ($p < 0.01$), indicating a protective effect of FMT against HUA-induced weight loss.

3.2 Regulation of serum biochemical indicators in hyperuricemic mice by FMT

UA was a critical biomarker for assessing HUA. Following FMT treatment, the mice exhibited a significant reduction of 26.00 ± 2.21 μmol/L in UA levels when compared to the MOD group (UA: 63.42 (MOD) vs. 37.42 (FMT); $p < 0.001$; Figure 2B). XOD, which catalyzes the conversion of xanthine to UA, demonstrated significantly diminished activity in the FMT group, with a decrease of 6.03 ± 1.38 U/L relative to the MOD group (XOD: 18.38 (MOD) vs. 12.35 (FMT); $p < 0.001$; Figure 2C). Cr and BUN are indicators of kidney damage. In the MOD group, which was subjected to a high-purine diet, these indicators were significantly elevated, indicating varying degrees of renal damage attributable to HUA. Cr and BUN levels in FMT-treated mice decreased significantly compared to the MOD group (Cr: 43.42 (MOD) vs. 24.74 (FMT), BUN: 21.50 (MOD) vs. 8.89 (FMT); $p < 0.001$), as also visualized in Figures 2D,E.

3.3 Amelioration of renal injury in HUA mice by FMT

The previously described HUA mouse model was established using PO and a high-purine diet. Following FMT treatment, we evaluated its protective effects against kidney injury induced by PO and a high-purine diet in HUA mice. H&E staining (Figure 3) revealed glomerular atrophy and reduced eosin staining intensity of epithelial cytoplasm in HUA mice compared to the CON group. More renal tubules were dilated with flattened epithelium, and eosinophilic proteinaceous fluid was observed within some of the tubular lumens, indicative of a proteinaceous casts in tubular lumens. Additionally, inflammatory infiltrates and necrotic debris were observed in certain renal tubules, signifying severe renal injury induced by PO and a high-purine diet in HUA mice. Importantly, the incidence of glomerular atrophy was diminished in HUA mice treated with FMT, while tubular dilatation was improved, suggesting that FMT treatment can enhance both glomerular and tubular structural integrity.

3.4 Treatment effects on gut microbiota diversity of mice by FMT

A Venn diagram was utilized to illustrate the overlap of OTUs among the groups, as well as those unique to each group. As depicted in Figure 4A, community diversity varied across the different mouse groups. Alpha diversity analysis was conducted to assess the microbial community diversity within each sample. The Shannon and Simpson indices, which reflect community richness and evenness, indicated that hyperuricemic mice exhibited higher gut microbiota richness compared to controls, whereas FMT-treated mice did not demonstrate a significant increase in gut flora richness (Figure 4B).

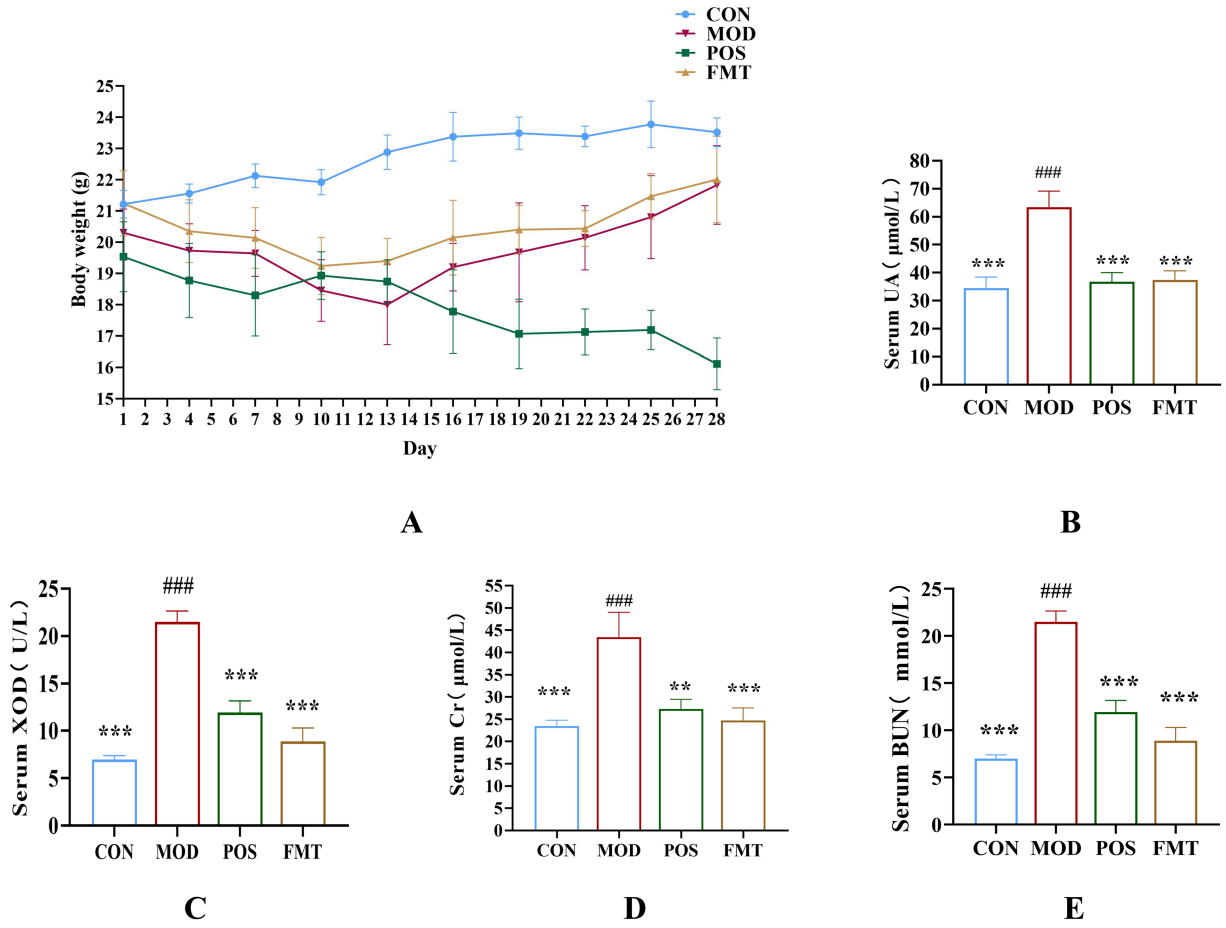


FIGURE 2
(A) Body weight (n = 6). **(B)** Concentration of UA from each group. **(C)** Activity of XOD from each group. **(D)** Concentration of creatinine from each group. **(E)** Concentration of BUN from each group. Data are presented as mean ± SD. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. the MOD group. ### $p < 0.001$ vs. the CON group.

The beta diversity of the gut microbiota of mice was assessed using PCoA and NMDS plots. Based on PCoA and NMDS revealed a clear separation among the four groups, indicating distinct differences in community composition (Figures 5A,B). The microbial community structures of the FMT group and the POS groups exhibited significant clustering, which was distinct from that of the MOD group and more closely aligned with the CON group. These results suggest that HUA induces alterations in the structure of the gut microbiota in mice, and that FMT treatment can restore and normalize the community composition of the gut microbiota.

3.5 Treatment effects on the taxonomic composition of the gut microbiota by FMT

At the phylum level, the gut microbiota of mice was predominantly composed of Bacteroidetes and Firmicutes. Notably, the abundance of Firmicutes in the MOD group was significantly elevated, while the abundance of Bacteroidetes was diminished in comparison to the CON group. In the study, the F/B ratio in the gut microbiota of MOD group mice was significantly higher than in the CON group (F/B: 1.16 (MOD) vs. 0.11 (CON); $p < 0.001$; Figure 6). Remarkably, FMT-treated mice showed a significant decrease in the F/B ratio, aligning more closely with the CON group (F/B: 1.16 (MOD) vs. 0.22 (CON); $p < 0.001$). In Akbar Hussain's experiment on HUA mice using *Limosilactobacillus reuteri*, the F/B ratio of the MOD group showed an upward trend, which reflects microbial imbalance. This implies that metabolic disorders may be impacting urea production and excretion. After administration of *Limosilactobacillus reuteri*, the F/B ratio tended to normalize, and the dysbiosis was adjusted (Hussain et al., 2024). These findings are consistent with our study. To elucidate the changes in bacterial taxa associated with HUA and after FMT treatment, heatmaps and genus-level LEfSe were compared for comparison (Figure 7). Box plots illustrating genus-level difference species across groups revealed that the abundances of *Muribaculaceae*, *Prevotellaceae_UCG-001*, and *Lachnospiraceae_NK4A136* were significantly lower in the gut microbiota of the MOD group compared to the CON group. Conversely, the abundances of *Desulfovibrio*, *Blautia*, *Ruminiclostridium_9*, *Bacteroides*, and *Parabacteroides* were significantly higher in abundance (Figure 8). The gut microbiota of mice treated with allopurinol and FMT exhibited improvements, aligning more closely with that of the CON group. These results suggest that FMT may rectify the dysbiosis of gut microbiota in mice induced by HUA, potentially serving as one of the

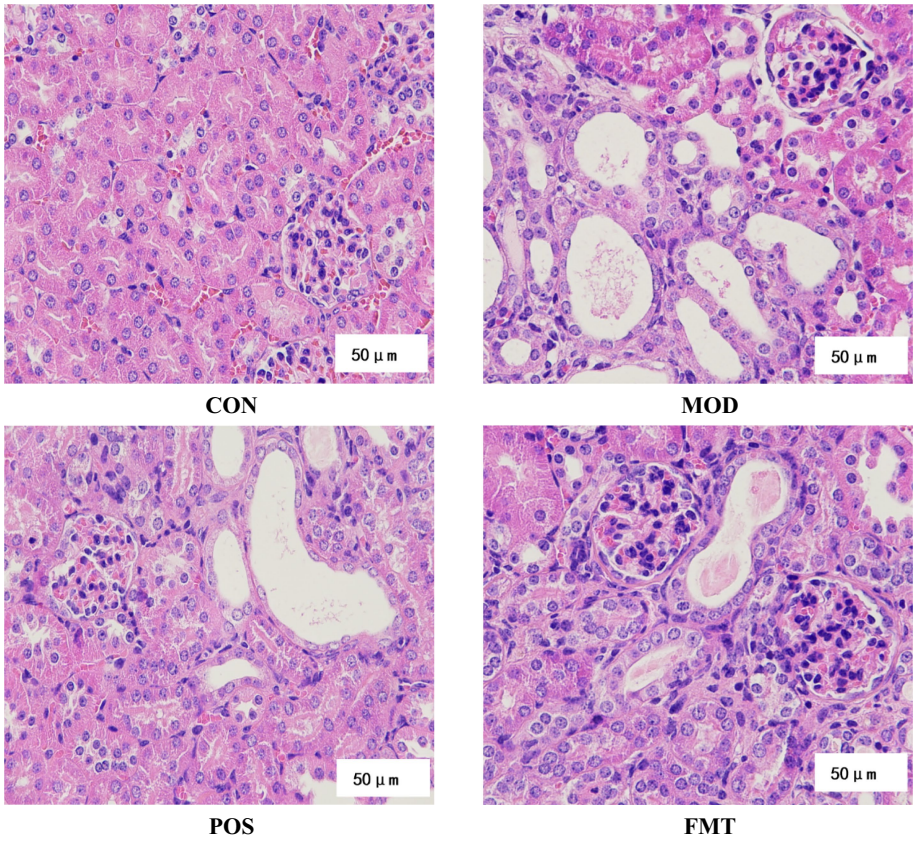


FIGURE 3
Histopathological analyses of H&E stained kidney sections (×400 magnification) from mice.

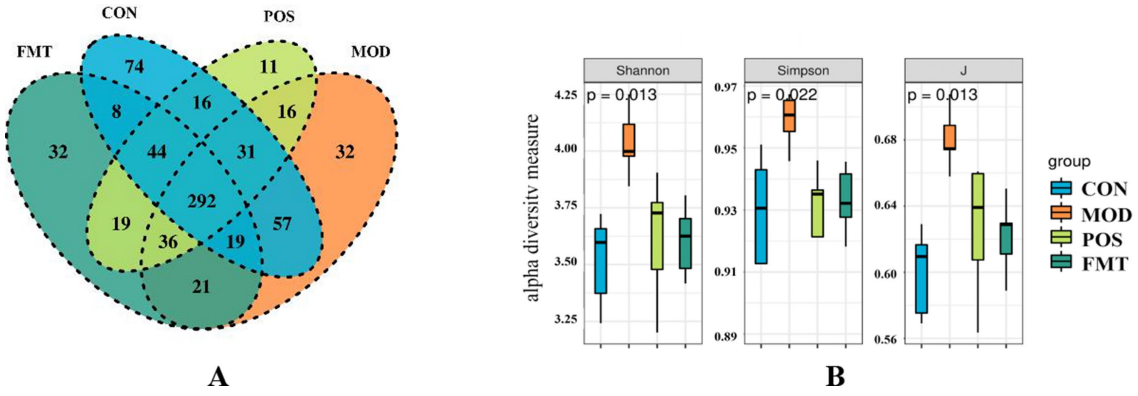


FIGURE 4
FMT alters the structure of the gut microbiota in hyperuricemic mice. **(A)** Venn diagram; **(B)** Alpha diversity based on the Shannon diversity index, the Simpson diversity index, and the inverse Simpson diversity index (J) in groups of mice.

mechanisms through which FMT exerts its therapeutic effects on HUA.

4 Discussion

The rising incidence of HUA can be attributed to improvements in living standards and changes in dietary habits. Recent statistics indicate that the overall prevalence of HUA in China is approximately 13.3%, with gout prevalence estimated at 1.1% (Tang et al., 2023). The diversification of dietary intake, particularly the excessive consumption of purine-rich foods such as animal offal, legumes, and sardines, contributes to the accumulation of purines within the body (Liu et al., 2021). Furthermore, prolonged fructose consumption has been shown to inhibit renal UA metabolism, leading to elevated UA levels (Caliceti et al., 2017). In addition, meat products contain

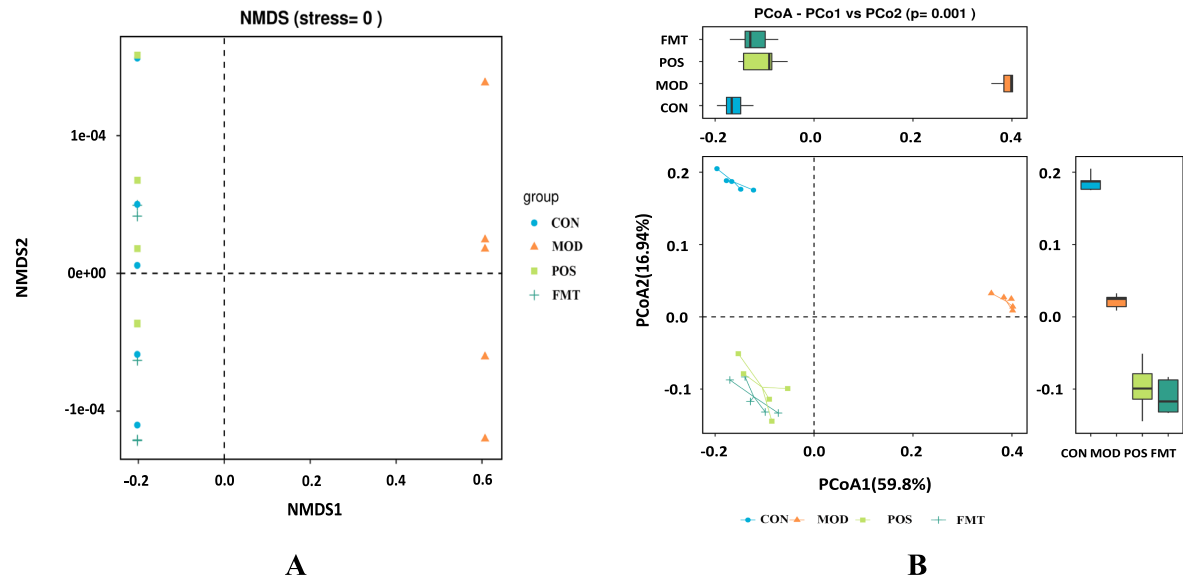


FIGURE 5
FMT alters the structure of the gut microbiota in hyperuricemic mice. **(A)** Non-metric multidimensional scaling plot (NMDS); **(B)** β-diversity based on the Bray-Curtis PCoA method analysis.

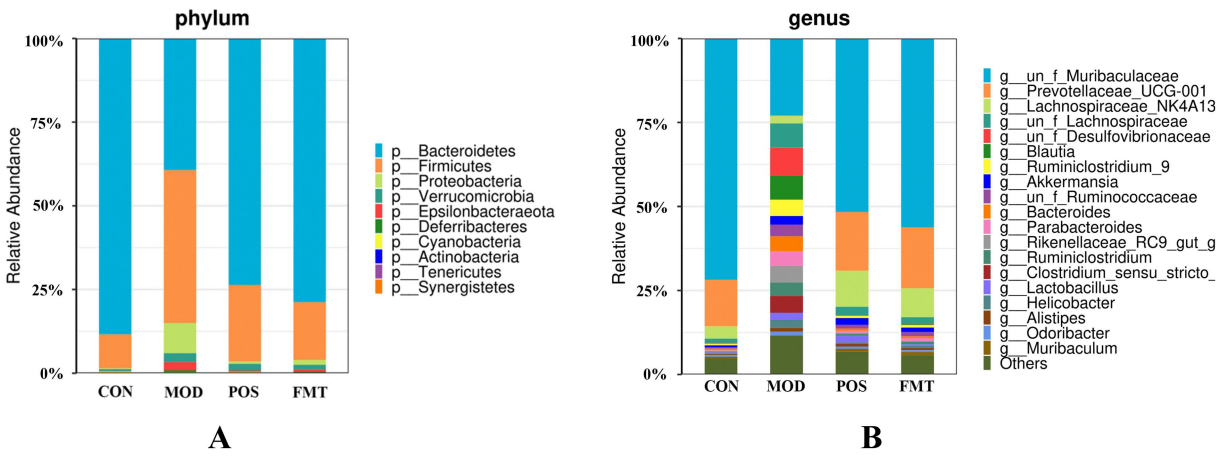


FIGURE 6
The taxonomic compositions of the gut microbiota in hyperuricemia mice. Relative abundance of the gut microbial community in each group at **(A)** The phylum level. **(B)** The genus level.

pro-inflammatory nutrients that may exacerbate HUA (She et al., 2022). Diets characterized by high purine, high fructose, and high-fat content are increasingly prevalent and serve as significant contributors to the development of HUA (Zhou et al., 2022). Consequently, HUA has gradually attracted the attention of the World Health Organization, presenting a global challenge for healthcare systems.

A concerning aspect of HUA management is the potential for serious adverse reactions associated with pharmacological treatments. Allopurinol, for instance, can lead to severe allergic skin reactions, hepatic and renal injuries, and hypersensitivity syndromes such as lethal exfoliative dermatitis. Similarly, febuxostat may induce gastrointestinal symptoms, while benzylbromarone is associated with diarrhoea, nausea, and skin sensitisation (Dubchak and Falasca, 2010). Although UA-lowering drugs are effective in lowering UA, discontinuation of these treatments often results in a rapid increase in UA concentrations (Cheng et al., 2015).

The pathogenesis of HUA is associated with disturbances in purine metabolism and UA excretion. The intestine is an important organ for UA excretion outside the kidney, primarily achieved through the catabolism of gut microbiota and the action of UA transporter proteins (Yin et al., 2022). Liu et al. demonstrated that antibiotic-induced dysbiosis of the gut microbiota alters its composition, elevates UA levels, and modulates purine metabolism in both the host and the gut microbiota (Liu et al., 2023). This suggests a potential link between alterations in the gut microbiota and the development of HUA.

In this study, we investigated the effects of FMT on body weight, modulation of serum biochemical indices, repair of kidney damage, and gut microbiota in hyperuricemic mice. During the modelling

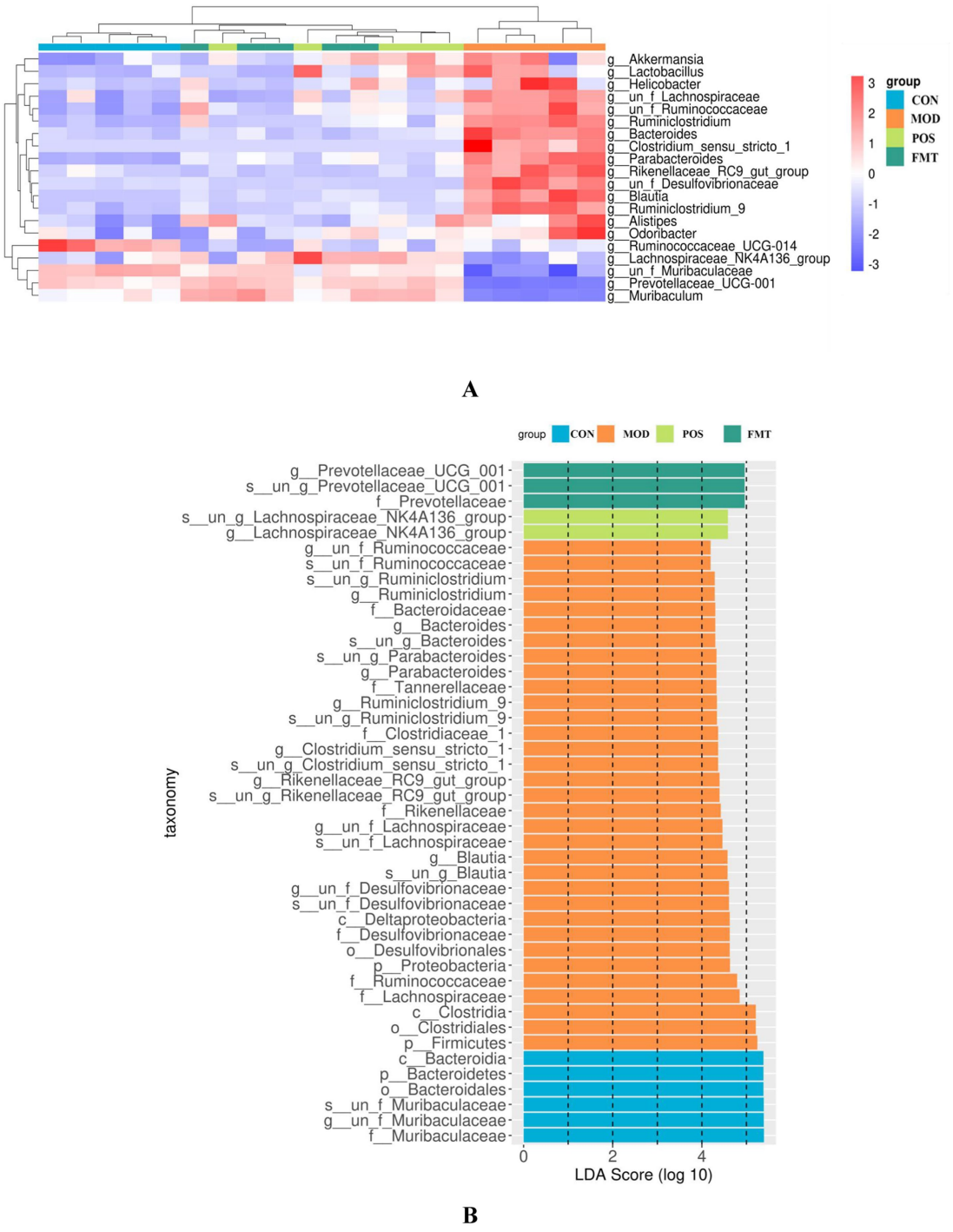


FIGURE 7
Effect of FMT on the gut microbiota at the genus level in hyperuricemic mice. **(A)** Heat maps of most of the selected differential features at the genus level; **(B)** Differences in gut microbiota composition at various levels derived from LEfSe analyses.

phase, the body weights of HUA mice in the MOD group were significantly lower than those in the CON group, indicating that HUA contributes to weight loss. After FMT treatment, the body weight of mice increased significantly compared with the MOD, indicating that FMT can effectively counteract the weight loss caused by HUA. Additionally, compared to the CON group, mice in the MOD group showed significantly higher UA and XOD levels, as well as elevated Cr and BUN levels, which are critical indicators of renal injury. This suggests that the PO-induced HUA model not only increased UA levels in the mice but also caused substantial renal damage. Notably, after 14 days of FMT treatment, there was significant reductions in UA, Cr, and BUN levels, as well as XOD activity, were observed in HUA mice compared to the MOD group, with FMT treatment effects being more pronounced than those observed in the POS group. XOD is an important enzyme that facilitates the synthesis of UA from xanthine, and its inhibition can effectively reduce UA

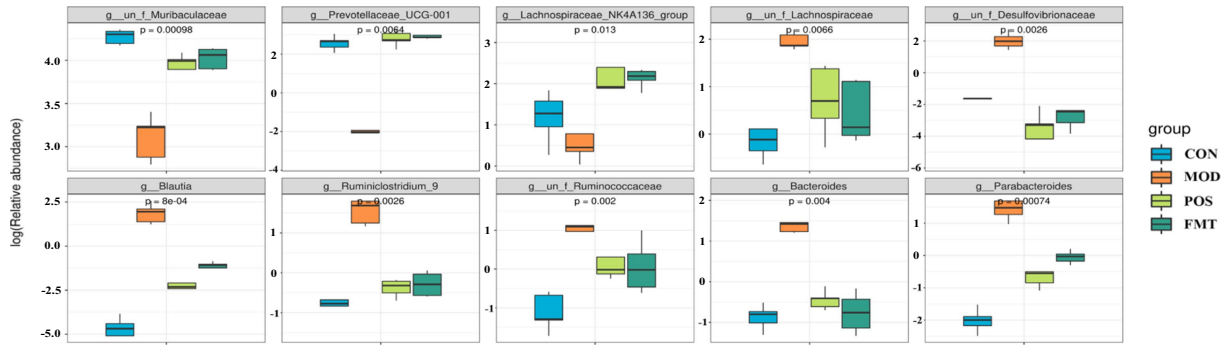


FIGURE 8
Box plots of genus-level differential species in each group.

production. Thus, FMT treatment appeared to reduces UA production and promotes its metabolism. H&E staining of renal sections revealed that chronic HUA modelling caused severe kidney damage, including glomerular atrophy, tubular dilatation, and inflammatory cell infiltration. However, FMT treatment significantly ameliorated the pathological structure of glomeruli and tubules in mice. The reduction in Cr and BUN levels further indicated that FMT could ameliorate renal injury associated with HUA. In alignment with our findings, Wang et al. demonstrated that fecal transplantation from guinea pigs into Sprague–Dawley rats, which were induced with 1.5% PO, effectively reduced urinary oxalate excretion, as well as lowered urea, UA, Cr, and BUN levels, suggesting that FMT can mitigate oxalate-mediated renal injury (Wang et al., 2023). H&E staining corroborated that chronic HUA modelling resulted in significant kidney damage, including glomerular atrophy, tubular dilatation and inflammatory cell infiltration. While FMT treatment improved the pathological structure of glomeruli and tubules, underscoring the potential of FMT in addressing HUA-induced renal injury.

To further elucidate the mechanisms underlying the effects of FMT treatment in HUA mice, we conducted 16S rRNA sequencing of the gut microbiota. PCoA revealed that the intestinal microbial diversity in the MOD group was significantly diminished compared to the CON group, exhibiting a distinct clustering pattern. Conversely, the microbiota of the FMT group exhibited greater similarity to that of the CON group. PO-induced HUA disrupted the gut microbiota in mice, with certain bacterial populations playing a pivotal role. At the phylum level, the MOD group demonstrated a significant decrease in the abundance of Bacteroidetes and a significant increase in the abundance of Firmicutes, resulting in a dysregulated F/B ratio. Firmicutes and Bacteroidetes constitute the primary components of the mouse gut microbiota, collectively accounting for approximately 90% of the fecal microbiome (Lee et al., 2021). An imbalance in the F/B ratio has been implicated in the development of metabolic syndrome (Wei et al., 2023). Firmicutes are enriched in genes associated with nutrient transporters and contain numerous carbohydrate-metabolizing enzymes that facilitate the absorption of calories from food, whereas the Bacteroidetes are capable of degrading complex glycans. Thus, Firmicutes are positively correlated with obesity, while Bacteroidetes exhibit a negative correlation, with the F/B ratio is positively correlated with body mass index (BMI) (Baek et al., 2023; Koliada et al., 2017). Research indicates that the abundance of Firmicutes and the F/B ratio are significantly elevated in obese individuals compared to their lean counterparts, while Bacteroidetes abundance is markedly reduced in obese individuals (Cheng et al., 2022). Obesity is closely linked to elevated UA levels, and numerous studies have shown that higher BMI correlates with an increased risk of HUA (Choi et al., 2020; Wang et al., 2022). Zhao et al. identified that stable unhealthy metabolic states and the transitions from metabolically healthy to unhealthy states heighten the risk of HUA among Chinese adults (Zhao and Zhao, 2022). Shailendra Kumar Singh et al. confirmed a robust positive correlation between obesity and UA levels in obese diabetic patients, noting increased XOD activity and active fatty acid synthesis in adipocytes (Singh et al., 2023). The F/B ratio may also serve as a potential biomarker of inflammation in type 2 diabetic patients, with alterations associated with various diseases (Petakh et al., 2023).

At the genus level (Figure 6B), beneficial bacterial populations such as *Muribaculaceae*, *Prevotellaceae_UCG-001*, and *Lachnospiraceae_NK4A136* were significantly diminished in the gut microbiota of the MOD group. *Muribaculaceae*, the dominant bacterial group in the mouse intestine, metabolizes and produces short-chain fatty acids (SCFAs), which are essential for the growth and development of intestinal epithelial cells. Byron J. Smith et al. demonstrated that treatment with acarbose in diabetic mice significantly increased the abundance of *Muribaculaceae*, which primarily produces propionate as a fermentation product, thereby promoting intestinal health in mice (Smith et al., 2021). An increase in the prevalence of *Muribaculaceae* may promote enhanced urinary excretion of UA or inhibit its synthesis, ultimately resulting in a decrease in UA concentrations. In a study by Shimasaki et al., fucoidan oligosaccharide (FOS) was utilized to increase the relative abundance of *Muribaculaceae* in a rat model. This intervention led to a subsequent reduction in serum levels of Cr, UA, and BUN, thereby alleviating renal damage (Shi et al., 2025). Additionally, specific strains within the *Lachnospiraceae* family, such as *Collinsella aerofaciens*, have been found to upregulate genes related to UA metabolism (e.g., *ygeX, ygeW, ygfK*) in uric acid-rich environments. The enzymes encoded by these genes facilitate the degradation of UA into SCFAs. These SCFAs play a role in lowering intestinal pH, which enhances the solubility and excretion of UA, thereby reducing its intestinal levels (Liu et al., 2023). Moreover, *Lachnospiraceae* engage in intricate metabolic interactions with other gut microbiota. Certain species within this family may collaborate with other butyrate-producing bacteria, such as *Roseburia*, to collectively modulate the gut environment, thereby indirectly influencing UA metabolism (Vacca

et al., 2020). In the context of harmful strains, the prevalence of *Bacteroides* and *Parabacteroides* was found to be significantly greater in the MOD group compared to the CON group. *Bacteroides*, a conditionally pathogenic bacterium, is known to be overrepresented in the gut microbiota of individuals suffering from HUA, especially those diagnosed with gout (Shao et al., 2017). This overrepresentation of *Bacteroides* has also been documented in various HUA mouse models (Amatjan et al., 2023). Furthermore, our investigation identified a significant increase in the abundance of *Desulfovibrio*, *Blautia*, and *Ruminiclostridium_9* in HUA mice. Numerous studies have established a positive correlation between *Desulfovibrio* and metabolic syndrome phenotypes, with elevated levels observed in patients with coronary artery disease with type 2 diabetes mellitus (Sanchez-Alcoholado et al., 2017; Singh et al., 2023), as well as in women diagnosed with gestational diabetes mellitus (Crusell et al., 2018). Given that HUA is a metabolic disorder characterized by dysregulated purine metabolism, we hypothesize that the heightened presence of *Desulfovibrio* in HUA patients may play a role in the pathogenesis of the condition. Following FMT treatment, the dysbiosis of the gut microbiota in mice was improved, resulting in a reduction of F/B ratio. The abundance of beneficial bacteria taxa, including *Muribaculaceae*, *Prevotellaceae_UCG-001*, and *Lachnospiraceae_NK4A136* increased significantly, while the abundance of harmful bacteria such as *Desulfovibrio*, *Blautia*, and *Ruminiclostridium_9* decreased significantly. The microbial composition shifted towards the composition observed in the CON. Su et al. demonstrated that FMT in patients with type 2 diabetes mellitus led to a reduction in the abundance of *Desulfovibrio* in their fecal samples (Su et al., 2022). Therefore, we speculate that FMT may mitigate the overexpression of harmful bacteria and restores the abundance of beneficial bacteria by enhancing the ecological balance of the gut microbiota in HUA mice, ultimately leading to an improvement in HUA.

In summary, the innovation of this study lies in its exploration of the potential therapeutic role of FMT in the management of HUA, thereby offering novel strategies that extend beyond traditional pharmacological and dietary approaches. FMT not only facilitated weight loss and mitigated renal damage in HUA-affected mice but also significantly influenced the composition and diversity of the gut microbiota, indicating a novel therapeutic target for HUA.

Although FMT has shown promise in alleviating HUA in murine models, its implementation in clinical practice faces numerous obstacles. The complex composition of the gut microbiota, along with individual differences among patients, complicates the ability to forecast treatment outcomes effectively (Yadegar et al., 2024). Various factors, including patients' genetic profiles, dietary practices, concurrent pharmacological treatments, and existing comorbidities, can significantly impact the effectiveness of FMT (Shao et al., 2023). Furthermore, there exists a potential risk of infection, as donors who appear healthy may harbor pathogens, with serious cases reported among immunocompromised patients (Green et al., 2023). Additionally, further investigation is necessary to assess the relative efficacy of different methods of FMT administration.

Future research should conduct a thorough investigation into the clinical potential of FMT. The integration of multi-omics technologies has the potential to significantly enhance our understanding of the gut microbiota, thereby clarifying the mechanisms that underpin FMT and improving the predictability of treatment outcomes. The development of personalized FMT protocols, along with advancements in donor screening and quality control measures, will be crucial. Additionally, the exploration of innovative administration routes and formulations may broaden the applicability of FMT. These initiatives will facilitate the wider and more effective application of FMT in the treatment of HUA and associated conditions, ultimately providing patients with safer and more effective therapeutic alternatives.

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/, PRJNA1135905.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Third People's Hospital Affiliated to Fujian University of Traditional Chinese Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by the Animal Ethics Committee of Fujian University of Traditional Chinese Medicine Laboratory Animal Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SY: Writing – original draft, Data curation. WJ: Validation, Writing – review & editing. XL: Conceptualization, Writing – review & editing. RL: Methodology, Writing – review & editing. MC: Formal analysis, Writing – review & editing. YW: Writing – review & editing, Visualization. YL: Project administration, Writing – review & editing. WX: Project administration, Writing – review & editing. CX: Funding acquisition, Writing – review & editing. ZH: Writing – review & editing, Funding acquisition. BZ: Project administration, Funding acquisition, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by Fujian University Industry-University-Research Joint Innovation Project (2022Y4007) and National Natural Science Foundation of China (U22A20376).

Conflict of interest

RL, MC, YW, YL, WX, CX, and BZ were employed by Xiamen Treatgut Biotechnology 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.

Generative AI statement

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1599107/full#supplementary-material

References

Amatjan, M., Li, N., He, P., Zhang, B., Mai, X., Jiang, Q., et al. (2023). A novel approach based on gut microbiota analysis and network pharmacology to explain the mechanisms of action of *cichorium intybus* l. formula in the improvement of hyperuricemic nephropathy in rats. *Drug Des. Devel. Ther.* 17, 107–128. doi: 10.2147/DDDT.S389811

Baek, G. H., Yoo, K. M., Kim, S. Y., Lee, D. H., Chung, H., Jung, S. C., et al. (2023). Collagen peptide exerts an anti-obesity effect by influencing the firmicutes/bacteroidetes ratio in the gut. *Nutrients* 15:2610. doi: 10.3390/nu15112610

Caliceti, C., Calabria, D., Roda, A., and Cicero, A. (2017). Fructose intake, serum uric acid, and cardiometabolic disorders: a critical review. *Nutrients* 9:395. doi: 10.3390/nu9040395

Cao, J., Liu, Q., Hao, H., Bu, Y., Tian, X., Wang, T., et al. (2022). *Lactobacillus paracasei* x11 ameliorates hyperuricemia and modulates gut microbiota in mice. *Front. Immunol.* 13:940228. doi: 10.3389/fimmu.2022.940228

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chen, C., Lü, J. M., and Yao, Q. (2016). Hyperuricemia-related diseases and xanthine oxidoreductase (xor) inhibitors: an overview. *Med. Sci. Monit.* 22, 2501–2512. doi: 10.12659/msm.899852

Cheng, L. C., Murugaiyah, V., and Chan, K. L. (2015). Flavonoids and phenylethanoid glycosides from lippia nodiflora as promising antihyperuricemic agents and elucidation of their mechanism of action. *J. Ethnopharmacol.* 176, 485–493. doi: 10.1016/j.jep.2015.11.025

Cheng, Z., Zhang, L., Yang, L., and Chu, H. (2022). The critical role of gut microbiota in obesity. *Front. Endocrinol. (Lausanne)* 13:1025706. doi: 10.3389/fendo.2022.1025706

Choi, H. K., McCormick, N., Lu, N., Rai, S. K., Yokose, C., and Zhang, Y. (2020). Population impact attributable to modifiable risk factors for hyperuricemia. *Arthritis Rheumatol.* 72, 157–165. doi: 10.1002/art.41067

Crusell, M., Hansen, T. H., Nielsen, T., Allin, K. H., Rühlemann, M. C., Damm, P., et al. (2018). Gestational diabetes is associated with change in the gut microbiota composition in third trimester of pregnancy and postpartum. *Microbiome* 6:89. doi: 10.1186/s40168-018-0472-x

Dhouibi, R., Affes, H., Salem, M. B., Moalla, D., Marekchi, R., Charfi, S., et al. (2021). Creation of an adequate animal model of hyperuricemia (acute and chronic hyperuricemia); study of its reversibility and its maintenance. *Life Sci.* 268:118998. doi: 10.1016/j.lfs.2020.118998

Dubchak, N., and Falasca, G. F. (2010). New and improved strategies for the treatment of gout. *Int. J. Nephrol. Renovasc. Dis.* 3, 145–166. doi: 10.2147/IJNRD.S6048

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than blast. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fu, Y., Chen, Y., Xia, D., Luo, X., Luo, H., Pan, J., et al. (2024). *Lactobacillus rhamnosus* gg ameliorates hyperuricemia in a novel model. *NPJ Biofilms Microbiomes* 10:25. doi: 10.1038/s41522-024-00486-9

Green, J. E., McGuinness, A. J., Berk, M., Castle, D., Athan, E., Hair, C., et al. (2023). Safety and feasibility of faecal microbiota transplant for major depressive disorder: study protocol for a pilot randomised controlled trial. *Pilot Feasibility Stud.* 9:5. doi: 10.1186/s40814-023-01235-z

He, J., He, X., Ma, Y., Yang, L., Fang, H., Shang, S., et al. (2021). A comprehensive approach to stool donor screening for faecal microbiota transplantation in China. *Microb. Cell Factories* 20:216. doi: 10.1186/s12934-021-01705-0

Hussain, A., Rui, B., Ullah, H., Dai, P., Ahmad, K., Yuan, J., et al. (2024). Limosilactobacillus reuteri hcs02-001 attenuates hyperuricemia through gut microbiota-dependent regulation of uric acid biosynthesis and excretion. *Microorganisms* 12:637. doi: 10.3390/microorganisms12040637

Kim, G. H., and Jun, J. B. (2022). Altered serum uric acid levels in kidney disorders. *Life (Basel)* 12:1891. doi: 10.3390/life12111891

Koliada, A., Syzenko, G., Moseiko, V., Budovska, L., Puchkov, K., Perederiy, V., et al. (2017). Association between body mass index and firmicutes/bacteroidetes ratio in an adult ukrainian population. *BMC Microbiol.* 17:120. doi: 10.1186/s12866-017-1027-1

Lee, Y. T., Mohd, I. N., and Wei, L. K. (2021). Microbiome and ischemic stroke: a systematic review. *PLoS One* 16:e245038. doi: 10.1371/journal.pone.0245038

Liu, Y., Jarman, J. B., Low, Y. S., Augustijn, H. E., Huang, S., Chen, H., et al. (2023). A widely distributed gene cluster compensates for uricase loss in hominids. *Cell* 186, 3400–3413.e20. doi: 10.1016/j.cell.2023.06.010

Liu, X., Ke, L., Lei, K., Yu, Q., Zhang, W., Li, C., et al. (2023). Antibiotic-induced gut microbiota dysbiosis has a functional impact on purine metabolism. *BMC Microbiol.* 23:187. doi: 10.1186/s12866-023-02932-8

Liu, J., Lin, H., Cao, M., Lin, T., Lin, A., Xu, W., et al. (2023). Shifts and importance of viable bacteria in treatment of dss-induced ulcerative colitis mice with fmt. *Front. Cell. Infect. Microbiol.* 13:1124256. doi: 10.3389/fcimb.2023.1124256

Liu, X., Lv, Q., Ren, H., Gao, L., Zhao, P., Yang, X., et al. (2020). The altered gut microbiota of high-purine-induced hyperuricemia rats and its correlation with hyperuricemia. *Peerj* 8:e8664. doi: 10.7717/peerj.8664

Liu, N., Xu, H., Sun, Q., Yu, X., Chen, W., Wei, H., et al. (2021). The role of oxidative stress in hyperuricemia and xanthine oxidoreductase (xor) inhibitors. *Oxidative Med. Cell. Longev.* 2021:1470380. doi: 10.1155/2021/1470380

Mehmood, A., Zhao, L., Wang, C., Nadeem, M., Raza, A., Ali, N., et al. (2019). Management of hyperuricemia through dietary polyphenols as a natural medicament: a comprehensive review. *Crit. Rev. Food Sci. Nutr.* 59, 1433–1455. doi: 10.1080/10408398.2017.1412939

Pan, L., Han, P., Ma, S., Peng, R., Wang, C., Kong, W., et al. (2020). Abnormal metabolism of gut microbiota reveals the possible molecular mechanism of nephropathy induced by hyperuricemia. *Acta Pharm. Sin. B* 10, 249–261. doi: 10.1016/j.apsb.2019.10.007

Petakh, P., Oksenych, V., and Kamyshnyi, A. (2023). The f/b ratio as a biomarker for inflammation in covid-19 and t2d: impact of metformin. *Biomed. Pharmacother.* 163:114892. doi: 10.1016/j.biopha.2023.114892

Qiu, J. R., Yang, M. Y., Ma, Y. L., and Yang, M. C. (2022). Effect of ejiao (asini corii colla) and turtle carapace glue on gut microbiota in nude mice with uterine fibroids based on high-throughput sequencing of 16srrna gene. *Evid. Based Complement. Alternat. Med.* 2022:3934877. doi: 10.1155/2022/3934877

Sanchez-Alcoholado, L., Castellano-Castillo, D., Jordán-Martínez, L., Moreno-Indias, I., Cardila-Cruz, P., Elena, D., et al. (2017). Role of gut microbiota on cardio-metabolic parameters and immunity in coronary artery disease patients with and without type-2 diabetes mellitus. *Front. Microbiol.* 8:1936. doi: 10.3389/fmicb.2017.01936

Sawada, S., Kajiyama, K., Shida, H., Kimura, R., Nakazato, Y., Iguchi, T., et al. (2023). Cardiovascular risk of urate-lowering drugs: a study using the national database of health insurance claims and specific health checkups of Japan. *Clin. Transl. Sci.* 16, 206–215. doi: 10.1111/cts.13439

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

Shaheen, M., McDougall, C., Chan, L., Franz, R., Wong, K., Giebelhaus, R. T., et al. (2025). Impact of fecal microbiota transplant formulations, storage conditions, and duration on bacterial viability, functionality, and clinical outcomes in patients with recurrent clostridioides difficile infection. *Microorganisms* 13:587. doi: 10.3390/microorganisms13030587

Shao, T., Hsu, R., Hacein-Bey, C., Zhang, W., Gao, L., Kurth, M. J., et al. (2023). The evolving landscape of fecal microbial transplantation. *Clin. Rev. Allergy Immunol.* 65, 101–120. doi: 10.1007/s12016-023-08958-0

Shao, T., Shao, L., Li, H., Xie, Z., He, Z., and Wen, C. (2017). Combined signature of the fecal microbiome and metabolome in patients with gout. *Front. Microbiol.* 8:268. doi: 10.3389/fmicb.2017.00268

She, D., Wang, Y., Liu, J., Luo, N., Feng, S., Li, Y., et al. (2022). Changes in the prevalence of hyperuricemia in clients of health examination in eastern China, 2009 to 2019. *BMC Endocr. Disord.* 22:202. doi: 10.1186/s12902-022-01118-z

Shi, J., Xu, Y., Zhang, K., Liu, Y., Zhang, N., Zhang, Y., et al. (2025). Fucoidan oligosaccharide supplementation relieved kidney injury and modulated intestinal homeostasis in d-galactose-exposed rats. *Nutrients* 17:325. doi: 10.3390/nu17020325

Singh, S. B., Carroll-Portillo, A., and Lin, H. C. (2023). Desulfovibrio in the gut: the enemy within? *Microorganisms* 11:1772. doi: 10.3390/microorganisms11071772

Singh, S. K., Singh, R., Singh, S. K., Iquebal, M. A., Jaiswal, S., and Rai, P. K. (2023). Prevalence of obesity in newly onset diabetes mellitus and its relationship with uric acid: an indian cross-sectional study. *Int. J. Gen. Med.* 16, 1217–1226. doi: 10.2147/IJGM.S402683

Skoczyńska, M., Chowaniec, M., Szymczak, A., Langner-Hetmańczuk, A., Maciążek-Chyra, B., and Wiland, P. (2020). Pathophysiology of hyperuricemia and its clinical significance – a narrative review. *Reumatologia* 58, 312–323. doi: 10.5114/reum.2020.100140

Smith, B. J., Miller, R. A., and Schmidt, T. M. (2021). *Muribaculaceae* genomes assembled from metagenomes suggest genetic drivers of differential response to acarbose treatment in mice. *mSphere* 6:e85121. doi: 10.1128/msphere.00851-21

Su, X., Gao, Y., and Yang, R. (2022). Gut microbiota-derived tryptophan metabolites maintain gut and systemic homeostasis. *Cells* 11:2296. doi: 10.3390/cells11152296

Su, L., Hong, Z., Zhou, T., Jian, Y., Xu, M., Zhang, X., et al. (2022). Health improvements of type 2 diabetic patients through diet and diet plus fecal microbiota transplantation. *Sci. Rep.* 12:1152. doi: 10.1038/s41598-022-05127-9

Sun, X., Wen, J., Guan, B., Li, J., Luo, J., Li, J., et al. (2022). Folic acid and zinc improve hyperuricemia by altering the gut microbiota of rats with high-purine diet-induced hyperuricemia. *Front. Microbiol.* 13:907952. doi: 10.3389/fmicb.2022.907952

Tang, J., Gao, H., Xu, Y., Chen, J., and Wu, B. (2023). The potential of Chinese medicines in the treatment of hyperuricemia. *Am. J. Transl. Res.* 15, 2291–2303.

Tien, Y. Y., Shih, M. C., Tien, C. P., Huang, H. K., and Tu, Y. K. (2022). To treat or not to treat? Effect of urate-lowering therapy on renal function, blood pressure and safety in patients with asymptomatic hyperuricemia: a systematic review and network meta-analysis. *J. Am. Board Fam. Med.* 35, 140–151. doi: 10.3122/jabfm.2022.01.210273

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., and De Angelis, M. (2020). The controversial role of human gut lachnospiraceae. *Microorganisms* 8:573. doi: 10.3390/microorganisms8040573

Wang, J., Chen, S., Zhao, J., Liang, J., Gao, X., Gao, Q., et al. (2022). Association between nutrient patterns and hyperuricemia: mediation analysis involving obesity indicators in the nhanes. *BMC Public Health* 22:1981. doi: 10.1186/s12889-022-14357-5

Wang, Y., Sun, J., Xie, S., Zhou, Y., Wang, T., Liu, Z., et al. (2023). Increased abundance of bacteria of the family Muribaculaceae achieved by fecal microbiome transplantation correlates with the inhibition of kidney calcium oxalate stone deposition in experimental rats. *Front. Cell. Infect. Microbiol.* 13:1145196. doi: 10.3389/fcimb.2023.1145196

Wang, K., Zhang, Y., Zhou, M., Du, Y., Li, P., Guan, C., et al. (2023). Hdac inhibitors alleviate uric acid-induced vascular endothelial cell injury by way of the hdac6/fgf21/pi3k/akt pathway. *J. Cardiovasc. Pharmacol.* 81, 150–164. doi: 10.1097/FJC.0000000000001372

Wang, J., Zhu, N., Su, X., Gao, Y., and Yang, R. (2023). Gut-microbiota-derived metabolites maintain gut and systemic immune homeostasis. *Cells* 12:793. doi: 10.3390/cells12050793

Wei, B., Ren, P., Yang, R., Gao, Y., Tang, Q., Xue, C., et al. (2023). Ameliorative effect of mannuronate oligosaccharides on hyperuricemic mice via promoting uric acid excretion and modulating gut microbiota. *Nutrients* 15:417. doi: 10.3390/nu15020417

Wei, J., Zhang, Y., Dalbeth, N., Terkeltaub, R., Yang, T., Wang, Y., et al. (2022). Association between gut microbiota and elevated serum urate in two independent cohorts. *Arthritis Rheumatol.* 74, 682–691. doi: 10.1002/art.42009

Xie, W. R., Yang, X. Y., Deng, Z. H., Zheng, Y. M., Zhang, R., Wu, L. H., et al. (2022). Effects of washed microbiota transplantation on serum uric acid levels, symptoms, and intestinal barrier function in patients with acute and recurrent gout: a pilot study. *Dig. Dis.* 40, 684–690. doi: 10.1159/000521273

Xu, J., Tong, L., and Mao, J. (2021). Hyperuricemia and associated factors in children with chronic kidney disease: a cross-sectional study. *Children (Basel)* 9:6. doi: 10.3390/children9010006

Yadegar, A., Bar-Yoseph, H., Monaghan, T. M., Pakpour, S., Severino, A., Kuijper, E. J., et al. (2024). Fecal microbiota transplantation: current challenges and future landscapes. *Clin. Microbiol. Rev.* 37, e0006022–e0006060. doi: 10.1128/cmr.00060-22

Yan, F., Xue, X., Lu, J., Dalbeth, N., Qi, H., Yu, Q., et al. (2022). Superiority of low-dose benzbromarone to low-dose febuxostat in a prospective, randomized comparative effectiveness trial in gout patients with renal uric acid underexcretion. *Arthritis Rheumatol.* 74, 2015–2023. doi: 10.1002/art.42266

Yin, H., Liu, N., and Chen, J. (2022). The role of the intestine in the development of hyperuricemia. *Front. Immunol.* 13:845684. doi: 10.3389/fimmu.2022.845684

Zhao, W., and Zhao, C. (2022). Association between metabolic obesity phenotype, transition of metabolic phenotypes and the risk of hyperuricemia in chinese adults: a cohort study. *Medicine (Baltimore)* 101:e32094. doi: 10.1097/MD.0000000000032094

Zhou, M., Huang, X., Li, R., Zhang, Z., Zhang, L., Gao, X., et al. (2022). Association of dietary patterns with blood uric acid concentration and hyperuricemia in northern chinese adults. *Nutr. J.* 21:42. doi: 10.1186/s12937-022-00789-7

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 25 September 2025
DOI 10.3389/fmicb.2025.1548027

Check for updates

OPEN ACCESS

EDITED BY
Tetyana Falalyeyeva,
Taras Shevchenko National University of Kyiv, Ukraine

REVIEWED BY
Mengmeng Cai,
Nanjing University of Chinese Medicine, China
Oleksandr Ivanovych Kovalchuk,
Taras Shevchenko National University of Kyiv, Ukraine

*CORRESPONDENCE
Xiaodong Tan
✉ tanxdcmu@163.com
Yixian Liu
✉ lyx9990226@163.com

RECEIVED 19 December 2024
ACCEPTED 26 May 2025
PUBLISHED 25 September 2025

CITATION
Li R, Hu Y, Liu Y and Tan X (2025) Fecal microbiota transplantation augments 5-fluorouracil efficacy in pancreatic cancer via gut microbiota modulation.
Front. Microbiol. 16:1548027.
doi: 10.3389/fmicb.2025.1548027

COPYRIGHT
© 2025 Li, Hu, Liu and Tan. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Fecal microbiota transplantation augments 5-fluorouracil efficacy in pancreatic cancer via gut microbiota modulation

Rui Li[1], Yaoyuan Hu[1], Yixian Liu[2]* and Xiaodong Tan[1]*

[1]Department of General Surgery, Shengjing Hospital of China Medical University, Shenyang, China, [2]Liaoning University of Traditional Chinese Medicine, Shenyang, China

Background: Pancreatic cancer is a highly aggressive malignancy with limited therapeutic options due to rapid tumor progression and poor prognosis. Fecal Microbiota Transplantation (FMT) has emerged as a promising approach to modulate gut microbiota, potentially enhancing the efficacy of conventional treatments.

Objectives: This study evaluates the combined effects of FMT and 5-fluorouracil (5FU) on gut microbiota composition, pancreatic tumor growth, and systemic immune responses in a murine model.

Methods: One hundred female C57BL/6 mice aged 6–8 weeks were randomly divided into five groups (n = 20 each): Sham, Model, FMT, 5FU, and FMT + 5FU. Pancreatic tumors were induced via orthotopic implantation of Pan02 cells. FMT was administered orally (0.2 g fecal material) three times per week, starting 2 weeks before tumor implantation. 5FU was administered intraperitoneally at 25 mg/kg body weight twice weekly, beginning one-week post-tumor implantation. Gut microbiota was analyzed via 16S rRNA gene sequencing of fecal samples after 10-week cell implantation. Tumor volumes were measured, and serum cytokine levels were assessed. Short-chain fatty acids (SCFAs) in blood and feces using gas chromatography–mass spectrometry (GC–MS).

Results: The FMT + 5FU group exhibited the smallest average tumor volume, significantly smaller than the Model ($p < 0.0001$) and 5FU groups ($p = 0.005$). FMT alone reduced tumor volume compared to the Model group ($p < 0.0001$). Gut microbiota analysis revealed increased α diversity in the FMT group compared to the Model group ($p < 0.0001$). The FMT + 5FU group showed a significant reduction in cytokine levels, including TNF-α ($p = 0.0001$) and IL-6 ($p = 0.012$) and increased IL-10 level ($p < 0.001$), compared to the Model group. Plasma and fecal SCFA concentrations were significantly higher in both FMT and FMT + 5FU groups relative to the Model group ($p < 0.001$). Additionally, the FMT + 5FU group had the highest survival rate (50%) after 10-week cell implantation, compared to the Model group (15%).

Conclusion: FMT significantly enhances the efficacy of 5FU in reducing pancreatic tumor growth through gut microbiota modulation.

KEYWORDS

fecal microbiota transplantation, 5-fluorouracil, pancreatic cancer, gut microbiota, short-chain fatty acids, mouse model

Introduction

The trillions of microbes inhabiting the gut regulate digestion, immunity and systemic metabolism—and their imbalance, or dysbiosis, contributes to obesity (Longo et al., 2023; Van Hul and Cani, 2023), diabetes (Bajinka et al., 2023; Crudele et al., 2023), cardiovascular diseases (Nesci et al., 2023), and various forms of cancer (Long et al., 2023; Wong and Yu, 2023). Experimental and clinical data link dysbiosis to disease progression and therapy resistance, suggesting that restoring microbial balance could bolster treatment efficacy (Chu et al., 2023; Hamjane et al., 2023).

Pancreatic ductal adenocarcinoma remains one of the deadliest malignancies, with a five-year survival under 10% despite surgery, targeted agents and chemotherapy (Halbrook et al., 2023; Strickler et al., 2023). 5-Fluorouracil (5FU) is a cornerstone drug (Kawakami et al., 2023), but its benefit is limited by toxicity and emerging resistance. Mounting evidence shows that gut microbes modulate both the efficacy and side-effect profile of chemotherapeutics (Chrysostomou et al., 2023; Lo et al., 2023), opening the door to microbiota-focused adjuvant strategies.

Specific genera—including Akkermansia, Bacteroides, Clostridium, Escherichia, Lactobacillus and Bifidobacterium—have been implicated in pancreatic tumor biology via effects on inflammation, bile-acid metabolism and genotoxin production (Abe et al., 2024; Bai et al., 2023; Kartal et al., 2022; Luo et al., 2020; Pahle et al., 2021; Saeed et al., 2024; Shim et al., 2021; Zhao et al., 2023). Their metabolites, notably short-chain fatty acids (acetate, propionate, butyrate), regulate gut barrier integrity, immune signaling and cancer cell proliferation (Murthy et al., 2024; Panebianco et al., 2022; Perazzoli et al., 2023; Zhong et al., 2024).

Fecal microbiota transplantation (FMT)—the transfer of stool from healthy donors—has proven effective against refractory Clostridioides difficile and shows promise in modulating chemotherapy response (Routy et al., 2023; Xu et al., 2023). Preclinical data suggest FMT can reshape microbial communities to enhance drug sensitivity and reduce toxicity (Bangolo et al., 2023). Here, using an orthotopic Pan02 mouse model, we test whether FMT augments 5FU's anti-tumor effects by increasing microbial diversity and beneficial taxa, thereby reducing tumor growth and improving survival (Bangolo et al., 2023; de Castilhos et al., 2024).

Our research addresses a critical gap how gut microbiota modulation can influence cancer treatment outcomes. By exploring the interaction between FMT and chemotherapy in a well-established rodent model, this study aims to explore the potential of microbiota-targeted therapies as adjuncts to conventional cancer treatments.

Methods

Animals and experimental design

The animal experiments were approved by the Animal Ethics Committee of Shengjing Hospital of China Medical University, Shenyang, China. All procedures were conducted in accordance with the ethical guidelines for the care and use of laboratory animals and were designed to minimize animal suffering. A total of 100 female C57BL/6 mice, aged 6–8 weeks and weighing approximately 20 grams each, were used per group. Initially, the study included 20 mice per group, providing a robust sample size for comparisons across the five groups. However, by the end of the study, the model group was reduced to only 3 surviving mice in the model group, necessitating the use of an equally reduced sample size of 3 mice per group for balanced comparisons. The animals were housed under controlled conditions with a 12-h light/dark cycle and were provided with food and water ad libitum. Before the commencement of the experiment, all animals were allowed to acclimate to their environment for 1 week. The study was divided into five groups, each receiving different treatments.

FMT

The antibiotic mixture consisted of ampicillin (1 g/L), vancomycin (0.5 g/L), neomycin (1 g/L), and metronidazole (1 g/L), dissolved in drinking water and administered ad libitum to all the mice for 7 days—to uniformly deplete the native gut flora. Antibiotics were withdrawn for 1 week to allow clearance of residual drugs before any downstream intervention. This broad-spectrum cocktail was selected to deplete the endogenous gut microbiota, creating a receptive environment for subsequent FMT engraftment. FMT was initiated 7 days after completing this antibiotic treatment to allow sufficient time for microbial depletion while minimizing residual antibiotic effects that could interfere with the donor microbiota's colonization. Except for the Sham, Model, and 5FU groups, all other groups received FMT starting 2 weeks before the orthotopic tumor implantation. FMT was administered orally using a gavage method, with each mouse receiving 0.2 grams of fecal material three times per week until the end of the experiment. The fecal samples used for transplantation were obtained from healthy, matched donor mice. These samples were aliquoted into 2 mL centrifuge tubes according to the required number of doses.

All donor mice are housed in the same experimental rooms under standardized conditions (22–24°C, humidity 40–60%, 12-h light/12-h dark cycle (lights on at 06:00, off at 18:00) with minimal-intensity lighting (~130–325 lux), and 10–15 air changes per hour (ACH) with HEPA-filtered airflow). Animals from different cages are not mixed during the study. Fecal samples are collected and processed per cage to avoid cross-contamination and maintain microbiota integrity. Based on past experience, the microbial composition of fecal samples from the same mouse may exhibit slight variations within a one-week timeframe. To mitigate this variability, fresh fecal samples from donor mice are pooled (within cages), homogenized, and processed into a single identical microbial inoculum. This inoculum is aliquoted and stored at −80°C immediately after preparation. Frozen aliquots are thawed once for administration to ensure consistency across experiments, eliminating batch-to-batch variability and temporal confounding factors.

Orthotopic tumor formation and monitoring

The Pan02 mouse pancreatic cancer cell line, known for its high tumor-forming efficiency, was cultured and used as the tumor source for orthotopic implantation. Mice were anesthetized using a suitable anesthetic protocol, such as a combination of xylazine and ketamine or ether, supplemented with subcutaneous injections of analgesics like

carprofen or meloxicam to minimize pain during surgery. For orthotopic tumor implantation, a transverse incision was made on the abdominal wall of each C57BL/6 mouse. Approximately 5×10^5 Pan02 cells were suspended in a mixture of 1/4 volume Matrigel and an equal volume of physiological saline. A volume of 40–60 μL containing around 2×10^5 cells was then injected directly into the pancreas. Tumor formation was typically observable 2–4 weeks post-implantation, with tumors being monitored weekly.

5-Fluorouracil treatment

In the 5FU and FMT + 5FU groups, 5-fluorouracil (5FU) was administered as a chemotherapeutic treatment to assess its impact on tumor growth and survival. The 5FU was administered intraperitoneally at a concentration of 25 mg/kg body weight. The treatment was initiated 1 week after the tumor cell transplantation and was continued twice weekly for a period of 10 weeks. This dosing regimen was designed to mimic clinical chemotherapy schedules and to evaluate the long-term effects of 5FU on tumor progression in the presence and absence of fecal microbiota transplantation.

Pancreatic cancer mouse model and treatment groups

In this study, a pancreatic cancer mouse model was established using tumor cell transplantation. Specifically, mice in the Model group received a surgical procedure during which tumor cells were directly transplanted onto the pancreas, facilitating the development of pancreatic tumors. The Sham group underwent the same surgical procedure without the transplantation of tumor cells, serving as a surgical control to isolate the effects of the surgery from tumor growth. The experimental groups were divided into five distinct cohorts: Sham, Model, FMT, 5FU, and FMT + 5FU. Mice in the FMT group received fecal microbiota transplantation (FMT) post-surgery to assess the influence of gut microbiota on tumor progression. The 5FU group was treated with 5-fluorouracil (5FU), a chemotherapeutic agent, following tumor cell transplantation to evaluate its effectiveness in inhibiting tumor growth. The FMT + 5FU group received a combination of FMT and 5FU treatment to explore the potential synergistic effects of microbiota modulation and chemotherapy on pancreatic cancer.

Microbiome analysis

To minimize diurnal variation and environmental contamination, fecal samples were collected from each mouse in the five experimental groups (Sham, Model, FMT, 5FU, and FMT + 5FU) at the same time each day (8:00 AM) during the tenth week of treatment. All samples were collected under sterile conditions using autoclaved collection tools and immediately transferred to cryovials, which were subsequently snap-frozen in liquid nitrogen. The samples were then stored at −80°C until DNA extraction, ensuring the preservation of microbial DNA integrity. Alongside the experimental samples, mock community standards (ZymoBIOMICS Microbial Community Standard, Zymo Research, Cat D6300) and blank extraction controls were included to monitor potential contamination during the DNA extraction and sequencing processes.

Genomic DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Cat 51,604) with an additional bead-beating step for 2 min at 6,000 rpm (using the MP Biomedicals FastPrep-24) to ensure efficient lysis of all bacterial cell types, including Gram-positive bacteria. DNA extraction efficiency was further assessed by comparing the yield and purity of DNA from the experimental samples to that from the mock community standards. DNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and fluorometric quantification with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat Q32854). The integrity of the DNA was confirmed via agarose gel electrophoresis on a 1% agarose gel.

The bacterial 16S rRNA gene, specifically the V3–V4 hypervariable regions, was amplified using universal primers (Forward: 5'-CCTACGGGNGGCWGCAG-3′, Reverse: 5'-GACTACHVGGGTATCTAATCC-3′) to target approximately 469 bp of the 16S gene. Each 25 μL PCR reaction contained 12.5 μL of 2X KAPA HiFi HotStart ReadyMix (Roche, Cat KK2601), 0.2 μM of each primer, and 20 ng of template DNA. The PCR conditions were set as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. Each sample was amplified in triplicate to reduce PCR bias, and the pooled PCR products were purified using AMPure XP beads (Beckman Coulter, Cat A63880). Sequencing libraries were prepared with the Nextera XT DNA Library Preparation Kit (Illumina, Cat FC-131-1024) and sequenced on an Illumina MiSeq platform, generating paired-end reads of 2 × 300 bp.

Raw sequencing data were processed using QIIME2 (version 2023.2), where quality control was implemented via the DADA2 plugin, which performs demultiplexing, quality filtering, chimera detection, and denoising. Reads with a Phred quality score below 30 were discarded, and sequences were truncated to 250 bp to ensure high-quality base calls throughout the dataset. Taxonomic assignment was carried out using a pre-trained Naive Bayes classifier based on the SILVA 138 99% OTUs database, with an additional decontamination step using the Decontam package to identify and remove any contaminant sequences. *A* diversity metrics, including Chao1, Shannon Index, and Faith's PD, were calculated to evaluate the richness and phylogenetic diversity within samples. *B* diversity was assessed using weighted UniFrac distances, and ordination was performed using Principal Coordinates Analysis (PCoA) with PERMANOVA employed to test for significant differences between groups.

Functional profiling was conducted using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), which predicts the metagenome based on the 16S rRNA gene data. The predicted metagenomes were then analyzed to identify pathways and functions that were significantly enriched across the different treatment groups. Statistical analyses of microbiota composition and functional profiles were performed using ANOVA, followed by *post hoc* Tukey's HSD test, with a significance threshold set at $p < 0.05$. False Discovery Rate (FDR) corrections were applied to control for multiple comparisons. Differential abundance analysis was further complemented by LEfSe (Linear discriminant analysis Effect Size) to identify key bacterial taxa associated with each experimental condition. Visualizations,

including heatmaps, bar plots, and microbial co-occurrence networks, were generated using the ggplot2 and pheatmap packages in R (version 4.3.1).

Tumor volume measurement and body weight monitoring

Tumor volume was measured at the conclusion of the 10-week study period to assess the extent of tumor growth across the different experimental groups. Due to high mortality in the Model group, only 3 mice survived until the 10-week endpoint following cell injection. To ensure balanced group sizes for comparative analyses, 3 mice were randomly selected from each of the other groups, resulting in a final sample size of $n = 3$ per group for tumor volume measurements. Tumor tissues were harvested from these selected mice, and their volumes were calculated using the ellipsoid formula: $V = \frac{4}{3}\pi \times \frac{l}{2} \times \frac{w}{2} \times \frac{h}{2}$, where l, w, and h represent the length, width, and height of the tumor, respectively, measured with rulers (10ths of a mm). All measurements were performed in triplicate by two independent researchers blinded to the group assignments, and the average values were used for analysis to minimize measurement bias. Tumor volume data are presented as mean ± standard deviation (SD) for each group, with the caveat that the limited sample size may affect the statistical power to detect differences.

Body weight was monitored weekly throughout the 10-week study period to evaluate the impact of tumor growth and the various treatments on the overall health and metabolism of the mice. Body weight measurements were recorded for all mice at baseline (week 0) and every 7 days thereafter, using a calibrated digital scale with a precision of 0.1 g. To account for the reduced sample size at the endpoint, body weight analyses were conducted in two stages: (1) weekly body weight progression was analyzed for all surviving mice in each group over the 10-week period to assess temporal trends in body mass, and (2) final body weights were recorded at the end of the study (week 10) for the randomly selected subset of $n = 3$ mice per group to evaluate cumulative weight changes and treatment effects. The selection of the subset of mice for final body weight analysis was performed randomly using a computer-generated randomization algorithm to avoid selection bias. Body weight data are expressed as mean ± SD, and the weight progression of each group was analyzed to determine the temporal effects of tumor burden and treatments on body mass.

Survival analysis

Survival rates were assessed over the 10-week period using Kaplan–Meier survival analysis. Mice were monitored daily for signs of morbidity and mortality, and the survival data were recorded accordingly. The Kaplan–Meier curves were generated for each group to visualize the impact of tumor growth and treatment on survival. For the purposes of Kaplan–Meier curve construction, the time-to-event data were grouped into 7-day increments (week 1: days 1–7, week 2: days 8–14, etc.). Comparisons between groups were made to determine the effectiveness of the treatments in extending survival, with particular focus on the potential benefits of FMT, 5FU, and their combination (FMT + 5FU) in improving outcomes in the pancreatic cancer model. The statistical significance of differences in survival between groups was analyzed to validate the results.

To evaluate the diagnostic potential of specific gut microbiota species in distinguishing between different experimental groups, receiver operating characteristic (ROC) curve analysis was conducted. Bacterial taxa were identified through 16S rRNA gene sequencing of fecal samples collected from the mice in the various treatment groups (Sham, Model, FMT, 5FU, and FMT + 5FU). Sequencing reads were processed and assigned to operational taxonomic units (OTUs) using the QIIME2 pipeline. The relative abundance of key bacterial species—Akkermansia, Clostridium, Escherichia, Bacteroides, Lactobacillus, and Bifidobacterium—was calculated for each sample. ROC curves were generated using the pROC package in R, with sensitivity plotted against 1-specificity to assess the species' ability to correctly classify samples according to their treatment group. The area under the curve (AUC) was calculated for each species to quantify its diagnostic accuracy, with higher AUC values indicating better discriminatory power.

To identify bacterial taxa that were differentially abundant between the experimental groups, a LEfSe (Linear Discriminant Analysis Effect Size) analysis was performed. The relative abundance data from 16S rRNA gene sequencing were processed through the LEfSe algorithm to compute Linear Discriminant Analysis (LDA) scores. These scores were used to rank bacterial genera based on their differential abundance across the groups, with positive LDA scores indicating genera more prevalent in the Model group and associated with disease progression, and negative LDA scores indicating genera more abundant in the treatment groups, associated with protective effects. The analysis was carried out using the LEfSe tool available in the Galaxy platform, with default parameters including an α value for the factorial Kruskal-Wallis test among classes set at 0.05 and a logarithmic LDA score threshold set at 2.0 for discriminative features. The results were visualized as a bar chart, highlighting the taxa with the most significant differences in abundance between the groups.

ELISA analysis

At the end of the 10-week experimental period, serum was extracted from the mice to evaluate circulating levels of inflammatory cytokines, following ethical euthanasia guidelines. Blood was collected via cardiac puncture, allowed to clot at room temperature for 30 min, and then centrifuged at 2,000 g for 10 min at 4°C to isolate the serum, which was carefully harvested (yielding approximately 100–200 μL per mouse) and stored at −80°C until analysis. ELISA kits from Sangon Biotech (Shanghai) Co., Ltd., China, were utilized to quantify the expression of key inflammatory cytokines in the serum, including TNF-α (Cat. No. D721217), IL-1β (Cat. No. D721017), IL-6 (Cat. No. D721022), and IL-10 (Cat. No. D721023).

Western blot analysis

Western blotting was conducted to assess the expression levels of key inflammatory markers and signaling proteins in the tumor tissues. Tumor tissues were lysed using RIPA buffer (Thermo Fisher Scientific, Cat 89,901) supplemented with protease and phosphatase inhibitors

(Sigma-Aldrich, Cat P8340). Protein concentrations were quantified using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Cat 23,225). Equal amounts of protein (30–50 μg) were loaded onto 10% SDS-PAGE gels and subjected to electrophoresis. After separation, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Cat IPVH00010). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature to prevent non-specific binding. The membranes were then incubated overnight at 4°C with primary antibodies specific to the proteins of interest, diluted 1:1000 in TBST with 1% non-fat milk. The primary antibodies used were as follows: TNF-α (rabbit polyclonal, Abcam, Cat ab6671), IL-1β (rabbit polyclonal, Abcam, Cat ab9722), IL-6 (rabbit monoclonal, Cell Signaling Technology, Cat 12,912), and IL-10 (rabbit monoclonal, Cell Signaling Technology, Cat 20,850).

After the overnight incubation with primary antibodies, the membranes were washed three times with TBST and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, HRP-linked, Cell Signaling Technology, Cat 7074S) at a dilution of 1:2000 in TBST for 1 h at room temperature. Following the secondary antibody incubation, the membranes were washed again with TBST, and the protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Cat 32,106). The intensity of the protein bands was quantified using ImageJ software (NIH, Bethesda, MD). The band intensities were normalized to the corresponding loading control, either β-actin (mouse monoclonal, Sigma-Aldrich, Cat A5441) or GAPDH (rabbit polyclonal, Abcam, Cat ab9485), to correct for variations in protein loading. The relative expression levels were calculated and compared across different experimental groups to elucidate the effects of the treatments on protein expression related to inflammation and signaling pathways in pancreatic cancer.

Measurement of SCFAs in serum and feces

Evaluating SCFAs in serum and feces provides critical insights into the interplay between gut microbiota, inflammation, and tumor suppression in pancreatic cancer mouse models, and involves systematic sample collection, preparation, extraction, and analysis using gas chromatography–mass spectrometry (GC–MS). For fecal sample collection, fresh fecal pellets should be obtained from each mouse at the end of the 10-week experimental period. Approximately 50–100 mg of fecal pellets per sample should be collected into sterile, pre-weighed microcentrifuge tubes and immediately frozen at −80°C to preserve SCFA integrity. For serum samples, blood should be collected via cardiac puncture or tail vein bleeding under terminal anesthesia at the 10-week endpoint, allowed to clot at room temperature for 30 min, and then centrifuged at 2,000 × g for 10 min at 4°C to separate serum. Approximately 100–200 μL of serum per sample should be transferred into sterile microcentrifuge tubes and stored at −80°C until analysis, ensuring the stability of SCFAs for subsequent processing.

Sample preparation and SCFA extraction differ slightly between fecal and serum samples but follow a standardized protocol to isolate SCFAs such as acetate, propionate, and butyrate for analysis. For fecal samples, thaw the pellets on ice, weigh them, and homogenize in 500 μL of ice-cold deionized water or 0.15 M phosphoric acid (to stabilize SCFAs) using a vortex mixer or bead beater. Centrifuge the homogenate at 13,000 × g for 10 min at 4°C to pellet debris, then transfer the supernatant to a new tube and add an internal standard (4-methylvaleric acid at 1 mM) to account for extraction variability. Acidify the supernatant with 100 μL of 50% sulfuric acid to protonate SCFAs, followed by extraction with 1 mL of diethyl ether or ethyl acetate, vortexing vigorously for 1 min, and centrifuging at 3,000 × g for 5 min to separate phases. The organic phase, containing SCFAs, should be transferred to a new tube, evaporated under a nitrogen stream to concentrate the sample, and resuspended in 100 μL of ethyl acetate for GC–MS analysis. This meticulous preparation ensures accurate detection of SCFAs in fecal samples.

For serum samples, the extraction process is adapted to the liquid matrix while maintaining precision in SCFA recovery. Thaw serum samples on ice, aliquot 100 μL into a microcentrifuge tube, and add 10 μL of the internal standard (4-methylvaleric acid at 1 mM) to normalize extraction efficiency. Acidify the serum with 20 μL of 50% sulfuric acid to protonate SCFAs, then extract them by adding 200 μL of diethyl ether or ethyl acetate, vortexing vigorously for 1 min, and centrifuging at 3,000 × g for 5 min to separate phases. Transfer the organic phase to a new tube, evaporate it under a nitrogen stream to concentrate the SCFAs, and resuspend the residue in 50 μL of ethyl acetate for GC–MS analysis. These steps ensure that SCFAs in serum, which are present at lower concentrations than in feces, are effectively isolated and concentrated for reliable quantification, providing insights into systemic SCFA levels influenced by gut microbiota activity.

GC–MS analysis requires precise instrumentation and calibration to quantify SCFAs accurately across both sample types. Use a gas chromatograph coupled with a mass spectrometer (Agilent 7890B GC with 5977A MSD) equipped with a capillary column suitable for SCFA analysis (Agilent DB-FFAP or HP-INNOWax, 30 m × 0.25 mm × 0.25 μm), with helium as the carrier gas at a flow rate of 1 mL/min and a splitless injection mode with a 1 μL injection volume. The temperature program should start at 50°C (held for 1 min), increase at 10°C/min to 180°C, then at 20°C/min to 240°C (held for 5 min) to elute all compounds, with injector and detector temperatures set to 250°C. Operate the mass spectrometer in electron ionization (EI) mode at 70 eV, using selected ion monitoring (SIM) mode to detect specific ions (m/z 60 for acetic acid, m/z 74 for propionic acid, m/z 88 for butyric acid, m/z 87 for valeric acid, and m/z 101 for 4-methylvaleric acid). Calibration involves preparing a standard mixture of SCFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids) at concentrations from 0.1 to 10 mM in ethyl acetate, including the internal standard, to establish retention times and generate calibration curves by plotting peak area ratios (SCFA/internal standard) against concentrations. Quantify SCFAs in samples by interpolating peak area ratios from these curves, normalizing to fecal weight (μmol/g) or serum volume (μmol/L).

Results

Pancreatic tumor development and progression in a mouse model

Figure 1A displays representative pancreatic tumors from different experimental groups after 10 weeks of model establishment, while Figure 1B presents the corresponding tumor volume data (mm^3). The

10.3389/fmicb.2025.1548027

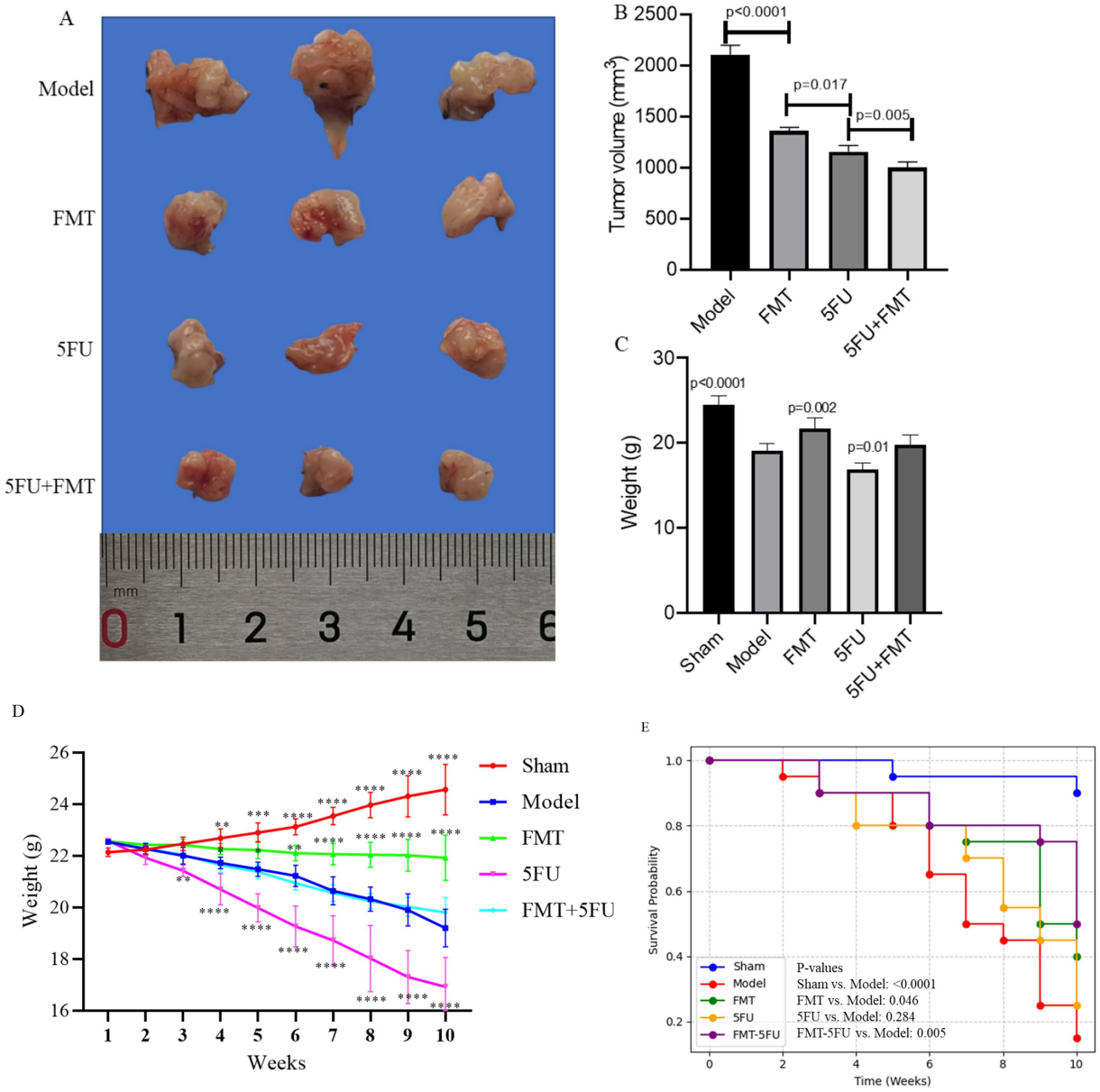


FIGURE 1
Overview of pancreatic tumor development and progression in a mouse model. **(A)** Representative images of pancreatic tumors isolated from mice after 10 weeks of model establishment, showing varying tumor sizes. **(B)** Quantification of tumor volume across different experimental groups, highlighting significant differences in tumor growth. **(C)** Final body weight of mice after the 10-week period, comparing weight changes among the groups. **(D)** Body weight progression from week 0 to week 10, illustrating weight changes over time in response to the different treatments or conditions. *$p < 0.05$, *$p < 0.01$, *$p < 0.001$ and *$p < 0.0001$ vs. the model group. **(E)** Kaplan–Meier survival curves representing survival rates of mice over the 10-week period, indicating differences in survival among the groups. The Sham group underwent pancreatic surgery without the transplantation of tumor cells, serving as a surgical control to evaluate the effects of the procedure itself. The Model group had tumor cells transplanted onto the pancreas, establishing a baseline for tumor development without any additional therapeutic intervention. The FMT group received fecal microbiota transplantation (FMT) following the tumor cell transplant, aiming to assess the influence of gut microbiota modulation on tumor growth. The 5FU group was treated with 5-fluorouracil (5FU), a chemotherapy agent, after tumor cell transplantation, to evaluate its effectiveness in inhibiting tumor progression. Finally, the FMT + 5FU group combined fecal microbiota transplantation with 5-fluorouracil treatment to explore the potential synergistic effects of microbiota modulation and chemotherapy in controlling pancreatic cancer. $n = 3$ for each group. There were significant differences if $p < 0.5$.

Model group exhibited the largest tumors, with an average volume of 2109.67 ± 94.05 mm^3, confirming the aggressive tumor growth in the absence of treatment and the successful establishment of the pancreatic cancer model. In contrast, the Sham group, subjected to pancreatic surgery without tumor cell transplantation, showed no tumor formation, verifying that the surgical procedure alone does not induce tumor growth. The FMT, 5FU, and FMT + 5FU groups displayed visibly smaller tumors, with average volumes of 1361.67 ± 34.93 mm^3, 1160.33 ± 59.60 mm^3, and 1006.00 ± 53.62 mm^3, respectively, indicating that fecal microbiota transplantation (FMT), 5-fluorouracil (5FU) treatment, or their combination effectively reduces tumor size. Statistical analysis revealed significant differences

in tumor volume: FMT significantly reduced tumor volume compared to the Model group ($p < 0.0001$), highlighting the potential role of gut microbiota in tumor suppression; 5FU further decreased tumor volume compared to FMT ($p = 0.017$), underscoring its efficacy as a chemotherapeutic agent; and the FMT + 5FU combination yielded the smallest tumor volumes ($p = 0.005$ compared to 5FU alone), suggesting a synergistic effect that enhances tumor suppression.

Figure 1C presents the final body weights of the mice after 10 weeks. The Sham group maintained the highest average body weight, reflecting the absence of tumor burden and the overall health of these mice. The Model group exhibited a significant reduction in body weight, likely due to the metabolic demands and cachexia associated with tumor growth ($p < 0.0001$). The FMT ($p = 0.002$) and 5FUgroups ($p = 0.01$) had higher or lower final body weights than the Model group, indicating that both treatments might have alleviated or induced some of the negative metabolic effects of the tumor. Although body weights in the FMT + 5FU group remained significantly lower than those in the Sham group, they were comparable to or slightly higher than those in the FMT or 5FU groups alone, indicating a potential synergistic effect in improving overall health.

Figure 1D tracks the body weight progression of the mice over the 10-week period. The Sham group consistently gained weight, reflecting normal growth and health. The Model group, however, showed a decline in weight gain, with some weight loss observed in the later weeks, indicating the impact of the tumor on the mice's metabolism and overall condition. The FMTgroup exhibited a more stable weight curve, with some weight gain, suggesting a protective effect of the gut microbiota against tumor-induced weight loss. The 5FU group showed an initial weight loss, indicating that while 5FU treatment helped control the tumor, it may have had some negative side effects on weight. The FMT + 5FU group had the most stable and steady weight gain, further supporting the potential benefits of combining FMT with chemotherapy.

Figure 1E illustrates the Kaplan–Meier survival curves for the different groups. The Sham group maintained a 90% survival rate by the end of the 10-week period, as expected given the absence of tumor burden. The Model group showed a steady decline in survival, with survival rates dropping to 15% by the end of the study, reflecting the lethal nature of the untreated tumor ($p < 0.0001$ vs. Sham group). The FMT group had improved survival compared to the Model group, with 40% of the mice surviving after 10 weeks, indicating a protective effect of the gut microbiota ($p = 0.046$ vs. Model group). The 5FU group showed a survival rate of 25%, but the difference was not statistically significant ($p = 0.284$ vs. Model group). The FMT-5FU group had the highest survival rate among the treated groups, with 50% of the mice surviving, suggesting that the combination of FMT and 5FU not only suppressed tumor growth but also significantly extended survival ($p = 0.005$ vs. Model group).

Serum inflammatory cytokines concentration

ELISA analysis of serum cytokine concentration reveals significant variations in the levels of TNF-α, IL-1β, IL-6, and IL-10 among the different experimental groups (Figure 2A). TNF-α, a key pro-inflammatory cytokine, was significantly elevated in the Model group compared to the Sham group, indicating a heightened inflammatory response due to tumor presence ($p < 0.0001$). The FMT group showed a notable reduction in TNF-α levels compared to the Model group $p < 0.05$, suggesting that FMT may have an anti-inflammatory effect ($p < 0.0001$). The 5FU group exhibited increased TNF-α levels relative to the Model group ($p = 0.003$). The combination treatment group (FMT + 5FU) demonstrated the reduced TNF-α levels, indicating a potential synergistic effect in reducing inflammation ($p = 0.001$).

The analysis of IL-1β, another pro-inflammatory cytokine, mirrored the trends observed with TNF-α. The Model group had significantly elevated IL-1β levels compared to the Sham group ($p < 0.0001$), further confirming the inflammatory milieu associated with tumor development. Treatment with FMT alone reduce IL-1β levels compared to the Model group ($p = 0.027$). The 5FU group also showed an insignificant change in IL-1β levels compared to the Model group ($p = 0.967$), with the FMT + 5FU combination treatment yielding no changes in IL-1β levels ($p = 0.096$).

IL-6, a cytokine often associated with chronic inflammation and cancer progression, was significantly elevated in the Model group relative to the Sham group ($p < 0.0001$). The FMT treatment led to an insignificant change in IL-6 levels ($p = 0.0008$), while the 5FU treatment resulted in no significant change either ($p = 0.234$). On the other hand, IL-10, an anti-inflammatory cytokine, was significantly lower in the Model group compared to the Sham group ($p < 0.0001$). FMT treatment slightly increased IL-10 levels ($p = 0.0004$), while 5FU treatment led to an insignificant reduction ($p = 0.123$). The combination treatment (FMT + 5FU) resulted in an insignificant increase ($p = 0.157$).

Relative protein levels of inflammatory cytokines

The Western blot analysis shows differential expression of key inflammatory markers and signaling proteins across the experimental groups (Figure 2B). The Model group displayed heightened expression of pro-inflammatory proteins, consistent with the elevated cytokine levels observed in the qPCR analysis. Both the FMT and 5FU groups showed reduced expression of these proteins compared to the Model group, with the FMT + 5FU group exhibiting the lowest expression levels, suggesting that the combination treatment most effectively downregulates inflammatory signaling pathways. The bar graphs quantifying the protein expression levels indicate statistically significant reductions in pro-inflammatory protein levels in the FMT, and FMT + 5FU groups compared to the Model group, with the combination treatment group showing the greatest reduction $p < 0.01$. These results further support the hypothesis that combining FMT with chemotherapy not only mitigates inflammation but also impacts key signaling pathways involved in tumor progression.

FMT improved SCFA profiles

Analysis of SCFA concentrations in fecal samples across the experimental groups provides valuable insights into the gut microbiota's role in pancreatic cancer progression and treatment response, supporting SCFA evaluation to better understand underlying mechanisms (Table 1). The Sham group exhibited the

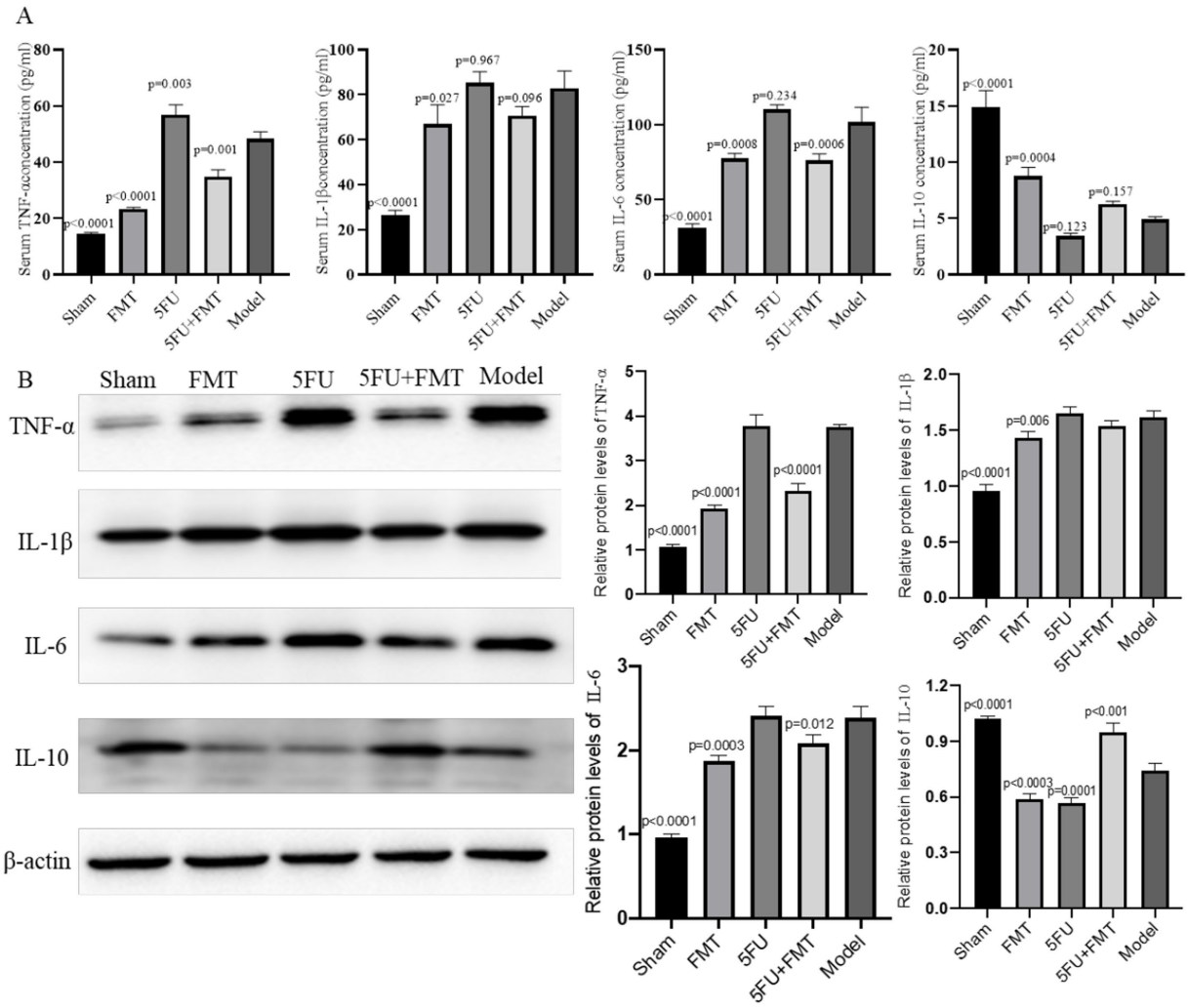


FIGURE 2
Analysis of inflammatory cytokines and protein expression in serum and tumor tissues. **(A)** Quantitative PCR (qPCR) analysis was performed to measure the levels of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-10 in the serum. The bar graphs represent the relative expression levels of these cytokines across different experimental groups, indicating the impact of various treatments on systemic inflammation. **(B)** Western blot analysis of protein expression levels in tumor tissues, with representative blots shown for key inflammatory markers and signaling molecules. The corresponding bar graphs quantify the relative expression levels, providing insights into the molecular effects of the treatments on tumor biology. $n = 3$ for each group. There were significant differences if $p < 0.5$.

TABLE 1 Short-chain fatty acid concentrations in feces (μmol/g wet weight).

Group	Acetate (μmol/L)	*p*-value	Propionate (μmol/L)	*p*-value	Butyrate (μmol/L)	*p*-value	Total SCFAs	*p*-value
Sham	65.3 ± 4.2	0.0002	25.8 ± 2.1	0.0007	20.4 ± 1.8	0.0003	111.5 ± 6.5	0.0001
Model	35.7 ± 3.1	–	15.2 ± 1.4	–	10.9 ± 1.2	–	61.8 ± 4.7	–
FMT	58.9 ± 3.8	0.0008	22.6 ± 1.9	0.0021	18.2 ± 1.6	0.0010	99.7 ± 5.9	0.0004
5FU	28.4 ± 2.5	0.0696	12.3 ± 1.1	0.1012	8.7 ± 0.9	0.1216	49.4 ± 3.6	0.0712
FMT + 5FU	52.1 ± 3.5	0.0034	20.1 ± 1.7	0.0118	16.8 ± 1.4	0.0042	89.0 ± 5.2	0.0016

Data are presented as mean ± standard error of the mean (SEM), $n = 3$ per group. $p < 0.05$ vs. the model group indicates statistical significance.

highest fecal SCFA levels, with acetate at 65.3 ± 4.2 μmol/g ($p = 0.0002$ vs. Model), propionate at 25.8 ± 2.1 μmol/g ($p = 0.0007$), butyrate at 20.4 ± 1.8 μmol/g ($p = 0.0003$), and total SCFAs at 111.5 ± 6.5 μmol/g ($p = 0.0001$), reflecting robust microbial fermentation in a healthy gut environment. In contrast, the Model group, representing untreated pancreatic cancer, showed significantly reduced SCFA concentrations (acetate: 35.7 ± 3.1 μmol/g; propionate: 15.2 ± 1.4 μmol/g; butyrate: 10.9 ± 1.2 μmol/g; total SCFAs: 61.8 ± 4.7 μmol/g), indicative of microbial dysbiosis linked to tumor progression. The FMT group

demonstrated a significant restoration of SCFA levels compared to the Model group (acetate: 58.9 ± 3.8 μmol/g, $p = 0.0008$; propionate: 22.6 ± 1.9 μmol/g, $p = 0.0021$; butyrate: 18.2 ± 1.6 μmol/g, $p = 0.0010$; total SCFAs: 99.7 ± 5.9 μmol/g, $p = 0.0004$), suggesting that fecal microbiota transplantation enhances SCFA production, potentially contributing to its anti-tumor effects as observed in tumor volume reductions. The 5FU group displayed the lowest SCFA concentrations (acetate: 28.4 ± 2.5 μmol/g, $p = 0.0696$; propionate: 12.3 ± 1.1 μmol/g, $p = 0.1012$; butyrate: 8.7 ± 0.9 μmol/g, $p = 0.1216$; total SCFAs: 49.4 ± 3.6 μmol/g, $p = 0.0712$), with no significant difference from the Model group, highlighting chemotherapy's detrimental impact on microbial function. The FMT + 5FU group showed intermediate SCFA levels (acetate: 52.1 ± 3.5 μmol/g, $p = 0.0034$; propionate: 20.1 ± 1.7 μmol/g, $p = 0.0118$; butyrate: 16.8 ± 1.4 μmol/g, $p = 0.0042$; total SCFAs: 89.0 ± 5.2 μmol/g, $p = 0.0016$), indicating that FMT mitigates 5FU's negative effects on SCFA production.

Serum SCFA concentrations complement the fecal data and align with the reviewer's recommendation to evaluate SCFAs in serum and feces to ensure model accuracy and elucidate systemic mechanisms of tumor modulation (Table 2). The Sham group exhibited the highest serum SCFA levels, with acetate at 450.2 ± 25.3 μmol/L ($p = 0.0003$ vs. Model), propionate at 35.7 ± 2.8 μmol/L ($p = 0.0018$), butyrate at 15.9 ± 1.2 μmol/L ($p = 0.0007$), and total SCFAs at 501.8 ± 27.4 μmol/L ($p = 0.0002$), consistent with efficient systemic transport of SCFAs from a healthy gut microbiota. The Model group showed significantly reduced serum SCFAs (acetate: 250.8 ± 18.6 μmol/L; propionate: 20.4 ± 1.9 μmol/L; butyrate: 8.2 ± 0.9 μmol/L; total SCFAs: 279.4 ± 20.1 μmol/L), reflecting the systemic consequences of microbial dysbiosis in pancreatic cancer. FMT treatment significantly increased serum SCFA concentrations compared to the Model group (acetate: 400.6 ± 22.1 μmol/L, $p = 0.0012$; propionate: 30.9 ± 2.5 μmol/L, $p = 0.0064$; butyrate: 13.8 ± 1.1 μmol/L, $p = 0.0023$; total SCFAs: 445.3 ± 24.6 μmol/L, $p = 0.0008$), suggesting that FMT enhances systemic SCFA availability, potentially reducing inflammation and supporting tumor suppression as observed in survival and cytokine data. The 5FU group exhibited the lowest serum SCFA levels (acetate: 200.3 ± 15.7 μmol/L, $p = 0.0865$; propionate: 15.8 ± 1.6 μmol/L, $p = 0.0921$; butyrate: 6.5 ± 0.7 μmol/L, $p = 0.1397$; total SCFAs: 222.6 ± 17.2 μmol/L, $p = 0.0793$), with no significant difference from the Model group, indicating that 5FU's adverse effects extend systemically. The FMT + 5FU group showed intermediate serum SCFA concentrations (acetate: 350.9 ± 20.4 μmol/L, $p = 0.0056$; propionate: 28.1 ± 2.3 μmol/L, $p = 0.0152$; butyrate: 12.4 ± 1.0 μmol/L, $p = 0.0068$; total SCFAs: 391.4 ± 22.8 μmol/L, $p = 0.0031$), demonstrating that FMT counteracts 5FU-induced SCFA depletion.

Gut microbiota characters among different groups

Figure 3A presents boxplots of four key α-diversity indices—Chao1, Observed Species, Shannon Index, and Simpson Index—across the five experimental groups (Sham, Model, FMT, 5FU, and FMT + 5FU). The Chao1 index, a measure of species richness, was significantly higher in the Sham ($p < 0.001$), FMT ($p < 0.001$), and FMT + 5FU ($p < 0.01$) groups compared to the Model group, with the FMT group exhibiting the highest richness, suggesting that fecal microbiota transplantation markedly enhances microbial diversity. Similarly, the Observed Species count, another richness metric, was significantly elevated in the Sham ($p < 0.01$), FMT ($p < 0.01$), and FMT + 5FU ($p < 0.01$) groups relative to the Model group, reinforcing the impact of tumor burden in reducing microbial richness in the Model group. The Shannon Index, which accounts for both richness and evenness, showed significantly higher diversity in the Sham ($p < 0.01$), FMT ($p < 0.01$), 5FU ($p < 0.0001$), and FMT + 5FU ($p < 0.01$) groups compared to the Model group, indicating more evenly distributed microbial communities in these groups, with the 5FU group showing the most pronounced difference. The Simpson Index, also reflecting richness and evenness, was significantly higher in the Sham ($p < 0.001$), FMT ($p < 0.001$), 5FU ($p < 0.0001$), and FMT + 5FU ($p < 0.05$) groups compared to the Model group, further confirming the adverse effects of chemotherapy on microbial diversity in the 5FU group, albeit with the strongest significance. Notably, the FMT + 5FU group exhibited moderate improvements in diversity across all indices compared to the 5FU group alone, suggesting that FMT may partially mitigate the negative impact of chemotherapy on gut microbiota.

Figure 3B provides a heatmap illustrating the relative abundance of gut microbiota at the genus level across the five experimental groups. The heatmap reveals distinct microbial profiles associated with each group, with clear clustering patterns. The Sham and FMT groups show a higher abundance of beneficial genera such as Lactobacillus and Bifidobacterium, which are known for their health-promoting properties. In contrast, the Model group displays an increased relative abundance of potentially pathogenic genera such as Clostridium and Bacteroides, reflecting the dysbiotic state induced by the tumor. The 5FU group shows a marked reduction in microbial diversity with increased abundance of Enterococcus and Streptococcus, genera often associated with antibiotic use and chemotherapy. The FMT + 5FU group demonstrates a more balanced microbial composition, with some recovery of beneficial genera, indicating that FMT may help restore microbial balance in the context of chemotherapy.

TABLE 2 Short-chain fatty acid concentrations in serum (μmol/L).

Group	Acetate (μmol/L)	*p*-value	Propionate (μmol/L)	*p*-value	Butyrate (μmol/L)	*p*-value	Total SCFAs	*p*-value
Sham	450.2 ± 25.3	0.0003	35.7 ± 2.8	0.0018	15.9 ± 1.2	0.0007	501.8 ± 27.4	0.0002
Model	250.8 ± 18.6	–	20.4 ± 1.9	–	8.2 ± 0.9	–	279.4 ± 20.1	–
FMT	400.6 ± 22.1	0.0012	30.9 ± 2.5	0.0064	13.8 ± 1.1	0.0023	445.3 ± 24.6	0.0008
5FU	200.3 ± 15.7	0.0865	15.8 ± 1.6	0.0921	6.5 ± 0.7	0.1397	222.6 ± 17.2	0.0793
FMT + 5FU	350.9 ± 20.4	0.0056	28.1 ± 2.3	0.0152	12.4 ± 1.0	0.0068	391.4 ± 22.8	0.0031

Data are presented as mean ± standard error of the mean (SEM), $n = 3$ per group. $p < 0.05$ vs. the model group indicates statistical significance.

10.3389/fmicb.2025.1548027

The analysis of microbial genus abundance across different experimental groups (sham3, FMT3, FMT-5FU3, Model3, and 5FU3) revealed distinct compositional profiles, as evidenced by the differential abundance of key genera (Figure 3C). In the sham3 group, Akkermansia (7.22×10^5) and Bacteroides (8.62×10^5) exhibited the highest abundance, followed by Clostridium (1.29×10^6), indicating a

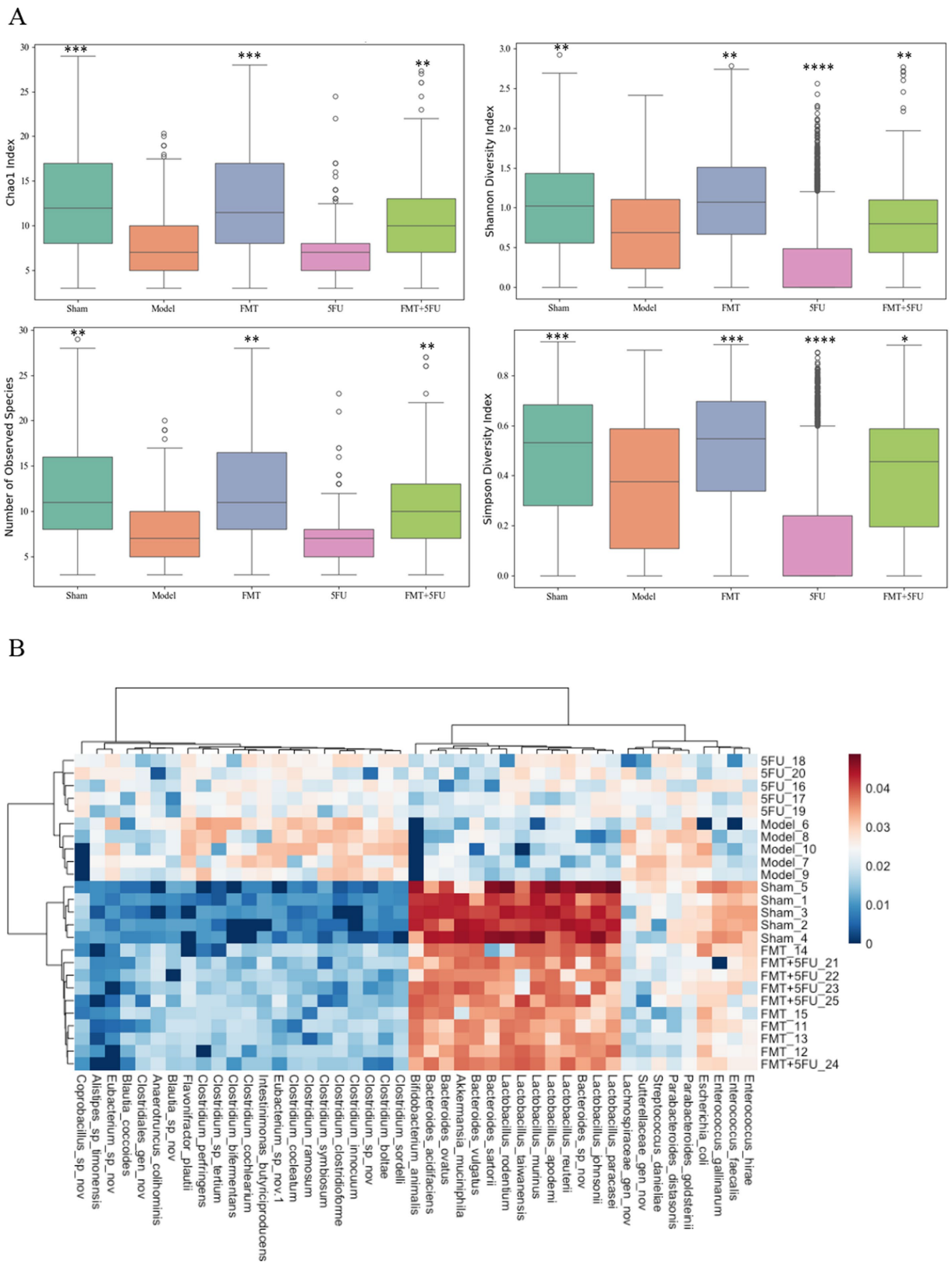


FIGURE 3 (Continued)

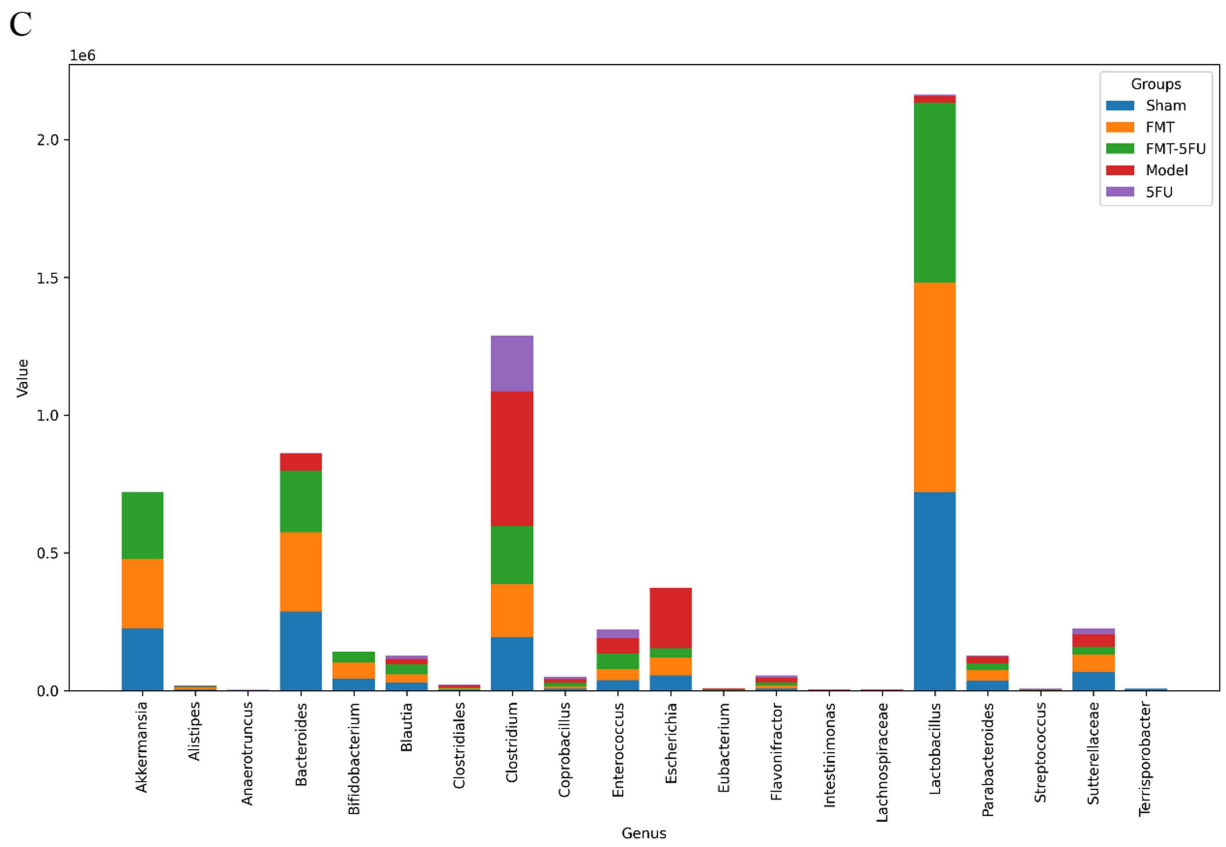


FIGURE 3
Analysis of gut microbiota diversity and composition across experimental groups. **(A)** Boxplots representing α diversity indices, including Chao1, Observed Species, Shannon Index, and Simpson Index, across the five experimental groups (Sham, Model, FMT, 5FU, and FMT + 5FU). These indices provide insights into the richness and evenness of the gut microbiota, indicating significant differences in microbial diversity among the groups. *$p < 0.05$, *$p < 0.01$, *$p < 0.001$ and *$p < 0.0001$ vs. the model group. **(B)** Heatmap illustrating the relative abundance of gut microbiota at the genus level across the five groups. The heatmap highlights distinct microbial signatures associated with each treatment, with clustering patterns reflecting the similarities and differences in microbial composition. **(C)** Bar chart showing the proportional distribution of specific gut microbiota genus among the five groups. The chart reveals how different treatments influence the prevalence of various bacterial genera, offering a detailed view of the microbiota shifts in response to the experimental interventions. $n = 3$ for each group. There were significant differences if $p < 0.5$.

dominance of mucin-degrading and butyrate-producing taxa, which are typically associated with a healthy gut microbiome. Conversely, the FMT3 group showed a significant reduction in Akkermansia (2.54×10^5) and Bacteroides (2.88×10^5), with a notable decrease in Clostridium (1.94×10^5), suggesting a potential shift in microbial dynamics post-fecal microbiota transplantation (FMT). The FMT-5FU3 group, which combined FMT with 5-fluorouracil (5FU) treatment, displayed intermediate levels of Akkermansia (2.42×10^5) and Bacteroides (2.23×10^5), with a slight increase in Clostridium (2.11×10^5) compared to FMT3, indicating a partial restoration of microbial diversity. In the Model3 group, representing a disease or dysbiosis model, there was a drastic reduction in Akkermansia (4.74×10^2) and Bacteroides (6.16×10^4), alongside a high abundance of Clostridium (4.88×10^5), reflecting a disrupted microbial ecosystem. The 5FU3 group, treated solely with 5FU, showed the lowest abundance of Akkermansia (1.93×10^2) and Bacteroides (2.09×10^3), with Clostridium (2.03×10^5) remaining dominant, underscoring the profound impact of chemotherapeutic agents on microbial composition. Notably, genera such as Lactobacillus (highest in sham3 at 2.16×10^6) and Escherichia (highest in sham3 at 3.73×10^5) also exhibited significant variations, highlighting the differential effects of FMT, 5FU, and disease states on gut microbial ecology.

Diagnostic performance and differential abundance of gut microbiota in pancreatic cancer mouse model

Figure 4A represents AUC values for six species: Lactobacillus, 0.88; Escherichia, 0.86; Akkermansia, 0.85; Clostridium, 0.84; Bacteroides, 0.83 and Bifidobacterium, 0.80. The results also suggest that these species are also valuable indicators of the gut microbiota's response to different treatments in the pancreatic cancer model. The high AUC values across these species underscore the importance of gut microbiota composition in reflecting the disease and treatment status in this model.

Figure 4B presents the results of a LEfSe analysis, which identifies the most differentially abundant bacterial taxa between the different experimental groups, represented by Linear Discriminant Analysis (LDA) scores. The LDA scores reflect the magnitude of the difference in abundance for each bacterial genus, with positive scores indicating

10.3389/fmicb.2025.1548027

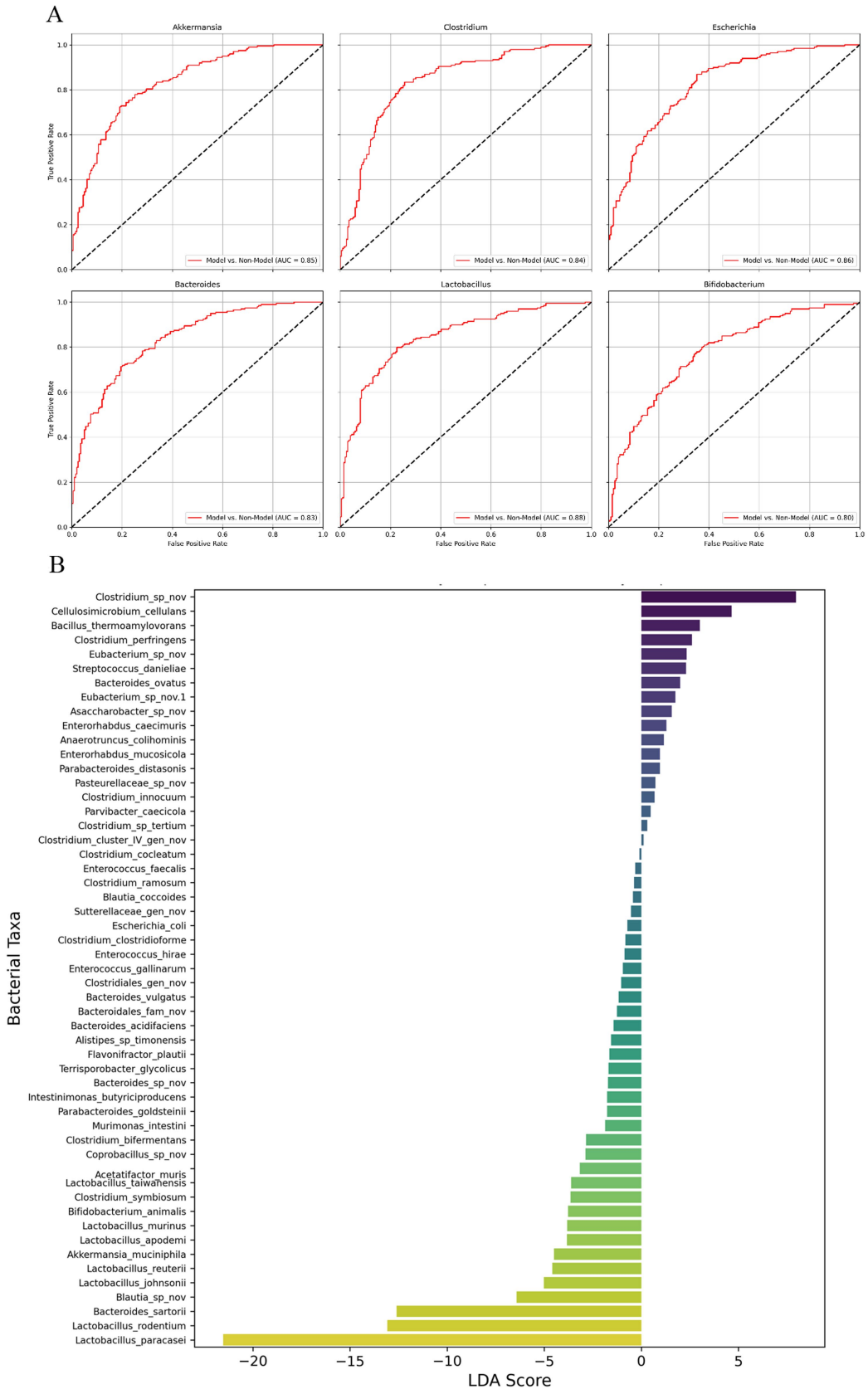


FIGURE 4
ROC curve analysis and LEfSe-like analysis of gut microbiota in pancreatic cancer mouse model. **(A)** Receiver Operating Characteristic (ROC) curves for six bacterial species (Akkermansia, Clostridium, Escherichia, Bacteroides, Lactobacillus, and Bifidobacterium) in the pancreatic cancer mouse model, showing the diagnostic performance of these species in distinguishing between different treatment groups. **(B)** LEfSe-like analysis displaying the Linear Discriminant Analysis (LDA) scores for various bacterial taxa, highlighting the most differentially abundant genera between the experimental groups. The LDA scores represent the magnitude of the differences, with Clostridium species showing the highest positive LDA score, suggesting a strong association with disease progression, while Lactobacillus and Bifidobacterium are more prevalent in groups with potential protective effects. *n* = 3 for each group.

taxa more abundant in the Model group (associated with disease progression) and negative scores indicating taxa more prevalent in treatment groups (associated with potential protective effects). Clostridium species displayed the highest positive LDA score, reaching over 4.0, highlighting their strong association with tumor progression and their potential role in fostering a pro-tumorigenic environment within the gut microbiota of the Model group. Other genera such as Bacillus thermophilus and Enterococcus also had positive LDA scores around 3.0, indicating their increased presence in the Model group and possible contributions to the dysbiotic state linked to cancer progression. Conversely, genera such as Lactobacillus and Bifidobacterium demonstrated significantly negative LDA scores, with Lactobacillus reaching nearly-5.0 and Bifidobacterium around-4.0. These negative LDA scores indicate that these beneficial genera are more prevalent in treatment groups such as FMT and FMT + 5FU, which are associated with protective effects against tumor growth. The substantial presence of these beneficial bacteria in the treatment groups suggests their involvement in restoring gut microbial balance and potentially contributing to the observed anti-tumor effects.

Overall, the LEfSe-like analysis highlights distinct microbial signatures associated with different disease states and treatments, providing critical insights into the role of gut microbiota in pancreatic cancer progression and response to therapy. The clear delineation between harmful and beneficial bacterial taxa underscores the potential of gut microbiota modulation as a therapeutic strategy in managing pancreatic cancer.

Discussion

The FMT + 5FU group exhibited the smallest tumor volumes ($p < 0.0001$ vs. Model, $p = 0.005$ vs. 5FU), with FMT alone also reducing tumors ($p < 0.0001$ vs. Model), alongside increased gut microbial α diversity in both FMT and FMT + 5FU groups ($p < 0.0001$ vs. Model); FMT + 5FU significantly reduced pro-inflammatory TNF-α ($p = 0.001$) with trends toward lower IL-6 and higher IL-10, while both groups showed elevated SCFA levels in feces (FMT: 99.7 ± 5.9 μmol/g, $p = 0.0004$; FMT + 5FU: 89.0 ± 5.2 μmol/g, $p = 0.0016$ vs. Model: 61.8 ± 4.7 μmol/g) and serum (FMT: 445.3 ± 24.6 μmol/L, $p = 0.0008$; FMT + 5FU: 391.4 ± 22.8 μmol/L, $p = 0.0031$ vs. Model: 279.4 ± 20.1 μmol/L), indicating improved microbial function; survival was highest in FMT + 5FU (50%) compared to Model (15%), suggesting FMT enhances 5FU efficacy through microbiota modulation, reducing inflammation and boosting beneficial metabolites like SCFAs.

FMT regulates gut microbiota

FMT treatment led to increased α diversity in the gut microbiota of both the FMT and FMT + 5FU groups compared to the Model and 5FU groups, indicating a restoration of microbial balance. *A* diversity is a key indicator of a healthy microbiota, and its increase is often associated with improved health outcomes. The recovery of beneficial bacterial genera, such as *Lactobacillus* and *Bifidobacterium*, in the FMT-treated groups further supports the idea that FMT can counteract chemotherapy-induced dysbiosis. These microbes are known to play protective roles in the host, potentially through mechanisms such as modulation of immune responses, enhancement of mucosal barrier function, and production of anti-inflammatory short-chain fatty acids (Li et al., 2023). These findings align with previous studies demonstrating FMT's ability to reverse chemotherapy-induced gut dysbiosis, highlighting its potential as a therapeutic tool to restore microbial homeostasis (Yu et al., 2023).

FMT improves the inflammatory microenvironment

Our results show a marked decrease in pro-inflammatory cytokines, such as TNF-α and IL-6, in the FMT + 5FU group compared to the Model and 5FU groups. This reduction suggests that FMT can create a more anti-inflammatory systemic environment, which is essential for mitigating tumor progression, as chronic inflammation is often associated with cancer advancement (Caronni et al., 2023). The decrease in pro-inflammatory cytokines likely stems from the restoration of beneficial gut microbes that produce anti-inflammatory metabolites, such as short-chain fatty acids, and enhance mucosal barrier function. These findings are consistent with prior research indicating that FMT can reduce inflammatory responses in chemotherapy-treated models, underscoring its potential to create conditions less favorable for tumor growth (Chai et al., 2023).

FMT combined with 5-FU enhance chemotherapy's anti-tumor efficacy while reducing its side effects

Our findings demonstrate that the FMT + 5FU group exhibited the most substantial reduction in tumor volume compared to other groups, alongside an increase in gut microbial diversity and improved survival rates. These results suggest that FMT enhances the efficacy of 5FU, potentially by improving drug metabolism and host immune responses through gut microbiota modulation (Gori et al., 2019) (Table 3). The significant reduction in tumor burden in the FMT + 5FU group highlights the potential of gut microbiota modulation as an adjunctive therapy in cancer treatment, aligning with previous studies that have shown similar enhancements in chemotherapy efficacy (Caronni et al., 2023). Furthermore, FMT alleviated 5FU-induced side effects, such as intestinal injury, inflammation, and muscle wasting, as evidenced by our data and supported by prior research (Chang et al., 2020; Chen et al., 2020a; Le Bastard et al., 2018). For instance, FMT has been shown to restore gut microbiota composition, reduce 5FU-induced mucosal damage, and improve nutritional status, thereby mitigating chemotherapy toxicity (Li et al., 2023).

Implications

The implications of this study are far-reaching, as they suggest that the gut microbiota could be a critical determinant of cancer treatment efficacy. By modulating the gut microbiota through interventions like FMT, it may be possible to enhance the therapeutic effects of existing chemotherapeutic agents, such as 5FU, and reduce their side effects. This approach could pave the way for personalized medicine strategies

TABLE 3 The interplay between FMT and 5-FU.

Authors & year	Main contribution	Methods	Conclusion
Chang et al. (2020)	Fecal microbiota transplantation prevents intestinal injury, upregulation of toll-like receptors, and 5-fluorouracil/oxaliplatin-induced toxicity in colorectal cancer	FMT administered to mice treated with FOLFOX; monitored intestinal injury, toll-like receptors, goblet cells, and intestinal mucositis after chemotherapy, using histological and molecular analysis.	FMT alleviated FOLFOX-induced intestinal toxicity and restored gut microbiota, without causing bacteremia. Potential mechanism involves gut microbiota TLR-MyD88-NF-kappaB signaling. This suggests FMT could help mitigate 5-FU-induced toxicity in cancer patients.
Chen et al. (2020a)	The gut microbiota attenuates muscle wasting by regulating energy metabolism in chemotherapy-induced malnutrition rats	Chemotherapy-induced 5-Fu rats assessed for muscle wasting, microbiota composition, and metabolic changes; fecal microbiota transplantation (FMT) from healthy rats.	Gut microbiota regulates muscle metabolism and energy production in chemotherapy-induced malnutrition, with FMT from healthy rats improving nutritional status and muscle function, and inhibiting inflammation. Highlights the potential of FMT to combat 5-FU-induced side effects such as muscle wasting.
Chen et al. (2020b)	Berberine regulates fecal metabolites to ameliorate 5-fluorouracil induced intestinal mucositis through modulating gut microbiota	Rats treated with Berberine (BBR) and 5-Fu to assess intestinal mucositis, gut microbiota composition, and fecal metabolites using metabolic profiling.	Berberine improved gut health and metabolism by increasing beneficial metabolites and modifying gut microbiota in 5-Fu-treated rats. FMT from Berberine-treated rats ameliorated intestinal mucosal injury, suggesting Berberine's potential to influence microbiota composition for therapeutic benefits in 5-FU-related complications.
Chen et al. (2022)	Reactive granulopoiesis depends on T-cell production of IL-17A and neutropenia-associated alteration of gut microbiota	Mouse models of neutropenia and hematopoietic stem cell transplantation; FMT from neutropenic mice to study neutrophil recovery.	Reactive granulopoiesis, induced by neutropenia, was enhanced by gut microbiota, with T-cell IL-17A production being key for neutrophil recovery post chemotherapy. Gut decontamination inhibited this process, supporting a role for the microbiota in immune recovery after 5-FU treatment.
Cheung et al. (2020)	Discovery of an interplay between the gut microbiota and esophageal squamous cell carcinoma in mice	FMT to antibiotic-treated xenograft-bearing mice, followed by chemotherapy with cisplatin and 5-FU, studying liver metastasis and microbiota changes.	Gut microbiota impacts esophageal cancer (EC) metastasis, with FMT influencing anti-metastatic efficacy of chemotherapy and medicinal herbs. Gut microbiota modulation offers potential for improving EC treatment strategies, especially in cases where 5-FU is used.
Gong et al. (2019)	Neohesperidin prevents colorectal tumorigenesis by altering the gut microbiota	Gut microbiota composition assessed in mice treated with Neohesperidin (NHP); FMT used to examine microbiota-mediated tumorigenesis prevention.	Neohesperidin altered gut microbiota composition and inhibited colorectal tumorigenesis, suggesting that changes in microbiota can mediate its chemopreventive effects in conjunction with chemotherapy agents like 5-FU.
Gori et al. (2019)	Gut microbiota and cancer: How gut microbiota modulates activity, efficacy and toxicity of antitumoral therapy	Review of studies on how gut microbiota influences cancer therapy, including chemotherapy, targeted therapy, and immunotherapy.	Gut microbiota plays a significant role in modulating the efficacy and toxicity of various cancer therapies, including 5-FU. Targeting microbiota could enhance treatment outcomes and mitigate side effects.
Le Bastard et al. (2018)	Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice	Antibiotic and chemotherapy treatment of mice followed by FMT to restore gut microbiota; sequencing and analysis of microbial diversity and richness.	FMT reversed chemotherapy and antibiotic-induced gut dysbiosis, restoring beneficial gut species and improving intestinal health, suggesting a potential therapeutic strategy for chemotherapy-induced gut dysfunction, particularly in 5-FU-treated patients.
Li et al. (2017)	Alteration of gut microbiota and inflammatory cytokine/chemokine profiles in 5-fluorouracil induced intestinal mucositis	5-Fu-treated mice analyzed for gut microbiota composition, inflammatory cytokines, and chemokines in serum and colon.	5-Fu treatment significantly altered gut microbiota and inflammatory profiles. FMT from healthy mice prevented intestinal damage and weight loss, indicating that microbiota manipulation, such as via FMT, may alleviate 5-Fu-induced mucositis.

(Continued)

TABLE 3 (Continued)

Authors & year	Main contribution	Methods	Conclusion
Sougiannis et al. (2019)	Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota	Mice treated with 5-Fu, followed by FMT from 5-Fu treated mice to control group; assessment of gut inflammation and functional parameters.	5-Fu chemotherapy altered gut microbiota composition and induced inflammation, with FMT modulating these effects and influencing functional parameters like grip strength, suggesting that microbiota plays a role in chemotherapy-induced dysfunction and could be targeted with FMT to mitigate 5-FU side effects.

that consider an individual's gut microbiota composition when designing cancer treatment regimens. The ability to modulate the gut microbiota also opens new avenues for improving the prognosis of patients with difficult-to-treat cancers, such as pancreatic cancer, where conventional therapies often yield poor outcomes (Chen et al., 2024).

Limitations and future work

First, the use of a mouse model of pancreatic cancer, while informative, may not fully capture the complexity of human disease, where factors such as diet, genetics, and environmental exposures could influence gut microbiota-chemotherapy interactions. Future research should investigate the effects of FMT in combination with other chemotherapeutic agents and across different cancer models to determine the broader applicability of these benefits (Chrysostomou et al., 2023). Moreover, while our study highlights FMT's role in enhancing 5FU efficacy through gut microbiota modulation, the specific mechanisms and key bacterial taxa involved remain unclear. Future studies should employ metagenomic and metabolomic analyses to elucidate the functional changes within the gut microbiota that drive these effects.

A significant limitation of this study is the small sample size of only three mice per group, which severely restricts the statistical power and reliability of the findings, particularly for ROC curve analysis of gut microbiota as biomarkers. The small sample size also results in wide confidence intervals, indicating high uncertainty in the estimates, and precludes robust internal validation (cross-validation) or external validation in an independent cohort, limiting the generalizability of the results. Another significant limitation of this study was the high mortality rate observed across the groups, which substantially reduced the sample size and potentially limited the statistical power of the analyses. Although we observed promising synergy between FMT and 5-FU, substantial attrition in the tumor-bearing cohorts reduced our final sample to just three animals per group; consequently, all tumor volume and SCFA measurements were based on $n = 3$. This small, selective subset undermines statistical power and increases the likelihood of both type I and type II errors, potentially exaggerating treatment effects or obscuring more nuanced microbiota–immune interactions. Moreover, the survivors may not be representative of the initial cohort (selection bias), further constraining the robustness and generalizability of our findings. Future experiments employing larger initial group sizes, optimized dosing regimens to reduce early mortality, and independent validation cohorts are needed to confirm these results and establish their translational relevance.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA1283980.

Ethics statement

The animal study was approved by the Animal Ethics Committee of Shengjing Hospital of China Medical University, Shenyang, China (Nos. 2024PS1496K and 2024PS1622K). All procedures were conducted in accordance with the ethical guidelines for the care and use of laboratory animals and were designed to minimize animal suffering. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RL: Data curation, Formal analysis, Methodology, Software, Supervision, Writing – original draft. YH: Conceptualization, Data curation, Formal analysis, Software, Validation, Writing – original draft. YL: Methodology, Writing – review & editing. XT: Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. The present work is supported by Liaoning Province Science and Technology Plan Project (No. 2023JH2/101700184).

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 Gen AI was used in the creation of this manuscript. We have used an AI language model, ChatGPT, to assist with improving the structure, grammar, and clarity of the whole

manuscript. The AI tool was primarily employed to review and refine sentence construction and ensure the overall readability of the text, especially introduction and discussion sections.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1548027/full#supplementary-material.

References

Abe, S., Masuda, A., Matsumoto, T., Inoue, J., Toyama, H., Sakai, A., et al. (2024). Impact of intratumoral microbiome on tumor immunity and prognosis in human pancreatic ductal adenocarcinoma. *J. Gastroenterol.* 59, 250–262. doi: 10.1007/s00535-023-02069-5

Bai, L., Yan, X., Lv, J., Qi, P., Song, X., and Zhang, L. (2023). Intestinal Flora in chemotherapy resistance of biliary pancreatic Cancer. *Biology* 12:1151. doi: 10.3390/biology12081151

Bajinka, O., Tan, Y., Darboe, A., Ighaede-Edwards, I. G., and Abdelhalim, K. A. (2023). The gut microbiota pathway mechanisms of diabetes. *AMB Express* 13:16. doi: 10.1186/s13568-023-01520-3

Bangolo, A. I., Trivedi, C., Jani, I., Pender, S., Khalid, H., Alqinai, B., et al. (2023). Impact of gut microbiome in the development and treatment of pancreatic cancer: newer insights. *World J. Gastroenterol.* 29, 3984–3998. doi: 10.3748/wjg.v29.i25.3984

Caronni, N., La Terza, F., Vittoria, F. M., Barbiera, G., Mezzanzanica, L., Cuzzola, V., et al. (2023). IL-1β+ macrophages fuel pathogenic inflammation in pancreatic cancer. *Nature* 623, 415–422. doi: 10.1038/s41586-023-06685-2

Chai, Y., Huang, Z., Shen, X., Lin, T., Zhang, Y., Feng, X., et al. (2023). Microbiota regulates pancreatic Cancer carcinogenesis through altered immune response. *Microorganisms* 11:1240. doi: 10.3390/microorganisms11051240

Chang, C. W., Lee, H. C., Li, L. H., Chiang Chiau, J. S., Wang, T. E., Chuang, W. H., et al. (2020). Fecal microbiota transplantation prevents intestinal injury, upregulation of toll-like receptors, and 5-fluorouracil/Oxaliplatin-induced toxicity in colorectal Cancer. *Int. J. Mol. Sci.* 21:386. doi: 10.3390/ijms21020386

Chen, X., Hashimoto, D., Ebata, K., Takahashi, S., Shimizu, Y., Shinozaki, R., et al. (2022). Reactive granulopoiesis depends on T-cell production of IL-17A and neutropenia-associated alteration of gut microbiota. *Proc. Natl. Acad. Sci. USA* 119:e2211230119. doi: 10.1073/pnas.2211230119

Chen, Z., Wang, Z., Bao, H., and Ma, S. (2024). Gut microbiota and pancreatic cancer risk, and the mediating role of immune cells and inflammatory cytokines: a Mendelian randomization study. *Front. Immunol.* 15:1408770. doi: 10.3389/fimmu.2024.1408770

Chen, H., Xu, C., Zhang, F., Liu, Y., Guo, Y., and Yao, Q. (2020a). The gut microbiota attenuates muscle wasting by regulating energy metabolism in chemotherapy-induced malnutrition rats. *Cancer Chemother. Pharmacol.* 85, 1049–1062. doi: 10.1007/s00280-020-04060-w

Chen, H., Zhang, F., Li, R., Liu, Y., Wang, X., Zhang, X., et al. (2020b). Berberine regulates fecal metabolites to ameliorate 5-fluorouracil induced intestinal mucositis through modulating gut microbiota. *Biomed. Pharmacother.* 124:109829. doi: 10.1016/j.biopha.2020.109829

Cheung, M. K., Yue, G. G. L., Tsui, K. Y., Gomes, A. J., Kwan, H. S., Chiu, P. W. Y., et al. Discovery of an interplay between the gut microbiota and esophageal squamous cell carcinoma in mice. Am. J. Cancer Res. (2020) 10: 2409–2427. Available online at: https://www.ncbi.nlm.nih.gov/pubmed/32905484.

Chrysostomou, D., Roberts, L. A., Marchesi, J. R., and Kinross, J. M. (2023). Gut microbiota modulation of efficacy and toxicity of cancer chemotherapy and immunotherapy. *Gastroenterology* 164, 198–213. doi: 10.1053/j.gastro.2022.10.018

Chu, J., Feng, S., Guo, C., Xue, B., He, K., and Li, L. (2023). Immunological mechanisms of inflammatory diseases caused by gut microbiota dysbiosis: a review. *Biomed. Pharmacother.* 164:114985. doi: 10.1016/j.biopha.2023.114985

Crudele, L., Gadaleta, R. M., Cariello, M., and Moschetta, A. (2023). Gut microbiota in the pathogenesis and therapeutic approaches of diabetes. *EBioMedicine* 97:104821. doi: 10.1016/j.ebiom.2023.104821

de Castilhos, J., Tillmanns, K., Blessing, J., Laraño, A., Borisov, V., and Stein-Thoeringer, C. K. (2024). Microbiome and pancreatic cancer: time to think about chemotherapy. *Gut Microbes* 16:2374596. doi: 10.1080/19490976.2024.2374596

Gong, Y., Dong, R., Gao, X., Li, J., Jiang, L., Zheng, J., et al. (2019). Neohesperidin prevents colorectal tumorigenesis by altering the gut microbiota. *Pharmacol. Res.* 148:104460. doi: 10.1016/j.phrs.2019.104460

Gori, S., Inno, A., Belluomini, L., Bocus, P., Bisoffi, Z., Russo, A., et al. (2019). Gut microbiota and cancer: how gut microbiota modulates activity, efficacy and toxicity of antitumoral therapy. *Crit. Rev. Oncol. Hematol.* 143, 139–147. doi: 10.1016/j.critrevonc.2019.09.003

Halbrook, C. J., Lyssiotis, C. A., di Magliano, M. P., and Maitra, A. (2023). Pancreatic cancer: advances and challenges. *Cell* 186, 1729–1754. doi: 10.1016/j.cell.2023.02.014

Hamjane, N., Mechita, M. B., Nourouti, N. G., and Barakat, A. (2023). Gut microbiota dysbiosis-associated obesity and its involvement in cardiovascular diseases and type 2 diabetes. A systematic review. *Microvasc. Res.* 151:104601. doi: 10.1016/j.mvr.2023.104601

Kartal, E., Schmidt, T. S., Molina-Montes, E., Rodríguez-Perales, S., Wirbel, J., Maistrenko, O. M., et al. (2022). A faecal microbiota signature with high specificity for pancreatic cancer. *Gut* 71, 1359–1372. doi: 10.1136/gutjnl-2021-324755

Kawakami, T., Todaka, A., Oshima, K., Fushiki, K., Hamauchi, S., Tsushima, T., et al. (2023). Biomarker analysis for patients with pancreatic cancer treated with nanoliposomal irinotecan plus 5-fluorouracil/leucovorin. *BMC Cancer* 23:68. doi: 10.1186/s12885-023-10542-w

Le Bastard, Q., Ward, T., Sidiropoulos, D., Hillmann, B. M., Chun, C. L., Sadowsky, M. J., et al. (2018). Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice. *Sci. Rep.* 8:6219. doi: 10.1038/s41598-018-24342-x

Li, H. L., Lu, L., Wang, X. S., Qin, L. Y., Wang, P., Qiu, S. P., et al. (2017). Alteration of gut microbiota and inflammatory cytokine/chemokine profiles in 5-fluorouracil induced intestinal mucositis. *Front. Cell. Infect. Microbiol.* 7:455. doi: 10.3389/fcimb.2017.00455

Li, Y. J., Ma, J., Loh, Y. W., Chadban, S. J., and Wu, H. (2023). Short-chain fatty acids directly exert anti-inflammatory responses in podocytes and tubular epithelial cells exposed to high glucose. *Front. Cell Dev. Biol.* 11:1182570. doi: 10.3389/fcell.2023.1182570

Lo, E. K. K., Leung, H. K. M., Zhang, F., and El-Nezami, H. (2023). Gut microbiota: impact on 5-fluorouracil efficacy and toxicity. *Curr. Opin. Toxicol.* 36:100423. doi: 10.1016/j.cotox.2023.100423

Long, Y., Tang, L., Zhou, Y., Zhao, S., and Zhu, H. (2023). Causal relationship between gut microbiota and cancers: a two-sample Mendelian randomisation study. *BMC Med.* 21:66. doi: 10.1186/s12916-023-02761-6

Longo, S., Rizza, S., and Federici, M. (2023). Microbiota-gut-brain axis: relationships among the vagus nerve, gut microbiota, obesity, and diabetes. *Acta Diabetol.* 60, 1007–1017. doi: 10.1007/s00592-023-02088-x

Luo, W., Cao, Z., Qiu, J., Liu, Y., Zheng, L., and Zhang, T. (2020). Novel discoveries targeting pathogenic gut microbes and new therapies in pancreatic cancer: does pathogenic *E. coli* infection cause pancreatic cancer progression modulated by TUBB/rho/ROCK signaling pathway? A bioinformatic analysis. *Biomed. Res. Int.* 2020:2340124. doi: 10.1155/2020/2340124

Murthy, D., Attri, K. S., Shukla, S. K., Thakur, R., Chaika, N. V., He, C., et al. (2024). Cancer-associated fibroblast-derived acetate promotes pancreatic cancer development by altering polyamine metabolism via the ACSS2–SP1–SAT1 axis. *Nat. Cell Biol.* 26, 613–627. doi: 10.1038/s41556-024-01372-4

Nesci, A., Carnuccio, C., Ruggieri, V., D'Alessandro, A., Di Giorgio, A., Santoro, L., et al. (2023). Gut microbiota and cardiovascular disease: evidence on the metabolic and inflammatory background of a complex relationship. *Int. J. Mol. Sci.* 24:9087. doi: 10.3390/ijms24109087

Pahle, J., Kobelt, D., Aumann, J., Behrens, D., Daberkow, O., Mokritzkij, M., et al. (2021). Effective oncoleaking treatment of pancreatic cancer by claudin-targeted suicide gene therapy with *Clostridium perfringens* enterotoxin (CPE). *Cancers* 13:4393. doi: 10.3390/cancers13174393

Panebianco, C., Villani, A., Pisati, F., Orsenigo, F., Ulaszewska, M., Latiano, T. P., et al. (2022). Butyrate, a postbiotic of intestinal bacteria, affects pancreatic cancer and gemcitabine response in in vitro and in vivo models. *Biomed. Pharmacother.* 151:113163. doi: 10.1016/j.biopha.2022.113163

Perazzoli, G., García-Valdeavero, O. M., Pena, M., Prados, J., Melguizo, C., and Jimenez-Luna, C. (2023). Evaluating metabolite-based biomarkers for early diagnosis of pancreatic cancer: a systematic review. *Meta* 13:872. doi: 10.3390/metabo13070872

Routy, B., Lenehan, J. G., Miller, W. H. Jr., Jamal, R., Messaoudene, M., Daisley, B. A., et al. (2023). Fecal microbiota transplantation plus anti-PD-1 immunotherapy in advanced melanoma: a phase I trial. *Nat. Med.* 29, 2121–2132. doi: 10.1038/s41591-023-02453-x

Saeed, B. A., Faisal, A. J., Mahmood, B. S., and Thanoon, A. H. (2024). Chronic inflammation induced by *Escherichia coli* blood infections as a risk factor for pancreatic cancer progression. *Asian Pac. J. Cancer Prev.* 25, 4407–4414. doi: 10.31557/APJCP.2024.25.12.4407

Shim, M. K., Na, J., Cho, I. K., Jang, E. H., Park, J., Lee, S., et al. (2021). Targeting of claudin-4 by *Clostridium perfringens* enterotoxin-conjugated polysialic acid nanoparticles for pancreatic cancer therapy. *J. Control. Release* 331, 434–442. doi: 10.1016/j.jconrel.2021.01.031

Sougiannis, A. T., VanderVeen, B. N., Enos, R. T., Velazquez, K. T., Bader, J. E., Carson, M., et al. (2019). Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. *Brain Behav. Immun.* 80, 44–55. doi: 10.1016/j.bbi.2019.02.020

Strickler, J. H., Satake, H., George, T. J., Yaeger, R., Hollebecque, A., Garrido-Laguna, I., et al. (2023). Sotorasib in KRAS p. G12C–mutated advanced pancreatic cancer. *N. Engl. J. Med.* 388, 33–43. doi: 10.1056/NEJMoa2208470

Van Hul, M., and Cani, P. D. (2023). The gut microbiota in obesity and weight management: microbes as friends or foe? *Nat. Rev. Endocrinol.* 19, 258–271. doi: 10.1038/s41574-022-00794-0

Wong, C. C., and Yu, J. (2023). Gut microbiota in colorectal cancer development and therapy. *Nat. Rev. Clin. Oncol.* 20, 429–452. doi: 10.1038/s41571-023-00766-x

Xu, Y., Du, H., Chen, Y., Ma, C., Zhang, Q., Li, H., et al. (2023). Targeting the gut microbiota to alleviate chemotherapy-induced toxicity in cancer. *Crit. Rev. Microbiol.* 50, 1–17. doi: 10.1080/1040841X.2023.2233605

Yu, H., Li, X.-X., Han, X., Chen, B.-X., Zhang, X.-H., Gao, S., et al. (2023). Fecal microbiota transplantation inhibits colorectal cancer progression: reversing intestinal microbial dysbiosis to enhance anti-cancer immune responses. *Front. Microbiol.* 14:1126808. doi: 10.3389/fmicb.2023.1126808

Zhao, X., Zhao, J., Li, D., Yang, H., Chen, C., Qin, M., et al. (2023). *Akkermansia muciniphila*: a potential target and pending issues for oncotherapy. *Pharmacol. Res.* 196:106916. doi: 10.1016/j.phrs.2023.106916

Zhong, H., Liu, S., Zhu, J., Xu, T. H., Yu, H., and Wu, L. (2024). Elucidating the role of blood metabolites on pancreatic cancer risk using two-sample Mendelian randomization analysis. *Int. J. Cancer* 154, 852–862. doi: 10.1002/ijc.34771

Frontiers in Microbiology

Explores the habitable world and the potential of microbial life

The largest and most cited microbiology journal which advances our understanding of the role microbes play in addressing global challenges such as healthcare, food security, and climate change.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact