GUT MICROBIOTA AND GASTROINTESTINAL DISORDERS

EDITED BY: Abbas Yadegar, Javier Ochoa-Repáraz, Toshifumi Ohkusa and Yan-Dong Wang

PUBLISHED IN: Frontiers in Medicine

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-807-7
DOI 10.3389/978-2-83250-807-7

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

GUT MICROBIOTA AND GASTROINTESTINAL DISORDERS

Topic Editors:
Abbas Yadegar, Shahid Beheshti University of Medical Sciences, Iran
Javier Ochoa-Repáraz, Boise State University, United States
Toshifumi Ohkusa, Juntendo University, Japan
Yan-Dong Wang, Beijing University of Chemical Technology, China

Citation: Yadegar, A., Ochoa-Repáraz, J., Ohkusa, T., Wang, Y.-D., eds. (2022). Gut Microbiota and Gastrointestinal Disorders. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-807-7

Table of Contents

frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 03 November 2022
DOI 10.3389/fmed.2022.1079787

OPEN ACCESS

EDITED AND REVIEWED BY
Angel Lanas,
University of Zaragoza, Spain

*CORRESPONDENCE
Abbas Yadegar
a.yadegar@sbmu.ac.ir;
babak_y1983@yahoo.com

SPECIALTY SECTION
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

RECEIVED 25 October 2022
ACCEPTED 26 October 2022
PUBLISHED 03 November 2022

CITATION
Yadegar A, Nabavi-Rad A, Ochoa-Repáraz J, Ohkusa T and Wang Y-D (2022) Editorial: Gut microbiota and gastrointestinal disorders. *Front. Med.* 9:1079787. doi: 10.3389/fmed.2022.1079787

Editorial: Gut microbiota and gastrointestinal disorders

Abbas Yadegar [1]*, Ali Nabavi-Rad [1], Javier Ochoa-Repáraz [2], Toshifumi Ohkusa[3,4] and Yan-Dong Wang [5]

[1]Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2]Department of Biological Sciences, Boise State University, Boise, ID, United States, [3]Department of Microbiota Research, Juntendo University Graduate School of Medicine, Tokyo, Japan, [4]Department of Gastroenterology and Hepatology, The Jikei University Kashiwa Hospital, Chiba, Japan, [5]State Key Laboratory of Chemical Resource Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China

KEYWORDS

gut microbiota, gastrointestinal disorders, non-alcoholic fatty liver disease, inflammatory bowel disease, fecal microbiota transplantation, short-chain fatty acid, predictive applications

Editorial on the Research Topic
Gut microbiota and gastrointestinal disorders

Observational outcomes of recent studies indicate the notion of the gut microbiota's contribution to the host's physiological, immune, neurological, and metabolic health. In dysbiotic conditions, gut microbes could contribute to the development of a wide spectrum of diseases including metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD) (1, 2). However, we are yet to fully understand the underlying mechanisms of the gut microbiota affecting host metabolisms and this field of study is still in its infancy; therefore, research is shifting toward cause-and-effect and multi-omics studies (3). Considering the translational applications of microbiome research, recent studies have demonstrated the great potential of gut microbiota manipulation in reducing the prevalence and severity of gastrointestinal disorders (4). Our growing knowledge about the human indigenous microbial species and the development of biotechnology and machine learning techniques are opening new avenues for the progression of microbiome-based therapeutics toward the application of precisely defined and targeted microbial consortia (5).

In this regard, the special issue of "Gut Microbiota and Gastrointestinal Disorders" appeared highly topical, wherein the aim was to provide novel insights on the central role of the gut microbiota in gastrointestinal disorders and seek innovative therapeutic and predictive applications of selected microbial species.

Considering the profound influence of the gut microbiota on the development and progression of ulcerative colitis (UC), Hu et al. reviewed the genetic, immunological, and microbial risk factors for UC pathogenesis. They enumerated

potential microbiome-based approaches to restore and preserve intestinal microbial homeostasis and subsequently resolve UC. In another review, Wang et al. focused on gastrointestinal microbial dysbiosis and its contribution to the development of NAFLD, ranging from steatosis to non-alcoholic steatohepatitis (NASH). In this study, the special association of periodontopathic bacteria, mainly *Porphyromonas gingivalis*, to the pathogenesis of NAFLD was highlighted through clinical and basic research.

The significance of microbial species in the progression and emission of gastrointestinal disorders has led to the evaluation of longitudinal bio-psychological dynamics by Tavakoli et al. during the treatment procedure of IBD patients. Observational findings suggested a substantial decrease in microbial diversity of Crohn's disease (CD) patients compared to UC patients. This, in principle, leads to the interdependence of treatment strategy and microbial dynamic comparing CD and UC patients. Although most studies associating the gut microbiota with human health and disease have focused on the analysis of bacterial and archaeal components, the importance of the mycobiota, the microbiota's fungal portion, is now recognized. The analysis of the mucosa-associated mycobiota in CD patients by Olaisen et al. revealed the enrichment of *Malassezia* and the depletion of *Saccharomyces*, along with a higher proportion of *Candida albicans* and *Malassezia restricta*, compared to healthy controls. Notably, the mycobiota structural differences between the inflamed and proximal non-inflamed ileum within the same individual may contribute to CD pathogenesis. Moreover, the identification of intraindividual and interindividual differences in microbial composition can be utilized as predictive biomarkers. Tian et al. exhibited the abundance of *Alistipes* and *Eubacterium* and the impoverishment of *Roseburia* species in patients suffering from slow transit constipation. The enrichment of fatty acid biosynthesis, butanoate metabolism, and methane metabolism pathways along with microbial alteration might be potential biomarkers for slow transit constipation. Another predicting strategy was provided by Vincentis et al., evaluating the clinical improvement of patients receiving rifaximin therapy. Although inaccurate in predicting gut microbiota alterations, their electronic multi-sensorial systems constituted of e-tongue and e-nose, could effectively predict clinical improvement.

In the context of IBD research, a treatment strategy for dextran sodium sulfate (DSS)-induced colitis was evaluated by Nakajima et al. in C57BL/6 male mice. Nicotine administration prior to the induction of DSS-induced colitis elevated indole concentration in the distal colon and rectum while short-chain fatty acid (SCFA) values presented insignificant differences, compared to the control cohort. The high level of indole concentration, as well as the increased proportion of *Clostridioides* and *Porphyromonas* genera, were presented as the underpinning mechanism of DSS-induced colitis suppression following nicotine administration. Similarly, Zhou et al. reported the potential for a ginger extract to attenuate the susceptibility to DSS-induced colitis by preventing weight loss, colon shortening, inflammation, and intestinal barrier dysfunction. Ginger administration to mice models for 4 weeks following antibiotic exposure increased bacterial diversity and the relative abundance of *Helicobacter* species while decreasing the relative abundance of *Peptococcaceae* rc4-4.

Another therapeutic approach for restoring the inherent microbial composition is fecal microbiota transplantation (FMT), which was proposed by Ishikawa et al. for UC patients following triple-antibiotic therapy. This study protocol presented a double-blinded, randomized, placebo-controlled, parallel assignment trial that primarily should evaluate the Total Mayo Score at 8 weeks after the FMT procedure. Furthermore, the comparison of clinical features, microbial structure, and metabolic profile, as well as post-FMT 2-year follow-up constitute other endpoints of this study protocol.

Yang et al. assessed the efficacy and safety of FMT combined with biofeedback for mixed constipation. They reported insignificant differences in side effects, yet the combined therapy presented a substantial enrichment in the proportion of probiotic species namely *Prevotella* and *Bifidobacterium*. Considering FMT as a stopgap, yet effective and safe, microbiome-based therapeutic strategy for the remission of gut dysbiosis and gastrointestinal disorders, Savigamin et al. presented four primary steps for initiating FMT in developing countries including: 1. Finding a perfect stool donor; 2. Initiating a clinical trial; 3. Establishing a stool standard for use in other research trials; 4. Establishing a clinical center for the transplantation of fecal microbiota. They further discussed and provided insightful suggestions for the first two steps.

In conclusion, the studies published in this Research Topic further shed light on the critical contribution of the gut microbiota in the pathogenesis of gastrointestinal disorders and its potential capacity in developing efficient biomarkers and therapeutic strategies. In this field of study, the knowledge gaps to be filled and the bottlenecks to be surpassed include deciphering the contribution of hundreds of as-yet-unknown metabolites and their related signaling pathways to the host's physiological and metabolic health and disease.

Author contributions

AN-R wrote the first draft of the manuscript. AY, JO-R, TO, and Y-DW revised the manuscript. All authors read and approved the submitted version.

Acknowledgments

We thank all of the authors involved in the Research Topic.

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

References

1. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. *Nat Rev Microbiol.* (2021) 19:55–71. doi: 10.1038/s41579-020-0433-9

2. Vijay A, Valdes AM. Role of the gut microbiome in chronic diseases: a narrative review. *Eur J Clin Nutr.* (2022) 76:489–501. doi: 10.1038/s41430-021-00991-6

3. de Vos WM, Tilg H, Van Hul M, Cani PD. Gut microbiome and health: mechanistic insights. *Gut.* (2022) 71:1020–32. doi: 10.1136/gutjnl-2021-326789

4. Hou K, Wu ZX, Chen XY, Wang JQ, Zhang D, Xiao C, et al. Microbiota in health and diseases. *Signal Transduct Target Ther.* (2022) 7:135. doi: 10.1038/s41392-022-00974-4

5. Sorbara MT, Pamer EG. Microbiome-based therapeutics. *Nat Rev Microbiol.* (2022) 20:365–80. doi: 10.1038/s41579-021-00667-9

frontiers in Medicine

ORIGINAL RESEARCH
published: 19 July 2021
doi: 10.3389/fmed.2021.655474

Gut Microbiota and Related Electronic Multisensorial System Changes in Subjects With Symptomatic Uncomplicated Diverticular Disease Undergoing Rifaximin Therapy

Antonio De Vincentis[1], Marco Santonico[2], Federica Del Chierico[3], Annamaria Altomare[4], Benedetta Marigliano[5], Alice Laudisio[6], Sofia Reddel[3], Simone Grasso[2], Alessandro Zompanti[7], Giorgio Pennazza[7], Lorenza Putignani[3,8], Michele Pier Luca Guarino[4], Michele Cicala[4] and Raffaele Antonelli Incalzi[1]*

[1] Unit of Internal Medicine, University Campus Bio Medico of Rome, Rome, Italy, [2] Unit of Electronics for Sensor Systems, Department of Science and Technology for Humans and the Environment, University Campus Bio-Medico di Roma, Rome, Italy, [3] Multimodal Laboratory Medicine Research Area, Unit of Human Microbiome, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy, [4] Unit of Gastroenterology, University Campus Bio Medico of Rome, Rome, Italy, [5] Emergency Department, Azienda Ospedaliera San Camillo Fornanini, Rome, Italy, [6] Unit of Geriatrics, University Campus Bio Medico of Rome, Rome, Italy, [7] Unit of Electronics for Sensor Systems, Department of Engineering, University Campus Bio-Medico di Roma, Rome, Italy, [8] Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Unit of Parasitology and Multimodal Laboratory Medicine Research Area, Unit of Human Microbiome, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carattere Scientifico, Rome, Italy

OPEN ACCESS

Edited by:
Giovanni Marasco, University of Bologna, Italy

Reviewed by:
Y. F. Gu, Zhejiang University, China
Francesco Di Mario, University of Parma, Italy

***Correspondence:**
Antonio De Vincentis
a.devincentis@unicampus.it

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *27 January 2021*
Accepted: *11 June 2021*
Published: *19 July 2021*

Citation:
De Vincentis A, Santonico M, Del Chierico F, Altomare A, Marigliano B, Laudisio A, Reddel S, Grasso S, Zompanti A, Pennazza G, Putignani L, Guarino MPL, Cicala M and Antonelli Incalzi R (2021) Gut Microbiota and Related Electronic Multisensorial System Changes in Subjects With Symptomatic Uncomplicated Diverticular Disease Undergoing Rifaximin Therapy. Front. Med. 8:655474. doi: 10.3389/fmed.2021.655474

Background: Intestinal dysbiosis might play a pathogenetic role in subjects with symptomatic uncomplicated diverticular disease (SUDD), but the effect of rifaximin therapy has been scantly explored with regard to gut microbiota variations in patients with SUDD.

Aims: To verify to which extent rifaximin treatment affects the gut microbiota and whether an electronic multisensorial assessment of stools and breath has the potential for detecting these changes.

Methods: Breath and stool samples were collected from consecutive patients with SUDD before and after a 7 days' therapy with rifaximin. Stool microbiota was assessed, and the electronic multisensorial assessment was carried out by means of the BIONOTE electronic (e-)tongue in stools and (e-)nose in breath.

Results: Forty-three subjects (female 60%, median age 66 years) were included, and 20 (47%) reported clinical improvement after rifaximin therapy. Alpha and beta diversity of stool microbiota did not significantly change after treatment, while a significant variation of selected taxa was shown (i.e., *Citrobacter*, *Coprococcus*, *Anaerotruncus*, *Blautia*, *Eggerthella lenta*, *Dehalobacterium*, *SMB53*, and *Haemophilus parainfluenzae*). Overall, the electronic multisensorial system suboptimally mirrored microbiota changes, but it was able to efficiently predict patients' clinical improvement after rifaximin with accuracies ranging from 0.81 to 0.98.

Conclusions: In patients with SUDD, rifaximin administration is associated with significant variation of selected taxa. While inaccurate in predicting gut microbiota change, an electronic multisensorial system, made up of e-tongue and e-nose, was able to predict clinical improvement, thus potentially qualifying as an easy and cheap tool to forecast subjects taking most likely benefit from rifaximin therapy.

Keywords: microbiota, e-tongue, e-nose, diverticular disease, rifaximin

INTRODUCTION

Colonic diverticulosis is a complex multifactorial disorder, in which dysbiosis could play a key role. It affects up to one third of people over the age of 60 years and causes symptoms including abdominal pain or bloating and changes in bowel habit (condition termed symptomatic uncomplicated diverticular disease—SUDD) in 20% of cases (1). Approximately 10–25% of patients with SUDD may develop acute diverticulitis (2). The fact that the small bowel diverticula are associated with bacterial overgrowth and most complications of colonic diverticular disease are bacterial in nature and may benefit from antibiotic therapy or fecal stream diversion further supports the importance of gut microbiota in diverticular disease pathogenesis (3). Fiber deficiency, attributed to a Western diet, plays a crucial role, considering that DNA sequencing confirms that fecal microbiota composition is affected by consumption of supplemental fibers (4). A low-fiber diet not only affects colonic motility but can account for the microbiota composition bending toward a prevalence of *Bacteroides* (5). Low-grade inflammation, altered intestinal microbiota, visceral hypersensitivity, and an abnormal colonic motility are likely to play a variable pathogenetic role (6). The presence of inflammation and dysbiosis in SUDD validates the responsiveness to anti-inflammatory medications like non-absorbable enteral antibiotics such as rifaximin, a semisynthetic antibiotic synthesized in 1982 from rifamycin (7). Rifaximin has a broad spectrum of antibacterial action, is unlikely to induce bacterial resistance (7), and decreases the metabolic activity of the intestinal bacterial flora and the degradation of dietary fiber (8). Rifaximin acts by binding to the beta subunit of bacterial DNA-dependent RNA polymerase resulting in the inhibition of bacterial RNA synthesis (9). It has *in vitro* bactericidal and bacteriostatic activity against aerobic and anaerobic gram-positive and gram-negative species, being also able to reduce bacterial virulence and translocation and to inhibit bacterial adherence to gut mucosa (10). In fact, cyclic administration of rifaximin with dietary fiber supplementation outperforms simple dietary fiber supplementation in reducing both symptoms and complication frequency of SUDD (11). In conclusion, the better comprehension of the inflammatory patterns and the gut microbiota has increased the therapeutic options: current evidence enhances the therapeutic role of rifaximin (as well as mesalazine that acts directly on the gastrointestinal epithelium) in the treatment of SUDD symptoms, and the fiber supplementation is still recommended in SUDD by much of the international guidelines even if its use is not supported by the recent evidence. Unfortunately, the only available study of rifaximin effects on gut microbiota refers to only four women, being thus less than exploratory in nature (12). Thus, we purposed to verify to which extent rifaximin treatment affects the gut microbiota and whether an electronic multisensorial assessment of stools and breath has the potential for detecting these changes. Indeed, the genetic study of microbiota is cumbersome and requires both time and money. Instead, both a gas sensor array (e-nose) and the liquid sensor array (e-tongue) qualify as very simple and inexpensive methods for assessing, respectively, the spectrum of volatile organic compounds and the electrical impedance of a given liquid, which clearly reflect its physical properties and chemical composition (13). Both e-nose and e-tongue have displayed a wide spectrum of diagnostic and classificatory properties in different conditions, e.g., in liver diseases, chronic obstructive pulmonary disease, asthma, and selected tumors for the e-nose (14–19), in ascites, pleural effusion, urinary tract infections, and wound infections for e-tongue (20–23). Thus, e-nose and e-tongue might be able to catch the rifaximin-induced changes in gut microbiota, allowing either to interpret or to monitor the response to rifaximin in SUDD patients.

METHODS

Study Setting and Participants

In this prospective longitudinal study, consecutive subjects with a diagnosis of SUDD were recruited at the Campus Bio-Medico University Hospital of Rome (Italy) from January to July 2017. Both design and size of this study are consistent with the intention of performing a proof-of-concept study testing the diagnostic properties of e-nose and e-tongue toward genetically proved changes in gut microbioma induced by rifaximin. SUDD was defined as the presence of abdominal pain, bloating, and/or bowel habit changes that include diarrhea, constipation, or a mixed bowel habit, in patients with diverticulosis in the absence of macroscopic inflammation and of any complications (stenosis, abscesses, fistula) (2, 24). Subjects referring allergy to rifaximin; or taking medication with a potential modifying role on microbiota (i.e., antibiotics, prebiotics, probiotics, proton pump inhibitors) in the previous month; or with active cancer, COPD exacerbation, end stage of liver, or kidney disease; or suffering from inflammatory bowel disease were excluded. Finally, considering the well-known influence of diet on intestinal microbiota, only subjects following a Mediterranean normocaloric omnivorous diet (2,000–2,100

and 2,500–2,800 kcal/diet for women and men, respectively) were included.

Since a reduction of potentially pathogenic components of the intestinal microbiota was expected after rifaximin therapy in at least the 70% of participants, the enrollment of 20 subjects was considered adequate to guarantee a statistical power of 80% assuming an alpha error of 0.05. Based on previous experience with multisensorial systems (14, 15, 20–23), this sample size was increased to at least 40 subjects in order to allow the investigation of the discriminative and classificatory capacities of these systems toward rifaximin-induced microbiome changes.

All the main socio-demographic and clinical variables along with blood tests were collected for each participant. Nutritional assessment was performed through the Mini Nutritional Assessment (MNA) (25), abdominal symptoms (i.e., pain and bloating) were scored as mild, moderate, or severe, and bowel habits were assessed through the Bristol Stool Scale. Eligible subjects were evaluated at baseline and, then, were prescribed with a cycle of 7 days of rifaximin 800 mg/diet. A follow-up evaluation was performed after the completion of the antibiotic therapy. Breath and fecal samples were collected for each participant at baseline and at follow-up for the multisensorial system analysis and for the gut microbiota analysis. The study protocol was approved by the local Ethical Committee (n 47/2016), and all participants signed an informed consent.

Gut Microbiota Analysis

Fecal samples were collected by each subject in the same morning of the outpatient visit. DNA was extracted from 200 mg of stools using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The 16S rRNA V3–V4 variable region (∼460 bp) was amplified by using the primer pairs described in the MiSeq rRNA Amplicon Sequencing protocol (Illumina, San Diego, CA, USA). The PCR reactions were set up using a 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems Inc., Wilmington, MA, USA) following the manufacturer's protocol. AMPure XP beads (Beckman Coulter Inc., Beverly, MA, USA) were employed to clean DNA amplicons from primers and dimer primers. A unique combination of Illumina Nextera adaptor-primers for each sample was incorporated in amplicons by a second amplification step. The final library was cleaned up and quantified using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were pooled together before the sequencing on an Illumina MiSeq™ platform according to the manufacturer's specifications to generate paired-end reads of 300 base-length. QIIME v1.9 software (26) was used to filter raw reads for quality, read length, and chimera presence. Cleaned sequences were then clustered into operational taxonomic units (OTUs) with a 97% clustering threshold of pairwise identity. OTUs' representative sequences were aligned using PyNAST v.0.1. software (27) against the Greengenes 13_08 database with a 97% similarity for bacterial sequences. All raw sequences have been archived in the NCBI database: PRJNA731467 (https://www.ncbi.nlm.nih.gov/bioproject).

Multisensorial System Analysis on Breath and Fecal Samples

The multisensorial system was made up of the BIONOTE e-Tongue for liquids and of the BIONOTE e-Nose for gases (13). Breath collection was performed in the morning by means of the Pneumopipe® (European Patent n 12425057.2), with all participants who have been fasting and smoke-free for at least 8 h. The breath samples were stored in adsorbent cartridges (Tenax GR by Supelco) at −20°C after collection. Before analysis, the VOC mixture was desorbed with a thermal procedure at four different temperatures (50, 100, 150, and 200°C) and, then, analyzed by the BIONOTE e-Nose (13). This instrument is composed of eight transducers consisting of quartz crystals with a resonance frequency of 20 MHz in thickness shear mode, functionalized with a combination of anthocyanins extracted from three different plant tissues: red rose, red cabbage, and blue hortensia. Each VOC in the mixture binds to the different anthocyanins in the measure cell, thus causing a frequency shift of the respective quartz forming the reference value: this frequency shift is acquired by the system as the sensor response. The final dataset is composed of a fingerprint of 32 responses for each sample, which derive from the registration of the eight-sensor behavior at the four different temperatures of VOC desorption from the cartridge.

Fecal samples were collected by each subject in the same morning of the outpatient visit and stored at +4°C. Before the analysis, feces were prepared for the analysis by diluting 50 mg in 5 ml of distilled water and subsequently centrifuged at 10°C at 1,000 relative centrifugal forces for 10 min. The supernatant was taken with a pipette and analyzed by the BIONOTE e-Tongue (13). This instrument consists of voltammetric screen-printed electrodes controlled by a high-stability electronic interface. The sensor probe is made up of three electrodes: a silver working, a platinum reference, and a gold counter electrode. The applied input signal consists of a triangular waveform with a working range from −1 to 1 V with 500 input voltages and 500 corresponding output current values. The time duration of the complete measurement process is of about 90 s, repeated five times for reproducibility assessment. Data array is made up by the characteristic fingerprints extracted by each voltammogram registered for the measured samples. The setup of the parameters for the acquisition, the number of samples, and the sampling interval are controlled by a dedicated software interface.

Analytical Approach

Data were presented by means of descriptive statistics and compared using non-parametric tests. Alpha diversity of the gut microbiota was measured on the raw data by the Good's coverage, Chao-1, and Shannon indices, using the Wilcoxon signed-rank test to assess differences after rifaximin therapy. For further analyses, OTUs not seen in at least 20% of the samples or with a relative abundance <1% in the total dataset were removed. Principal component analysis (PCoA) on unweighted and weighted UniFrac distance matrices was used as ordination method to compare gut microbiota of subjects pre- and post-rifaximin therapy by permutational multivariate

analysis of variance (PERMANOVA). In addition, the differential abundance analysis of gut bacteria was conducted through the negative binomial distribution on raw counts normalized by "size factors," taking into account the sequencing depth between the samples. The differences in bacterial abundance were expressed as $\log_2$ fold change ($\log_2$FC). Analyses of gut microbiota changes were stratified by gender and by presence of abdominal pain, and linear mixed models were applied to verify the related impact in the amount of alpha diversity change after rifaximin therapy.

The ability of e-tongue and e-nose to predict gut microbiota changes or clinical improvement after rifaximin therapy was verified using partial-least-squared discriminant analysis (PLS-DA) with 10-fold cross-validation. Predictive capacities were expressed with the root-mean-square-error cross validation (RMSECV) to aggregate in a single measure of predictive power the magnitudes of the machine errors in prediction of continuous variables (e.g., Shannon and Chao-1 indices). Since RMSECV expresses the prediction error in the same unit of the original measurement, it cannot be compared between different variables. To allow comparability, RMSECV% was computed standardizing RMSECV by the 95% interval of the variable-specific distribution. Conversely, for dichotomous outcomes (i.e., clinical improvement), overall accuracy, sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values were computed. All the analyses were performed using R version 4.0.2 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Pre–post Rifaximin Clinical and Biochemical Characteristics

Forty-three subjects with SUDD were included in the study (**Table 1**). The median age was 66 years, and 26 (60%) were women, with a median BMI of 24.5 kg/m^2. Sixteen subjects (37%) were at risk of malnutrition, and no malnourished subjects were identified. At baseline, all subjects complained about abdominal bloating [mild: 29 (67%); moderate: 4 (9.3%); and severe: 10 (23%)] and 18 (42%) referred also abdominal pain [mild: 13 (72.2%); moderate: 5 (27.8%)]. According to the Bristol Stool Scale, 12 participants (28%) had diarrheal stool, while only 2 (4.7%) were constipated.

After rifaximin therapy, no significant differences were observed regarding the main inflammatory markers (leukocytes, C-reactive protein, and erythrocyte sedimentation rate). Conversely, patients reported a significant improvement of symptoms in terms of abdominal pain ($p = 0.05$), bloating ($p = 0.003$), and bowel habits ($p = 0.011$—**Table 1**). Overall, 20 subjects (47%) experienced a clinical improvement after therapy, defined as relief of abdominal pain or any improvement of abdominal bloating or normalization of bowel habits.

TABLE 1 | Main clinical and biochemical characteristics of the study cohort at baseline and after rifaximin therapy (follow-up).

Characteristic	*N* = 43		
Age (years)	66 (61, 73)		
Sex (female)	26 (60%)		
BMI (kg/m^2)	24.5 (22.6, 27.4)		
Malnutrition (MNA)			
Well-nourished	27 (63%)		
At risk of malnutrition	16 (37%)		
Malnourished	0 (0%)		
DICA classification			
1	40 (93%)		
2	3 (7%)		
3	0 (0%)		
Clinical improvement after rifaximin*	20 (47%)		
	Baseline	**Follow-up****	***p***
Hemoglobin (g/dl)	14.1 (12.8, 14.9)	14.1 (13.2, 14.9)	0.357
Leukocytes (/mm^3)	6,000 (5,110, 7,165)	6,050 (4,930, 7,205)	0.947
Lymphocytes (/mm^3)	1,970 (1,560, 2,315)	1,990 (1,500, 2,425)	0.476
C-reactive protein (mg/dl)	1.4 (0.5, 3.7)	0.5 (0.5, 3.0)	0.194
ESR (mm/h)	36 (26, 42)	33 (20, 43)	0.101
Bristol Stool Scale (linear)	5 (4, 6)	4 (3, 5)	<0.001
Bristol Stool Scale (categories)			0.011
1–2 (constipation)	2 (4.7%)	6 (14%)	
3–5 (normal)	29 (67%)	35 (81%)	
6–7 (diarrhea)	12 (28%)	2 (4.7%)	
Abdominal pain (presence)	18 (42%)	10 (23%)	0.05
Abdominal pain (severity)			0.05
Absence	25 (58%)	33 (77%)	
Mild	13 (30%)	10 (23%)	
Moderate	5 (12%)	0 (0%)	
Severe	0 (0%)	0 (0%)	
Abdominal bloating (presence)	43 (100%)	43 (100%)	1
Abdominal bloating (severity)			0.003
Absence	0 (0%)	0 (0%)	
Mild	29 (67%)	42 (98%)	
Moderate	4 (9.3%)	1 (2.3%)	
Severe	10 (23%)	0 (0%)	

*Continuous variables are expressed as median with interquartile range, while categorical variables are displayed as numbers with percentages. p-values are from the Wilcoxon signed-rank test for continuous variables or from McNemar's or marginal homogeneity test for categorical variables. *Clinical improvement defined as relief of abdominal pain or any improvement of abdominal bloating or normalization of bowel habits with a Bristol Stool Scale 3–5. **After a 1-week course of rifaximin 800 mg/die.*

MNA, Mini Nutritional Assessment; BMI, body mass index; ESR, erythrocyte sedimentation rate; DICA classification, "Diverticular Inflammation and Complication Assessment" classification.

Influence of Rifaximin Therapy on Gut Microbiota

Good's coverage averaged 96% in baseline samples and 97% in follow-up, indicating that most of the OTUs in the samples were detected. Alpha diversity measures did not significantly change after rifaximin therapy [Shannon index: baseline 4.5 (4.0–4.8) vs. follow-up 4.1 (3.6–4.6), p 0.06; Chao-1 index: baseline

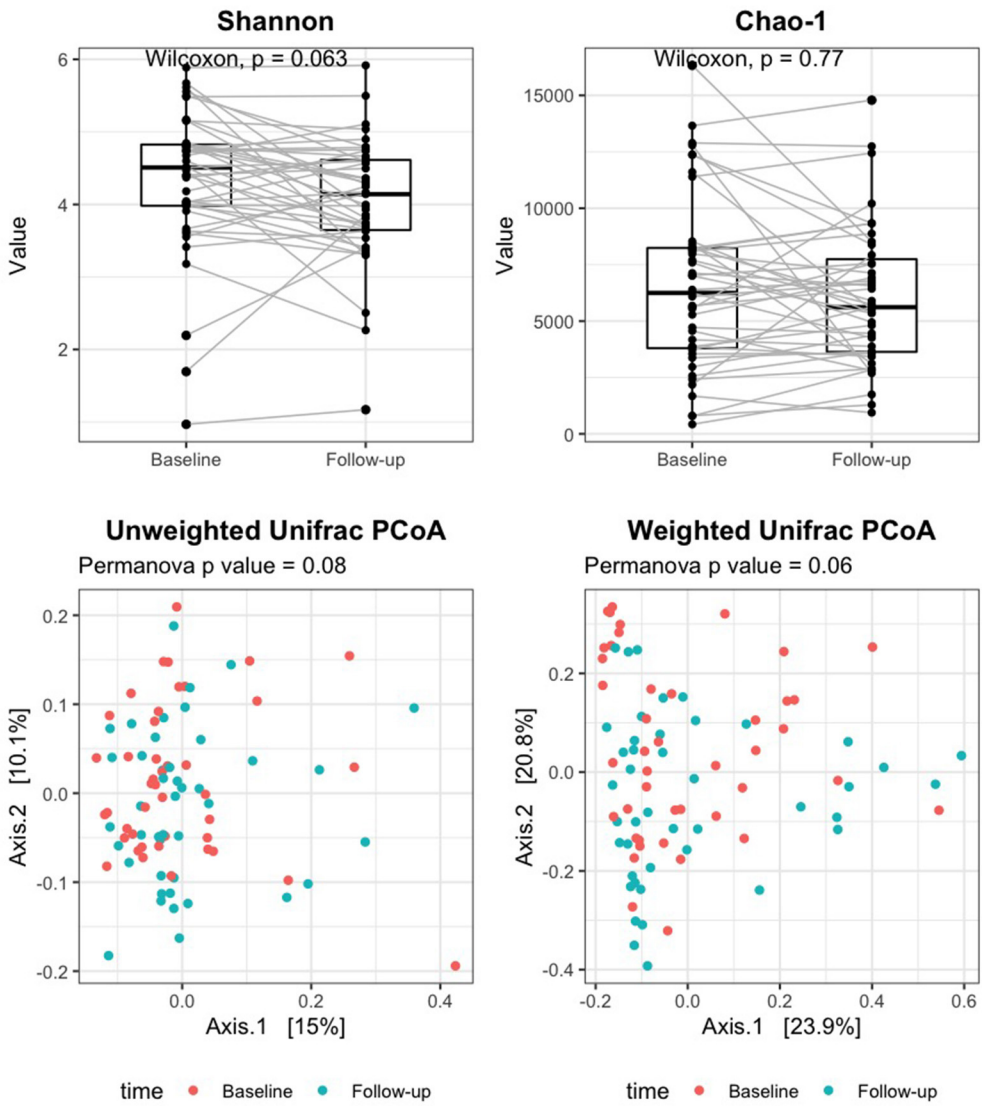


FIGURE 1 | Change of alpha and beta diversity measures after rifaximin therapy in SUDD patients. Alpha diversity of the gut microbiota was measured on the raw data by the Shannon and Chao-1 indices (upper panels). Beta diversity was assessed by principal component analysis (PCoA) on unweighted and weighted UniFrac distance matrices (lower panels). The Wilcoxon signed-rank test and permutational multivariate analysis of variance (PERMANOVA) were applied to assess differences in alpha and beta diversity measures after rifaximin therapy, respectively.

6,246.1 (3,796.5–8,239.8) vs. follow-up 5,615.7 (3,634.5–7,741), p 0.77; **Figure 1**, upper panels]. Similarly, the UniFrac PCoA plot did not show a shift in the overall gut microbiota composition from baseline to post-rifaximin treatment (PERMANOVA p 0.08 for unweighted and 0.06 for weighted analysis; **Figure 1**, lower panels). Similar findings were observed after stratification according to gender and to the presence of abdominal pain (**Supplementary Figure 1**), and no difference was found in the amount of alpha diversity change after rifaximin therapy across gender (p for linear mixed model 0.40 and 0.92 for Shannon and Chao-1 indices, respectively) and across patients with/out abdominal pain (p for linear mixed model 0.26 and 0.42 for Shannon and Chao-1 indices, respectively).

The relative abundance analysis revealed significant changes in selected families and genera (**Figure 2**). In particular, after rifaximin therapy the gut microbiota was enriched in Bacteroidaceae, *Citrobacter*, and *Coprococcus* and deficient in Mogibacteriaceae, Christensenellaceae, Dehalobacteriaceae, Pasteurellaceae, *Anaerotruncus*, *Blautia*, *Eggerthella lenta*, *Dehalobacterium*, *SMB53*, and *Haemophilus parainfluenzae* (p-adj < 0.05) at the family and genus levels, respectively (**Figure 2**). Other selected families and genera showed large $\log_2$FC (>2 or <-2), but without reaching statistical significance: Peptostreptococcaceae, EtOH8, Leuconostocaceae, Eubacteriaceae, *Clostridium*, *Bifidobacterium*, and *Klebsiella* (**Figure 2**). Subgroup analyses revealed a significant decrease of

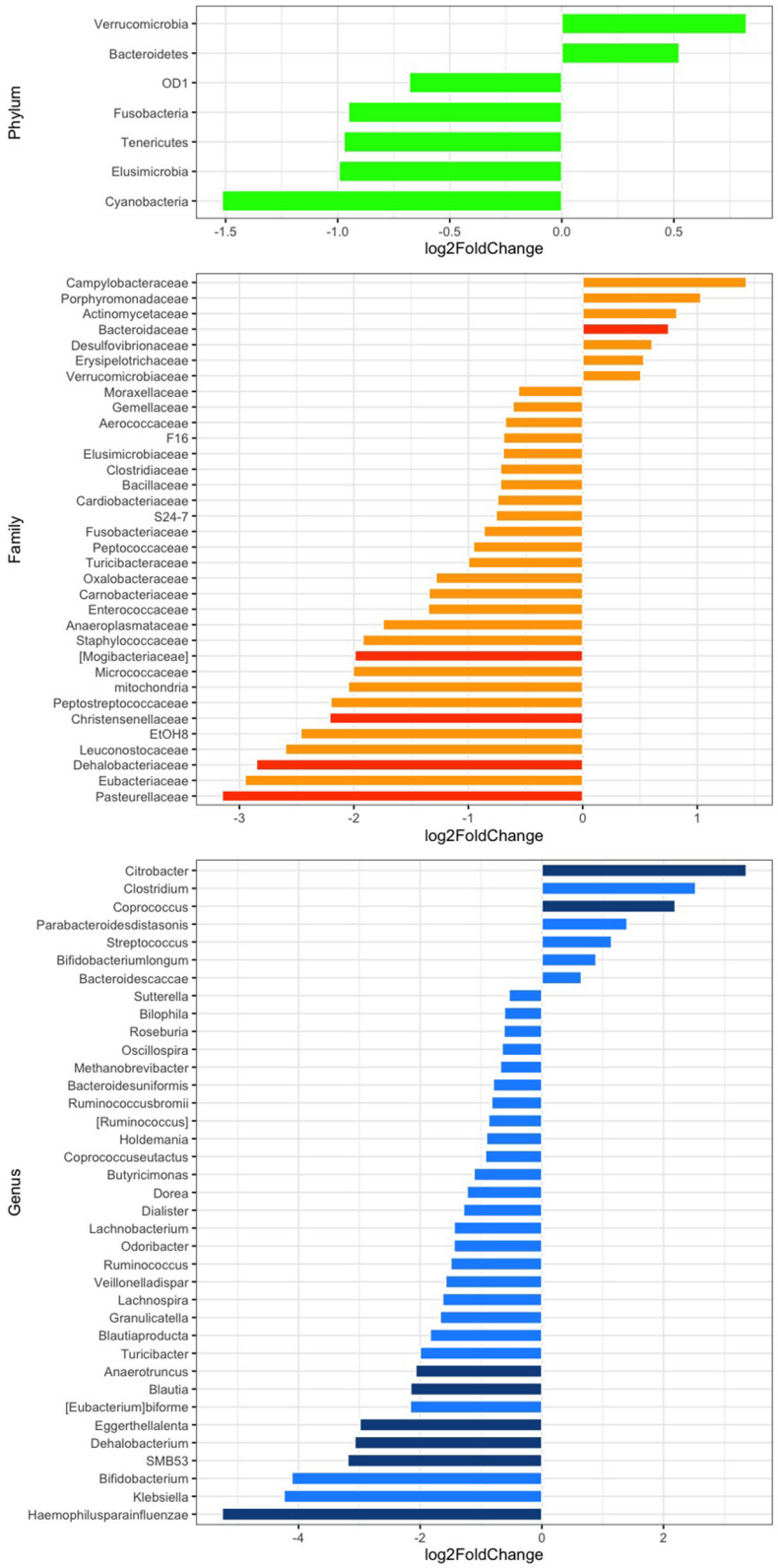


FIGURE 2 | Differential abundance analysis of the gut microbiota composition at the phylum (green), family (orange), and genus (blue) levels after rifaximin therapy. Differential bacterial abundance is expressed as log2 fold change (log2FC); positive or negative values indicate an increase or decrease proportional to the absolute value of log2FC. Comparisons with a log2FC higher or lower than 0.5 are displayed. $p < 0.05$ adjusted for multiple comparisons with the Benjamini-Hochberg method (*p*-adj) are considered significant and represented by a darker color.

TABLE 2 | E-tongue and e-nose prediction of alpha measure variation in patients with SUDD undergoing rifaximin therapy.

	E-tongue		E-nose		E-tongue + E-nose	
	RMSECV	**RMSECV %***	**RMSECV**	**RMSECV %***	**RMSECV**	**RMSECV %***
Prediction of Shannon index	0.88	22	0.95	24	0.80	20
Prediction of Shannon index change after rifaximin	0.62	20	0.78	25	0.50	16
Prediction of Chao-1 index	3,226	25	3,314	26	3,222	25
Prediction of Chao-1 index change after rifaximin	2,292	19	2,844	23	2,356	20

**RMSECV% represents the ratio between the root mean squared error in cross validation (RMSECV) and the 95% interval of the distribution of each index/index variation.*

Pasteurellaceae, Clostridiaceae, *Blautia*, *Veillonella dispar*, and *Haemophilus parainfluenzae* in men and of Christensenellaceae in women. *Parabacteroides* were significantly increased in men (**Supplementary Figures 2A,B**). Selected variations at the phylum, family, and genus levels according to the presence of abdominal pain have been also evidenced and are reported in **Supplementary Figures 2C,D**.

Electronic Multisensorial System Prediction of Gut Microbiota and of Clinical Outcomes

Predictive performances of the electronic multisensorial system toward alpha diversity measures of gut microbiota in pre–post rifaximin samples are reported in **Table 2**. E-tongue and e-nose predicted Shannon index and Shannon index variation after rifaximin with a RMSECV% between 20 and 25 (RMSECV 0.62–0.95). A similar range of RMSECV% was observed for the prediction of Chao-1 index and Chao-1 index variation after rifaximin (19–26) with RMSECV between 2,292 and 3,314. The integration of e-tongue and e-nose data only minimally improved predictive performances (**Table 2**). The abilities of both e-tongue and e-nose to predict the main phyla, families, and genera variation were generally suboptimal, with exception made for a few selected cases (**Supplementary Table 1**).

Conversely, the accuracy for the discrimination of pre/post rifaximin samples was 0.81 (0.72–0.89) for e-tongue, 0.9 (0.81–0.95) for e-nose, and 0.87 (0.78–0.93) for the integration of both techniques (**Table 3**). Both e-tongue and e-nose evidenced good to excellent capacities to predict clinical outcome after rifaximin therapy. Two different potential predictors were tested to this purpose, i.e., the sensors' responses obtained from the analysis of only pre-rifaximin samples, or the change in sensors' responses obtained from the analysis of both pre- and post-rifaximin samples. Indeed, the analysis of only pre-rifaximin samples could correctly predict the occurrence of clinical improvement after treatment in 36/43 subjects with e-tongue [accuracy 0.84 (0.69–0.93), sensitivity 0.91, specificity 0.75, PPV 0.81, NPV 0.88] and in 41/43 subjects with e-nose [accuracy 0.95 (0.84–0.99), sensitivity 1.00, specificity 0.90, PPV 0.92, NPV 1.00]. The integration of both techniques did not lead to an improvement of the predictive performances [accuracy 0.81 (0.67–0.92), sensitivity 0.87, specificity 0.75, PPV 0.80, NPV 0.83]. Similarly, the change in sensors' responses after rifaximin could efficiently predict clinical improvement [accuracy 0.84 (0.69–0.93) for e-tongue; accuracy 0.81 (0.67–0.92) for e-nose]. In this case, the integration of both techniques could further increase the accuracy to 0.98 (0.88–1) (sensitivity 1.00, specificity 0.95, PPV 0.96, NPV 1.00).

DISCUSSION

In this study, we comprehensively explored the effects of rifaximin on fecal microbiota in SUDD patients, correlating them with clinical and laboratory data and assessing whether sensor-based methods can gain insight into microbiota status and its rifaximin-related changes. We showed that rifaximin significantly affects the relative abundance of selected bacteria and that an electronic multisensorial system (e-nose and e-tongue) has the potential for predicting and discriminating rifaximin-induced clinical response.

Several studies have demonstrated that rifaximin, a poorly absorbed oral antibiotic with an activity against anaerobic, gram-positive, and gram-negative bacteria, generates an "eubiotic" effect, also promoting the growth of beneficial bacteria such as *Lactobacillus* and Bifidobacteria (28, 29). Moreover, it has been reported that rifaximin decreases the metabolic activity of intestinal microbiota, increases fecal mass, and reduces bacterial overgrowth (30), and its very low absorption rates imply a low level of bacterial resistance onset (31). However, the effects of rifaximin on the intestinal microbiota are largely unknown. In fact, it seems to not affect the overall microbiome composition (32) but to induce selective depletion of a few taxa involved in the regulation of inflammation and mucosal barrier functionality (33).

In the present study, after treatment of rifaximin, an increase of Bacteroidaceae was observed in fecal microbiota of SUDD patients; even if the impact of these bacteria on SUDD pathogenesis is not known, in several studies using animal models of colitis, Bacteroidaceae or their metabolites seem to exert a protective role against inflammation (34, 35). Actually, a significant increase of *Bacteroidetes* spp., which usually represent a significant and stable part of the GI microbiota, plays an important metabolic role with the production of succinic acid, acetic acid, and in some cases propionic acid. Interestingly, propionic acid is mainly produced by the fermentation of indigested food by the microbiota in the colon, but can reach the blood compartment and the adipose tissue, where it reduces fatty acid levels in plasma *via* inhibition of lipolysis and induction of lipogenesis in adipose tissue and suppression of fatty acid production in liver (36). Moreover, after rifaximin treatment, we

TABLE 3 | E-tongue and e-nose discrimination of pre/post-rifaximin sample and prediction of clinical improvement.

Discrimination of pre/post-rifaximin samples

E-tongue					E-nose					E-tongue + E-nose				
		Reference					Reference					Reference		
		Baseline	*Follow-up*				*Baseline*	*Follow-up*				*Baseline*	*Follow-up*	
Predicted	*Baseline*	34	7		**Predicted**	*Baseline*	39	5		**Predicted**	*Baseline*	38	6	
	Follow-up	9	36			*Follow-up*	4	38			*Follow-up*	5	37	
Accuracy	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
0.81 (0.72–0.89)	0.79	0.84	0.83	0.8	*0.9 (0.81–0.95)*	*0.91*	*0.88*	*0.89*	*0.9*	0.87 (0.78–0.93)	0.88	0.86	0.86	0.88

Prediction of clinical improvement by the analysis of only pre-rifaximin samples*

E-tongue					E-nose					E-tongue + E-nose				
		Reference					Reference					Reference		
		No	*Yes*				*No*	*Yes*				*No*	*Yes*	
Predicted	*No*	21	5		**Predicted**	*No*	23	2		**Predicted**	*No*	20	5	
	Yes	2	15			*Yes*	0	18			*Yes*	3	15	
Accuracy	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
0.84 (0.69–0.93)	0.91	0.75	0.81	0.88	*0.95 (0.84–0.99)*	*1*	*0.9*	*0.92*	*1*	0.81 (0.67–0.92)	0.87	0.75	0.8	0.83

Prediction of clinical improvement by the analysis of both pre- and post-rifaximin samples*

E-tongue					E-nose					E-tongue + E-nose				
		Reference					Reference					Reference		
		No	*Yes*				*No*	*Yes*				*No*	*Yes*	
Predicted	*No*	21	5		**Predicted**	*No*	19	4		**Predicted**	*No*	23	1	
	Yes	2	15			*Yes*	4	16			*Yes*	0	19	
Accuracy	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
0.84 (0.69–0.93)	0.91	0.75	0.81	0.88	0.81 (0.67–0.92)	0.83	0.8	0.83	0.8	*0.98 (0.88–1)*	*1*	*0.95*	*0.96*	*1*

**In both cases, partial-least-squared discriminant analyses were run to predict the same outcome, i.e., clinical improvement after rifaximin therapy. In the first model, only sensors' responses obtained from the analysis of pre-rifaximin samples were entered as predictor, whereas, in the second model, the change in sensors' responses obtained from the analysis of both pre- and post-rifaximin samples was tested as potential predictor of clinical improvement.*
PPV, positive predictive value; NPV, negative predictive value.

observed a significant reduction of Christensenellaceae, which are usually present, in a large amount, in patients with a previous history of diverticulitis, suggesting a possible role of this bacterial family in the pathogenesis of SUDD (37). Moreover, after rifaximin treatment a significant decrease of several microbial species was also observed such as *Eggerthella lenta*, which is an emerging pathogen responsible for bacteremia in several pathological conditions, among which is diverticular disease (38).

Overall, we did not observe a significant variation of serum inflammatory markers, and only less than half of the participants reported a clinical improvement after rifaximin. This could be likely due to the fact that the participants were not naïve to rifaximin. Furthermore, nearly two-thirds of enrolled subjects had normal bowel habits or absence of abdominal pain or only mild bloating at baseline. As such, the impact of rifaximin therapy in this specific cohort of not-naïve and pauci-symptomatic subjects could have been less evident.

Both e-nose and e-tongue were able to distinguish patients who ultimately will benefit from rifaximin from those who will not. However, the integration of the two methods did not improve the discrimination (prediction), as if the information inherent to (in) each technique was in itself complete and could not benefit from any integration. We remind that e-nose assesses the pattern of VOCs, while e-tongue assesses the bioelectric properties of a liquid, which reflects the chemical composition. It is noteworthy that all the components of the liquid contribute to shape its e-tongue pattern, whereas only volatile compounds contribute to the e-nose pattern. Accordingly, it is not surprising that adding information based on selected components (e-nose) to that derived from the whole set of liquid components (e-tongue) does not significantly improve the prognostic and discriminatory properties of the latter. The same consideration likely applies to the lack of improvement in the prediction of alpha diversity measures of gut microbiota by integrating e-nose and e-tongue. Unfortunately, the available literature does not provide any example of integration of e-tongue and e-nose, making our hypothesis worthy of validation in other settings. Instead, the integration of the two methods significantly improved the discrimination based on both baseline and post-rifaximin data. This seemingly contradictory (variant) finding is likely due to the distinctive effects of rifaximin on bacterial phyla. Indeed, the observed changes in loads of individual phyla might account for changing proportions of volatile compounds after rifaximin, making thus a "repeated information," the one on volatile compounds collected by e-nose and, in their liquid form, by e-tongue, more representative of the biological changes underlying clinical changes. Finally, both e-nose and e-tongue were weakly correlated with changes in the vast majority of bacterial taxa, with only a few exceptions. Given, we found relevant changes in 7 phyla, 34 families, and 37 genera; the few significant correlations might be chance findings. However, it is of interest that they pertain to bacteria with plausible biological importance.

The present study has some limitations. First, the limited sample size makes the obtained results exploratory in nature, thus requiring confirmation in largest studies. Subgroup analyses did not reveal differences in diversity measures according to gender and to the presence of abdominal pain, and selected families and genera were found increased or decreased across strata. However, the reduced statistical power hampers sensible speculations on gender- and symptom-specific findings, and a wider cohort should be enrolled to highlight such subtle microbiological differences at the family and genus levels. Secondly, the studied cohort of SUDD subjects was not naïve to rifaximin therapy, and this might have smoothed the discriminative properties of multisensory systems. However, the fact that these properties were evident even in this "difficult" population testifies to the potentialities of the proposed method. Then, we did not assess intestinal permeability or inflammatory status, which might have helped our understanding of observed changes in microbioma. However, interpreting changes was out of the scope of our study, which was designed to test whether e-nose and e-tongue could assess rifaximin-induced changes in microbioma. Finally, the lack of concomitant stool metabolomic data prevents a more comprehensive understanding of the impact of the observed microbiota changes after therapy.

However, strengths also are worthy of mention. This is the first study characterizing the microbiota in SUDD subjects before and after rifaximin, and, as such, its results should be regarded with attention. In particular, the time by dose cumulative exposition to rifaximin chosen for treatment corresponds to the minimal one known to be clinically effective. Thus, the multisensorial system showed discriminative properties in a difficult experimental condition. It is likely that expanding the research toward stronger therapeutic regimens will further disclose the diagnostic potential of this method. Moreover, it tests a very innovative approach based on different sensor methods (electronic multisensorial system made up of e-nose and e-tongue) in the search for an easy and inexpensive method to surrogate microbiota genetic study. Finally, the e-tongue method can be easily standardized, given its dependence upon the operating electrical field, which guarantees for its reproducibility.

In conclusion, the present study highlights specific rifaximin-related changes in stool microbiota of SUDD patients in the selected bacterial population. Moreover, it shows that an electronic multisensorial system is able to efficiently predict rifaximin-induced clinical response. These preliminary results need to be confirmed and expanded in other SUDD and not SUDD populations. If confirmed, they might open the way to a fast and low-cost stool characterization with many potential perspectives of use not only in gastroenteric diseases.

DATA AVAILABILITY STATEMENT

All sequences have been archived in NCBI database: PRJNA731467 (https://www.ncbi.nlm.nih.gov/bioproject).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Campus Bio-Medico. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RA, BM, MC, LP, AD, MS, and GP conceived the study. BM, MG, and AA recruited participants. AD, FD, SR, LP, and RA conducted the analysis. SG, GP, MS, and AZ analyzed breath and fecal samples. AD, RA, AA, and AL wrote the article. All authors revised and approved the written manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.655474/full#supplementary-material

REFERENCES

1. Barbara G, Scaioli E, Barbaro MR, Biagi E, Laghi L, Cremon C, et al. Gut microbiota, metabolome and immune signatures in patients with uncomplicated diverticular disease. *Gut.* (2017) 66:1252–61. doi: 10.1136/gutjnl-2016-312377
2. Cuomo R, Barbara G, Pace F, Annese V, Bassotti G, Binda GA, et al. Italian consensus conference for colonic diverticulosis and diverticular disease. *United Euro Gastroenterol J.* (2014) 2:413–42. doi: 10.1177/2050640614547068
3. Jacobs DO. Clinical practice. *Diverticulitis. N Engl J Med.* (2007) 357:2057–66. doi: 10.1056/NEJMcp073228
4. Hooda S, Boler BMV, Serao MCR, Brulc JM, Staeger MA, Boileau TW, et al. 454 pyrosequencing reveals a shift in fecal microbiota of healthy adult men consuming polydextrose or soluble corn fiber. *J Nutr.* (2012) 142:1259–65. doi: 10.3945/jn.112.158766
5. Tap J, Furet J-P, Bensaada M, Philippe C, Roth H, Rabot S, et al. Gut microbiota richness promotes its stability upon increased dietary fibre intake in healthy adults. *Environ Microbiol.* (2015) 17:4954–64. doi: 10.1111/1462-2920.13006
6. Seth A, Yan F, Polk DB, Rao RK. Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol.* (2008) 294:G1060–9. doi: 10.1152/ajpgi.00202.2007
7. Calanni F, Renzulli C, Barbanti M, Viscomi GC. Rifaximin: beyond the traditional antibiotic activity. *J Antibiot.* (2014) 67:667–70. doi: 10.1038/ja.2014.106
8. Scaioli E, Colecchia A, Marasco G, Schiumerini R, Festi D. Pathophysiology and therapeutic strategies for symptomatic uncomplicated diverticular disease of the colon. *Dig Dis Sci.* (2016) 61:673–83. doi: 10.1007/s10620-015-3925-0
9. Kruis W, Germer C-T, Leifeld L, German Society for Gastroenterology, Digestive and Metabolic Diseases, and the German Society for General and Visceral Surgery. Diverticular disease: guidelines of the german society for gastroenterology, digestive and metabolic diseases and the german society for general and visceral surgery. *Digestion.* (2014) 90:190–207. doi: 10.1159/000367625
10. Bielaszewska M, Idelevich EA, Zhang W, Bauwens A, Schaumburg F, Mellmann A, et al. Effects of antibiotics on Shiga toxin 2 production and bacteriophage induction by epidemic *Escherichia coli* O104:H4 strain. *Antimicrob Agents Chemother.* (2012) 56:3277–82. doi: 10.1128/AAC.06315-11
11. Bianchi M, Festa V, Moretti A, Ciaco A, Mangone M, Tornatore V, et al. Meta-analysis: long-term therapy with rifaximin in the management of uncomplicated diverticular disease. *Aliment Pharmacol Ther.* (2011) 33:902–10. doi: 10.1111/j.1365-2036.2011.04606.x
12. Laghi L, Mastromarino P, Elisei W, Capobianco D, Zhu CL, Picchio M, et al. Impact of treatments on fecal microbiota and fecal metabolome in symptomatic uncomplicated diverticular disease of the colon: a pilot study. *J Biol Regul Homeost Agents.* (2018) 32:1421–32.
13. Santonico M, Pennazza G, Grasso S, D'Amico A, Bizzarri M. Design and test of a biosensor-based multisensorial system: a proof of concept study. *Sensors.* (2013) 13:16625–40. doi: 10.3390/s131216625
14. De Vincentis A, Pennazza G, Santonico M, Vespasiani-Gentilucci U, Galati G, Gallo P, et al. Breath-print analysis by e-nose for classifying and monitoring chronic liver disease: a proof-of-concept study. *Sci Rep.* (2016) 6:25337. doi: 10.1038/srep25337
15. De Vincentis A, Pennazza G, Santonico M, Vespasiani-Gentilucci U, Galati G, Gallo P, et al. Breath-print analysis by e-nose may refine risk stratification for adverse outcomes in cirrhotic patients. *Liver Int.* (2017) 37:242–50. doi: 10.1111/liv.13214
16. De Vincentis A, Vespasiani-Gentilucci U, Sabatini A, Antonelli-Incalzi R, Picardi A. Exhaled breath analysis in hepatology: state-of-the-art and perspectives. *World J Gastroenterol.* (2019) 25:4043–50. doi: 10.3748/wjg.v25.i30.4043
17. Scarlata S, Pennazza G, Santonico M, Santangelo S, Rossi Bartoli I, Rivera C, et al. Screening of obstructive sleep apnea syndrome by electronic-nose analysis of volatile organic compounds. *Sci Rep.* (2017) 7:11938. doi: 10.1038/s41598-017-12108-w
18. Finamore P, Pedone C, Lelli D, Costanzo L, Bartoli IR, De Vincentis A, et al. Analysis of volatile organic compounds: an innovative approach to heart failure characterization in older patients. *J Breath Res.* (2018) 12:026007. doi: 10.1088/1752-7163/aa8cd4
19. Rocco R, Incalzi RA, Pennazza G, Santonico M, Pedone C, Bartoli IR, et al. BIONOTE e-nose technology may reduce false positives in lung cancer screening programmes†. *Eur J Cardiothorac Surg.* (2016) 49:1112–7; discussion: 1117. doi: 10.1093/ejcts/ezv328
20. Muley M, Vespasiani-Gentilucci U, De Vincentis A, Santonico M, Pennazza G, Sanguedolce S, et al. Voltammetric analysis for distinguishing portal hypertension-related from malignancy-related ascites: a proof of concept study. *PLoS ONE.* (2020) 15:e0233350. doi: 10.1371/journal.pone.0233350
21. Pipita ME, Santonico M, Pennazza G, Zompanti A, Fazzina S, Cavalieri D, et al. Integration of voltammetric analysis, protein electrophoresis and pH measurement for diagnosis of pleural effusions: a non-conventional diagnostic approach. *Sci Rep.* (2020) 10:15222. doi: 10.1038/s41598-020-71542-5
22. Lelli D, Pedone C, Alemanno P, Bertini A, Di Gioia C, Fazzina S, et al. Voltammetric analysis for fast and inexpensive diagnosis of urinary tract infection: a diagnostic study. *J Transl Med.* (2018) 16:17. doi: 10.1186/s12967-018-1393-y
23. Santonico M, Frezzotti E, Antonelli Incalzi R, Pedone C, Lelli D, Zompanti A, et al. Non-invasive monitoring of lower-limb ulcers via exudate fingerprinting using BIONOTE. *Sens Actuators B Chem.* (2016) 232:68–74. doi: 10.1016/j.snb.2016.03.101
24. Maconi G. Diagnosis of symptomatic uncomplicated diverticular disease and the role of Rifaximin in management. *Acta Biomed.* (2017) 88:25–32. doi: 10.23750/abm.v88i1.6360
25. Rubenstein LZ, Harker JO, Salvà A, Guigoz Y, Vellas B. Screening for undernutrition in geriatric practice: developing the short-form mini-nutritional assessment (MNA-SF). *J Gerontol A Biol Sci Med Sci.* (2001) 56:M366–72. doi: 10.1093/gerona/56.6.M366
26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
27. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics.* (2010) 26:266–7. doi: 10.1093/bioinformatics/btp636
28. Lopetuso LR, Petito V, Scaldaferri F, Gasbarrini A. Gut microbiota modulation and mucosal immunity: focus on rifaximin. *Mini Rev Med Chem.* (2015) 16:179–85. doi: 10.2174/1389557516031511261216 33
29. Ponziani FR, Scaldaferri F, Petito V, Paroni Sterbini F, Pecere S, Lopetuso LR, et al. The role of antibiotics in gut microbiota modulation: the eubiotic effects of rifaximin. *Dig Dis.* (2016) 34:269–78. doi: 10.1159/000443361

30. Lanas A, Abad-Baroja D, Lanas-Gimeno A. Progress and challenges in the management of diverticular disease: which treatment? *Therap Adv Gastroenterol.* (2018) 11:1756284818789055. doi: 10.1177/1756284818789055
31. Scarpignato C, Barbara G, Lanas A, Strate LL. Management of colonic diverticular disease in the third millennium: highlights from a symposium held during the United European Gastroenterology week 2017. *Therap Adv Gastroenterol.* (2018) 11:1756284818771305. doi: 10.1177/1756284818771305
32. Kaji K, Takaya H, Saikawa S, Furukawa M, Sato S, Kawaratani H, et al. Rifaximin ameliorates hepatic encephalopathy and endotoxemia without affecting the gut microbiome diversity. *World J Gastroenterol.* (2017) 23:8355–66. doi: 10.3748/wjg.v23.i47.8355
33. Kawaguchi T, Suzuki F, Imamura M, Murashima N, Yanase M, Mine T, et al. Rifaximin-altered gut microbiota components associated with liver/neuropsychological functions in patients with hepatic encephalopathy: an exploratory data analysis of phase II/III clinical trials. *Hepatol Res.* (2019) 49:404–18. doi: 10.1111/hepr.13300
34. Delday M, Mulder I, Logan ET, Grant G. Bacteroides thetaiotaomicron ameliorates colon inflammation in preclinical models of Crohn's disease. *Inflamm Bowel Dis.* (2019) 25:85–96. doi: 10.1093/ibd/izy281
35. Brown EM, Ke X, Hitchcock D, Jeanfavre S, Avila-Pacheco J, Nakata T, et al. Bacteroides-derived sphingolipids are critical for maintaining intestinal homeostasis and symbiosis. *Cell Host Microbe.* (2019) 25:668–80.e7. doi: 10.1016/j.chom.2019.04.002
36. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. *Biochim Biophys Acta.* (2010) 1801:1175–83. doi: 10.1016/j.bbalip.2010.07.007
37. Kvasnovsky CL, Leong LEX, Choo JM, Abell GCJ, Papagrigoriadis S, Bruce KD, et al. Clinical and symptom scores are significantly correlated with fecal microbiota features in patients with symptomatic uncomplicated diverticular disease: a pilot study. *Eur J Gastroenterol Hepatol.* (2018) 30:107–12. doi: 10.1097/MEG.0000000000000995
38. Gardiner BJ, Tai AY, Kotsanas D, Francis MJ, Roberts SA, Ballard SA, et al. Clinical and microbiological characteristics of eggerthella lenta bacteremia. *J Clin Microbiol.* (2015) 53:626–35. doi: 10.1128/JCM.02926-14

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 20 October 2021
doi: 10.3389/fmed.2021.746990

The Efficacy and Safety of Fecal Microbiota Transplantation Combined With Biofeedback for Mixed Constipation: A Retrospective Cohort Study

Bo Yang[1†], Hongliang Tian[1†], Chen Ye[1†], Zhiliang Lin[1], Di Zhao[1], Chunlian Ma[1], Jiangman Zhao[2], Shouxin Wu[2], Rongfeng Jiang[2], Ning Li[1], Huanlong Qin[1] and Qiyi Chen[1]*

[1] *Intestinal Microenvironment Treatment Center of General Surgery, Shanghai Tenth People's Hospital, Tenth People's Hospital of Tongji University, Shanghai, China,* [2] *Shanghai Zhangjiang Institue of Medical Innovation, Shanghai Biotecan Pharmaceuticals Co., Ltd., Shanghai, China*

OPEN ACCESS

Edited by:
Toshifumi Ohkusa, Juntendo University, Japan

Reviewed by:
Dai Ishikawa, Juntendo University, Japan
Naoki Ohmiya, Fujita Health University, Japan

***Correspondence:**
Qiyi Chen
qiyichen2011@163.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *25 July 2021*
Accepted: *20 September 2021*
Published: *20 October 2021*

Citation:
Yang B, Tian H, Ye C, Lin Z, Zhao D, Ma C, Zhao J, Wu S, Jiang R, Li N, Qin H and Chen Q (2021) The Efficacy and Safety of Fecal Microbiota Transplantation Combined With Biofeedback for Mixed Constipation: A Retrospective Cohort Study. Front. Med. 8:746990. doi: 10.3389/fmed.2021.746990

This study aims to assess the effectiveness and safety of fecal microbiota transplantation (FMT) combined with biofeedback for patients with mixed constipation. Patients who received biofeedback (biofeedback group, $n = 40$) and those who received FMT combined with biofeedback (FMT combination group, $n = 45$) were enrolled. Spontaneous bowel movements (SBMs) frequency, Bristol Stool Form Scale (BSFS), and Patient Assessment of Constipation Symptoms (PAC-SYM) score were analyzed to evaluate the effect of treatment. Gastrointestinal Quality of Life Index (GIQLI) scores of patients were used to assess the quality of life, and the safety of FMT combination therapy was evaluated by the presence of adverse events. The 16S rRNA gene sequencing was performed on the fecal samples of 12 donors, feces of 31 patients before and after receiving FMT combination treatment. Comparing the biofeedback group and the FMT combination group 1 month after the treatment, significant differences were observed in the mean value of SBM frequency, BSFS, and PAC-SYM scores, which were 2.15 ± 1.05 vs. 3.61 ± 0.89 ($p = 0.0031$), 2.1 ± 0.9 vs. 2.5 ± 1.2 ($p = 0.008$), and 2.4 ± 0.5 vs. 2.2 ± 0.6 ($p = 0.0021$), respectively. Meanwhile, FMT combination therapy had long-term beneficial effects according to the data collected at six months and 12 months after the treatment. With respect to the quality of life, GIQLI scores were higher in the FMT combination group (103.6 ± 15.1) compared with that in the biofeedback group (88.7 ± 10.1) one month after administration ($p = 0.0042$). In addition, there were no significant differences between the two groups in adverse events, including abdominal pain, diarrhea, dizziness, nausea, vomiting, and other side effects. Results of 16S rRNA gene sequencing showing some well-known probiotics had significantly increased after FMT combination treatment compared with pre-FMT samples, such as Prevotella and Bifidobacterium. Findings of this study suggested that FMT combined with biofeedback could be effective and safe for patients with mixed constipation.

Keywords: mixed constipation, fecal microbiota transplantation, biofeedback - BFB, spontaneous bowel movements, 16S rRNA gene sequencing, gut microbiota diversity

BACKGROUND

Chronic constipation is a common gastrointestinal disease with an estimated prevalence of 5 to 20% worldwide based on the Rome IV criteria (1, 2). Chronic constipation is characterized by trouble defecating, reduced stool frequency, or perceived incomplete evacuation of bowel movements (3). In the light of the changes in gastrointestinal structure and function, constipation can be categorized into slow-transit constipation (STC), outlet obstructive constipation, and mixed constipation (STC and outlet obstructive constipation) (4). Evidence showed that most patients who suffered from STC had associated outlet obstruction, and would develop to mixed constipation as the disease progresses (5, 6). Fecal microbiota transplantation (FMT) has been proposed as a therapeutic approach for functional gastrointestinal disease, especially for recurrent *Clostridium difficile* infection (7, 8). Our previous studies suggested that FMT was effective and safe for STC (9), with 66.7 and 42.9% clinical improvement and remission rates at our hospital, respectively (10). Meanwhile, we found that FMT in combination with soluble dietary fiber (pectin) had both short-term and long-term efficacy in treating STC (11). In addition, biofeedback has been demonstrated as a safe and effective treatment for mixed constipation secondary to slow colonic transport (12), since biofeedback therapy works primarily through strengthening pelvic floor muscles (13). In addition, it was demonstrated that the FMT could improve the clinical phenotype of constipation by affecting gastrointestinal motility (14). Whether FMT combined with biofeedback could give more benefits for the patients with mixed constipation aroused our curiosity. This study aims to explore the clinical efficacy and safety of FMT combined with biofeedback in mixed constipation.

MATERIALS AND METHODS

Patients and Data Collection

This single-center retrospective cohort study includes patients who were treated with biofeedback or FMT combined with biofeedback therapy for mixed constipation between June 2017 and June 2019 at the Tenth People's Hospital Affiliated to the Tongji University. The diagnosis of mixed constipation is based on Rome IV diagnostic criteria (15). Patients with mixed constipation were divided into two groups mainly based on their willingness. The clinicians did not intervene in the grouping of patients to minimize the selection bias. The flow diagram of this study is shown in **Supplementary Figure 1**.

Patients were assessed for inclusion according to the following criteria: (1) aged 18 to 70 years; (2) body mass index (BMI) 18 to 25 kg/m^2. Patients were excluded if they met either of the following criteria: (1) patients lacking data on spontaneous bowel movements (SBMs) frequency, Bristol Stool Form Scale (BSFS), Patient Assessment of Constipation Symptoms (PAC-SYM) score, Gastrointestinal Quality of Life Index (GIQLI) score, and adverse events in the electronic medical record; (2) patients who had a history or evidence of the gastrointestinal organic diseases or metabolic or endocrine diseases.

Following the aforementioned inclusion and exclusion criteria, 40 patients in the biofeedback group and 45 patients in the FMT combination group were recruited in this study. The following data were retrospectively extracted from the electronic medical records of the patients: clinical characteristics (age, sex), underlying disease, and concomitant drugs.

Bristol Stool Form Scale (16) was used to measure the stool form. PAC-SYM questionnaire (17) and GIQLI (18) were applied to evaluate the constipation-related symptoms, and the quality of life of patients in the past two wk, respectively. PAC-SYM consists of 12 symptoms, which are grouped into the three subscales: stool, abdominal, and rectal symptoms. For each item, scores range from 0 (not at all) to 4 (all the time), with the higher scores indicating a worse symptom. The GIQLI was assessed using a 36-item questionnaire on the aspects of emotional, social, and physical states and also gastrointestinal health. The score for each item on the questionnaire is calculated using a 5-point Likert scale, ranging from 0 (the least desirable) to 4 (the most desirable), with a maximum score of 144. The reference value of GIQLI for healthy people is 125.8 ± 13.0.

This study was conducted with the approval from the Ethics Committee of the Tenth People's Hospital Affiliated to Tongji University and in accordance with the ethical principles for the medical research outlined in the Declaration of Helsinki 1964 as modified by the subsequent revisions.

Donor Screening and Stool Processing

Potential donors were screened by strict criteria to minimize risks of disease transmissions, according to Evaluation criteria for FMT of the donor (T/SBIAORG 001-2020) issued by the Shanghai Biopharmaceutics Industry Association (19). In this study, 12 donors were enrolled through a series of questionnaires and medical examinations, including but not limited to the etiology detection of blood and stool, underlying disease, medication history, defecation status, psychological state, sleep quality, age, and BMI (10, 19). A total of 40 fresh feces were donated by the 12 donors, producing 40 batches of the fecal suspension, as we described previously (10). Quality control of the donated feces referenced the diagnostic criteria for the samples of facial microbiota transplantation (T/SBIAORG 002-2020) (20). Each donor's stool suspension provided the treatment for one to eight recipients. Each fresh feces underwent 16S rRNA gene sequencing.

Treatment

The way of administration of FMT was described as a previous study (11). Briefly, vancomycin (500 mg, two times per day) was given orally for three consecutive days. On the last day of antibiotic treatment, bowel lavage with two liters of macrogol solution was applied. The next day, fecal suspension (100 ml, once per day) was infused within 10 min through a nasoduodenal tube. The infusion was performed for 6 consecutive days. Patients who were treated with biofeedback therapy learned gradually to eliminate the inadequate sphincter contraction guided by the therapist and by the visual and auditory feedback of the electromyographic activity of the external anal sphincter during simulated defecation (12). They received 20 treatment sessions (five per week) across one month. After the discharge, the Kegel exercise was performed twice a day for 30 min each time.

Fecal DNA Extraction and 16S rRNA Gene Sequencing

Fresh feces samples were collected by sterile collection tubes before and two months after FMT, then stored at −80°C until further analysis. Fecal DNA was extracted using the QIAamp PowerFecal Pro DNA kit (Qiagen, Hilden, Germany) following the instructions. The quantity and quality of the DNAs were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and agarose gel electrophoresis was performed. PCR amplification of the bacterial 16S rDNA genes V4 region was performed using the forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA−3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR reaction volumes were 50: 25 μl of Phusion High-Fidelity PCR Master Mix with HF Buffer, 3 μl (10 uM) of each forward and reverse primer, 10 μl of DNA template, and 6 μl of ddH_2O. The reactions were set up to perform the PCR amplification using the following program: initial denaturation at −98°C for 30 sec, followed by 25 cycles consisting of denaturation at 98°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 15 sec with a final extension at 72°C for one min. The PCR amplicons were purified using an AMPure XP Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, California, USA). After the quantification step, pair-end 2 × 150 bp sequencing was performed using the Illumina HiSeq4000 platform.

Bioinformatics

After the quality control, the operational taxonomic unit (OTU) was analyzed using Vsearch-1.11, sequences were grouped into OTUs with a sequence identity similarity threshold of 97%. An OTU table was further generated to record the community composition of each sample at taxonomic ranks: kingdom, phylum, class, order, family, and genus. Sequence data analyses were mainly performed using the QIIME and R packages (v3.2.0). Use QIIME to calculate OTU-level alpha diversity indices such as Chao1, ACE, Shannon, and Simpson index. To compare the richness and evenness of OTUs among samples, OTU level ranked abundance curves were generated, dilution curves were drawn, and Alpha diversity index between groups analyses were carried out. Beta diversity analysis was performed by the QIMME to measure the UniFrac distance metrics and visualized *via* principal-component analysis (PCoA) to investigate the compositional differences among the microbial habitats across samples. To identify taxa with differing relative abundances between the two groups, linear discriminant analysis (LDA) effect size (LEfSe) analyses were performed. Microbial functions were predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt).

Outcomes

The primary outcome was the frequency of bowel movements per week. Secondary outcomes included the BSFS, PAC-SYM score, GIQLI score, and the presence of the main adverse events.

TABLE 1 | Clinical characteristics of patients in Biofeedback groups and FMT combination group.

	Biofeedback group ($n = 40$)	FMT combination group ($n = 45$)	p-Value
Age	45.5 ± 7.6	46.1 ± 7.1	>0.05
Sex (Male/Female)	14/26	14/31	>0.05
BMI (kg/m^2)	21.5 ± 3.4	21.6 ± 2.7	>0.05
Underlying disease			
Diabetes	6 (15%)	8 (17.8%)	>0.05
Cardiovascular disease	5 (12.5%)	7 (15.6%)	>0.05
Parkinson's disease	2 (5%)	1 (2.2%)	>0.05
Concomitant laxative			
Senna extract	19 (47.5%)	23 (51.1%)	>0.05
Polyethylene glycol-electrolyte powder	29 (72.5%)	19 (42.2%)	**0.008**
Bisacodyl	11 (27.5%)	21 (46.7%)	>0.05
Aole paidu capsule	14 (40%)	11 (24.4%)	>0.05
Phenolphthalein tablets	15 (37.5%)	5 (11.1%)	**0.005**
Rhubarb	6 (15%)	10 (22.2%)	>0.05

Statistical Analysis

Statistical analysis was performed to compare bowel movement frequency, BSFS, PAC-SYM score, and GIQLI score between the FMT combined with biofeedback (FMT combination) group and biofeedback (biofeedback) group using t-test. To compare the presence of adverse events, Fisher's exact probability test was used. The significance level was set as $p < 0.05$.

RESULTS

Baseline Characteristics of the Patients

A total of 40 patients administered with biofeedback and 45 patients administered with FMT combined with biofeedback were included in this study. There were no significant differences between the FMT combination group and the biofeedback group with respect to age, sex, and BMI (**Table 1**). The mean disease course of the FMT combination group and biofeedback group was 7.2 ± 3.1 and 7.4 ± 3.5 years, respectively. There were no significant differences between the groups in the aspect of complicated cardiovascular diseases, diabetes, or Parkinson's disease. Furthermore, there were no significant differences between the groups with respect to the periodical oral dosing of senna extract, bisacodyl, aole paidu capsule, and rhubarb used for the treatment of constipation. However, polyethylene glycol-electrolyte powder and phenolphthalein tablets were also used significantly more in the biofeedback group as compared with that used in the FMT combination group ($p = 0.008$ and $p = 0.005$, respectively, **Table 1**).

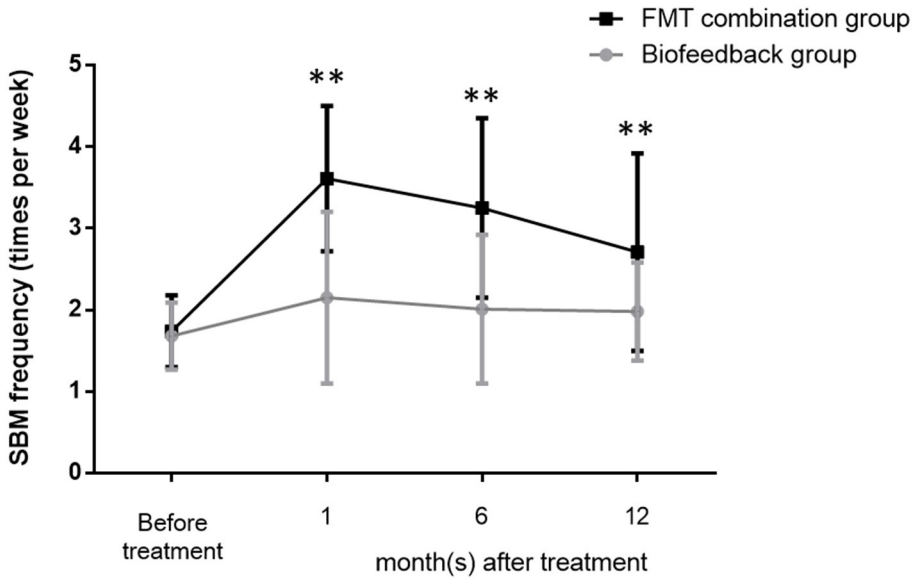


FIGURE 1 | Changes in the frequency of bowel movement per week before and after treatment in the biofeedback group and the FMT combination group. The number of bowel movements per week before administration and up to 12 months after treatment is indicated by the mean ± SD. $p < 0.01$ according to the *t*-test are indicated by **.

Effects of FMT Combined With Biofeedback

The mean values of SBMs frequency within one wk before treatment were 1.68 ± 0.41 and 1.74 ± 0.44 in the biofeedback group and the FMT combination group, respectively ($p > 0.05$). The frequency of SBMs (times per week) at one month after treatment were 2.15 ± 1.05 in the biofeedback group and 3.61 ± 0.89 in the FMT combination group, respectively, which were significantly different ($p = 0.0031$). Furthermore, the mean values of SBMs frequency at 6 and 12 months after treatment were 2.01 ± 0.91 and 1.98 ± 0.6 in the biofeedback group. In comparison, the frequency of SBMs 6 and 12 months after FMT combined with biofeedback was 3.25 ± 1.1 and 2.71 ± 1.21 times per week ($p = 0.0024$ and $p = 0.0046$, respectively, **Figure 1**). According to the exclusion criteria, the number of patients in the biofeedback group and the FMT combination group when 1/6/12 month(s) after the corresponding therapy were 37 and 38/32 and 36/16 and 25, respectively.

TABLE 2 | Adverse Events after treatment in the Biofeedback group and FMT combination group.

	Biofeedback group ($n = 40$)	FMT combination group ($n = 45$)	*p*-Value
Abdominal pain	3 (7.5%)	7 (15.6%)	0.209
Abdominal distension	3 (7.5%)	5 (11.1%)	0.425
Anal pain	3 (7.5%)	4 (8.9%)	0.567
Diarrhea	0 (0%)	4 (8.9%)	0.074
Nausea	1 (2.5%)	3 (6.7%)	0.354
Fever	0 (0%)	3 (6.7%)	0.144
Palpitations	1 (2.5%)	2 (4.4%)	0.545
Dizziness	1 (2.5%)	2 (4.4%)	0.545
Allergies	0 (0%)	2 (4.4%)	0.277
Vomiting	0 (0%)	1 (2.2%)	0.524
Chest tightness	0 (0%)	1 (2.2%)	0.524

Adverse Events in FMT Combined With Biofeedback

Table 2 describes the adverse events between the biofeedback group and the FMT combination group. The adverse events included abdominal pain, abdominal distension, anal pain, diarrhea, nausea, fever, palpitations, dizziness, allergies, vomiting, and chest tightness. The results showed that there was no significant difference in the adverse events between the two groups. Abdominal pain (7.5 vs. 15.6%, $p = 0.209$), abdominal distension (7.5 vs. 11.1%, $p = 0.425$), and anal pain (7.5 vs. 8.9, $p = 0.567$) were the Top 3 frequency adverse events both in the biofeedback groups and FMT combination groups. In addition, diarrhea ($n = 4$), fever ($n = 3$), allergies ($n = 2$), vomiting ($n = 1$), and chest tightness ($n = 1$) only appeared in the FMT combination groups, but not in the biofeedback group.

Secondary Outcomes of FMT Combined With the Biofeedback

In addition, we also analyzed the secondary outcomes data of patients, including BSFS, PAC-SYM score, GIQLI score at one, six, and 12 months after the treatment. The mean values of

TABLE 3 | The comparison of Bristol score between Biofeedback group and FMT combination group before and after treatment.

	Bristol stool form scale			
	Before administration	**1 M**	**6 M**	**12 M**
Biofeedback group	1.5 ± 0.5	2.1 ± 0.9	1.9 ± 0.8	1.8 ± 0.5
FMT combination group	1.6 ± 0.5	2.5 ± 1.2	2.4 ± 0.9	2.3 ± 1.1
p-Value	>0.05	**0.008**	**0.0163**	**0.047**

TABLE 4 | The comparison of PAC-SYM score between Biofeedback group and FMT combination group before and after treatment.

	PAC-SYM score			
	Before administration	**1 M**	**6 M**	**12 M**
Biofeedback group	2.8 ± 0.4	2.4 ± 0.5	2.6 ± 0.4	2.5 ± 0.4
FMT combination group	2.9 ± 0.5	2.2 ± 0.6	2.4 ± 0.5	2.3 ± 0.6
p-Value	>0.05	**0.0021**	**0.0023**	**0.001**

BSFS (average ± SD) before the treatment were 1.5 ± 0.5 and 1.6 ± 0.5 in the biofeedback group and the FMT combination group, respectively ($p> > 0.05$). One month after treatment, BSFS were 2.1 ± 0.9 in the biofeedback group and 2.5 ± 1.2 in the FMT combination group ($p = 0.008$). BSFS at 6 and 12 months after treatment were 1.9 ± 0.8 and 1.8 ± 0.5 in the biofeedback group, and 2.4 ± 0.9 and 2.3 ± 1.1 in the FMT combination group, which was significantly different ($p = 0.0163$ and $p = 0.047$, respectively, **Table 3**). The PAC-SYM score before the treatment were 2.8 ± 0.4 and 2.9 ± 0.5 in the biofeedback group and the FMT combination group, respectively ($p > 0.05$). The PAC-SYM score was 2.4 ± 0.5 in the biofeedback group and 2.2 ± 0.6 in the FMT combination group at one month after treatment ($p = 0.0021$). The PAC-SYM score at six and 12 months after the treatment were 2.6 ± 0.4 and 2.5 ± 0.4 in the biofeedback group, and 2.4 ± 0.5 and 2.3 ± 0.6 in the FMT combination group ($p = 0.0023$ and $p = 0.001$, respectively, **Table 4**). Combined with the results of SBMs, these results indicated that the FMT combination therapy had a better effect on mixed constipation as compared with the biofeedback treatment. With respect to the quality of life, GIQLI scores before the treatment were 80.5 ± 7.8 and 85.4 ± 13.2 in the biofeedback group and the FMT combination group, respectively ($p > 0.05$), while after the treatment, the GIQLI scores were higher in the FMT combination group as compared with that in the biofeedback group. As in **Table 5**, the GIQLI scores at 1 month were 88.7 ± 10.1 in the biofeedback group and 103.6 ± 15.1 in the FMT combination group ($p = 0.0042$), and GIQLI scores at 6 and 12 months were 86.2 ± 11.3 and 85.7 ± 10.8 in the biofeedback group, and 98.4 ± 13.2 and 95.6 ± 11.6 in the FMT combination group ($p = 0.0035$ and $p = 0.0024$, respectively).

TABLE 5 | The comparison of GIQLI score between Biofeedback group and FMT combination group before and after treatment.

	GIQLI score			
	Before administration	**1 M**	**6 M**	**12 M**
Biofeedback group	80.5 ± 7.8	88.7 ± 10.1	86.2 ± 11.3	85.7 ± 10.8
FMT combination group	85.4 ± 13.2	103.6 ± 15.1	98.4 ± 13.2	95.6 ± 11.6
p-Value	>0.05	**0.0042**	**0.0035**	**0.0024**

Gut Microbiota Diversity Elevated of Patients Receiving FMT Combination Treatment

To further investigate the role of the gut microbiota on the patients of FMT combination treatment, feces of the donor and feces of the patient before and 2 months after FMT underwent 16S rRNA gene sequencing. **Figure 2** showed the microbiota composition at the genus level. Bacteroides, Prevotella, and Faecalibacterium were the major components in the feces of the donors (**Figure 2**). **Figure 3** compared the alpha diversity (the ACE, Chao1, Shannon, and Simpson) of feces of the donors and pre- and post-FMT feces of the patients. We found that there were no significant differences on ACE ($p = 0.66$, **Figure 3A**), Chao ($p = 0.47$, **Figure 3B**), Shannon ($p = 0.11$, **Figure 3C**), and Simpson ($p = 0.071$, **Figure 3D**) index between the donors and patients with mixed constipation. After receiving FMT combination therapy, ACE ($p = 0.05$, **Figure 3A**) and Chao ($p = 0.026$, **Figure 3B**) were significantly higher in the post-FMT samples as compared with that in the pre-FMT samples. In addition, the patients after FMT combination therapy have a higher ACE ($p = 0.031$), Shannon ($p = 0.0081$), and lower Simpson ($p = 0.012$) as compared with the donors. For the beta diversity, principal-component analysis (PCoA) of a non-metric multidimensional scaling plot (on a Bray-Curtis distance matrix) and an unweighted UniFrac distance revealed significant qualitative differences in the gut microbial community structure before and after FMT combination therapy (**Figure 4**).

Differences in Microbial Communities After FMT

Cladograms were plotted by the LEfSe analysis, which presented the most significant difference at the taxonomic levels between pre- and post-FMT groups (**Figure 5A**). **Figures 5B–F**, respectively, showed the high- and low-dimensional biomarkers at phylum, class, order, family, and genus levels in the patients with post-FMT gut microbiota as compared with that of the patients with pre-FMT. At the phylum level, the abundance of Actinobacteria significantly increased, while Lentisphaerae decreased (**Figure 5B**). At the class level, Actinobacteria, Coriobacteriia increased, while Lentisphaeria decreased (**Figure 5C**). At the order level, Bifidobacteriales, Coriobacteriales, and Actinobacteria increased, while Victivallales decreased (**Figure 5D**). At the family level, Prevotellaceae, Bifidobacteriaceae, Coriobacteriaceae,

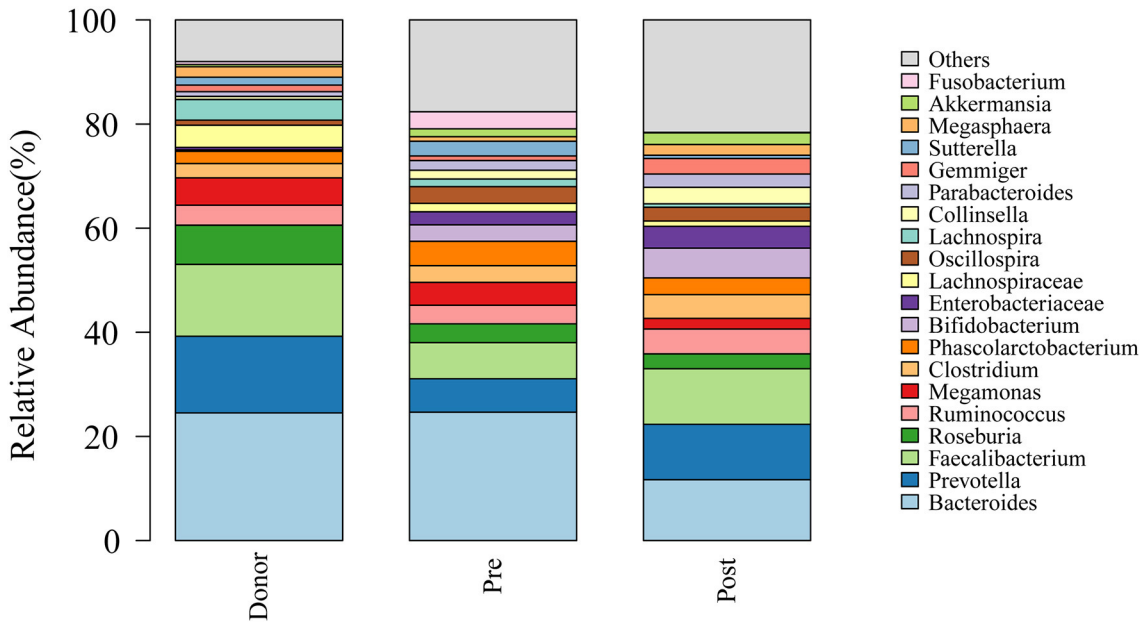


FIGURE 2 | Genus distribution of the gut microbiomes of the donors, mixed constipated patients before and after receiving FMT combination therapy.

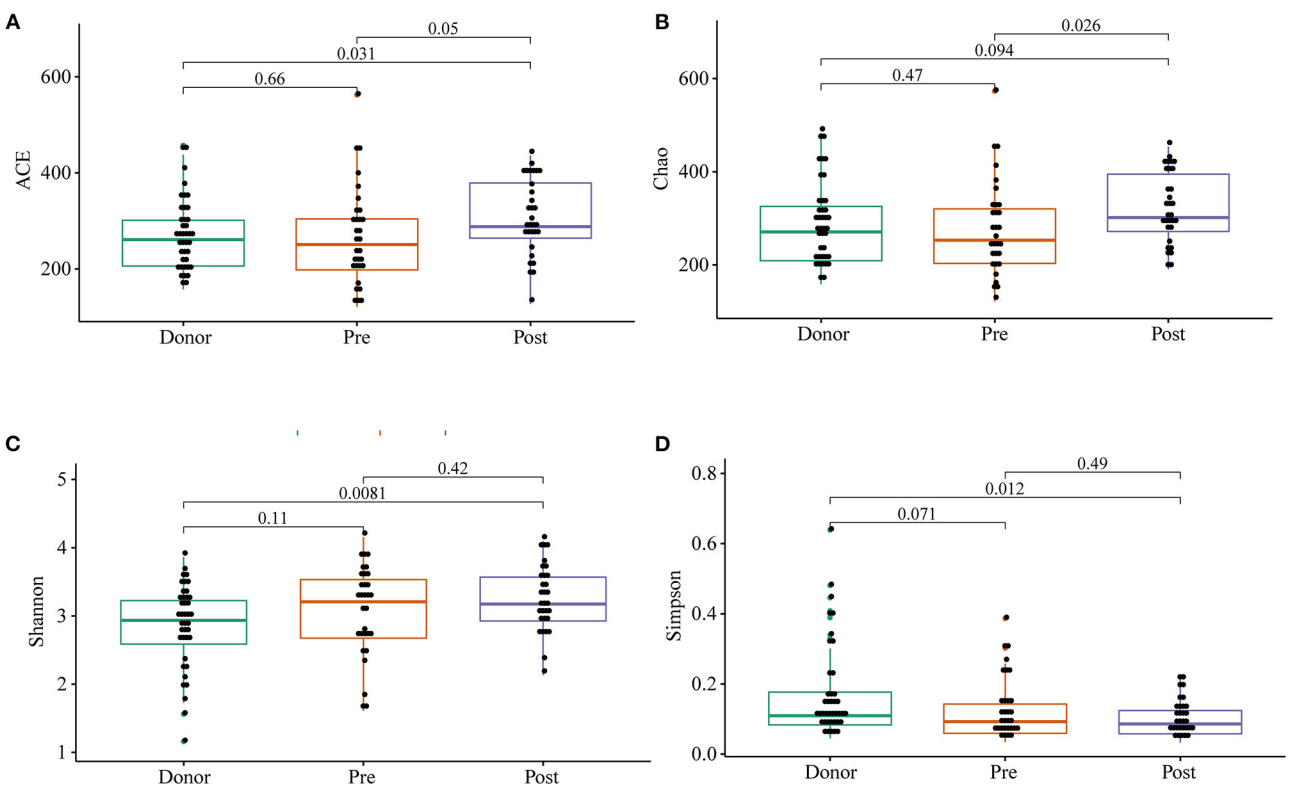


FIGURE 3 | ACE **(A)**, Chao **(B)**, Shannon **(C)**, and Simpson **(D)** were used to analyze the alpha diversity of the gut microbiomes of the donors, as well as patients before and after receiving an FMT combination therapy. Statistical analysis used Wilcoxon rank-sum test.

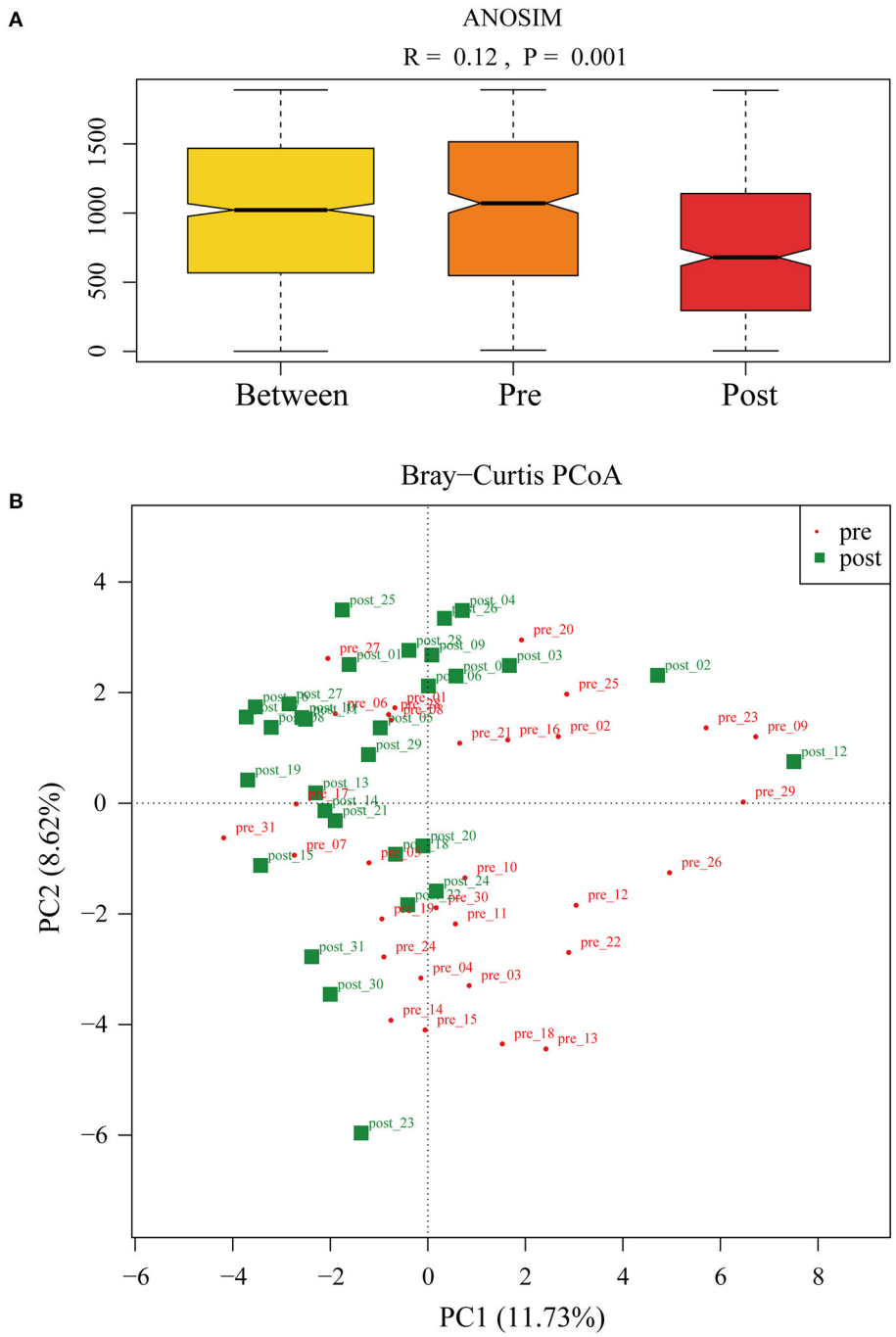


FIGURE 4 | (A) ANalysis Of Similarities was used to compare the differences in each group and between the two groups (pre- and post-FMT) in the microbiota structure. **(B)** Principal-component analysis of the fecal microbiota between pre- and post-FMT groups.

Barnesiellaceae, Actinobacteria, and Enterococcaceae increased, while Bacteroidaceae, Victivallaceae decreased (**Figure 5E**). At the genus level, Prevotella, Bifidobacterium, Paraprevotella, Collinsella, Barnesiellaceae, Actinobacteria, Slackia, Adlercreutzia, and Enterococcus increased, while Bacteroides decreased (**Figure 5F**).

Figure 6A presents the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation (**Figure 6B**) and Clusters of Orthologous Genes (COG) function (**Figure 6B**) predicted by PICRUSt based on the sequencing data of microbiome from all the patients. KEGG pathway (**Figure 6C**) and COG function (**Figure 6D**) in the second-level classification comparisons were

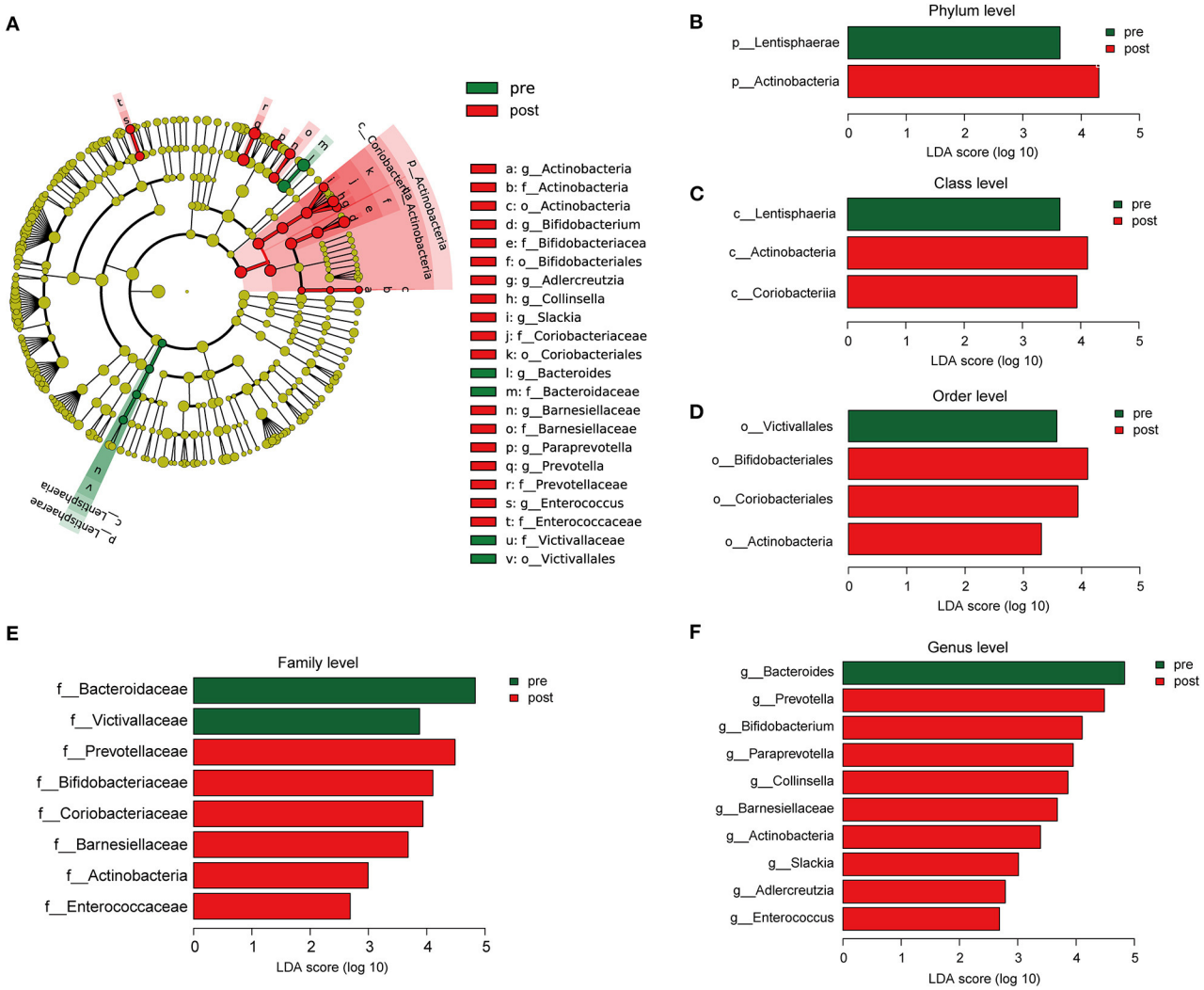


FIGURE 5 | The different composition of the gut microbiota between the patients with pre- and post-FMT. **(A)** The cladogram depicts the phylogenetic distribution of microbial lineages in fecal samples taken before and after 2-month FMT combination treatment. **(B–F)** Key altered phylotypes of the gut microbiota after FMT treatment in phylum **(B)**, class **(C)**, order **(D)**, family **(E)**, and genus levels **(F)**.

performed to explore potential differences in the functional composition of the microbiome between pre- and post-FMT patients by the STAMP software based on the results of PICRUSt. Genetic information processing (transcription and translation) of the gut microbiota was more active after receiving the FMT combination therapy (**Figure 6C**). Inorganic ion transport and metabolism, coenzyme transport, and metabolism in the gut microbiota of the patients with post-FMT were not as active as that in the patients with pre-FMT.

DISCUSSION

Mixed constipation is a kind of chronic constipation with STC and outlet obstruction, difficult to treat in clinical practice (21). In this study, we aim to assess the efficacy and safety of FMT combined with biofeedback on mixed constipation. Based on the results in our study, FMT combination therapy possibly has a superior long-term effect over the biofeedback treatment.

Fecal microbiota transplantation refers to the transplantation of the functional flora from a healthy donor feces into the intestine of the recipient, which aims to restore the diversity of the intestinal flora and achieve the purpose of treating intestinal and extra-intestinal diseases. In the late 1980s, Borody et al. (22) performed FMT on four patients with chronic constipation and demonstrated a significant improvement in the defecation frequency, as well as immediate improvements in symptoms, such as abdominal pain, early satiety, and nausea after FMT. A prospective study reported that FMT might play its role through the restoration and colonization of the donor microbiota in the gut of the recipient up to one year after FMT (23). It has been considered that the FMT could be a promising choice for patients who are refractory to conventional therapeutic strategies.

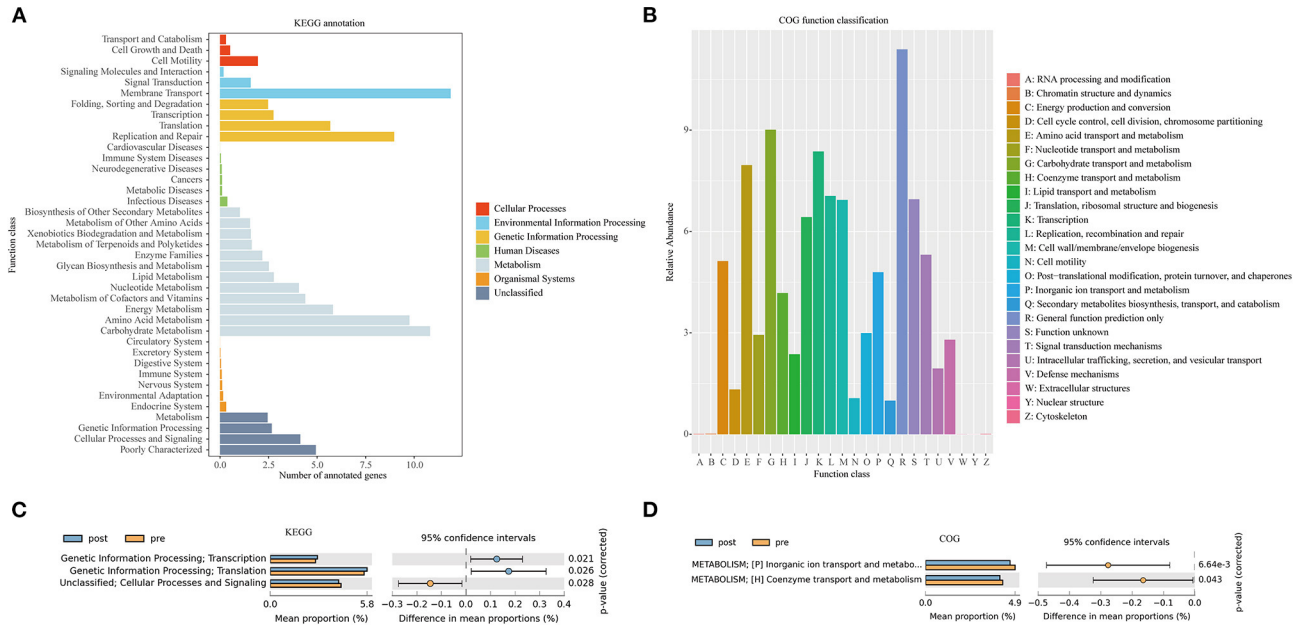


FIGURE 6 | Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations **(A)** and Clusters of Orthologous Genes (COG) function classification **(B)** by PICRUSt-predicted based on 16S rDNA gene sequencing data of the patients with mixed constipation. Differential KEGG pathways **(C)** and COG functions **(D)** in the patients with mixed constipation before and after 2-month FMT combination therapy.

In our previous randomized controlled trial (RCT) study, patients who underwent FMT had significantly improved symptoms, including fecal properties and defecation frequency, compared with the conventional treatment group (24). Meanwhile, biofeedback therapy has been demonstrated to be more effective with good long-term results as compared with sham therapy, laxatives, or the anti-anxiety drug diazepam for patients with constipation associated with a rectal evacuation disorder (25–27). In this study, we found that FMT combined with biofeedback had a sustained effect on mixed constipation. After one year of follow-up, patients who received FMT combination therapy showed relatively more frequent defecation, milder constipation-related symptoms, and better quality of life, compared with those who were treated with the biofeedback only.

Microorganism residing in the gut is believed to have a profound impact on the human physiology and nutrition, and are of the essence for human life. It has been found that the disruption of the homeostasis between the microbiota and the host has a more vital role as compared with the host genetics in the development of a range of diseases, such as inflammatory bowel disease, obesity, and type two diabetes (28). Increasing evidence uncovered that chronic constipation is associated with striking changes in the gut flora (29). Although without significance, we found that the patients with constipation had an increased quantity and diversity of bacteria as compared with the healthy donors, which has been reported in our previous study (14) and other researches (30). The study by Mattea Müller (31) had verified that slow distal colonic transit and hard stools are associated with an increased gut microbiota diversity. Although lack of clear consensus, patients with constipation have a lower abundance of Actinobacteria (32), including Bifidobacteria (32–34) and Prevotella (35) in their feces as compared with the healthy controls (29). Bifidobacterium is commonly used as a probiotic in adults (36, 37) and children (38) with constipation. In this study, the abundance of Prevotella, Bifidobacterium, and Actinobacteria have increased significantly in the guts of the patients after receiving FMT combination therapy. These results indicated that the FMT could remodel the gut microbiota composition of patients with mixed constipation, especially upregulate the beneficial bacterium that mainly explained the efficacy of FMT. Beyond that, some other significantly altered bacteria were also found. For example, Bacteroides was the most significantly downregulated community after an FMT combination therapy, but interestingly, healthy donors also have a relatively high abundance of Bacteroides (**Figures 2**, **5F**). Previous studies give conflicting results of Bacteroides abundance, some reported higher (39, 40) and others reported lower (32) in the patients with constipation. We speculated Bacterioides have the highest abundance in gut microbiota that was susceptible to composition change of other bacteria but have slight effects on constipation.

Regarding safety, FMT has been generally considered a safe and well-tolerated treatment (41). In this study, there was little difference in adverse events between FMT combination groups and biofeedback groups. The adverse events in the FMT combination group were mostly short-term and mild, which were known to be associated with the delivery methods. We noticed that the low-grade fever appeared in three patients of the FMT combination group, but not in the biofeedback group. The appearance of fever is probably because of the result of

a temporary systemic immune response to the transplanted bacteria, other researchers have also reported low-grade fever as the side effect of FMT (42). Besides, the long-term immunologic effects of FMT should also be concerned in the follow-up period after an FMT. Importantly, we should pay extra attention to the possible uncommon severe side effects following FMT, mainly referring to the risk of the infection transmission. Food and Drug Administration (FDA) have reported two cases of Extended Spectrum Beta Lactamase-producing *Escherichia* coli (*E.* coli) infection resulting in one death (43) and enteropathogenic *E.* coli in two cases and Shigatoxin-producing *E.* coli in four cases by donor stool (https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/safety-alert-regarding-use-fecal-microbiota-transplantation-and-risk-serious-adverse-events-likely). Rigorous donor screening and testing could be mandated to minimize the risk of FMT. In recent years, management practices of FMT (44) and evaluation criteria for donors (19) have been issued in China, which were the safeguards of an FMT clinical application. Considering that mixed constipation is a kind of combination of STC and outlet obstructive constipation, which severely impacts the quality of life of those affected, findings of this study suggested that FMT combined with biofeedback would be beneficial to the patients relieving their symptoms and improving their quality of life.

The main limitation of this study is that it was in a retrospective setting. Strictly randomized control was difficult when patients were divided into two groups with a different therapy, which might cause selection bias. Meanwhile, there is a possibility that the diagnosis of chronic constipation and the indication of the treatment may vary among prescribing physicians. Thus, further cases are necessary for an in-depth evaluation. Since the mechanism of an FMT efficacy is comprehensive and 16S rRNA gene sequencing only explained it at the microbiome level, metabolome should be applied to the further probe into an FMT mechanism in the future study. At last, each donor provided stool suspension for one to eight patients, and the patients showed various degrees of efficacy. So, it was difficult to define the more effective donors.

In conclusion, FMT combined with biofeedback showed better effects and equal adverse events on mixed constipation, compared with biofeedback treatment. It also alleviated the constipation symptoms and improved the quality of life of the patients. However, a large-scale prospective study is still to be required to further assess the benefits and risks of an FMT combination therapy for mixed constipation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/750874 (BioProject ID: PRJNA750874).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai 10th People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QC and BY have made substantial contribution to the conception or design of the work. HT, DZ, CY, ZL, RJ, CM, and BY have made the acquisition, analysis, or interpretation of data for the work. NL, HQ, JZ, and SW have drafted the work or revised it critically for important intellectual content. BY have approved the final version to be published. BY agree to be accountable for all aspects of the work in ensuring the questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by National Natural Science Foundation of China (No. 8210031248), Clinical Research Plan of SHDC (No. SHDC2020CR4026), Clinical Research Plan of SHDC (No. SHDC2020CR1030B), and the Shanghai Strategic New Development Project (BT-FGW-2019-06).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.746990/full#supplementary-material

Supplementary Figure 1 | Flow diagram of this study.

REFERENCES

1. Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. *Clin Gastroenterol Hepatol*. (2012) 10:712–21 e4. doi: 10.1016/j.cgh.2012.02.029
2. Suares NC, Ford AC. Prevalence of, and risk factors for, chronic idiopathic constipation in the community: systematic review and meta-analysis. *Am J Gastroenterol*. (2011) 106:1582–91. doi: 10.1038/ajg.2011.164
3. Mearin F, Lacy BE, Chang L, Chey WD, Lembo AJ, Simren M, et al. Bowel Disorders. *Gastroenterology*. (2016) 150:1393–407. doi: 10.1053/j.gastro.2016.02.031
4. Zhu X, Li J, Fu T, Sun P, Jing Y, Tian W. Laparoscopic-assisted subtotal colectomy combined with modified Duhamel procedure for mixed constipation. *JSLS*. (2015) 19:e2014. doi: 10.4293/JSLS.2014.00131
5. Ragg J, McDonald R, Hompes R, Jones OM, Cunningham C, Lindsey I. Isolated colonic inertia is not usually the cause of chronic constipation. *Colorectal Dis*. (2011) 13:1299–302. doi: 10.1111/j.1463-1318.2010.02455.x
6. Li N, Jiang J, Ding WW, Feng XB, Liu JL, Hu XH, et al. Novel surgery for refractory mixed constipation: analysis of the technical notes and outcome to Jinling procedure. *Zhonghua Wai Ke Za Zhi*. (2012) 50:509–13. doi: 10.3760/cma.j.issn.0529-5815.2012.06.011
7. Cammarota G, Ianiro G, Gasbarrini A. Fecal microbiota transplantation for the treatment of Clostridium difficile infection: a systematic review.

J Clin Gastroenterol. (2014) 48:693–702. doi: 10.1097/MCG.0000000000000046
8. Stollman N, Smith M, Giovanelli A, Mendolia G, Burns L, Didyk E, et al. Frozen encapsulated stool in recurrent Clostridium difficile: exploring the role of pills in the treatment hierarchy of fecal microbiota transplant nonresponders. *Am J Gastroenterol.* (2015) 110:600–1. doi: 10.1038/ajg.2015.81
9. Ding C, Fan W, Gu L, Tian H, Ge X, Gong J, et al. 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.* (2018) 6:101–7. doi: 10.1093/gastro/gox036
10. Tian H, Ding C, Gong J, Ge X, McFarland LV, Gu L, et al. Treatment of slow transit constipation with fecal microbiota transplantation: a pilot study. *J Clin Gastroenterol.* (2016) 50:865–70. doi: 10.1097/MCG.0000000000000472
11. Xueying Z, Hongliang T, Lili G, Yongzhan N, Chao D, Xiaolong G, et al. Long-term follow-up of the effects of fecal microbiota transplantation in combination with soluble dietary fiber as a therapeutic regimen in slow transit constipation. *Sci China Life Sci.* (2018) 61:779–86. doi: 10.1007/s11427-017-9229-1
12. Rao SS, Benninga MA, Bharucha AE, Chiarioni G, Di Lorenzo C, Whitehead WE, et al. position paper and consensus guidelines on biofeedback therapy for anorectal disorders. *Neurogastroenterol Motil.* (2015) 27:594–609. doi: 10.1111/nmo.12520
13. Wang J, Luo MH Qi QH, Dong ZL. Prospective study of biofeedback retraining in patients with chronic idiopathic functional constipation. *World J Gastroenterol.* (2003) 9:2109–13. doi: 10.3748/wjg.v9.i9.2109
14. Ge X, Zhao W, Ding C, Tian H, Xu L, Wang H, et al. Potential role of fecal microbiota from patients with slow transit constipation in the regulation of gastrointestinal motility. *Sci Rep.* (2017) 7:441. doi: 10.1038/s41598-017-00612-y
15. Drossman DA, Chang L, Chey WD, Kellow J. *Rome IV Functional Gastrointestinal Disorders*. 4th edn. (2016). doi: 10.24890/pc
16. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology.* (2006) 130:1480–91. doi: 10.1053/j.gastro.2005.11.061
17. Frank L, Kleinman L, Farup C, Taylor L, Miner P. Psychometric validation of a constipation symptom assessment questionnaire. *Scand J Gastroenterol.* (1999) 34:870–7. doi: 10.1080/003655299750025327
18. Borgaonkar MR, Irvine EJ. Quality of life measurement in gastrointestinal and liver disorders. *Gut.* (2000) 47:444–54. doi: 10.1136/gut.47.3.444
19. Shanghai Biopharmaceutics Industry Association. *Evaluation criteria for faecal microbiota transplantation of donor*. T/SBIAORG 001-2020.
20. Shanghai Biopharmaceutics Industry Association. *Diagnostic criteria for samples of facial microbiota transplantation.* T/SBIAORG 002-2020.
21. Ding W, Jiang J, Feng X, Ni L, Li J, Li N. Clinical and pelvic morphologic correlation after subtotal colectomy with colorectal anastomosis for combined slow-transit constipation and obstructive defecation. *Dis Colon Rectum.* (2015) 58:91–6. doi: 10.1097/DCR.0000000000000222
22. Borody TJ, George L, Andrews P, Brandl S, Noonan S, Cole P, et al. Bowel-flora alteration: a potential cure for inflammatory bowel disease and irritable bowel syndrome? *Med J Aust.* (1989) 150:604. doi: 10.5694/j.1326-5377.1989.tb136704.x
23. Ohara T. Identification of the microbial diversity after fecal microbiota transplantation therapy for chronic intractable constipation using 16s rRNA amplicon sequencing. *PLoS ONE.* (2019) 14:e0214085. doi: 10.1371/journal.pone.0214085
24. Tian H, Ge X, Nie Y, Yang L, Ding C, McFarland LV, et al. Fecal microbiota transplantation in patients with slow-transit constipation: a randomized, clinical trial. *PLoS ONE.* (2017) 12:e0171308. doi: 10.1371/journal.pone.0171308
25. Lee HJ, Boo SJ, Jung KW, Han S, Seo SY, Koo HS, et al. Long-term efficacy of biofeedback therapy in patients with dyssynergic defecation: results of a median 44 months follow-up. *Neurogastroenterol Motil.* (2015) 27:787–95. doi: 10.1111/nmo.12552
26. Rao SS, Seaton K, Miller M, Brown K, Nygaard I, Stumbo P, et al. Randomized controlled trial of biofeedback, sham feedback, and standard therapy for dyssynergic defecation. *Clin Gastroenterol Hepatol.* (2007) 5:331–8. doi: 10.1016/j.cgh.2006.12.023
27. Chiarioni G, Whitehead WE, Pezza V, Morelli A, Bassotti G. Biofeedback is superior to laxatives for normal transit constipation due to pelvic floor dyssynergia. *Gastroenterology.* (2006) 130:657–64. doi: 10.1053/j.gastro.2005.11.014
28. Blaser M, Bork P, Fraser C, Knight R, Wang J. The microbiome explored: recent insights and future challenges. *Nat Rev Microbiol.* (2013) 11:213–7. doi: 10.1038/nrmicro2973
29. Ohkusa T, Koido S, Nishikawa Y, Sato N. Gut microbiota and chronic constipation: a review and update. *Front Med (Lausanne).* (2019) 6:19. doi: 10.3389/fmed.2019.00019
30. Mancabelli L, Milani C, Lugli GA, Turroni F, Mangifesta M, Viappiani A, et al. Unveiling the gut microbiota composition and functionality associated with constipation through metagenomic analyses. *Sci Rep.* (2017) 7:9879. doi: 10.1038/s41598-017-10663-w
31. Müller M, Hermes GD, Canfora EE, Smidt H, Masclee AA, Zoetendal EG, et al. Distal colonic transit is linked to gut microbiota diversity and microbial fermentation in humans with slow colonic transit. *Am J Physiol Gastrointest Liver Physiol.* (2020) 318:G361–G9. doi: 10.1152/ajpgi.00283.2019
32. Rajilić-Stojanović M, Biagi E, Heilig HG, Kajander K, Kekkonen RA, Tims S, et al. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology.* (2011) 141:1792–801. doi: 10.1053/j.gastro.2011.07.043
33. Kim SE, Choi SC, Park KS, Park MI, Shin JE, Lee TH, et al. Change of fecal flora and effectiveness of the short-term vsl#3 probiotic treatment in patients with functional constipation. *J Neurogastroenterol Motil.* (2015) 21:111–20. doi: 10.5056/jnm14048
34. Chassard C, Dapoigny M, Scott KP, Crouzet L. Del'Homme C, Marquet P, et al. Functional dysbiosis within the gut microbiota of patients with constipated-irritable bowel syndrome. *Aliment Pharmacol Ther.* (2012) 35:828–38. doi: 10.1111/j.1365-2036.2012.05007.x
35. Zhu L, Liu W, Alkhouri R, Baker RD, Bard JE, Quigley EM, et al. Structural changes in the gut microbiome of constipated patients. *Physiol Genomics.* (2014) 46:679–86. doi: 10.1152/physiolgenomics.00082.2014
36. Waller PA, Gopal PK, Leyer GJ, Ouwehand AC, Reifer C, Stewart ME, et al. Dose-response effect of Bifidobacterium lactis HN019 on whole gut transit time and functional gastrointestinal symptoms in adults. *Scand J Gastroenterol.* (2011) 46:1057–64. doi: 10.3109/00365521.2011.584895
37. Yoon JY, Cha JM, Oh JK, Tan PL, Kim SH, Kwak MS, et al. Probiotics ameliorate stool consistency in patients with chronic constipation: a randomized, double-blind, placebo-controlled study. *Dig Dis Sci.* (2018) 63:2754–64. doi: 10.1007/s10620-018-5139-8
38. Tabbers MM, Chmielewska A, Roseboom MG, Crastes N, Perrin C, Reitsma JB, et al. Fermented milk containing Bifidobacterium lactis DN-173 010 in childhood constipation: a randomized, double-blind, controlled trial. *Pediatrics.* (2011) 127:e1392–9. doi: 10.1542/peds.2010-2590
39. Parthasarathy G, Chen J, Chen X, Chia N, O'Connor HM, Wolf PG, et al. Relationship between microbiota of the colonic mucosa vs feces and symptoms, colonic transit, and methane production in female patients with chronic constipation. *Gastroenterology.* (2016) 150: 367–79e1. doi: 10.1053/j.gastro.2015.10.005
40. Durbán A, Abellán JJ, Jiménez-Hernández N, Salgado P, Ponce M, Ponce J, et al. Structural alterations of faecal and mucosa-associated bacterial communities in irritable bowel syndrome. *Environ Microbiol Rep.* (2012) 4:242–7. doi: 10.1111/j.1758-2229.2012.00327.x
41. Green JE, Davis JA, Berk M, Hair C, Loughman A, Castle D, et al. Efficacy and safety of fecal microbiota transplantation for the treatment of diseases other than Clostridium difficile infection: a systematic review and meta-analysis. *Gut Microbes.* (2020) 12:1–25. doi: 10.1080/19490976.2020.1854640
42. Wang JW, Kuo CH, Kuo FC, Wang YK, Hsu WH Yu FJ, et al. Fecal microbiota transplantation: review and update. *J Formos Med Assoc.* (2019) 118:S23–31. doi: 10.1016/j.jfma.2018.08.011
43. DeFilipp Z, Bloom PP, Torres Soto M, Mansour MK, Sater MR, Huntley MH, et al. Drug-Resistant E coli bacteremia transmitted by fecal microbiota transplant. *N Engl J Med.* (2019) 381:2043–50. doi: 10.1056/NEJMoa1910437
44. Shanghai Muicipal Health Commission. *Managerial technique standard of faecal microbiota transplantation in Shanghai* (2021).

Conflict of Interest: Authors Jiangman Zhao, Shouxin Wu and Rongfeng Jiang were employed by company Shanghai Biotecan Pharmaceuticals 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.

frontiers in Medicine

REVIEW
published: 13 December 2021
doi: 10.3389/fmed.2021.766126

The Communication Between Intestinal Microbiota and Ulcerative Colitis: An Exploration of Pathogenesis, Animal Models, and Potential Therapeutic Strategies

Yu Hu[1†], Zhen Ye[1†], Mingquan Wu[2], Yingqi She[1], Linzhen Li[3], Yujie Xu[4], Kaihua Qin[5], Zhipeng Hu[3], Maoyi Yang[3], Fating Lu[1] and Qiaobo Ye[1]*

[1] School of Basic Medical Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2] Department of Pharmacy, Sichuan Provincial Orthopedic Hospital, Chengdu, China, [3] Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, China, [4] Guangzhou University of Chinese Medicine, Guangzhou, China, [5] Health Preservation and Rehabilitation College, Chengdu University of Traditional Chinese Medicine, Chengdu, China

OPEN ACCESS

Edited by:
Yan-Dong Wang,
Beijing University of Chemical Technology, China

Reviewed by:
Hong Nie,
Jinan University, China
Jianping Chen,
The University of Hong Kong, China

***Correspondence:**
Qiaobo Ye
yeqiaobo@cdutcm.edu.cn

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 28 August 2021
Accepted: 18 November 2021
Published: 13 December 2021

Citation:
Hu Y, Ye Z, Wu M, She Y, Li L, Xu Y, Qin K, Hu Z, Yang M, Lu F and Ye Q (2021) The Communication Between Intestinal Microbiota and Ulcerative Colitis: An Exploration of Pathogenesis, Animal Models, and Potential Therapeutic Strategies. Front. Med. 8:766126. doi: 10.3389/fmed.2021.766126

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease. The prolonged course of UC and the lack of effective treatment management make it difficult to cure, affecting the health and life safety of patients. Although UC has received more attention, the etiology and pathogenesis of UC are still unclear. Therefore, it is urgent to establish an updated and comprehensive understanding of UC and explore effective treatment strategies. Notably, sufficient evidence shows that the intestinal microbiota plays an important role in the pathogenesis of UC, and the treating method aimed at improving the balance of the intestinal microbiota exhibits a therapeutic potential for UC. This article reviews the relationship between the genetic, immunological and microbial risk factors with UC. At the same time, the UC animal models related to intestinal microbiota dysbiosis induced by chemical drugs were evaluated. Finally, the potential value of the therapeutic strategies for restoring intestinal microbial homeostasis and treating UC were also investigated. Comprehensively, this study may help to carry out preclinical research, treatment theory and methods, and health management strategy of UC, and provide some theoretical basis for TCM in the treatment of UC.

Keywords: ulcerative colitis, pathogenesis, intestinal microbiota, animal model, treatment strategy, fecal microbiota transplantation, probiotic, traditional Chinese medicine

INTRODUCTION

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease (IBD) that involves the rectum and colonic mucosal layer, leading to superficial damage to the intestinal wall (1). Chronic diarrhea, fecal blood or rectal bleeding are the main clinical manifestations of these patients. Approximately 15% of UC patients develop severe illnesses (2). More importantly, chronic UC is associated with an increased risk of colorectal cancer. Yet the pathogenesis of UC has not been fully elucidated, it is mainly related to genetic, immunological, microbial and other risk factors. The intestinal

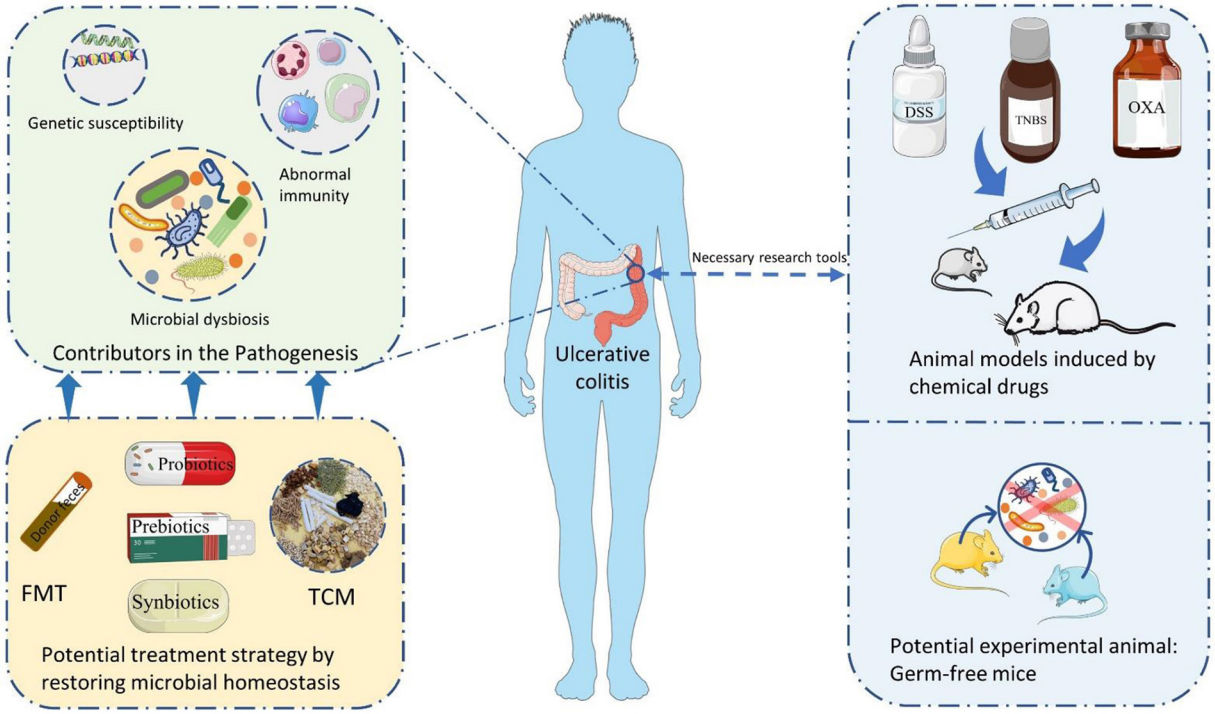


FIGURE 1 | The summary of themes in this review. FMT, Fecal microbiota transplantation; TCM, traditional Chinese medicine; DSS, dextran sulfate sodium; TNBS, 2,4,6-trinitrobenzene sulfonic acid; OXA, oxazolone.

microbiota is considered as a kind of "mysterious organization" in human body and has been proved to play an essential role in the pathogenesis of UC (3). In recent years, based on the development of high-throughput sequencing technology, related significant progress has been made in this research field, which is helpful for understanding the microbiota on human mucosal surface (4). Under physiological conditions, the interactions between bacteria, fungi, and other members maintain a dynamic balance called intestinal microbial homeostasis. Once the homeostasis is broken, the links between microbiota will change, resulting in a decreasing microbial diversity and increasing opportunistic pathogens (5). These changes further induce an abnormal immune response in the host intestine and eventually led to UC (6).

Abbreviations: UC, Ulcerative Colitis; IBD, Inflammatory bowel disease; 5-ASA, 5-aminosalicylic acid; TCM, Traditional Chinese medicine; CAMs, Complementary and alternative medicines; CD, Crohn's disease; IL, Interleukin; TLR, Toll-like receptor; NF-κB, Nuclear factor κB; TNF-α, Tumor necrosis factor-α; Th, T helper type; CpG-DNA, Non-methylated bacterial DNA; MyD88, Myeloid differentiation factor 88; IRAK, IL-1 receptor-related kinase; SCFAs, Short-chain fatty acids; DSS, Dextran sulfate sodium; SYK, Spleen tyrosine kinase; CARD9; Caspase recruitment domain family member 9; TNBS, 2,4,6-trinitrobenzene sulfonic acid; OXA, Oxazolone; SASP, Sulfasalazine; FMT, Fecal microbiota transplantation; RCTs, Randomized controlled trials; DAI, Disease activity index; IFN-γ, Interferon-gamma; MAPK, Mitogen-activated protein kinase; Akt, Protein kinase B; MPO, Myeloperoxidase; IP10, gamma-induced protein 10; CXCR3, Chemokine (cys-x-cys motif) receptor 3; STAT3, Signal transducer and activator of transcription 3.

At present, anti-inflammatory and immunosuppressive therapies are the important treating method for UC. 5-aminosalicylic acid (5-ASA), corticosteroids, and thiopurines are commonly used drugs. However, steroid dependence and side effects of thiopurine make long-term use of the drug at high risk (7). Moreover, even with medication, 20–25% of patients eventually need surgery (8). As a consequence, it is imminent to find effective and safe treatment strategies. Of note, the therapeutic approaches aimed to improve microbial dysbiosis has shown great potential for the treatment of UC. Similarly, traditional Chinese medicine (TCM), as an important part of complementary and alternative medicines (CAMs), has been used in China for more than 2,000 years. At present, various scholars have begun to pay attention to the therapeutic effect of TCM on UC, and have made some progress in China (9, 10). Increasing evidence shows that the efficacy of TCM in the treatment of UC dependents on its effect on intestinal microbiota (11, 12).

In this review, the essential components involved in the pathogenesis of UC are firstly summarized, and the critical role of intestinal microbiota is further discussed. Secondly, the potential of UC animal models applied in studying the intestinal microbiota is also addressed and evaluated. At last, potential therapeutic strategies which exerting a therapeutic role in UC by modulating the intestinal microbiota are exposed and revealed as much as possible (**Figure 1**). This work is expected to theoretical support

and inspiration for the drug selection and development for treating UC.

THE RISK FACTORS WITH THE OCCURRENCE OF UC

Genetic Factors

Although certain studies have shown that the heritable risk of Crohn's disease (CD) is greater than that of UC, the risk of the disease in first-degree relatives of UC patients is still four times greater than that of the general population, suggesting that the risk of genetic factors remains a contributor to the occurrence of UC (13, 14). The first genome-wide association studies based on IBD identified IL23R, a gene encoding the pro-inflammatory cytokine interleukin (IL)-23, whose abnormalities and mutations are associated with the development of CD and UC (15). To date, more than 240 risk loci have been identified to be associated with IBD (16). It was interesting that only 4–7% of UC occurrence can be explained through known risk loci, but genetic factors appear to be more important for early IBD that develops in children (17, 18).

The disease-associated loci of involving genes with different functions, such as the innate and adaptive immune systems, cytokine signaling, lymphocyte activation, and response function to microbial molecules (17). For example, variants in CARD9, a bridging protein involved in antifungal innate immunity, enhance the host response to the fungus and increase the production of inflammatory cytokines, and is also considered as one of the genetic risk factors for CD and UC (19). Moreover, the ADCY7 gene is expressed in hematopoietic cells and encodes a protein, adenylate cyclase 7, which converts ATP to cAMP to participate in the regulation of host innate and adaptive immunity (20, 21). And it is reported that the missense variant of ADCY7 increases the risk of UC. For other IBD risk loci, a complete description was given in the research article published in 2017 (16).

So far, the genetic risk of CD has been well studied, and a large number of studies have shown a strong genetic predisposition for CD. For example, disorders of the NOD2 gene have been identified as an essential causative factor for CD occurrence (22). At the same time, researches on the genetic risk of UC and IBD is continuing, which not only facilitates the development of the pathogenesis of IBD and the differentiation of IBD subtypes but also contributes to the updating of therapeutic targets and drugs or programs for the disease.

Immunological Factors

Genetic, environmental and microbial factors have been identified as risk factors of UC (22). However, UC is ultimately linked with immune abnormalities, which indicated immunological factors may be the central link (1). The human immune system can be divided into innate immunity and adaptive immunity according to functions, and the available evidence points to the involvement of both innate and adaptive immunity in the pathogenesis of UC (23).

The innate immune response is the first line of defense against any attack, and the neutrophils, dendritic cells or macrophages that mediate innate immunity has been confirmed to be involved in the pathogenesis of UC. The earliest feature of intestinal inflammation is the infiltration of neutrophils into the mucosa and epithelium, and is present throughout the period of active inflammation of the intestine. The tissue and epithelial barrier disruption are caused by neutrophils with oxidative and proteolytic damage, as well as promote the release of pro-inflammatory cytokines to perpetuate inflammation (24). Dendritic cells are also involved in the expression of Toll-like receptors (TLRs) and the recognition of microbiota (25). Increased activation and sensitivity of mature dendritic cells and significantly higher the level of TLR2 and TLR4 expressed by mucosal dendritic cells are observed in UC patients, which lead to abnormal activation of signaling pathways such as the nuclear factor κB (NF-κB) to promote the inflammatory cascade (26). Macrophages can be polarized into classically activated pro-inflammatory or alternatively activated anti-inflammatory macrophages depending on the stimulus (27). The pro-inflammatory macrophages are induced by pathogen-associated molecular patterns and involved in the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β and IL-6 to intensify inflammation (28). In addition, the polarization of pro-inflammatory macrophages has been found to promote the development of colitis. In conclusion, excessive abnormalities in innate immunity and the occurrence of inflammatory cascade aggravate the emergence and persistence of local inflammation in the colon, which is closely associated with the occurrence of UC.

After recognizing of antigens, the dendritic cells and macrophages will present antigens to T cells and B cells, leading to the activation of adaptive immunity (29). And the abnormalities of adaptive immunity are another risk factor of UC (30). UC is thought to be a disease mediated by T helper type (Th) 2 cells, which are involved in the secretion of IL-4, IL-5 and IL-13. Published studies have shown the increase in IL-5 and IL-13 secretion in UC patients, with IL-13 affecting the intestinal epithelium and disrupting tight junctions to cause an inflammatory state (31, 32). In addition, abnormalities in Th9 and Th17 cells provide evidence for the involvement of adaptive immunity in UC. Th9 cells are associated with the release of IL-9, which can inhibit the repair of intestinal epithelial cells and increase the concentration of TNF-α (33). Similarly, Th17 cells are also involved in the release of pro-inflammatory cytokines such as IL-17A, IL-17F, IL-21 and IL-22, and increased Th17 cells expression has been observed in UC patients (34, 35). In summary, current evidence suggests that both innate immunity and adaptive immunity are critical in the pathogenesis of UC (1).

Microbial Factors

The Role of Intestinal Bacteria in the Pathogenesis of UC

The intestinal microbiota mainly settles in the gastrointestinal tract of humans, and bacteria occupy the primary advantage in the composition (36). The intestinal bacteria are comprised of three types: anaerobic bacteria, facultative anaerobic bacteria and aerobic bacteria, with anaerobic bacteria being the dominance (37). At the phylum level, the intestinal bacteria are

mainly composed of *Firmicutes*, *Bacteroides*, *Proteobacteria* and *Actinobacteria* (38). Under physiological conditions, intestinal bacteria play an essential role in stimulating the absorption of nutrients and minerals, breaking down protein compounds, synthesizing amino acids and vitamins, promoting intestinal cell renewal, and maintaining immune function (39).

Heredity, age, environment and dietary structure can influence the composition of intestinal bacteria. For example, a high-fat diet reduces the abundance of *Bifidobacterium spp* in the intestinal tract of mice (40). Various types of studies have shown that the abundance of *Bacteroides* in the elderly is greater than that of the young (41). Other studies reported that the intestinal bacteria's amount and diversity of UC patients are significantly decreased (42). At the same time, the changes in flora composition were also found in the pathological state. At the phylum level, the abundance of *Bacteroidetes* and *Proteobacteria* increased, while the abundance of *Firmicutes* decreased (5). In specific microbes, the number of beneficial bacteria such as the *Roseburia spp* and *lactobacillus* in the intestine decreased, while the number of destructive bacteria such as *Escherichia coli*, *Bacteroides fragilis*, and *Helicobacter* increased (5, 43, 44). So far, many experts believe that the dysbiosis of intestinal microbiota can lead to the dysregulation of the immune response to bacterial antigens, and ultimately leads to the occurrence of IBD (45, 46).

The intestinal epithelial barrier is mainly composed of the intestinal epithelial cells, tight junctions (connecting the epithelial cells), goblet cells, and mucus (secreted by goblet cells). This barrier is the first defensing phase to ensure the normal physiological function of the intestinal tract and prevent pathogenic microbes from crossing the intestinal mucosa. As an integral part of the barrier, intestinal mucus can limit the direct contact between host and intestinal bacteria, promote bacteria clearance, and inhibit inflammation and infection (47). Furthermore, dysbiosis of intestinal microbiota can affect the function of epithelial barrier. Firstly, the tight junction is damaged, resulting in increased intestinal mucosa permeability (48). Secondly, the dysbiosis of bacteria will significantly impact intestinal mucus. For example, *Akkermansia muciniphila* and *Enterorhabdus mucosicola* can degrade intestinal mucus and proliferate in the mucus layer (42), *Escherichia coli* and *Gardnerella* can form adherent biofilms on the surface of intestinal epithelium, destroy intestinal mucus, and allow other commensal bacteria to migrate to the mucosa (49). Muc2 protein, secreted by goblet cells, is the primary source of mucus. This protein is mediated by intestinal microbes to maintain the balance of the mucus layer. However, loss of goblet cells and Muc2 protein is a typical feature of epithelial barrier changes after the dysbiosis of intestinal bacteria in UC patients (50).

When the epithelial barrier is disrupted, symbiotic bacteria are allowed to flow in the epithelial layer. Invasion of pathogenic bacteria and opportunistic pathogens activate the host's maladaptive immune response (51). TLR is a protein molecule involved in nonspecific immunity, while lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria, can bind to TLR4 (52). Similarly, peptidoglycan, lipoprotein, and lipoteichoic acid in the bacterial cell wall can also bind to TLR2/TLR6 complex. TLR9 responds to non-methylated bacterial DNA (CpG-DNA) (53). After recognizing the components of the bacterial cell wall, TLRs interact with Myeloid differentiation factor 88 (MyD88), up-regulated the activation signal of IL-1 receptor-related kinase (IRAK) of related member, activated the NF-κB signaling pathway, released pro-inflammatory cytokines, resulting in abnormal intestinal inflammation and occurrence of UC (**Figure 2**) (54).

As well, dysbiosis of intestinal microbiota also affects the regulation of bacterial metabolites (55). Short-chain fatty acids (SCFAs) are produced by symbiotic bacteria that digest dietary fiber in the intestine, including acetate, propionate, and butyrate, etc. (56). SCFAs can maintain the balance of regulatory T cells number to regulate intestinal inflammation (57). It is worth noting that the disorder of SCFAs' generation occurs in UC patients, which is related to the significant reduction of butyrate-producing bacteria (5). Therefore, the dysbiosis of intestinal bacteria further leads to the disorder of its metabolites and products, destroys normal immune response of the host intestine, and is also an important factor leading to UC.

The Role of Intestinal Fungi in the Pathogenesis of UC

Fungi are also normal inhabitants of the human intestine. In healthy individuals, the number of intestinal fungi only accounts for 0.1% of intestinal microorganisms, among which the most dominant fungi are *Candida*, *Saccharomyces* and *Cladosporium* (4). The interaction between symbiotic fungi in the intestine and the other microbial members remain balanced, but risk factors can cause changes in the composition of fungi in the human intestine. High-carbohydrate diets increase the number of *Candida albicans* in the intestine, while high-protein diets are the opposite. And meat-based diets promote the abundance of *Penicillium* fungi (58, 59). An analysis showed different intestinal fungal spectrums in UC patients and healthy people, and the diversity of intestinal fungi in UC patients was significantly reduced (60). In terms of its composition, intestinal fungi of UC patients also changed. The abundance of *Candida albicans* increased, and the abundance of *Wickerhamomyces* fungi was positively correlated with the severity of UC patients (61). *Saccharomyces cerevisiae*, a fungus that can stimulate the release of IL-10 to inhibit intestinal inflammation, and its abundance is absolutely decreased in the acute phase of intestinal inflammation (62).

Intestinal fungi imbalance can reduce host immune tolerance and activate abnormal immune responses. The main component of the fungal cell wall, β-glucan, α-mannan and α-mannose, can be identified by TLRs and C-type lectin receptors (Dectin-1, Dectin-2, and Mincle) (63). Dectin-1 is a key molecule involved in fungi immune response, and the polymorphism of the Dectin-1 gene is closely related to the severity of UC. Mice without Dectin-1 were more susceptible to dextran sulfate sodium (DSS)-induced colitis (63). After recognition, spleen tyrosine kinase (SYK) and caspase recruitment domain family member 9 (CARD9) were activated, thereby activating the NF-kB pathway, releasing pro-inflammatory cytokines, and inducing inflammatory response (**Figure 2**) (64).

In addition, the DSS-induced mouse antifungal drug experiment reported that antifungal drugs could aggravate the

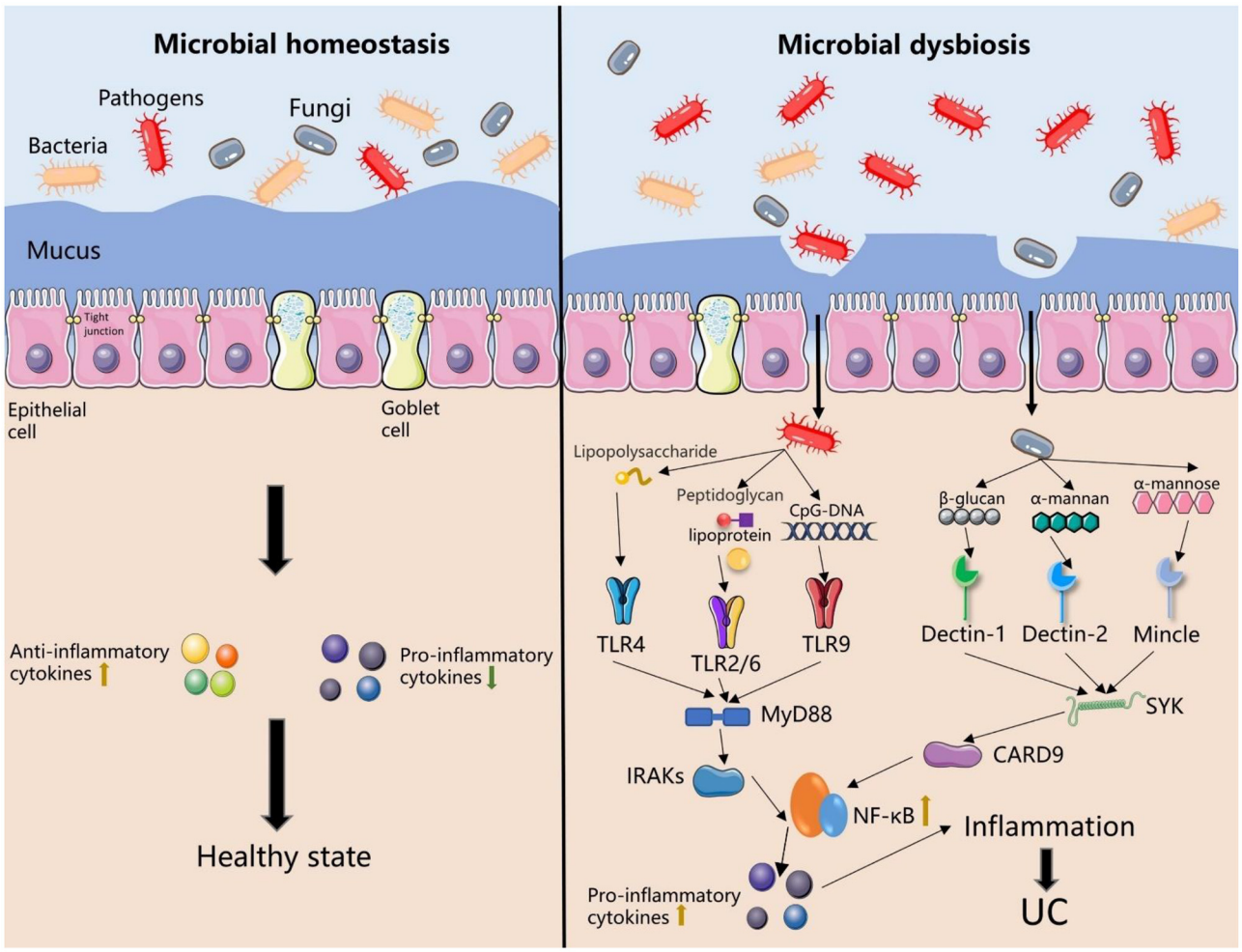


FIGURE 2 | The dysbiosis of intestinal microbiota (bacteria and fungi) results in the occurrence of UC. Under the influence of risk factors, intestinal microbial homeostasis is disrupted. The dysbiosis of microbiota leads to changes in the tight junctions between intestinal epithelial cells, damage of intestinal epithelial barrier, disruption of the mucus layer, and an increase of intestinal permeability. Symbiotic microorganisms and pathogens cause direct damage to the intestinal wall. Furthermore, the main components of the bacterial cell wall (lipopolysaccharide, peptidoglycan, and lipoprotein) are recognized by TLRs, and C-type lectin receptors recognize the components of the fungal cell wall (β-glucan, α-mannan, and α-mannose). After recognition of the antigen, these receptors induce downstream signaling and activate the NF-κB signaling pathways, further induce abnormal immune responses in the host and ultimately leading to the occurrence of UC.TLR, Toll-like receptor; MyD88, Myeloid differentiation factor 88; IRAK, interleukin-1 receptor-associated kinase; NF-κB, nuclear factor κB; Mincle, Macrophage-inducible C-type lectin; SYK, spleen tyrosine kinase; CARD9, caspase recruitment domain family member nine.

inflammation response in mice (65). The expression of tumor necrosis TNF-α and IL-17A in the colon of UC patients is positively correlated with *Wickerhamomyces* and *Penicillium* fungi (61). These studies also provide evidence that intestinal fungal dysbiosis is associated with the occurrence of UC.

Intestinal Microbial Homeostasis and UC

Intestinal microbiota is a complex and extensive system composed of bacteria, fungi, archaea, viruses and protozoa, *etc*. Interactions between the microbiome maintain homeostasis, help host resist pathogen infection, and promote host immunity and health (66).

The interaction between intestinal fungi and bacteria plays a key role in maintaining intestinal microbial homeostasis. A study found that after antibiotic treatment, the number of intestinal fungi in mice increased as bacteria continued to decrease. This change returned to the initial state after the antibiotics stopped (67).

However, after the use of antifungal drugs, the decrease of intestinal symbiotic fungi promotes the growth of pathogenic bacteria in the intestinal tract, thereby aggravating intestinal inflammation (68). Bacteria can limit the effective colonization of fungi in the intestinal tract by producing antifungal compounds and competing nutrients. This phenomenon reflects the competitive relationship between bacteria and fungi due to the limited intestinal resource. In fact, there is also a synergistic relationship between them (69). *Candida albicans* is the most common human infectious fungi, mainly affecting

immunologically impaired individuals (70). In another study, researchers found that the harmful effects of *Candida albicans* depended on the presence of colistin-sensitive bacteria in the intestine (71). At the same time, *Candida albicans* can form mixed biofilms with other intestinal bacteria, which can surround anaerobic bacteria to protect them from the effects of an oxygenated environment (72).

Under different interactions, intestinal microbiota maintains balance and stability in the intestine. Affected by risk factors, the dysbiosis of intestinal bacteria and fungi leads to susceptibility of the host to UC (73). The positive and negative correlations between intestinal bacteria and fungi in UC patients are higher than those in healthy individuals (62). Therefore, the disruption of intestinal microbial homeostasis is tightly related to the occurrence of UC. With the increase of relevant evidence, the relationship between microbial homeostasis and the pathogenesis of UC is increasingly obvious.

The Importance of the Intestinal Microbiota in UC's Occurrence

Abnormalities of the immune system are certainly important for the pathogenesis of UC, which is also a major reason for the appearance and persistence of local inflammation in UC patients. However, except for the immune system, the mucus and epithelial barriers are the first physical and chemical lines of defense protecting the intestinal epithelium from pathogens and antigens. Mucus barrier disorders in the colon are also a possible cause of UC. Moreover, a recent study has shown that the disorders of mucus barrier were not related to local inflammation and immune response, but the response of goblet cells to microbial alterations (74). In addition, defects in the mucus barrier occur early in the onset of UC, further leads to increased intestinal permeability and exposure to antigens, thus activating abnormal immune responses (22, 23). This suggests that it was necessary to consider other risk factors. Notably, there is growing evidence that dysbiosis of intestinal microbiota is associated with disruption of the intestinal barrier, increased permeability, and increased antigen exposure.

Bacteria occupy the absolute advantage of intestinal microbiota, but fungi, viruses, and archaea are also important parts of the intestinal microbiota. Microbiota maintains a dynamic balance through complex interactions and connections, and the homeostasis is a key to maintaining host healthy. The composition of intestinal microbiota and the interactions between microorganisms are often altered by the risk factors such as genes, eating habits, unhealthy lifestyles, and drug abuse. For example, a long-term high-fat diet thins intestinal mucus, increases intestinal permeability, and impairs the tight junctions of intestinal epithelial cells, leading in intestinal barrier dysfunction (75). Additionally, the intestinal barrier function tends to deteriorate as the body matures (76). Increased intestinal permeability and breakdown of the intestinal barrier allow bacteria to readily pass past the epithelium and colonize the mucosa, hence amplifying their effect on the intestine (77). Although intestinal commensal microbiota contributes to the regulation of intestinal epithelial and immune function in the physiological state, risk factors such as diet, environment, and age can also result in dysbiosis of the intestinal microbiota, as revealed by decreased diversity and changes in specific microbial species and abundance (3). Inequality within each microbial community and changed interactions contribute to overall microbial homeostasis imbalance (78). Reduced probiotic bacteria and a rise in pathogenic and opportunistic pathogenic bacteria are significant aspects of microbial homeostasis imbalance in UC patients (79–81). The intestinal microbiota can directly interact with the immune system of the body (82). Certain *Clostridium* bacteria demonstrate pro-inflammatory properties by penetrating and mucosalizing the intestinal mucosa. Similarly, increasing *bifidobacteria* abundance has been shown to stimulate Th1 cell-mediated immune responses (83). Additionally, TLRs identification of bacterial and fungal surface components results in excessive activation of TLRs signaling pathways, triggering innate and adaptive immunity and chronic inflammation when the gut barrier is disrupted and pathogenic bacteria colonize. The metabolites generated by intestinal microbiota play a role in modulating host immune responses, such as SCFAs and bile acids, which are critical for sustaining anti-inflammatory effects and safeguarding intestinal barrier function (84, 85). Likewise, a decrease in the absolute abundance of SCFAs-producing bacteria is another unique symptom of microbial dysregulation in UC patients. Thus, the combination of reduced intestinal barrier function, microbial homeostasis imbalance, and lack of beneficial microbial products results in disruption of the intestinal epithelium and an abnormal immune response, which is a significant contributor to the development of UC.

The intestinal microbiota is a very complex and extensive system. Although the mechanisms of bacteria and fungi in the occurrence of UC are increasingly obvious, members such as viruses and archaea are also related to UC. Therefore, more in-depth studies are needed to explore the relationship between the intestinal microbiota and UC. At the same time, drugs and treatment methods focusing on restoring intestinal microbiota homeostasis may be the key to the treatment of UC.

COMMON ANIMAL MODELS OF UC

Establishing an appropriate animal model helps to study the pathogenesis of UC and investigate potential therapeutic drugs. UC animal models can be established with chemical drug induction, pathogen colonization, genetic engineering modification, and adoptive cell transfer (86). Notably, due to the limitations of modeling methods and prices, experimental colitis induced by the chemical drugs has become the most common animal model of UC (87). DSS, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and oxazolone (OXA) are three commonly used chemical drugs. Therefore, it is necessary to review the advantages and limitations of these three models to provide a scientific basis for the reasonable selection of UC animal models.

DSS

DSS is normally used to induce experimental colitis by dissolving it in water and provided ad libitum for several days to animals.

It is generally believed that DSS can induce acute UC in SD rats and Winstar rats at a concentration of 2–5% added to drinking water for 5–9 days. At the same time, adding 3–5% of DSS in drinking water for 5–8 days can induce acute UC in C57BL/6 mice and BALB/C mice (88). Multiple repeated drinking cycles are required if model is necessary to induce chronic UC (89). DSS can cause complete loss of intestinal epithelial cells to destroy the epithelial barrier. The lamina propria and submucosa are subsequently exposed to antigens and microbiota in the intestinal lumen, which eventually induced inflammation (90). During the administration of DSS, animals showed weight loss, thickening of stool, bloody stool and diarrhea *etc*. (91). Histopathological changes such as epithelial denuding, loss of mucin and goblet cells, submucosal edema, hyperemia and erosions, infiltration of inflammatory cells in the lamina propria and loss of crypt structures could be observed (92, 93).

The DSS-induced UC model has the characteristics of simple administration, easy control of drug dose and duration. Moreover, researchers can design acute or chronic experimental colitis by controlling the dose and duration of administration. Meanwhile, due to the induction of severe intestinal ulcer in animals, this model is also been interpreted as the animal model closest to human UC (94). Similarly, DSS-induced model also showed changes in the intestinal microbiota. The decrease in microbial diversity and abundance of beneficial symbiotic bacteria (*Lactobacillus* and *Alistipes*), and the increase of the abundance of pathogenic bacteria (*Oscillibacter*, *Streptococcus*, and *Escherichia–Shigella*) are similar to the changes of intestinal microbiota in UC patients (87, 95), which makes it also has advantages in studying the effects of drugs on the intestine microbiota. However, DSS-induced UC model also has obvious limitations. It is reported that DSS-induced inflammatory response began to ease naturally after 7 days, and the changes in the intestinal microbiota returned to normal levels after 21 days (87, 95). Besides, the sensitivity of certain types of mice to DSS is also different. After 5 days of acute administration, C57BL/6 mice induced colon inflammation, while BALB/C mice recovered simultaneously (94). Since UC itself is a chronic disease, DSS-induced model still lacks sufficient representation for UC.

TNBS

Rats were first used in TNBS-induced experimental colitis modeling. 5-30 mg TNBS into 0.25 ml of 50% ethanol, and the colon ulcer and inflammation in rats were successfully induced by intraluminal drip administration. The BALB/C and C57BL/6 mice are also used for colitis models by 0.3–5.0 mg of TNBS mixed with 50% ethanol (96). In TNBS-induced animal model, the use of ethanol is not only a solvent or carrier but also can destroy epithelial barrier of animals. TNBS is a hapten reagent that induces the immune response of T cells to tentacle proteins and luminal antigens through acute oxidative stress, resulting in intestinal wall necrosis (97). After administration of TNBS, animals showed bloody diarrhea and weight loss. Histopathological changes can be observed in intestinal wall thickening, edema, bleeding, and ulcer (98). Chronic TNBS colitis is manifested with diffuse necrosis of the intestinal wall, involving the mucosa, submucosa and muscle layer. At the same time, there are significant edema and immune cell infiltration in submucosa (99).

The TNBS-induced colitis model has the advantages of rapid disease development, localized colon injury and low cost. Compared with DSS-induced UC model, animal inflammation does not begin to relieve until the 15th day, which seems to be more conducive to researchers to ensure the accuracy of their research results (97). However, TNBS-induced animal model is associated with the immune response mediated by Th1 cells, which is considered closer to the immunological changes of CD. Moreover, the intestinal microbial changes in TNBS-induced mice showed a decrease in α-diversity, possibly beneficial bacteria decreased and harmful bacteria increased, similar to those in patients with Crohn's disease (100). Interestingly, some experts believe that it causes intestinal ulcer in animals to be similar to UC (101). Hence, it is still doubtful whether the TNBS-induced colitis model can represent human UC.

OXA

OXA is also a hapten reagent that needs to be administered with ethanol to induce colitis in animals (102). Before administration, 3% OXA (100–150 μl) was dissolved in 40–50% ethanol to sensitize the skin of mice and induce experimental colitis (103). The sensitivity of different strains of mice to OXA is also different. SJL/J mice and C57BL/10 mice are highly susceptible to OXA, but C57BL/10 mice are more resistant than SJL/J mice. BALB/C mice are more suitable for inducing chronic colitis (104).

The OXA-induced mice have symptoms of diarrhea, bloody stool, and weight loss. Histopathological changes are mainly characterized by epithelial cell loss, inflammatory cell infiltration, edema, and occasionally crypt abscess (105). As a kind of hapten reagent, OXA-induced colitis in mice is closely related to Th2 cell-related immune response, which is similar to human UC (91, 106). Compared with other animal models induced by chemical drugs, The OXA-induced model is more suitable for studying the pathology of UC. However, due to the high mortality rate of OXA, it is rarely applied in practical research (94, 105). In experimental animals, whether OXA can cause microbial disorders similar to human UC still needs more research.

The Role of Microbiota in Different UC Animal Models

As is well known, the application of drugs is the key to successful modeling of chemically induced animal models. However, intestinal microbiota has also been found to play a role in different animal models. A study found that during the induction of colonic inflammation in mice with DSS, an increase in the proportion of cells expressing CD11c and TLR4 in the mesenteric lymph nodes of mice was consistent with an increase in the number of *Enterobacteriaceae* and *Akkermansia* on the colonic mucosa, implying that the response of the immune system to microbial recognition and the induction of inflammatory responses (107). Similarly, in TNBS and OXA-induced inflammation models, the abundance of Sulfate-reducing bacteria, which produce hydrogen sulfide that damages the colon, causes epithelial damage and inflammation, and

TABLE 1 | The difference of three types of commonly used drugs in treating UC.

	5-ASA	Corticosteroids	Thiopurines
Applicable stage	Mild to moderate UC patients	Mild to moderate UC patients who are unresponsive or intolerant to 5-ASA treatment Moderate to severe UC patients	Steroid-dependent and Steroid-refractory UC patients
Mechanism of action	Acting on the colonic epithelium, exerting local mucosal anti-inflammatory effects	Inhibition of gene expression in the nucleus to suppress pro-inflammatory signaling pathway activation and limit immune cell translocation to sites of inflammation	As an immunosuppressant that inhibits inflammatory gene expression.
Advantages	Safety and Efficacy are proven	Available for UC patients who do not respond to 5-ASA therapy	Available as a maintenance treatment option for UC patients
Limitations	Some patients do not respond to 5-ASA treatment Not suitable for the treatment of moderate to severe UC patients	Long-term use will increase the risk of steroid dependence and steroid refractory	Obvious adverse events and serious potential side effects

5-ASA, 5-aminosalicylic acid; UC, ulcerative colitis.

induces a Th17 cell-associated immune response, was increased (108, 109).

All of these studies provide that microbiota is also vital in UC animal models. However, there are existed those different conclusions were from other experts. Gancarcikova et al. found the absence of microbiota did not affect the inflammatory effects of DSS when exposed antibiotic-treated germ-free mice to DSS (110). Another study found that in the absence of intestinal microbiota, the function of the intestinal barrier was also impaired although intestinal inflammation was significantly reduced in animals exposed to DSS (111). Obviously, the intestinal microbiota is a very complex organization, and different microorganisms play different roles. Therefore, further studies on the specific effects of microbiota in animal models are still necessary to resolve the current controversies, but this does not affect the use of animal models as a tool for UC research in subsequent studies.

Selection of Animal Models

The establishment of animal models is necessary to study the pathogenesis and pathological changes of UC. So far, the DSS-induced mice model has been most widely used. The intestinal microbiota of mice showed similar diversity to humans at the levels of *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. And the DSS-induced model is considered to be similar to the changes in the intestinal microbiota in UC patients. Therefore, DSS may be the current advantageous animal model for studying the relationship between intestinal microbiota and UC. However, the DSS-induced model may still be a limited result of obvious inflammation and rapid recovery of intestinal microbiota disorder, which still needs to be improved and developed.

Notably, many researchers have begun to use germ-free mice as an important tool to mimic the close relationship between intestinal microbiota and host (112). Studies ranged from single microorganisms to whole microbiota can be carried out in germ-free mice (23). For example, the intestinal bacteria highly coated with immunoglobulin A in IBD patients were isolated and cultured, and then transplanted into germ-free mice, which were eventually found to exhibit a high susceptibility to DSS-induced colitis (113).Consequently, germ-free mice may be a promising UC animal model, and the studies based on germ-free mice are also more conducive to revealing the specific link between intestinal microbiota and UC pathogenesis.

THE MEDICATION OF UC

The disease progression with outpatient medications can be managed successfully in most UC patients (114). The choice of specific medications should be evaluated according to the severity of disease, with 5-ASA preferred for patients with mild to moderate UC, and corticosteroids for patients who do not respond to or are intolerant of 5-ASA medications (1, 115). For patients with moderate to severe UC, systemic corticosteroids are the first-line induction therapy. Thiopurines are indicated for patients with steroid-refractory or steroid-dependent UC (116, 117). Moreover, current guidelines also recommend thiopurines as corticosteroid-free maintenance therapy (118). The mechanism of action, advantages and limitations of each of the three classes of drugs are different and compared (**Table 1**).

5-ASA

The use of 5-ASA dates back to 1941 when sulfasalazine (SASP) was used for the treatment of UC. SASP is composed of 5-ASA linked to sulfapyridine via a diazo bond, which can be cleaved by bacteria in the colon. After colonic bacterial azoreductase enzyme cleaves the diazo bond, 5-ASA achieves a high intraluminal concentration in the colon, makes it the active moiety of SASP (119, 120). To date, mesalazine and SASP are the main 5-ASA drugs used in the treatment of UC.

5-ASA acts on the colonic epithelium and exerts local mucosal anti-inflammatory effects by inhibiting cyclooxygenase and lipoxygenase, which subsequently leads to a decrease in prostaglandin and leukotriene production (121, 122). It has also been reported that 5-ASA inhibits the activation of the NF-κB signaling pathway, which promotes the transcription of pro-inflammatory cytokines and is an important mechanism in the

pathogenesis of UC (123). In addition, 5-ASA also inhibits the function of active lymphocytes, macrophages, and natural killer cells in the inflammatory process, which can scavenge the reactive oxygen metabolites (122). In conclusion, the local mucosal anti-inflammatory effect of 5-ASA is the main mechanism of action for treating UC.

Both American Gastroenterology Association and European Crohn's and Colitis Organization recommend 5-ASA as the first-line therapy for mild to moderate UC (116, 117). And the 5-ASA compound is a pillar for patients with mild to moderate ulcerative colitis, both as induction and maintenance therapy. Studies have shown that one-third of mild to moderate UC patients achieved clinical remission and half of the patients had mucosal healing after 8 weeks of oral 5-ASA treatment (124). Moreover, up to three-fifths of patients showed significant clinical remission and endoscopic improvement with 5-ASA maintenance therapy, which strongly supports the effectiveness of 5-ASA (125). In terms of specific drug use, there was no significant difference in efficacy between mesalazine and SASP, but patients with SASP often experienced adverse events (124, 125). However, 5-ASA still has a high safety profile compared to corticosteroids and thiopurines, and long-term 5-ASA therapy may have a preventive effect on colorectal cancer (126, 127). Notably, several studies have demonstrated that 5-ASA (including SASP and mesalazine) can restore the microbial diversity and the abundance of beneficial bacteria and fungi, reduce the abundance of pathogenic bacteria, and increase the production of SCFAs in experimental colitis and UC patients, which implying that 5-ASA also has the potential to modulate gut microbial homeostasis (80, 128, 129). Unfortunately, 5-ASA is not available for all UC patients. First, there are still some patients who do not respond to or are intolerant of 5-ASA therapy, and such patients usually dependent on corticosteroids. Second, for patients with moderate to severe UC, guidelines suggest that 5-ASA should not be used for induction or maintenance therapy (116, 117). Thus, corticosteroids, thiopurines, and even advanced therapies are in development after 5-ASA.

Corticosteroids

Corticosteroids are used in patients with mild to moderate UC who are unresponsive or intolerant to 5-ASA therapy. In addition, corticosteroids are the treatment of choice for patients with moderate to severe UC. The first use of corticosteroids in treating UC was reported in 1955, with the surgical resection rate of the colon was significantly lower in UC patients treated with cortisone than in the placebo group (130). After that, the first generation of corticosteroids such as prednisone and hydrocortisone began to be widely used in UC. Corticosteroids act in the cell nucleus and ultimately play a role in regulating the immune response by inhibiting gene expression during transcription, down-regulating the production of transcription factor NF-κB and the expression of pro-inflammatory cytokines, and causing a decrease in the expression of adhesion molecules to limit the transfer of immune-inflammatory cells to inflammatory areas (131–133).

Since the immunosuppressive effects of first-generation corticosteroids are non-specific, inevitably, other body parts besides the intestinal inflammatory sites are also affected. As a result, up to 90% of UC patients experienced adverse effects after corticosteroid treatment (134), which led to the creation and development of second-generation corticosteroids such as beclomethasone dipropionate and budesonide. Compared to first-generation corticosteroids, second-generation corticosteroids can target the site of inflammation to exert local anti-inflammatory effects, to potentially reduce systemic corticosteroid concentrations (135). Similarly, the use of second-generation corticosteroids has an overall better safety profile and a reduced incidence of adverse events (136). However, all corticosteroids are absorbed by the body to some extent, and this leads to a continued occurrence of adverse events. In a study evaluating the safety of budesonide MMX, a novel Multimatrix formulation of budesonide, adverse events were reported in 31.8% of patients (137). In conclusion, as hormonal drugs, the therapeutic risk of corticosteroids rises with increasing dose and duration of exposure (138). In addition, steroid dependence is an issue that has to be considered (139). Therefore, corticosteroids are not recommended as the first choice for maintenance therapy, and corticosteroid use should be tapered or discontinued after clinical remission.

Thiopurines

The first application of thiopurine for UC was in 1962 (140). To date, thiopurines are the most commonly used drug for maintenance treatment of UC after 5-ASA (141). Thiopurines are recommended for maintenance therapy without corticosteroids. In addition, the use of thiopurines is necessary for patients with moderate to severe UC who have developed steroid-refractory or steroid-dependent. Thiopurine analogs include azathioprine, mercaptopurine, and thioguanine, of which thioguanine is considered an atypical thiopurine drug, and is used only in those that have failed to respond to mercaptopurine and azathioprine in a few countries and regions (121). As a type of immunosuppressants, the mechanism of action of thiopurines may be the incorporation of their pharmacologically active metabolite, 6-thioguanine nucleotides, into DNA or RNA as false purine analogs, which lead to DNA damage, cell cycle arrest and apoptosis, and inhibition of nucleotide and protein synthesis (142). Ultimately, the expression of inflammatory genes can be inhibited (142).

Although thiopurines are thought to play a corticosteroid-sparing role in the treatment of steroid-dependent UC patients, it remains controversial in using thiopurines. Firstly, thiopurines have significant toxic effects, including bone marrow suppression, impaired red blood cell regeneration, and death in rare cases (143). Secondly, the use of thiopurines may increase the incidence of lymphoma and non-melanoma skin cancer in patients (144). Therefore, the treatment of thiopurines needs to be effective in a way that ensures a reduced risk of side effects, which often requires a rigorous evaluation by the physician.

Potential Treatment Strategies of UC

Undoubtedly, 5-ASA, corticosteroids, and thiopurines have controlled the course of UC, and even saved the lives of a large number of UC patients. But the limitations of these drugs are

also obvious and led to the search for more effective and safer drugs, which is an important reason for the rapid development of more advanced treatment strategies such as biologics and small molecules products. In addition, the microbial dysbiosis often occurs at the early stage in the occurrence of UC, suggesting that the therapeutic strategy based on restoring the microbial balance may be beneficial (145). Therefore, fecal microbiota transplantation (FMT), probiotics, prebiotics and synbiotics have become potential therapeutic approaches for UC and researched in fronter. It is also mentioned that TCM has potential advantages in the treatment of UC and should be paid more attention to (146).

FMT

FMT, originally used in the treatment of *Clostridium difficile* infection, is a method of regulating microbial dysbiosis by transplanting healthy donors' feces into patients (147). In 2013, the U.S. Food and Drug Administration officially approved FMT as a clinical treatment for recurrent or refractory *Clostridium difficile* infection (148). Moreover, studies have shown that its success rate is as high as 90%, which means that FMT can restore healthy microbial ecology (149). Therefore, FMT is extended to treat other microbial-related diseases, including UC.

To date, multiple randomized controlled trials (RCTs) have explored the efficacy and safety of FMT in the treatment of UC (**Table 2**). Among them, 5 RCTs showed that the clinical remission rate of FMT in the treatment of UC was significantly different from that of placebo or autologous fecal transplantation, suggesting that FMT has therapeutic effect on UC (150, 152–155). On the contrast, one study showed that there was no significant difference in the efficacy between FMT and autologous fecal transplantation (156). In addition, another study showed that in UC patients, FMT had worse clinical remission outcomes than 5-ASA, though both had the same clinical response rate (151). These studies seem inconsistent in evaluating the efficacy of FMT. It is worth noting that a meta-analysis involving four FMT-related RCTs finally found that the overall clinical remission rate of FMT group was 28%, which was significantly better than of placebo group, providing some evidence to support the effectiveness of FMT for UC (157). Regarding the different results obtained from these RCTs, it seems biased in relation to specific trials undertaken. For example, experts in a clinical trial found that only one female patient responded to fecal microbiota from male donors, implying that gender may have an impact on the specific efficacy of FMT (151). Second, due to the increased microbial diversity associated with multiple donors, the treatment with multiple donors is more effective than that of individual donor (154, 158). In addition, enema administration is more effective than naso-duodenal tube administration (154). In conclusion, the curative effect of FMT may be influenced by various factors, and more strict and standardized research is needed to explore more standardized strategies for FMT use.

In terms of safety evaluation, although 4 RCTs reported severe adverse reactions in some patients during treatment, there was no significant statistical difference between FMT and placebo groups (152, 154–156). Furthermore, according to the assessment from principal investigators, the occurrence of severe adverse reactions was not related to FMT treatment. It is worth noting that some experts pointed out that the risk of long-term use of FMT infection transmission is still unclear. In theory, the risk increases with the increase of multiple donors' infusions times, which indicates that the long-term safety of FMT still needs to be studied and evaluated (154).

In addition to adequate evaluating the efficacy and safety, it is also essential to study the effect of FMT interventions on the microbiota of UC patients. Studies have shown that both OUT diversity and Shannon diversity of intestinal microbiota in UC patients after FMT are enhanced (159). Moreover, the abundance of *Bifidobacteriaceae* and *Coriobacteriaceae* increased at the phylum level (160). *Bifidobacteriaceae* has been used as probiotics for the treatment of UC, and *Coriobacteriaceae* plays a significant role in the transformation of bile salts and steroids (161). Furthermore, as an important component of SCFAs, butyrate can regulate intestinal homeostasis. After FMT treatment, the abundance of butyrate-producing bacteria increased significantly and SCFAs recovered (162). In short, FMT can change the microbiota composition of patients, correct microbial dysbiosis, and ultimately exert therapeutic effects for UC by transplanting the microbiota from healthy people to patients. Nevertheless, some problems needed to solved to make FMT a regular treatment strategy for UC. Firstly, a large number of studies are needed to critically evaluate the clinical efficacy and safety of FMT for long-term use. Secondly, the selection of donor sources, whether gender factors and donor diversity need to be considered, and how to avoid the potential infection risks also need to be explored in subsequent studies. Furthermore, there is a lack of standard guidance on specific transplant modalities and treatment periods. In summary, it is undeniable that FMT is a promising treatment for UC. Focusing on FMT research will help its development and maturity.

Probiotics, Prebiotics and Synbiotics

Probiotics are active microorganisms that have beneficial effects on the host health at sufficient and accurate doses. A large number of functional foods containing probiotics are safe to eat and have some health effects, suggesting that probiotics are usually safe. At the same time, plenty of studies began to evaluate the therapeutic effects of probiotics on UC. So far, some probiotic species have been proved to have therapeutic potential for UC (**Table 3**). Among these probiotics, *Escherichia coli* Nissle 1917 and VSL # 3, a probiotic mixture containing *L. paracasei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii*, *B. longum*, *B. breve*, *B. infantis* and *Streptococcus thermophilus*, are two probiotic products that have been studied more. Studies have shown that VSL # 3 has therapeutic effect on patients with mild to moderate UC, whether for induction therapy or maintenance therapy (174). Moreover, VSL # 3 also has the effect of preventing disease progression (175). The European Society of Nutrition and Metabolism also recommended VSL # 3 and *Escherichia coli* Nissle 1917 as drug for the treatment of mild to moderate UC, suggesting that probiotics do have therapeutic potential for UC (176).

The mechanism of probiotics improving UC has not been determined, but different probiotics may play a role in different

TABLE 2 | The characteristics of some randomized controlled trials of FMT for treating UC.

Study	N (FMT/Control)	Control	Delivery	Primary end point	Efficacy	Safety
Crothers et al. (150)	12 (6/6)	Sham colonoscopic infusion and sham capsules	Initial colonoscopy then enema and oral maintenance therapy with frozen FMT Capsules.	A mayo score $\leq$ 2 and an endoscopic sub-score of $\leq$ 1 at week 12.	FMT group: two subjects, control group: none. (95% CI = 0.38-infinity, $p = 0.45$)	None of the subjects experienced FMT-associated adverse events.
Schierová et al. (151)	16 (8/8)	5-ASA	Enema	A Mayo score $\leq$ 2, with no subscore $>$ 1 at week 12.	FMT group: 37.5%, control group: 50.0% ($P = 0.51$)	No adverse events were reported during the treatment and 6 weeks after treatment.
Costello et al. (152)	73 (38/35)	Autologous FMT	Initial colonoscopy then enema	A total Mayo score of $\leq$2 (range, 0–12) with an endoscopic Mayo score of $\leq$1 (range, 0–3) at week 8.	FMT group: 32%, control group: 9% (OR, 5.0, 95% CI, 1.2–20.1, $P = 0.03$)	Three serious adverse events in the FMT group and two in the control group with no significant differences.
Sood et al. (153)	61 (31/30)	Saline	Colonoscopic infusion at weeks 0, 8, 16, 24, 32, 40 and 48.	A mayo score $\leq$2, all sub-scores $\leq$ 1 at week 48.	FMT group: 87.1%, control group: 66.7% (RR 2.2, 95% CI 1.1–4.5; $p = 0.021$)	There were no serious adverse events in FMT group.
Paramsothy et al. (154)	81 (41/40)	Isotonic saline	Initial colonoscopy then enema	A total Mayo score $\leq$ 2, with all Mayo subscores $\leq$ 1, and at least a 1-point reduction from baseline in the endoscopy subscore at week 8.	FMT group: 44%, control group: 20% (RR 2.2, 95% CI 1.1–4.5; $p = 0.021$)	Mild adverse events: 78% in the FMT group vs. 83% in the control group with no significant difference. Serious adverse events: two patients in the FMT group vs. one patient in the control group which is not associated with the individual donor or donor batch.
Moayyedi et al. (155)	75 (38/37)	Water	Initial colonoscopy then enema	A full Mayo score <3 and complete healing of the mucosa at flexible sigmoidoscopy at week 7.	FMT group: 24%, control group: 5% ($p = 0.3$).	No difference in serious adverse events between the FMT and placebo groups.
Rossen et al. (156)	48 (23/25)	Autologous FMT	Naso-duodenal tube	A SCCAI score of $\leq$2 in combination with $\geq$1 point improvement on the combined Mayo endoscopic score of the sigmoid and rectum at week 12.	FMT group: 30.4%, control group: 20.0% ($P = 0.51$)	Mild adverse events: 78.3% in the FMT group vs. 64.0% in the control group ($p = 0.28$) Serious adverse events: two patients in the FMT group vs. two patients in the control group.

FMT, fecal microbiota transplantation; SCCAI, Simple Clinical Colitis Activity Index.

TABLE 3 | Probiotics with proven therapeutic potential for UC.

Probiotic	Reference
Escherichia coli Nissle 1917	Kruis et al., Rembacken et al., Kruis et al., (163–165).
Saccharomyces boulardii	Guslandi et al., (166).
Bifidobacteria	Ishikawa et al., Kato et al., (167, 168).
Lactobacillus GG	Zocco et al., (169).
VSL # 3 (a probiotic mixture containing *L. paracasei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp *bulgaricus*, *B. longum*, *B. breve*, *B. infantis* and *Streptococcus thermophilus*	Sood et al., Miele et al., Tursi et al., (170, 171).
Lactobacillus reuteri ATCC 55730	Oliva et al., (172).
Symprove (a probiotic mixture containing *Lactobacillus rhamnosus* NCIMB 30174, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus acidophilus* NCIMB 30175 and *Enterococcus faecium* NCIMB 30176)	Bjarnason et al., (173).

ways. VSL # 3 inhibits the activation of NF-κB signaling pathway, which is a key factor in the development and persistence of chronic inflammation in UC (177). Another probiotic mixture, Symprove is thought to improve UC by restoring microbial homeostasis, increasing SCFAs production, and restoring epithelial tight junctions (178). In summary, the mechanism of action of probiotics depends on the strains used, and the specificity of some probiotic properties means that often multiple species of probiotics have better therapeutic effects than single probiotics (179). At the same time, a specific probiotic may not be suitable for all UC patients. Therefore, the evaluation of the selection and utilization of probiotics still need to be continued.

Probiotics have been widely studied and gradually applied. Prebiotics are substrates that host probiotic bacteria can selectively utilized and produce health benefits (180). Consequently, prebiotics are used to increase the abundance and activity of probiotics, prolong their lifespan, change the composition of intestinal microbiota, and improve the intestinal barrier function, which is also considered as a mechanism for prebiotics to treat UC (181, 182). Lactulose, inulin, fructooligosaccharide and malt are the most studied prebiotics (183). Although the number of RCTs evaluating the efficacy of prebiotics in the treatment of UC is limited, some preclinical studies and clinical trials have shown their therapeutic potential. Malt is effective for UC to alleviate clinical symptoms and reduce clinical activity index in UC patients (184). Lactulose can improve the quality of life of UC patients (185). Similarly, preclinical studies have found that after treatment with fructans and resveratrol, the abundance of probiotics *Bifidobactrium* and *Lactobacillus* in IBD model rats increase, and starch can induce beneficial changes in the microbiota composition by promoting butyrate production and inhibiting the growth of potentially harmful bacteria (186–188). This evidence supports that prebiotics have the potential to treat UC. However, more well-controlled and high-quality RCTs are urgently needed to further evaluate the therapeutic effects and safety of prebiotics.

Given that both probiotics and prebiotics may have therapeutic potential, the role of synbiotics is also being evaluated. Considering that the probiotic intake can help the host correct the dysbiosis of microbiota, the use of synbiotics, a mixture of probiotics and prebiotics, seems reasonable and prebiotics can also increase the abundance of probiotics and restore the intestinal microbiota (189). Studies have shown that the clinical efficacy of synbiotics in the treatment of UC is significant. The combined application of *bifidobacterial* strains and galactooligosaccharide improved the colonoscopy score and inflammatory markers in UC patients (190). Similarly, a comprehensive meta-analysis showed that synbiotics improved colonoscopy score, clinical activity index and inflammation-related indicators in UC patients (191). In addition, synbiotics can also increase the abundance of probiotics in the intestinal tract of UC patients, and these studies also provide evidence for the treatment of UC by synbiotics.

Since the microbiota is mainly concentrated in the colon, the therapeutic strategies aimed at restoring intestinal microbial homeostasis have a more obvious effect on the colonic microbiota (192). Therefore, probiotics, prebiotics, and synbiotics treatment have more therapeutic value for UC patients than CD patients. In addition, the therapeutic potential of probiotics, prebiotics and synbiotics cannot be ignored in the urgent search for more efficient and safer alternatives to UC. However, the evaluation of their effectiveness and safety is remain inadequate. First, the number of studies on probiotics, prebiotics and synbiotics seems to be decreasing in recent years (**Table 4**). Second, researchers do not always get positive reactions or conclusions in these RCTs. Some studies have shown that there is no significant different between the efficacy of probiotics and placebo. The underlying reasons may be related to the selection of specific probiotic strains, dosage and the duration of treatment, which also suggests that there is a lack of standard protocol for the use of probiotics. Fortunately, a meta-analysis showed that the use of probiotics increased the risk of side effects compared with placebo, but these symptoms were limited to gastrointestinal reactions and abdominal pain (192). Therefore, the use of probiotics seems to be safer than corticosteroids, thiopurines and other drugs. These drugs always produce severe adverse effects and requires strict follow-up RCTs to re-evaluation.

The Application of TCM in Treating UC

The Potential Effect of TCM on Intestinal Microbiota

Due to the complex active ingredients, unclear pharmacological effects, and low oral availability of Chinese medicinals, it is difficult to fully clarify its effective mechanism, which is a great challenge to TCM. However, with intestinal microbiota becoming an emerging field to understand the occurrence and development of diseases in recent years, researchers begin to pay attention to TCM. On this basis, it is found that the pharmacological effects of TCM are related to the intestinal microbiota (12).

Most medicinals are oral. Low oral availability compounds from medicinals can reach the colon, which is the most concentrated part of the intestinal microbiota (197). Medicinals will inevitably be exposed to intestinal microbiota, providing the necessary conditions to affect the intestinal microbiota (198).

TABLE 4 | The characteristics of some clinical trials of probiotics, prebiotics and synbiotics for treating UC.

Study	N (Treatment/ Control)	Treatment	Species	Control	Efficacy	Safety
Chen et al. (193)	25 (12/13)	Probiotic	A probiotic product that contained *L. casei* Zhang, *L. plantarum P*-8 and *B. animalis subsp. lactis* V9	Dextrin	The overall remission rate was 91.67% for the probiotic group vs. 69.23% for the placebo group ($P = 0.034$)	-
Bjarnason et al. (173)	81 (40/41)	Probiotic	Symprove (contains *Lactobacillus rhamnosus* NCIMB 30174, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus acidophilus* NCIMB 30175 and *Enterococcus faecium* NCIMB 30176	Water and flavoring	The calprotectin levels were significantly decreased following 4 weeks in the probiotic group ($p = 0.011$ and 0.001, *t*-test and Wilcoxon's, respectively)	-
Yilmaz et al. (194)	25 (15/10)	Probiotic	Kefir (*Lactobacillus* Bacteria)	-	No statistically significant difference was found between weeks 1 and 2 in patients with UC in terms of abdominal pain, bloating, frequency of stools, defecation consistency, and feeling good.	No adverse events were reported.
Kamarli et al. (183)	36 (18/18)	Synbiotic	A symbiotic which concluded six probiotics: *Enterococcus faecium*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Bifidobacterium longum* and fructooligosaccharide.	Placebo product which has the same taste and appearance	The change in the CRP and sedimentation values had a statistically significant decrease in the synbiotic group ($P = 0.003$). The improvement in the clinical activity was significantly higher in the synbiotic group ($p < 0.05$).	-
Yoshimatsu et al. (195)	46 (23/23)	Probiotic	A tablet contains *Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A and *Bacillus mesentericus* TO-A	A placebo tablet which contains starch	The relapse rates in the treatment and placebo groups were 0.0% vs. 17.4% at months ($p = 0.036$). At 12 months, the remission rate was 69.5% in the treatment group and 56.6% in the placebo group ($p = 0.248$).	-
Matsuoka et al. (196)	192 (97/95)	Probiotic	Mil–Mil (a fermented milk product containing *B. breve* strain Yakult and *Lactobacillus acidophilus*	-	Relapse-free survival was not significantly different between the treatment and placebo groups ($P = 0.643$)	Three mild adverse events occurred which could not be ruled out whether is associated with the probiotic.

After TCM compounds enter the intestinal tract, the composition and metabolism of the intestinal microbiota are regulated directly and indirectly (12).

The intestinal microbiota participates in the transformation of TCM compounds by expressing corresponding biological enzymes to activate, inactivate, or reactivate TCM compounds (199). Berberine is the primary pharmacological component of *huáng lián* (the dried rhizome of *Coptis chinensis* Franch.), with low oral availability and maximum blood concentration in human body. Interestingly, the berberine ethanol extracts significantly reduced the abundance of *Firmicutes* and *Bacteroides* in the feces of HFD mice (200). Meanwhile, the diversity and total number of intestinal microbiotas treated with berberine were also significantly reduced (201). The use of TCM is often not only an herb blindly but also a combination of medicinals with different effects through compatibility. *Huáng Qín Tāng* (Scutellaria Decoction), widely used in the treatment of gastrointestinal diseases, has been found that its improvement effect on UC is related to intestinal microbiota regulation (202). In short, some medicinals with low oral availability can exert sound effects in the treatment of various diseases, which is closely related to intestinal microbiota.

The Preclinical Study of Application TCM in Animal Models of UC

TCM is characterized by abundant resources and clinical safety. Numbers of researchers have invested in TCM for treating UC and made some progress (9, 10). The research on animal experiments is essential in discovering suitable therapeutic drugs and studying their possible mechanisms. Consequently, recent animal studies have shown that TCM may have a potential to be applied to UC patients. According to the different TCM selected by the researchers in animal experiments, we categorized the investigated medicinals into three parts: compound extracted from Chinese medicinals and single medicinal, couplet medicinal, and Chinese medicinal formula.

Compound Extracted From Chinese Medicinals and Single Medicinal. Although a single medicinal is rarely used in clinical practice, relative studies can identify its unilateral effectiveness in UC. The selection of specific medicinals, animal models, and study results of these experiments are shown in **Table 5**. Under the guidance of TCM theory, the particular efficacy of TCM and pharmacological research will become the essential reference for researchers to choose TCM. On this basis, choosing appropriate TCM for animal experiments is more likely to find medicinals with obvious therapeutic effects on UC.

Qīng dài (the dried processed product of leaf or stem and leaf of *Strobilanthes cusia* (Nees) Kuntze), *qiàn cǎo* (the dried root and rhizome of *Rubia cordifolia* L.) and *huáng qín* (the dried root of *Scutellaria baicalensis* Georgi) have the effect of clearing heat, and its pharmacological effects with anti-inflammatory and antibacterial have also been proved (210, 211). In animal experiments, it was found that these medicinals have an improvement effect on the general symptoms such as diarrhea, bloody stool of animals and colon inflammation in experimental colitis. At the same time, the disease activity index (DAI) score and histopathological score also showed a downward trend.

The release of pro-inflammatory cytokines plays an essential role during the development of UC (212). Compared with the model group, the expressions of pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-18, TNF-α and interferon-gamma (IFN-γ) decreased obviously after administration. The levels of IL-4 and IL-10, considered to be anti-inflammatory cytokines, increased after administration. The use of medicinals also reduced the levels of pro-inflammatory cytokines in animals to alleviate experimental colitis.

Abnormal activation of signaling pathways is often the key to induce inflammation. The activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways have been found to be important factors leading to the occurrence and development of UC (213). Similarly, medicinals also inhibit the signaling pathways such as NF-κB and MAPK in animal experiments. The phosphorylation of protein kinase B (Akt) was increased in mice after the induction of DSS, while *dà xuè téng* (the dried stem of *Sargentodoxa cuneata* (Oliv.) Rehder & E.H.Wilson) reversed this alteration (10).

Chuān xīn lián (the dried above-ground part of *Andrographis paniculata* (Burm.f.) Nees) not only down-regulates the expression of NF-κB p65 and p-IκBα, inhibits the activation of NF-κB, but also down-regulates the phosphorylation of MAPK subfamily-related kinases (extracellular signal-regulated kinase, p38mapk, and c-Jun amino-terminal kinase), thereby inhibiting the activation of MAPK signaling pathway to alleviate DSS-induced UC (206). Besides, the application of *qīng dài* (203, 204), *huáng qín* (205), and *shí hú* (the fresh or dried stem of *Dendrobium nobile* Lindl.) (209) also showed an inhibitory effect on the NF-κB signaling pathway. Briefly, the inhibition of key signaling pathways can improve or relieve UC, which also offers a theoretical basis for the efficacy of TCM.

The pharmacological effects of medicinals in the human body lie in their interaction with the intestinal microbiota. The abnormal host immune response caused by the dysbiosis of intestinal microbiota is considered to be the critical mechanism for UC (46), so the essential role of intestinal microbiota in the pathogenesis of UC has been attracted more and more attention (214). Of course, we also found that some scholars have focused their researches on intestinal microbiota with TCM in treating UC. DSS-induced rats have a dysbiosis of the intestinal microbiota, including a decrease in the diversity of the microbiota, a reduction in the abundance of *Firmicutes,* and an increase in the abundance of *Bacteroidetes*. After the treatment of *qing dài*, the diversity of the rat's bacteria and the balance between the two microbiotas were restored. It is considered that *qing dài* modulates its immune response by changing the intestinal microbiota, finally reduce DSS-induced colitis (203).

Many animal experiments conducted on a single medicinal show that TCM can treat experimental colitis by inhibiting the activation of related inflammatory signal pathways and the release of pro-inflammatory cytokines. Besides, some researchers have noticed that it has a therapeutic effect on experimental colitis by improving the intestinal microbiota, indicating that the potential of TCM in the treatment of UC has been proved.

TABLE 5 | Application of compound extracted from Chinese medicinal (or single medicinals) in animal models of UC.

Medicinals	Compounds	Animals	Experimental methods	Results (symptoms, cytokines and pathways)	Results(Intestinal Microbiota)	References
qīng dài (the dried processed product of leaf or stem and leaf of *Strobilanthes cusia* (Nees) Kuntze)	Indirubin Indigo	BALB/c mice SD rats	DSS	It inhibited the loss of bodyweight, reversed the elevation of DAI store, alleviated crypt distortion and mucosal injury, and reduced inflammatory cell infiltration in the colon mucosa. TNF-α, IFN-γ, IL-2, MPO were decreased. IL-4, IL-10 were increased. Few CD4+ T cells were observed in colon tissues. The activation of NF-κB signaling was inhibited.	α-diversity was increased. At the phylum level, *Firmicutes* and *Actinobacteria* were increased, *Bacteroidetes* was decreased. At the family level, the abundance of *bifidobacteriaceae* and *Ruminococcaceae* was increased.	(203, 204)
huáng qín (the dried root of *Scutellaria baicalensis* Georgi)	Oroxindin	C57BL/6 mice	DSS	Oroxindin suppressed massive macrophages infiltration and attenuated pathological changes in colonic tissue. The expression of IL-1β, IL-18, caspase-1 and p-p65 were decreased, it suggested that Oroxindin inhibited NLRP3 inflammasome formation and NF-κB activation.	-	(205)
dà xuè téng (the dried vine stems of *Sargentodoxa cuneata* (Oliv.) Rehder & E.H.Wilson)	Liriodendrin	BALB/c mice	DSS	Liriodendrin improved DAI, colon length and histological damage in colon of mice. MPO, IL-6, TNF-α, and IL-1β were reduced. It also suppressed the activation of Akt and NF-κB pathways and up-regulated the expression of Erβ.	-	(10)
chuān xin lián (the dried above-ground part of *Andrographis paniculata* (Burm.f.) Nees)	3,14,19-triacetyl andrographolide	BALB/c mice	DSS	It reduced body weight loss, colon length shortening, colon weight, the spleen index, and DAI store, and alleviated histological damage in the colon. MPO, TNF-α, and IL-6 were decreased. It could inhibit the activation of NF-κB and MAPK pathways.	-	(206)
qiàn cǎo (the dried root and rhizome of *Rubia cordifolia* L.)	Mollugin	C57BL/6 mice	DSS	Mollugin decreased the DAI scores and histological score. IL-1β and TNF-α were decreased. The level of TLR4 was decreased.	-	(207)
mù xiāng (the dried root of *Aucklandia costus* Falc.)	-	SD rats	TNBS	*Mù xiāng* ameliorated stomachache, diarrhea and hematochezia of rats. Body weight was increased. IL-1β, TNF-α, caspase-3, BAD were decreased. IL-6, IL-10, p53 and Bcl-2 were upregulated. The activity level of PI3K and Akt was increased.	-	(208)
shí hú (the fresh or dried stem of *Dendrobium nobile* Lindl.)	Polysaccharides	BALB/C mice	DSS	Polysaccharides of *shí hú* could improve clinical signs and symptoms, decrease mortality, alleviate colonic pathological damage. IL-1β, IL-6, IL-18, TNF-α, and IFN-γ were decreased. IL-10 was increased. It could also suppress the activation of NLRP3 inflammasome and β-arrestin1.	-	(209)

DAI, disease activity index; TNF-α, tumor necrosis factor-α; IL, interleukin; MPO, myeloperoxidase; NF-κB, nuclear factor κB; Akt, protein kinase B; TLR, Toll-like receptor; Bcl-2, apoptosis regulator Bcl-2; BAD, Bcl2-associated agonist of cell death; PI3K, phosphatidylinositol 3-kinase; IFN-γ, interferon-gamma.

Couplet Medicinal. Couplet medicinal is a combination of two medicinal that enhances curative effect or reduces toxicity according to the principle of TCM treatment. It is also the basic unit of Chinese medicinal formula (215). Researches on couplet medicinals help to dispel the interactions between medicinals and explain the mechanism behind them. The corresponding couplet medicinals are shown in **Table 6**.

Huáng qín and *huáng lián* is a combination commonly used in the treatment of intestinal diseases. Baicalin and berberine are two main chemical constituents extracted from these medicinals. And the baicalin-berberine complex was found in *Huáng qín-huáng lián* decoction (218). After administration of baicalin-berberine hybrid compound to DSS-induced mice, the myeloperoxidase (MPO) activity in colon tissue and the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were inhibited, and the development of inflammation was prevented. Besides, the therapeutic effect of baicalin-berberine complex was significantly better than that of baicalin and berberine (215).

Another couplet medicinal, *dǎng shēn* (the dried root of *Codonopsis pilosula* (Franch.) Nannf.) and *huáng qí* (the dired root of *Astragalus mongholicus* Bunge), is also commonly used in the Chinese medicinal formula for treating UC. The polysaccharides in this combination improved the symptoms of experimental colitis mice and alleviated the intestinal mucosa injury. At the same time, the diversity of intestinal microbiota recovered with the increase of *Bacteroidetes* abundance as well as the decrease of *Firmicutes* and *Proteobacteria* (216). Moreover, the therapeutic effects of the polysaccharides of *dǎng shēn* and *huáng qí* on UC are better than that of a single polysaccharide.

In addition to enhancing the efficacy of medicinals, the couplet medicinals can also reduce the toxicity of TCM. *fù zi* (the processed lateral root of *Aconitum carmichaelii* Debeaux) and *gān jiāng* (the dried rhizome of *Zingiber officinale* Roscoe) are commonly used to treat diarrhea (219). However, hypaconitine, mesaconitine, and aconitine are the main toxic chemical composition in *fù zi* (220). After the co-use of *fù zi* and *gān jiāng*, the dissolution of hypaconitine and mesaconitine significantly reduced, indicating that the compatibility is beneficial to reduce the toxicity of *fù zi*. Meanwhile, the combination can reduce the release of pro-inflammatory cytokines by inhibiting the MAPK, NF-κB, and signal transducer and activator of transcription 3 (STAT3) signaling pathways in the DSS-induced colitis (217).

The use of couplet medicinals can often improve the efficacy of TCM for experimental colitis. Besides, the reasonable compatibility of some toxic medicinals can also reduce its harmful effects, which also provides strong evidence for the treatment of UC by TCM.

Chinese Medicinal Formula. Under the guidance of the TCM theory, the Chinese medicinal formula is to combine different single medicinal or couplet medicinals into a complete formula with corresponding effects (221). The outstanding multi-target synergistic effect of Chinese medicinal formula provides a certain basis for its rationality (222). The selection of specific formulas and study results are shown in **Table 7**. Each Chinese medicinal formula usually comprises different medicinals, but we found that *huáng qín*, *huáng lián*, *huáng qí, dǎng shēn* and *qīng dài* were widely used in these selected formulas in animal experiments. In the DSS-induced colitis in rats or mice, the general symptoms of the animals were improved compared with the model groups. The DAI score and histopathological score were decreased. Meanwhile, the decreased expression of various pro-inflammatory cytokines also suggests the effect of Chinese medicinal formulas in reducing UC.

The diversity of the constituent medicinals of a Chinese medicinal formula determines the difference in its mechanism of action. *Bàn Xià Xiè Xīn Tang* (Pinellia Heart-Draining Decoction) (221), *Shēn Líng Bái Zhú Sǎn* (Ginseng, Poria and Atractylodes Macrocephalae Powder) (224), *Jiàn Pí Qīng Cháng Tāng* (Spleen-Fortifying and Intestine-Clearing Decoction) (222), *Sān Huáng Shú Ài Tāng* (Scutellaria, Coptis, Phellodendron bark and Mugwort Decoction) (228) and *Huáng Lián Jiě Dú Tāng* (Coptis Toxin-Resolving Decoction) (229) can inhibit the signal of NF-κB Pathways to reduce colon inflammation. *Qīng Cháng Wēn Zhong Tāng* (Intestine-Clearing and Center-WarmingDecoction) is related to the inflammation mediated by the interferon gamma-induced protein 10(IP10)/Chemokine (cys-x-cys motif) receptor 3 (CXCR3) axis (208). Moreover, *Suqīng Wán* is thought to be involved in the up-regulation of anti-inflammatory cytokines and down-regulation of pro-inflammatory and oxidative factors (230).

The effect of Chinese medicinal formula on intestinal microbiota has also been confirmed. The microbial dysbiosis in UC patients includes the decreased diversity and microbiota composition disorder (231). *Dà Huáng Mǔ Dān Tāng* (Rhubarb and peony bark Decoction) (223), *Píng Wèi Sǎn* (Stomach-Calming Powder) (225), *Qing Cháng Huà Shī Tāng* (Intestine-Clearing and Dampness-Removing Decoction) (226), *Sān Huáng Shú Ài Tāng* (228) and *Bā wèi Xī lèi Sǎn* (232) all could restore the diversity of the intestinal microbiota in DSS-treated mice. Meanwhile, it was found that the intestinal bacteria in DSS-treated mice also showed a recovery after administration: the abundance of *Firmicutes* increased, the amount of *Bacteroides* and *Proteobacteria* decreased, and the amount of *lactobacillus* increased.

Intestinal bacteria are responsible for the biotransformation of bile acids and the production of SCFAs in the intestinal lumen. The dysbiosis of intestinal bacteria will be secondary to the imbalance of the microbiota metabolites and products in the development of UC (5). Interestingly, *Qīng Cháng Huà Shi Tāng* restores the balance between BAs metabolism and SCFAs production (226).

Single medicinal, couplet medicinal and Chinese medicinal formula all can play an obvious therapeutic effect on UC through animal experiments, which proves the effectiveness of TCM. The inhibition of inflammatory signaling pathways, the reduction of pro-inflammatory cytokines expression, and the restoration of intestinal microbiota disorders also indicate the critical mechanism of TCM in treating UC.

The Clinical Application of TCM in Treating UC

The most common clinical manifestations of UC are mucus, bloody stool, and diarrhea (1), which correspond to diseases such

TABLE 6 | Application of couplet medicinals in animal models of UC.

Medicinals	Compounds	Animals	Experimental methods	Results (symptoms, cytokines and pathways)	Results(Intestinal Microbiota)	References
huáng qín (the dried root of *Scutellaria baicalensis* Georgi) and *huáng lián* (the dried rhizome of *Coptis chinensis* Franch.)	Baicalin and berberine hybrid compound	BALB/c mice	DSS	It ameliorated the disease symptoms and prevented the colon damage of mice. Expression of MPO, IL-1β, TNF-α and IL-6 were decreased. Level of SOD was increased.	-	(215)
dǎng shēn (the dried root of *Codonopsis pilosula* (Franch.) Nannf.) and *huáng qí* (the dired root of *Astragalus mongholicus* Bunge)	Total polysaccharides of *huáng qí* and total polysaccharides of *dǎng shēn*	C57BL/6 mice	DSS	It alleviated weight loss and DAI score of mice. The level of MLN coefficient, MDA, IL-1β, TNF-α, IL-6 were decreased. In contract, SOD, IL-10, IL-22 were increased.	The level of *Firmicutes* and *Proteobacteria* was down-regulated. *Bacteroidetes* was up-regulated. Also, production of butyrate and overall microbiota structure were improved.	(216)
fù zi (the processed lateral root of *Aconitum carmichaelii* Debeaux) and *gān jiāng* (the dried rhizome of *Zingiber officinale* Roscoe)	-	C57BL/6 mice	DSS	*Fù zi* and *gān jiāng* significantly ameliorated the clinical symptoms of body weight loss, colonic shortening, increased DAI and splenomegaly, as well as histological scores of UC mice. IFN-γ, TNF-α, IL-1β, IL-6, IL-10 and IL-17A were suppressed. The levels of MPO, iNOS and COX-2 mRNA were suppressed too. The activation of MAPK, NF-κB and STAT3 signaling pathways were inhibited.	-	(217)

MPO, myeloperoxidase; IL, interleukin; TNF-α, tumor necrosis factor-α; SOD, superoxide dismutase; MLN, mesenteric lymph node; DAI, disease activity index; IFN-γ, interferon-gamma; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB.

TABLE 7 | Application of Chinese medicinal formulas in animal models of UC.

Formulas	Medicinals	Animals	Experimental methods	Results (symptoms, cytokines and pathways)	Results(Intestinal Microbiota)	References
Bàn Xià Xiè Xīn Tang (Pinellia Heart-Draining Decoction)	*bàn xià* (the dried tuber of *Pinellia ternata* (Thunb.) Makino), *huáng qín* (the dried root of *Scutellaria baicalensis* Georgi), *huáng lián* (the dried rhizome of *Coptis chinensis* Franch.), *gān jiāng* (the dried rhizome of *Zingiber officinale* Roscoe), *rén shēn* (the dried root and rhizome of *Panax ginseng* C.A.Mey.), *gān cǎo* (the dried root and rhizome of *Glycyrrhiza uralensis* Fisch. ex DC.), *dà zǎo* (the dried ripe fruit of *Ziziphus jujuba* Mill.)	C57BL/6 mice	DSS	It ameliorates body weight loss, DAI and histology score. TNF-α, IL-1β, IL-17, IL-23, COX-2, p-p65, MPO and 8-Oxoguanine were decreased. IL-10, SOD activity and Nrf2 expression were elevated.	-	(221)
Dà Huáng Mǔ Dān Tāng (Rhubarb and peony bark Decoction)	*dà huáng* (the dried root and rhizome of *Rheum palmatum* L.), *mǔ dān pí* (the dried velamen of *Paeonia* × *suffruticosa* Andrews), *táo rén* (the dried ripe seed of *Prunus persica* (L.) Batsch), *máng xiāo* (Natrii Sulfas), *dōng guā zi* (the dried ripe seed of *Benincasa hispida* (Thunb.) Cogn.)	C57BL/6 mice	DSS	*Dà Huáng Mu Dān Tāng* rescued the inflammation-related reduction of colon length, ameliorated body weight loss and damaged tissue of mice. The level of IL-6, TNF-α, IFN-γ, IL-10, IL-17A, IL-21, IL-22 in colon was decreased. The Th17/Treg balance was restored.	α-diversity of gut microbiota was restored. Abundance of *Firmicutes* and *Actinobacteria* was increased. *Proteobacteria* was decreased. The content of SCFA in intestinal tract was restored.	(223)
Shēn Líng Bái Zhú Sǎn (Ginseng, Poria and Atractylodes Macrocephalae Powder)	*rén shēn*, *fú líng* (the dried sclerotia of *Poria cocos* (Schw.) Wolf), *bái zhú* (the dried rhizome of *Atractylodes macrocephala* Koidz.), *shān yào* (the dried rhizome of *Dioscorea oppositifolia* L.), *bái biǎn dòu* (the dried ripe seed of *Lablab purpureus subsp. purpureus)*, *lián zi* (the dried ripe seed of *Nelumbo nucifera* Gaertn.), *yì yi rén* (the dried ripe seed kernel of *Coix lacryma-jobi var. ma-yuen* (Rom.Caill.) Stapf), *shā rén* (the dried ripe fruit of *Wurfbainia villosa* (Lour.) Skornick. & A.D.Poulsen), *jié gěng* (the dried root of *Platycodon grandiflorus* (Jacq.) A.DC.), *gān cǎo*	C57BL/6 mice	DSS	It could increase body weight and colon length of UC mice, decrease the DAI score and improve colonic injury. The production of IL-1β, IL-18, and TNF-α was decreased. It also inhibited the MAPK and NF-kB signaling pathways.	-	(224)

(Continued)

TABLE 7 | Continued

Formulas	Medicinals	Animals	Experimental methods	Results (symptoms, cytokines and pathways)	Results(Intestinal Microbiota)	References
Jiàn Pí Qīng Cháng Tāng (Spleen-Fortifying and Intestine-Clearing Decoction)	*huáng qí* (the dired root of *Astragalus mongholicus* Bunge), *dǎng shēn* (the dried root of *Codonopsis pilosula* (Franch.) Nannf.), *mǎ chǐ xiàn* (the dried above-ground part of *Portulaca oleracea* L.), *dì yú* (the dried root of *Sanguisorba officinalis* L.), *sān qi* (the dried root and rhizome of *Panax notoginseng* (Burkill) F.H.Chen), *bái jí* (the dried tuber of *Bletilla striata* (Thunb.) Rchb.f.), *mù xiāng* (the dried root of *Aucklandia costus* Falc.), *huáng lián*, *gān cǎo*	C57BL/6 mice	DSS	*Jiàn Pí Qīng Cháng Tāng* increased body weight and colon length of UC mice and decreased DAI score. TNF-α and IL-1β were decreased. It could inhibit the NF-κB/HIF-1α signaling pathway.	-	(222)
Píng Wèi Sǎn (Stomach-Calming Powder)	*cāng zhú* (the dried rhizome of *Atractylodes lancea* (Thunb.) DC.), *hòu pò* (the dried bark of *Magnolia officinalis* Rehder & E.H.Wilson), *chén pí* (the dried ripe pericarp of *Citrus reticulata* Blanco), *gān cǎo*, *shēng jiāng* (the fresh rhizome of *Zingiber officinale* Roscoe), *dà zǎo*	C57BL/6 mice	DSS	*Píng Wèi Sǎn* improved body weight, bloody feces and diarrhea of UC mice. MPO, IL-17A and IFN-γ mRNA levels were decreased.	The abundance of microbiota in mice was restored. In the phylum level, *Firmicutes* was increased and *Bacteroidetes* was decreased. Besides, the abundance of *Lactobacillus* was restored. The level of LPS in serum was reduced.	(225)
Qīng Cháng Huà Shi Tāng (Intestine-Clearing and Dampness-Removing Decoction)	*huáng qí*, *bái sháo* (the dried root of *Paeonia lactiflora* Pall.), *bái tóu wēng* (the dried root of *Pulsatilla chinensis* (Bunge) Regel), *bái zhǐ* (the dried root of *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav.), *huáng qín*, *dì yú*	C57BL/6 mice	DSS	It could improve weight loss, diarrhea, and rectal bleeding of DSS-treated mice. Expression of IL-1β, IL-6, TNF-α, NLRP3, caspase1, and IL-18 were decreased, Muc2 and Reg3γ were increased.	α-diversity of intestinal microbiota was improved. The level of *Firmicutes* was increased. In contrast, the level of *Bacteroidetes* was decreased.	(226)
Qīng Cháng Wēn Zhōng Tāng (Intestine-Clearing and Center-Warming Decoction)	*huáng lián*, *páo jiāng* (the processed product of root of Zingiber officinale Roscoe), *kǔ shēn* (the dried root of *Sophora flavescens* Aiton), *qing dài*, *dìyútàn* (the processed product of root of *Sanguisorba officinalis* L.), *mù xiāng*, *sān qī*, *gān cǎo*	SD rats	DSS	*Qīng Cháng Wēn Zhōng Tāng* improved weight loss, DAI score, and histological score of DSS-induced rats. Expression of IP10, CXCR3 and NF-κB p65 were decreased.	-	(227)

(Continued)

TABLE 7 | Continued

Formulas	Medicinals	Animals	Experimental methods	Results (symptoms, cytokines and pathways)	Results(Intestinal Microbiota)	References
Sān Huáng Shú Ài Tāng (Scutellaria, Coptis, phellodendron bark and Mugwort Decoction)	*huáng lián*, *huáng qín*, *huáng bǎi* (the dried bark of *Phellodendron chinense* C.K.Schneid.), *ài yè (*the dried leaf of *Artemisia argyi* H.Lév. & Vaniot)	BALB/C mice	DSS	*Sān Huáng Shú Ài Tāng* restored weight loss, colon length of UC mice. DAI score wad decreased. IL-1β, IL-6, TNF-α, P-p65, MPO and MDA were reduced.	Species of intestinal flora were restored. The abundance of *Lactobacillus sp*. was increased.	(228)
Huáng Lián Jiě Dú Tāng (Coptis Toxin-Resolving Decoction)	*huáng lián*, *huáng qín*, *huáng bǎi*, *zhi zi* (the dried ripe fruit of *Gardenia jasminoides* J.Ellis)	BABL/c mice	DSS	*Huáng Lián Jiě Dú Tāng* decreased DAI score, inhibited weight loss and colon shortening of UC mice. IL-1β and TNF-α were reduced, IL-10 was increased. It could suppress NF-κB signaling pathway, activating Nrf2 signaling pathway, and enhancing intestinal barrier function.	-	(229)
Sūqīng Wán	*jin yín huā* (the dried bud of flower of *Lonicera japonica* Thunb.), *lián qiào* (the dried fruit of *Forsythia suspensa* (Thunb.) Vahl), *pú gōng yīng* (the dried whole grass of *Taraxacum mongolicum* Hand. -Mazz.), *dì yú*, *tiān huā fen* (the dried root of *Trichosanthes kirilowii* Maxim.), *bái zhǐ*, *shēng dì* (the fresh or dried tuberous root of *Rehmannia glutinosa* (Gaertn.) DC.), *shēng má* (the dried rhizome of *Actaea cimicifuga* L.), *huáng qí*, *dāng guī* (the dried root of *Angelica sinensis* (Oliv.) Diels), *ji nèi jīn* (Endothelium Corneum Gigeriae Galli), *xuán shēn* (the dried root of *Scrophularia ningpoensis* Hemsl.), *gān cǎo*	Kunming mice	DSS	*Sūqīng Wán* decreased DAI score and attenuated symptoms of UC mice. TNF-α, IL-1β, IL-6, MPO, and MDA were reduced.IL-4 and IL-10 were increased.	-	(230)
Bā wèi Xi lèi Săn	*xi guā shuāng* (Mirabilitum Praeparatum), *hán shuǐ shí* (Glauberitum), *niú huáng* (Calculus Bovis), *zhēn zhū* (Margarita), *péng shā* (Borax), *bing piàn* (Borneolum Syntheticum), *náo shā* (Sal Ammoniacum), *qīng dài*	C57BL/6 mice	DSS	*Bā wèi Xi lèi Săn* improved body weight loss and colon length of DSS-treated mice. The expression level of Th17-related cytokines IL-17A/F and IL-22 was significantly reduced, resulting in the restoration of Th17/Treg balance.	The level of *Lactobacillus* was improved.	(46)

DAI, disease activity index; TNF-α, tumor necrosis factor-α; IL, interleukin; COX-2, cyclooxygenase-2; MPO, myeloperoxidase. SOD, superoxide dismutase; Nrf2, Nuclear factor E2-related factor 2; IFN-γ, interferon-gamma; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; HIF-1α, Hypoxia-inducible factor-1α; Reg3γ, regenerating gene 3γ; IP10, Interferon gamma-induced protein 10; CXCR3, Chemokine (cys-x-cys motif) receptor 3; MDA, malondialdehyde.

as "dysentery," "chronic dysentery," and "intestinal diarrhea" in TCM (233). According to the consensus opinion of the TCM diagnosis and treatment of UC issued by the Spleen and Stomach Diseases Branch of the China Association of Chinese Medicine in 2017, it is believed that TCM should diagnose and treat UC with "chronic dysentery" because that UC also has the characteristics of recurring attacks and difficult to be cured.

According to the principle of pattern differentiation and treatment and the latest consensus opinion, UC can be divided into seven syndromes characterized by large intestinal damp-heat, spleen deficiency and damp accumulation, intense heat toxin, cold and heat in complexity, liver constraint and spleen deficiency, spleen-kidney yang deficiency, and yin-blood depletion. In clinical practice, intestinal damp-heat spleen deficiency and damp accumulation are the two most common syndromes in UC patients (234). Patients with intestinal damp-heat syndrome can use the prescription *Sháo Yào Tāng* (Peony Decoction), while patients with spleen deficiency and damp accumulation syndrome can use *Shēn Líng Bái Zhú Sǎn* for treatment. Interestingly, syndrome differentiation determines that the use of TCM is individualized. Doctors always prescribe the appropriate formulas or medicinals according to the symptoms and signs of patients. When conducting animal experiments to evaluate the therapeutic effects of TCM, researchers often choose medicinals and formulas with efficacy of clearing heat, strengthening the spleen, and drying dampness. Moreover, the main chemical constituents of these medicinals have been proven to have antibacterial and anti-inflammatory effects similar to antibiotics. Through the analysis of medication rules of UC in TCM from 2000 to 2020, it was found that medicinals such as *huáng lián*, *ku shēn* (the dried root of *Sophora flavescens* Aiton), *huáng qín*, *mù xiāng* (the dried root of *Aucklandia costus* Falc.), and *bái zhú* (the dried rhizome of *Atractylodes macrocephala* Koidz.) are used frequently (235).

The heat-clearing medicinals are the most widely used for classification based on efficacy. Similar results were obtained by analyzing the formulas related to the treatment of UC in the Chinese patent database. Among these formulas, *huáng lián* is the most frequently used medicinal (236). Notably, the formula *Sháo Yào Tāng* and *Shēn Líng Bái Zhú Sǎn* mentioned in the consensus opinion also contain medicinals such as *huáng lián*, *huáng qín*, and *bái zhú*. Thus, it can be found that medicinals with heat-clearing effects are critical among the specific medications currently used in TCM to treat UC.

TCM has been widely used in the clinical treatment of UC in China. In the retrospective study on 247 UC patients, TCM was chosen in mild to moderate UC patients and used as an adjuvant treatment for severe UC. It was finally found that TCM treatment is indeed effective (237). At the same time, the application of TCM showed the same therapeutic effect as that of mesalazine with fewer adverse reactions (238, 239). Another medicinal, *qing dài*, was found to alleviate moderate active UC significantly (240). The results of a randomized controlled trial on the treatment of UC by *Jiàn Pí Qing Cháng Tāng* showed that it could improve the clinical symptoms of patients with mild to moderate UC and improve the quality of life (241). Undoubtedly, these studies provide more evidence for the effectiveness of TCM in treating UC. However, sufficient evidence-based medical evidence is needed to support its effectiveness and safety for long-term use. Moreover, given the typically low quality of existing TCM-related randomized controlled trials, there remains an urgent need for more rigorous randomized controlled trials to offer high-quality evidence for the appropriate application of various TCM.

Potential and Deficiency of TCM

In a series of animal experiments, alleviation of animal symptoms, reducing inflammation, and decreasing expression of pro-inflammatory cytokines all suggest the therapeutic potential of TCM on experimental colitis. Most researchers focus on the critical inflammatory signal pathways affected by TCM, including NF-κB and MAPK pathways, and study the mechanism of action. Interestingly, some medicinals have also shown significant regulatory effects on the intestinal microbiota, including increasing the diversity and changing the composition of the microbiota. Therefore, TCM can restore the intestinal microbial homeostasis, reduce the damage of pathogenic bacteria and opportunistic pathogens to the epithelial barrier, inhibit the abnormal activation of key inflammatory signaling pathways, and ultimately reduce the abnormal immune response of the host, which may be the key mechanism of TCM in treating UC.

Moreover, medicinals with heat-clearing effects prioritized treating UC in the animal experiment stage and specific clinical practice. Heat-clearing medicinals have cold or cool properties in nature according to the theory of TCM. Pharmacological studies have shown that they have antibacterial and anti-inflammatory effects. Notably, excessive and long-term use of heat-clearing herbs can damage the "yang" of the stomach and spleen, resulting in abdominal pain, and diarrhea and other gastrointestinal symptoms. To a certain extent, it is still unclear whether the irrational use of heat-clearing drugs in UC patients will further destroy the intestinal microbial homeostasis and eventually lead to the aggravation of UC. Consequently, more preclinical studies and evidence-based clinical studies are needed to support whether heat-clearing medicinals as critical drugs for treating UC and establishing reasonable use specifications to obtain better clinical benefits.

CONCLUSION

According to current evidence, the occurrence of UC is associated with multiple risk factors such as heredity, immunity and microorganisms. It is worth noting that more and more studies have pointed out that the intestinal microbiota may play a key role in the development of UC. What's more, the microbial disorders are also related to abnormal immune system. Therefore, further study of intestinal microbiota may be a prospective direction to elucidate the pathogenesis of UC. Similarly, it is found that treatments aimed at correcting the imbalance and restoring the homeostasis of intestinal microbiota may be an essential strategy for the treatment of UC. In many animal studies and clinical practices, FMT, probiotics, prebiotics and synbiotics treatment have shown great therapeutic potential for UC. At the same time, more researches are needed to support their efficacy and safety, and to explore more standardized approaches in their application.

Likewise, TCM has obvious advantages in the treatment of UC, and it has systematic and comprehensive advantages in regulating intestinal microbiota and human body, and has potential and practical value in the treatment of UC. In addition, animal models are basic tools for studying the pathogenesis of diseases and exploring therapeutic strategies. In the most commonly used models, the DSS-induced UC model is closer to the occurrence and development of UC, but the role of microbiota in it seems unclear or even controversial. In conclusion, it is necessary to study the roles of microbiota in the pathogenesis of UC and the improvement of UC by microbial therapy.

AUTHOR CONTRIBUTIONS

YH, ZY, and MW proposed the idea. YH and ZY searched the literature. YH, ZY, MW, YS, KQ, and YX reviewed and summarized the literature. ZH, MY, FL, and QY modify the manuscript. LL was contributed to the figures and tables. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (no. 81973742), the Science and Technology Department of Sichuan Province (nos. 2020YJ0383 and 20YYJC0910), and the Sichuan Youth Science and Technology Innovation Research Team Project (no. 2020JDTD0022).

ACKNOWLEDGMENTS

Figures 1, **2** were modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Genetic License. (http://creativecommons.org/licenses/by/3.0/).

REFERENCES

1. Ungaro R, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF. Ulcerative colitis. *Lancet.* (2017) 389:1756–70. doi: 10.1016/S0140-6736(16)32126-2
2. Dignass A, Eliakim R, Magro F, Maaser C, Chowers Y, Geboes K, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohns Colitis.* (2012) 6:965–90. doi: 10.1016/j.crohns.2012.09.003
3. Guo XY, Liu XJ, Hao JY. Gut microbiota in ulcerative colitis: insights on pathogenesis and treatment. *J Dig Dis.* (2020) 21:147–59. doi: 10.1111/1751-2980.12849
4. Underhill DM, Iliev ID. The Mycobiota: Interactions Between Commensal Fungi and the Host Immune System. *Nat Rev Immunol.* (2014) 14:405–16. doi: 10.1038/nri3684
5. Imhann F, Vich VA, Bonder MJ, Fu J, Gevers D, Visschedijk MC, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut.* (2018) 67:108–19. doi: 10.1136/gutjnl-2016-312135
6. Gersemann M, Wehkamp J, Stange EF. Innate immune dysfunction in inflammatory bowel disease. *J Intern Med.* (2012) 271:421–8. doi: 10.1111/j.1365-2796.2012.02515.x
7. Kobayashi T, Matsuoka K, Yokoyama Y, Nakamura T, Ino T, Numata T, et al. A multicenter, retrospective, observational study of the clinical outcomes and risk factors for relapse of ulcerative colitis at 1 year after leukocytapheresis. *J Gastroenterol.* (2018) 53:387–96. doi: 10.1007/s00535-017-1356-8
8. Samuel S, Ingle SB, Dhillon S, Yadav S, Harmsen WS, Zinsmeister AR, et al. Cumulative incidence and risk factors for hospitalization and surgery in a population-based cohort of ulcerative colitis. *Inflamm Bowel Dis.* (2013) 19:1858–66. doi: 10.1097/MIB.0b013e31828c84c5
9. Luo CX, Wen ZH, Zhen Y, Wang ZJ, Mu JX, Zhu M, et al. Chinese research into severe ulcerative colitis has increased in quantity and complexity. *World J Clin Cases.* (2018) 6:35–43. doi: 10.12998/wjcc.v6.i3.35
10. Zhang Z, Yang L, Wang B, Zhang L, Zhang Q, Li D, et al. Protective role of liriodendrin in mice with dextran sulphate sodium-induced ulcerative colitis. *Int Immunopharmacol.* (2017) 52:203–10. doi: 10.1016/j.intimp.2017.09.012
11. Chang CJ, Lin CS, Lu CC, Martel J, Ko YF, Ojcius DM, et al. Ganoderma lucidum reduces obesity in mice by modulating the composition of the gut microbiota. *Nat Commun.* (2015) 6:7489. doi: 10.1038/ncomms8489
12. Feng W, Ao H, Peng C, Yan D. Gut microbiota, a new frontier to understand traditional Chinese medicines. *Pharmacol Res.* (2019) 142:176–91. doi: 10.1016/j.phrs.2019.02.024
13. Graham DB, Xavier RJ. Pathway paradigms revealed from the genetics of inflammatory bowel disease. *Nature.* (2020) 578:527–39. doi: 10.1038/s41586-020-2025-2
14. Moller FT, Andersen V, Wohlfahrt J, Jess T. Familial risk of inflammatory bowel disease: a population-based cohort study 1977-2011. *Am J Gastroenterol.* (2015) 110:564–71. doi: 10.1038/ajg.2015.50
15. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science.* (2006) 314:1461–3. doi: 10.1126/science.1135245
16. de Lange KM, Moutsianas L, Lee JC, Lamb CA, Luo Y, Kennedy NA, et al. Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. *Nat Genet.* (2017) 49:256–61. doi: 10.1038/ng.3760
17. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature.* (2012) 491:119–24. doi: 10.1038/nature11582
18. Crowley E, Muise A. Inflammatory bowel disease: what very early onset disease teaches us. *Gastroenterol Clin North Am.* (2018) 47:755–72. doi: 10.1016/j.gtc.2018.07.004
19. Limon JJ, Tang J, Li D, Wolf AJ, Michelsen KS, Funari V, et al. Malassezia is associated with crohn's disease and exacerbates colitis in mouse models. *Cell Host Microbe.* (2019) 25:377–88. doi: 10.1016/j.chom.2019.01.007
20. Luo Y, de Lange KM, Jostins L, Moutsianas L, Randall J, Kennedy NA, et al. Exploring the genetic architecture of inflammatory bowel disease by whole-genome sequencing identifies association at ADCY7. *Nat Genet.* (2017) 49:186–92. doi: 10.1038/ng.3761
21. Dahle MK, Myhre AE, Aasen AO, Wang JE. Effects of Forskolin on Kupffer cell production of interleukin-10 and tumor necrosis factor alpha differ from those of endogenous adenylyl cyclase activators: possible role for adenylyl cyclase 9. *Infect Immun.* (2005) 73:7290–6. doi: 10.1128/IAI.73.11.7290-7296.2005
22. de Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol.* (2016) 13:13–27. doi: 10.1038/nrgastro.2015.186
23. Chang JT. Pathophysiology of inflammatory bowel diseases. *N Engl J Med.* (2020) 383:2652–64. doi: 10.1056/NEJMra2002697
24. Brazil JC, Louis NA, Parkos CA. The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease. *Inflamm Bowel Dis.* (2013) 19:1556–65. doi: 10.1097/MIB.0b013e318281f54e

25. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3. (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun.* (2000) 68:7010–7. doi: 10.1128/IAI.68.12.7010-7017.2000
26. Hart AL, Al-Hassi HO, Rigby RJ, Bell SJ, Emmanuel AV, Knight SC, et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology.* (2005) 129:50–65. doi: 10.1053/j.gastro.2005.05.013
27. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: different gene signatures in M1(LPS+) vs. Classically and M2(LPS-) Vs. alternatively activated macrophages. *Front Immunol.* (2019) 10:1084. doi: 10.3389/fimmu.2019.01084
28. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity.* (2010) 32:593–604. doi: 10.1016/j.immuni.2010.05.007
29. Kim DH, Cheon JH. Pathogenesis of inflammatory bowel disease and recent advances in biologic therapies. *Immune Netw.* (2017) 17:25–40. doi: 10.4110/in.2017.17.1.25
30. Du L, Ha C. Epidemiology and pathogenesis of ulcerative colitis. *Gastroenterol Clin North Am.* (2020) 49:643–54. doi: 10.1016/j.gtc.2020.07.005
31. Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, et al. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-Gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol.* (1996) 157:1261–70. doi: 10.1097/00024382-199703001-00528
32. Inoue S, Matsumoto T, Iida M, Mizuno M, Kuroki F, Hoshika K, et al. Characterization of cytokine expression in the rectal mucosa of ulcerative colitis: correlation with disease activity. *Am J Gastroenterol.* (1999) 94:2441–6. doi: 10.1111/j.1572-0241.1999.01372.x
33. Gerlach K, Hwang Y, Nikolaev A, Atreya R, Dornhoff H, Steiner S, et al. TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis *via* IL-9 receptor signaling in intestinal epithelial cells. *Nat Immunol.* (2014) 15:676–86. doi: 10.1038/ni.2920
34. Galvez J. Role of Th17 cells in the pathogenesis of human IBD. *ISRN Inflamm.* (2014) 2014:928461. doi: 10.1155/2014/928461
35. Pene J, Chevalier S, Preisser L, Venereau E, Guilleux MH, Ghannam S, et al. Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J Immunol.* (2008) 180:7423–30. doi: 10.4049/jimmunol.180.11.7423
36. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. *Science.* (2005) 308:1635–8. doi: 10.1126/science.1110591
37. Sommer F, Backhed F. The gut microbiota–masters of host development and physiology. *Nat Rev Microbiol.* (2013) 11:227–38. doi: 10.1038/nrmicro2974
38. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature.* (2010) 464:59–65. doi: 10.1038/nature08821
39. Ekmekciu I, von Klitzing E, Neumann C, Bacher P, Scheffold A, Bereswill S, ET AL. Fecal microbiota transplantation, commensal *Escherichia coli* and lactobacillus johnsonii strains differentially restore intestinal and systemic adaptive immune cell populations following broad-spectrum antibiotic treatment. *Front Microbiol.* (2017) 8:2430. doi: 10.3389/fmicb.2017.02430
40. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes.* (2008) 57:1470–81. doi: 10.2337/db07-1403
41. Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci U S A.* (2011) 108 Suppl 1:4586–91. doi: 10.1073/pnas.1000097107
42. Lepage P, Hasler R, Spehlmann ME, Rehman A, Zvirbliene A, Begun A, et al. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology.* (2011) 141:227–36. doi: 10.1053/j.gastro.2011.04.011
43. Jess T, Simonsen J, Nielsen NM, Jorgensen KT, Bager P, Ethelberg S, et al. Enteric salmonella or campylobacter infections and the risk of inflammatory bowel disease. *Gut.* (2011) 60:318–24. doi: 10.1136/gut.2010.223396
44. Tuci A, Tonon F, Castellani L, Sartini A, Roda G, Marocchi M, et al. Fecal detection of mycobacterium avium paratuberculosis using the IS900 DNA sequence in crohn's disease and ulcerative colitis patients and healthy subjects. *Dig Dis Sci.* (2011) 56:2957–62. doi: 10.1007/s10620-011-1699-6
45. Becker C, Neurath MF, Wirtz S. The intestinal microbiota in inflammatory bowel disease. *Ilar J.* (2015) 56:192–204. doi: 10.1093/ilar/ilv030
46. Hegazy AN, West NR, Stubbington M, Wendt E, Suijker K, Datsi A, et al. Circulating and tissue-resident CD4(+) T cells with reactivity to intestinal microbiota are abundant in healthy individuals and function is altered during inflammation. *Gastroenterology.* (2017) 153:1320–37. doi: 10.1053/j.gastro.2017.07.047
47. Fu J, Wei B, Wen T, Johansson MEV, Liu X, Bradford E, et al. Loss of intestinal core 1–derived O-glycans causes spontaneous colitis in mice. *J Clin Invest.* (2011) 121:1657–66. doi: 10.1172/JCI45538
48. Llopis M, Antolin M, Carol M, Borruel N, Casellas F, Martinez C, et al. Lactobacillus casei downregulates commensals' inflammatory signals in Crohn's disease mucosa. *Inflamm Bowel Dis.* (2009) 15:275–83. doi: 10.1002/ibd.20736
49. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The Inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A.* (2008) 105:15064–9. doi: 10.1073/pnas.0803124105
50. Gersemann M, Becker S, Kubler I, Koslowski M, Wang G, Herrlinger KR, et al. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation.* (2009) 77:84–94. doi: 10.1016/j.diff.2008.09.008
51. Nakanishi Y, Sato T, Ohteki T. Commensal gram-positive bacteria initiates colitis by inducing monocyte/macrophage mobilization. *Mucosal Immunol.* (2015) 8:152–60. doi: 10.1038/mi.2014.53
52. Yue B, Ren J, Yu Z, Luo X, Ren Y, Zhang J, et al. Pinocembrin alleviates ulcerative colitis in mice via regulating gut microbiota, suppressing TLR4/MD2/NF-KappaB pathway and promoting intestinal barrier. *Biosci Rep.* (2020) 40. doi: 10.1042/BSR20200986
53. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A toll-like receptor recognizes bacterial DNA. *Nature.* (2000) 408:740–5. doi: 10.1038/35047123
54. Lu Y, Li X, Liu S, Zhang Y, Zhang D. Toll-like receptors and inflammatory bowel disease. *Front Immunol.* (2018) 9:72. doi: 10.3389/fimmu.2018.00072
55. Sui Y, Wu J, Chen J. The role of gut microbial beta-glucuronidase in estrogen reactivation and breast cancer. *Front Cell Dev Biol.* (2021) 9:631552. doi: 10.3389/fcell.2021.631552
56. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell.* (2016) 165:1332–45. doi: 10.1016/j.cell.2016.05.041
57. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. *Science.* (2013) 341:569–73. doi: 10.1126/science.1241165
58. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature.* (2014) 505:559–63. doi: 10.1038/nature12820
59. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, et al. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *Plos ONE.* (2013) 8:E66019. doi: 10.1371/journal.pone.0066019
60. Ott SJ, Kuhbacher T, Musfeldt M, Rosenstiel P, Hellmig S, Rehman A, et al. Fungi and inflammatory bowel diseases: alterations of composition and diversity. *Scand J Gastroenterol.* (2008) 43:831–41. doi: 10.1080/00365520801935434
61. Qiu X, Ma J, Jiao C, Mao X, Zhao X, Lu M, et al. Alterations in the mucosa-associated fungal microbiota in patients with ulcerative colitis. *Oncotarget.* (2017) 8:107577–88. doi: 10.18632/oncotarget.22534
62. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, et al. Fungal microbiota dysbiosis in IBD. *Gut.* (2017) 66:1039–48. doi: 10.1136/gutjnl-2015-310746
63. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor dectin-1 influence colitis. *Science.* (2012) 336:1314–7. doi: 10.1126/science.1221789
64. Romani L. Immunity to fungal infections. *Nat Rev Immunol.* (2011) 11:275–88. doi: 10.1038/nri2939

65. Wheeler ML, Limon JJ, Bar AS, Leal CA, Gargus M, Tang J, et al. Immunological consequences of intestinal fungal dysbiosis. *Cell Host Microbe.* (2016) 19:865–73. doi: 10.1016/j.chom.2016.05.003
66. Sun Z, Pei W, Guo Y, Wang Z, Shi R, Chen X, et al. Gut microbiota-mediated NLRP12 expression drives the attenuation of dextran sulphate sodium-induced ulcerative colitis by Qingchang Wenzhong decoction. *Evid Based Complement Alternat Med.* (2019) 2019:9839474. doi: 10.1155/2019/9839474
67. Dollive S, Chen YY, Grunberg S, Bittinger K, Hoffmann C, Vandivier L, et al. Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. *Plos ONE.* (2013) 8:E71806. doi: 10.1371/journal.pone.0071806
68. Qiu X, Zhang F, Yang X, Wu N, Jiang W, Li X, et al. Changes in the composition of intestinal fungi and their role in mice with dextran sulfate sodium-induced colitis. *Sci Rep.* (2015) 5:10416. doi: 10.1038/srep10416
69. Seelbinder B, Chen J, Brunke S, Vazquez-Uribe R, Santhaman R, Meyer AC, et al. Antibiotics create a shift from mutualism to competition in human gut communities with a longer-lasting impact on fungi than bacteria. *Microbiome.* (2020) 8:133. doi: 10.1186/s40168-020-00899-6
70. Allison DL, Willems H, Jayatilake J, Bruno VM, Peters BM, Shirtliff ME. Candida-bacteria interactions: their impact on human disease. *Microbiol Spectr.* (2016) 4. doi: 10.1128/microbiolspec.VMBF-0030-2016
71. Sovran B, Planchais J, Jegou S, Straube M, Lamas B, Natividad JM, et al. Enterobacteriaceae are essential for the modulation of colitis severity by fungi. *Microbiome.* (2018) 6:152. doi: 10.1186/s40168-018-0538-9
72. Fox EP, Cowley ES, Nobile CJ, Hartooni N, Newman DK, Johnson AD. Anaerobic bacteria grow within Candida albicans biofilms and induce biofilm formation in suspension cultures. *Curr Biol.* (2014) 24:2411–6. doi: 10.1016/j.cub.2014.08.057
73. Sam QH, Chang MW, Chai LY. The fungal mycobiome and its interaction with gut bacteria in the host. *Int J Mol Sci.* (2017) 18. doi: 10.3390/ijms18020330
74. van der Post S, Jabbar KS, Birchenough G, Arike L, Akhtar N, Sjovall H, et al. Structural weakening of the colonic mucus barrier is an early event in ulcerative colitis pathogenesis. *Gut.* (2019) 68:2142–51. doi: 10.1136/gutjnl-2018-317571
75. Mizuno S, Ono K, Mikami Y, Naganuma M, Fukuda T, Minami K, et al. 5-aminosalicylic acid intolerance is associated with a risk of adverse clinical outcomes and dysbiosis in patients with ulcerative colitis. *Intest Res.* (2020) 18:69–78. doi: 10.5217/ir.2019.00084
76. Liu A, Lv H, Wang H, Yang H, Li Y, Qian J. Aging increases the severity of colitis and the related changes to the gut barrier and gut microbiota in humans and mice. *J Gerontol A Biol Sci Med Sci.* (2020) 75:1284–92. doi: 10.1093/gerona/glz263
77. Xu N, Bai X, Cao X, Yue W, Jiang W, Yu Z. Changes in intestinal microbiota and correlation with TLRs in ulcerative colitis in the coastal area of Northern China. *Microb Pathog.* (2021) 150:104707. doi: 10.1016/j.micpath.2020.104707
78. Ahlawat S, Kumar P, Mohan H, Goyal S, Sharma KK. Inflammatory bowel disease: tri-directional relationship between microbiota, immune system and intestinal epithelium. *Crit Rev Microbiol.* (2021) 47:254–73. doi: 10.1080/1040841X.2021.1876631
79. Zakerska-Banaszak O, Tomczak H, Gabryel M, Baturo A, Wolko L, Michalak M, et al. Dysbiosis of gut microbiota in polish patients with ulcerative colitis: a pilot study. *Sci Rep.* (2021) 11:2166. doi: 10.1038/s41598-021-81628-3
80. Jun X, Ning C, Yang S, Zhe W, Na W, Yifan Z, et al. Alteration of fungal microbiota after 5-ASA treatment in UC patients. *Inflamm Bowel Dis.* (2020) 26:380–90. doi: 10.1093/ibd/izz207
81. Seidelin JB, Bahl MI, Licht TR, Mead BE, Karp JM, Johansen JV, et al. Acute experimental barrier injury triggers ulcerative colitis-specific innate hyperresponsiveness and ulcerative colitis-type microbiome changes in humans. *Cell Mol Gastroenterol Hepatol.* (2021) 12:1281–96. doi: 10.1016/j.jcmgh.2021.06.002
82. Beheshti-Maal A, Shahrokh S, Ansari S, Mirsamadi ES, Yadegar A, Mirjalali H, et al. Gut mycobiome: the probable determinative role of fungi in IBD patients. *Mycoses.* (2021) 64:468–76. doi: 10.1111/myc.13238
83. Maldonado-Arriaga B, Sandoval-Jimenez S, Rodriguez-Silverio J, Lizeth AS, Cortes-Espinosa T, Perez-Cabeza DVR, et al. Gut dysbiosis and clinical phases of pancolitis in patients with ulcerative colitis. *Microbiologyopen.* (2021) 10:E1181. doi: 10.1002/mbo3.1181
84. Lavelle A, Sokol H. Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol.* (2020) 17:223–37. doi: 10.1038/s41575-019-0258-z
85. Yoo JY, Groer M, Dutra S, Sarkar A, McSkimming DI. Gut microbiota and immune system interactions. *Microorganisms.* (2020) 8. doi: 10.3390/microorganisms8101587
86. Martin R, Chain F, Miquel S, Motta JP, Vergnolle N, Sokol H, et al. Using murine colitis models to analyze probiotics-host interactions. *Fems Microbiol Rev.* (2017) 41:S49–70. doi: 10.1093/femsre/fux035
87. Jialing L, Yangyang G, Jing Z, Xiaoyi T, Ping W, Liwei S, et al. Changes in serum inflammatory cytokine levels and intestinal flora in a self-healing dextran sodium sulfate-induced ulcerative colitis murine model. *Life Sci.* (2020) 263:118587. doi: 10.1016/j.lfs.2020.118587
88. Randhawa PK, Singh K, Singh N, Jaggi AS. A review on chemical-induced inflammatory bowel disease models in rodents. *Korean J Physiol Pharmacol.* (2014) 18:279–88. doi: 10.4196/kjpp.2014.18.4.279
89. Giner E, Recio MC, Rios JL, Giner RM. Oleuropein protects against dextran sodium sulfate-induced chronic colitis in mice. *J Nat Prod.* (2013) 76:1113–20. doi: 10.1021/np400175b
90. Low D, Nguyen DD, Mizoguchi E. Animal models of ulcerative colitis and their application in drug research. *Drug Des Devel Ther.* (2013) 7:1341–57. doi: 10.2147/DDDT.S40107
91. Kim JJ, Shajib MS, Manocha MM, Khan WI. Investigating intestinal inflammation in DSS-induced model of IBD. *J Vis Exp.* (2012). doi: 10.3791/3678
92. Dharmani P, Leung P, Chadee K. Tumor necrosis factor-alpha and Muc2 mucin play major roles in disease onset and progression in dextran sodium sulphate-induced colitis. *Plos ONE.* (2011) 6:E25058. doi: 10.1371/journal.pone.0025058
93. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran Sulfate Sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol.* (2014) 104:15–25. doi: 10.1002/0471142735.im1525s104
94. Bilsborough J, Fiorino MF, Henkle BW. Select animal models of colitis and their value in predicting clinical efficacy of biological therapies in ulcerative colitis. *Expert Opin Drug Discov.* (2020) 1–11. doi: 10.1080/17460441.2021.1851185
95. Nagalingam NA, Kao JY, Young VB. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. *Inflamm Bowel Dis.* (2011) 17:917–26. doi: 10.1002/ibd.21462
96. Silva I, Pinto R, Mateus V. Preclinical study *in vivo* for new pharmacological approaches in inflammatory bowel disease: a systematic review of chronic model of TNBS-induced colitis. *J Clin Med.* (2019) 8. doi: 10.3390/jcm8101574
97. Seoane-Viano I, Gomez-Lado N, Lazare-Iglesias H, Barreiro-De AM, Silva-Rodriguez J, Luzardo-Alvarez A, et al. Longitudinal PET/CT evaluation of TNBS-induced inflammatory bowel disease rat model. *Int J Pharm.* (2018) 549:335–42. doi: 10.1016/j.ijpharm.2018.08.005
98. El-Salhy M, Hatlebakk JG. Changes in enteroendocrine and immune cells following colitis induction by TNBS in rats. *Mol Med Rep.* (2016) 14:4967–74. doi: 10.3892/mmr.2016.5902
99. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, et al. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis.* (2009) 15:341–52. doi: 10.1002/ibd.20753
100. He Q, Li X, Liu C, Su L, Xia Z, Li X, et al. Dysbiosis of the fecal microbiota in the TNBS-induced Crohn's disease mouse model. *Appl Microbiol Biotechnol.* (2016) 100:4485–94. doi: 10.1007/s00253-015-7205-x
101. Sadar SS, Vyawahare NS, Bodhankar SL. Ferulic acid ameliorates TNBS-induced ulcerative colitis through modulation of cytokines, oxidative stress, INOs, COX-2, and apoptosis in laboratory rats. *Excli J.* (2016) 15:482–99. doi: 10.17179/excli2016-393
102. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-Producing NK-T Cells. *Immunity.* (2002) 17:629–38. doi: 10.1016/S1074-7613(02)00453-3

103. Nascimento R, Machado A, Galvez J, Cazarin C, Marostica JM. Ulcerative colitis: gut microbiota, immunopathogenesis and application of natural products in animal models. *Life Sci.* (2020) 258:118129. doi: 10.1016/j.lfs.2020.118129
104. Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, et al. Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc.* (2017) 12:1295–309. doi: 10.1038/nprot.2017.044
105. Kojima R, Kuroda S, Ohkishi T, Nakamaru K, Hatakeyama S. Oxazolone-induced colitis in BALB/C mice: a new method to evaluate the efficacy of therapeutic agents for ulcerative colitis. *J Pharmacol Sci.* (2004) 96:307–13. doi: 10.1254/jphs.FP0040214
106. Hoving JC, Kirstein F, Nieuwenhuizen NE, Fick LC, Hobeika E, Reth M, et al. B cells that produce immunoglobulin E mediate colitis in BALB/c mice. *Gastroenterology.* (2012) 142:96–108. doi: 10.1053/j.gastro.2011.09.044
107. Hakansson A, Tormo-Badia N, Baridi A, Xu J, Molin G, Hagslatt ML, et al. Immunological alteration and changes of gut microbiota after dextran sulfate sodium (DSS) administration in mice. *Clin Exp Med.* (2015) 15:107–20. doi: 10.1007/s10238-013-0270-5
108. Figliuolo VR, Dos SL, Abalo A, Nanini H, Santos A, Brittes NM, et al. Sulfate-reducing bacteria stimulate gut immune responses and contribute to inflammation in experimental colitis. *Life Sci.* (2017) 189:29–38. doi: 10.1016/j.lfs.2017.09.014
109. Kozik AJ, Nakatsu CH, Chun H, Jones-Hall YL. Comparison of the fecal, cecal, and mucus microbiome in male and female mice after TNBS-induced colitis. *Plos ONE.* (2019) 14:E225079. doi: 10.1371/journal.pone.0225079
110. Gancarcikova S, Lauko S, Hrckova G, Andrejcakova Z, Hajduckova V, Madar M, et al. Innovative animal model of DSS-induced ulcerative colitis in pseudo germ-free mice. *Cells-Basel.* (2020) 9. doi: 10.3390/cells9122571
111. Hernandez-Chirlaque C, Aranda CJ, Ocon B, Capitan-Canadas F, Ortega-Gonzalez M, Carrero JJ, et al. Germ-free and antibiotic-treated mice are highly susceptible to epithelial injury in DSS colitis. *J Crohns Colitis.* (2016) 10:1324–35. doi: 10.1093/ecco-jcc/jjw096
112. Round JL, Palm NW. Causal effects of the microbiota on immune-mediated diseases. *Sci Immunol.* (2018) 3. doi: 10.1126/sciimmunol.aao1603
113. Reinshagen M. Immunoglobulin a coating identifies colitogenic bacteria in inflammatory bowel disease. *Z Gastroenterol.* (2015) 53:53. doi: 10.1055/s-0034-1385651
114. Kobayashi T, Siegmund B, Le Berre C, Wei SC, Ferrante M, Shen B, et al. Ulcerative colitis. *Nat Rev Dis Primers.* (2020) 6:74. doi: 10.1038/s41572-020-0205-x
115. Sandborn WJ, Travis S, Moro L, Jones R, Gautille T, Bagin R, et al. Once-daily budesonide MMX(R) extended-release tablets induce remission in patients with mild to moderate ulcerative colitis: results from the core i study. *Gastroenterology.* (2012) 143:1218–26. doi: 10.1053/j.gastro.2012.08.003
116. Harbord M, Eliakim R, Bettenworth D, Karmiris K, Katsanos K, Kopylov U, et al. Third European evidence-based consensus on diagnosis and management of ulcerative colitis. part 2: current management. *J Crohns Colitis.* (2017) 11:769–84. doi: 10.1093/ecco-jcc/jjx009
117. Ko CW, Singh S, Feuerstein JD, Falck-Ytter C, Falck-Ytter Y, Cross RK. AGA clinical practice guidelines on the management of mild-to-moderate ulcerative colitis. *Gastroenterology.* (2019) 156:748–64. doi: 10.1053/j.gastro.2018.12.009
118. Bressler B, Marshall JK, Bernstein CN, Bitton A, Jones J, Leontiadis GI, et al. Clinical practice guidelines for the medical management of nonhospitalized ulcerative colitis: the Toronto consensus. *Gastroenterology.* (2015) 148:1035–58. doi: 10.1053/j.gastro.2015.03.001
119. Peppercorn MA, Goldman P. The role of intestinal bacteria in the metabolism of salicylazosulfapyridine. *J Pharmacol Exp Ther.* (1972) 181:555–62.
120. Gisbert JP, Gomollon F, Mate J, Pajares JM. Role of 5-aminosalicylic acid (5-ASA) in treatment of inflammatory bowel disease: a systematic review. *Dig Dis Sci.* (2002) 47:471–88. doi: 10.1023/a:1017987229718
121. Berends SE, Strik AS, Lowenberg M, D'Haens GR, Mathot R. Clinical pharmacokinetic and pharmacodynamic considerations in the treatment of ulcerative colitis. *Clin Pharmacokinet.* (2019) 58:15–37. doi: 10.1007/s40262-018-0676-z
122. Tavares JA, de Araujo J, Meneguin AB, Chorilli M. Characteristics, properties and analytical/bioanalytical methods of 5-aminosalicylic acid: a review. *Crit Rev Anal Chem.* (2020) 1–15. doi: 10.1080/10408347.2020.1848516
123. Kaiser GC, Yan F, Polk DB. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor KappaB activation in mouse colonocytes. *Gastroenterology.* (1999) 116:602–9. doi: 10.1016/S0016-5085(99)70182-4
124. Murray A, Nguyen TM, Parker CE, Feagan BG, MacDonald JK. Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev.* (2020) 8:D543. doi: 10.1002/14651858.CD000543.pub5
125. Sutherland L, Macdonald JK. Oral 5-aminosalicylic acid for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev.* (2006) D544. doi: 10.1002/14651858.CD000544.pub2
126. Lyakhovich A, Gasche C. Systematic review: molecular chemoprevention of colorectal malignancy by mesalazine. *Aliment Pharmacol Ther.* (2010) 31:202–9. doi: 10.1111/j.1365-2036.2009.04195.x
127. Le Berre C, Roda G, Nedeljkovic PM, Danese S, Peyrin-Biroulet L. Modern use of 5-aminosalicylic acid compounds for ulcerative colitis. *Expert Opin Biol Ther.* (2020) 20:363–78. doi: 10.1080/14712598.2019.1666101
128. Zheng H, Chen M, Li Y, Wang Y, Wei L, Liao Z, et al. Modulation of gut microbiome composition and function in experimental colitis treated with sulfasalazine. *Front Microbiol.* (2017) 8:1703. doi: 10.3389/fmicb.2017.01703
129. Olaisen M, Spigset O, Flatberg A, Granlund A, Brede WR, Albrektsen G, et al. Mucosal 5-aminosalicylic acid concentration, drug formulation and mucosal microbiome in patients with quiescent ulcerative colitis. *Aliment Pharmacol Ther.* (2019) 49:1301–13. doi: 10.1111/apt.15227
130. Dubois-Camacho K, Ottum PA, Franco-Munoz D, De la Fuente M, Torres-Riquelme A, Diaz-Jimenez D, et al. Glucocorticosteroid therapy in inflammatory bowel diseases: from clinical practice to molecular biology. *World J Gastroenterol.* (2017) 23:6628–38. doi: 10.3748/wjg.v23.i36.6628
131. Barnes PJ. Molecular mechanisms and cellular effects of glucocorticosteroids. *Immunol Allergy Clin North Am.* (2005) 25:451–68. doi: 10.1016/j.iac.2005.05.003
132. Ford AC, Bernstein CN, Khan KJ, Abreu MT, Marshall JK, Talley NJ, et al. Glucocorticosteroid therapy in inflammatory bowel disease: systematic review and meta-analysis. *Am J Gastroenterol.* (2011) 106:590–9. doi: 10.1038/ajg.2011.70
133. Silverman J, Otley A. Budesonide in the treatment of inflammatory bowel disease. *Expert Rev Clin Immunol.* (2011) 7:419–28. doi: 10.1586/eci.11.34
134. Curtis JR, Westfall AO, Allison J, Bijlsma JW, Freeman A, George V, et al. Population-based assessment of adverse events associated with long-term glucocorticoid use. *Arthritis Rheum.* (2006) 55:420–6. doi: 10.1002/art.21984
135. Saibeni S, Meucci G, Papi C, Manes G, Fasci-Spurio F. Low bioavailability steroids in inflammatory bowel disease: an old chestnut or a whole new ballgame? *Expert Rev Gastroenterol Hepatol.* (2014) 8:949–62. doi: 10.1586/17474124.2014.924396
136. D'Haens G. Systematic review: second-generation vs. conventional corticosteroids for induction of remission in ulcerative colitis. *Aliment Pharmacol Ther.* (2016) 44:1018–29. doi: 10.1111/apt.13803
137. Rubin DT, Cohen RD, Sandborn WJ, Lichtenstein GR, Axler J, Riddell RH, et al. Budesonide multimatrix is efficacious for mesalamine-refractory, mild to moderate ulcerative colitis: a randomised, placebo-controlled trial. *J Crohns Colitis.* (2017) 11:785–91. doi: 10.1093/ecco-jcc/jjx032
138. Gross V, Bunganic I, Belousova EA, Mikhailova TL, Kupcinskas L, Kiudelis G, et al. 3g Mesalazine granules are superior to 9mg budesonide for achieving remission in active ulcerative colitis: a double-blind, double-dummy, randomised trial. *J Crohns Colitis.* (2011) 5:129–38. doi: 10.1016/j.crohns.2010.11.006
139. Travis S. How do you avoid and treat steroid side effects? *Inflamm Bowel Dis.* (2008) 14 Suppl 2:S214–5. doi: 10.1002/ibd.20649
140. Bean RH. The treatment of chronic ulcerative colitis with 6-mercaptopurine. *Med J Aust.* (1962) 49:592–3. doi: 10.5694/j.1326-5377.1962.tb20590.x
141. Vegh Z, Burisch J, Pedersen N, Kaimakliotis I, Duricova D, Bortlik M, et al. Incidence and initial disease course of inflammatory bowel diseases in 2011 in Europe and Australia: results of the 2011 ECCO-epicom inception cohort. *J Crohns Colitis.* (2014) 8:1506–15. doi: 10.1016/j.crohns.2014.06.004

142. Coskun M, Steenholdt C, de Boer NK, Nielsen OH. Pharmacology and optimization of thiopurines and methotrexate in inflammatory bowel disease. *Clin Pharmacokinet.* (2016) 55:257–74. doi: 10.1007/s40262-015-0316-9
143. Gisbert JP, Nino P, Cara C, Rodrigo L. Comparative effectiveness of azathioprine in Crohn's disease and ulcerative colitis: prospective, long-term, follow-up study of 394 patients. *Aliment Pharmacol Ther.* (2008) 28:228–38. doi: 10.1111/j.1365-2036.2008.03732.x
144. van Gennep S, de Boer NK, D'Haens GR, Lowenberg M. Thiopurine treatment in ulcerative colitis: a critical review of the evidence for current clinical practice. *Inflamm Bowel Dis.* (2017) 24:67–77. doi: 10.1093/ibd/izx025
145. Li Q, Ding X, Liu K, Marcella C, Liu X, Zhang T, et al. Fecal microbiota transplantation for ulcerative colitis: the optimum timing and gut microbiota as predictors for long-term clinical outcomes. *Clin Transl Gastroenterol.* (2020) 11:E224. doi: 10.14309/ctg.0000000000000224
146. Zhang Q, Yang H, An J, Zhang R, Chen B, Hao DJ. Therapeutic effects of traditional chinese medicine on spinal cord injury: a promising supplementary treatment in future. *Evid Based Complement Alternat Med.* (2016) 2016:8958721. doi: 10.1155/2016/8958721
147. Borody TJ, Brandt LJ, Paramsothy S. Therapeutic faecal microbiota transplantation: current status and future developments. *Curr Opin Gastroenterol.* (2014) 30:97–105. doi: 10.1097/MOG.0000000000000027
148. Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, et al. Guidelines for diagnosis, treatment, and prevention of clostridium difficile infections. *Am J Gastroenterol.* (2013) 108:478–98. doi: 10.1038/ajg.2013.4
149. Quraishi MN, Widlak M, Bhala N, Moore D, Price M, Sharma N, et al. Systematic review with meta-analysis: the efficacy of faecal microbiota transplantation for the treatment of recurrent and refractory clostridium difficile infection. *Aliment Pharmacol Ther.* (2017) 46:479–93. doi: 10.1111/apt.14201
150. Crothers JW, Chu ND, Nguyen L, Phillips M, Collins C, Fortner K, et al. Daily, Oral FMT for long-term maintenance therapy in ulcerative colitis: results of a single-center, prospective, randomized pilot study. *Bmc Gastroenterol.* (2021) 21:281. doi: 10.1186/s12876-021-01856-9
151. Schierova D, Brezina J, Mrazek J, Fliegerova KO, Kvasnova S, Bajer L, et al. Gut microbiome changes in patients with active left-sided ulcerative colitis after fecal microbiome transplantation and topical 5-aminosalicylic acid therapy. *Cells-Basel.* (2020) 9. doi: 10.3390/cells9102283
152. Costello SP, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, et al. Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: a randomized clinical trial. *JAMA.* (2019) 321:156–64. doi: 10.1001/jama.2018.20046
153. Sood A, Mahajan R, Singh A, Midha V, Mehta V, Narang V, et al. Role of Faecal microbiota transplantation for maintenance of remission in patients with ulcerative colitis: a pilot study. *J Crohns Colitis.* (2019) 13:1311–7. doi: 10.1093/ecco-jcc/jjz060
154. Paramsothy S, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, et al. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet.* (2017) 389:1218–28. doi: 10.1016/S0140-6736(17)30182-4
155. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology.* (2015) 149:102–9. doi: 10.1053/j.gastro.2015.04.001
156. Rossen NG, Fuentes S, van der Spek MJ, Tijssen JG, Hartman JH, Duflou A, et al. Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis. *Gastroenterology.* (2015) 149:110–8. doi: 10.1053/j.gastro.2015.03.045
157. Costello SP, Soo W, Bryant RV, Jairath V, Hart AL, Andrews JM. Systematic review with meta-analysis: Faecal microbiota transplantation for the induction of remission for active ulcerative colitis. *Aliment Pharmacol Ther.* (2017) 46:213–24. doi: 10.1111/apt.14173
158. Levy AN, Allegretti JR. Insights into the role of fecal microbiota transplantation for the treatment of inflammatory bowel disease. *Therap Adv Gastroenterol.* (2019) 12:321941539. doi: 10.1177/1756284819836893
159. Jacob V, Crawford C, Cohen-Mekelburg S, Viladomiu M, Putzel GG, Schneider Y, et al. Single delivery of high-diversity fecal microbiota preparation by colonoscopy is safe and effective in increasing microbial diversity in active ulcerative colitis. *Inflamm Bowel Dis.* (2017) 23:903–11. doi: 10.1097/MIB.0000000000001132
160. Venturi A, Gionchetti P, Rizzello F, Johansson R, Zucconi E, Brigidi P, et al. Impact on the composition of the Faecal Flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment Pharmacol Ther.* (1999) 13:1103–8. doi: 10.1046/j.1365-2036.1999.00560.x
161. Lee T, Clavel T, Smirnov K, Schmidt A, Lagkouvardos I, Walker A, et al. Oral versus intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD. *Gut.* (2017) 66:863–71. doi: 10.1136/gutjnl-2015-309940
162. Fuentes S, Rossen NG, van der Spek MJ, Hartman JH, Huuskonen L, Korpela K, et al. Microbial shifts and signatures of long-term remission in ulcerative colitis after faecal microbiota transplantation. *Isme J.* (2017) 11:1877–89. doi: 10.1038/ismej.2017.44
163. Kruis W, Schutz E, Fric P, Fixa B, Judmaier G, Stolte M. Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther.* (1997) 11:853–8. doi: 10.1046/j.1365-2036.1997.00225.x
164. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT. Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet.* (1999) 354:635–9. doi: 10.1016/S0140-6736(98)06343-0
165. Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, ET AL. Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 Is as Effective as With Standard Mesalazine. *Gut.* (2004) 53:1617–23. doi: 10.1136/gut.2003.037747
166. Guslandi M, Giollo P, Testoni PA. A Pilot Trial of Saccharomyces Boulardii in Ulcerative Colitis. *Eur J Gastroenterol Hepatol.* (2003) 15:697–8. doi: 10.1097/00042737-200306000-00017
167. Ishikawa H, Akedo I, Umesaki Y, Tanaka R, Imaoka A, Otani T. Randomized controlled trial of the effect of bifidobacteria-fermented milk on ulcerative colitis. *J Am Coll Nutr.* (2003) 22:56–63. doi: 10.1080/07315724.2003.10719276
168. Kato K, Mizuno S, Umesaki Y, Ishii Y, Sugitani M, Imaoka A, et al. Randomized placebo-controlled trial assessing the effect of bifidobacteria-fermented milk on active ulcerative colitis. *Aliment Pharmacol Ther.* (2004) 20:1133–41. doi: 10.1111/j.1365-2036.2004.02268.x
169. Zocco MA, dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, et al. Efficacy of Lactobacillus GG in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther.* (2006) 23:1567–74. doi: 10.1111/j.1365-2036.2006.02927.x
170. Sood A, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, et al. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. *Clin Gastroenterol Hepatol.* (2009) 7:1202–9. doi: 10.1016/j.cgh.2009.07.016
171. Tursi A, Brandimarte G, Papa A, Giglio A, Elisei W, Giorgetti GM, et al. Treatment of relapsing mild-to-moderate ulcerative colitis with the probiotic VSL#3 as adjunctive to a standard pharmaceutical treatment: a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol.* (2010) 105:2218–27. doi: 10.1038/ajg.2010.218
172. Oliva S, Di Nardo G, Ferrari F, Mallardo S, Rossi P, Patrizi G, et al. Randomised clinical trial: the effectiveness of lactobacillus reuteri ATCC 55730 rectal enema in children with active distal ulcerative colitis. *Aliment Pharmacol Ther.* (2012) 35:327–34. doi: 10.1111/j.1365-2036.2011.04939.x
173. Bjarnason I, Sission G, Hayee B. A randomised, double-blind, placebo-controlled trial of a multi-strain probiotic in patients with asymptomatic ulcerative colitis and crohn's disease. *Inflammopharmacology.* (2019) 27:465–73. doi: 10.1007/s10787-019-00595-4
174. Miele E, Pascarella F, Giannetti E, Quaglietta L, Baldassano RN, Staiano A. Effect of a probiotic preparation (VSL#3) on induction and maintenance of remission in children with ulcerative colitis. *Am J Gastroenterol.* (2009) 104:437–43. doi: 10.1038/ajg.2008.118

175. Derwa Y, Gracie DJ, Hamlin PJ, Ford AC. Systematic review with meta-analysis: the efficacy of probiotics in inflammatory bowel disease. *Aliment Pharmacol Ther.* (2017) 46:389–400. doi: 10.1111/apt.14203
176. Akutko K, Stawarski A. Probiotics, prebiotics and synbiotics in inflammatory bowel diseases. *J Clin Med.* (2021) 10. doi: 10.3390/jcm10112466
177. Jakubczyk D, Leszczynska K, Gorska S. The effectiveness of probiotics in the treatment of inflammatory bowel disease (IBD)-A Critical Review. *Nutrients.* (2020) 12. doi: 10.3390/nu12071973
178. Ghyselinck J, Verstrepen L, Moens F, Van den Abbeele P, Said J, Smith B, et al. A 4-strain probiotic supplement influences gut microbiota composition and gut wall function in patients with ulcerative colitis. *Int J Pharm.* (2020) 587:119648. doi: 10.1016/j.ijpharm.2020.119648
179. Darb EA, Rajabpour M, Ghanavati R, Asadolahi P, Farzi S, Sobouti B, et al. The effects of probiotics, prebiotics and synbiotics on the reduction of IBD complications, a periodic review during 2009-2020. *J Appl Microbiol.* (2021) 130:1823–38. doi: 10.1111/jam.14907
180. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol.* (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75
181. Pandey KR, Naik SR, Vakil BV. Probiotics, prebiotics and synbiotics- a review. *J Food Sci Technol.* (2015) 52:7577–87. doi: 10.1007/s13197-015-1921-1
182. Rasmussen HE, Hamaker BR. Prebiotics and inflammatory bowel disease. *Gastroenterol Clin North Am.* (2017) 46:783–95. doi: 10.1016/j.gtc.2017.08.004
183. Kamarli AH, Akal YE, Akin M. Effects of synbiotic therapy in mild-to-moderately active ulcerative colitis: a randomized placebo-controlled study. *Turk J Gastroenterol.* (2019) 30:313–20. doi: 10.5152/tjg.2019.18356
184. Kanauchi O, Mitsuyama K, Homma T, Takahama K, Fujiyama Y, Andoh A, et al. Treatment of ulcerative colitis patients by long-term administration of germinated barley foodstuff: multi-center open trial. *Int J Mol Med.* (2003) 12:701–4. doi: 10.3892/ijmm.12.5.701
185. Hafer A, Kramer S, Duncker S, Kruger M, Manns MP, Bischoff SC. Effect of oral lactulose on clinical and immunohistochemical parameters in patients with inflammatory bowel disease: a pilot study. *Bmc Gastroenterol.* (2007) 7:36. doi: 10.1186/1471-230X-7-36
186. Rose DJ, Venema K, Keshavarzian A, Hamaker BR. Starch-entrapped microspheres show a beneficial fermentation profile and decrease in potentially harmful bacteria during *in vitro* fermentation in faecal microbiota obtained from patients with inflammatory bowel disease. *Br J Nutr.* (2010) 103:1514–24. doi: 10.1017/S0007114509993515
187. Damaskos D, Kolios G. Probiotics and prebiotics in inflammatory bowel disease: microflora 'on the Scope'. *Br J Clin Pharmacol.* (2008) 65:453–67. doi: 10.1111/j.1365-2125.2008.03096.x
188. Larrosa M, Yanez-Gascon MJ, Selma MV, Gonzalez-Sarrias A, Toti S, Ceron JJ, et al. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. *J Agric Food Chem.* (2009) 57:2211–20. doi: 10.1021/jf803638d
189. Oka A, Sartor RB. Microbial-Based and microbial-targeted therapies for inflammatory bowel diseases. *Dig Dis Sci.* (2020) 65:757–88. doi: 10.1007/s10620-020-06090-z
190. Ishikawa H, Matsumoto S, Ohashi Y, Imaoka A, Setoyama H, Umesaki Y, et al. Beneficial effects of probiotic bifidobacterium and galacto-oligosaccharide in patients with ulcerative colitis: a randomized controlled study. *Digestion.* (2011) 84:128–33. doi: 10.1159/000322977
191. Rufino MN, Da CA, Jorge EN, Paiano VF, Camparoto ML, Keller R, et al. Synbiotics improve clinical indicators of ulcerative colitis: systematic review with meta-analysis. *Nutr Rev.* (2021) nuab017. doi: 10.1093/nutrit/nuab017
192. Dore MP, Bibbo S, Fresi G, Bassotti G, Pes GM. Side effects associated with probiotic use in adult patients with inflammatory bowel disease: a systematic review and meta-analysis of randomized controlled trials. *Nutrients.* (2019) 11. doi: 10.3390/nu11122913
193. Chen P, Xu H, Tang H, Zhao F, Yang C, Kwok LY, et al. Modulation of gut mucosal microbiota as a mechanism of probiotics-based adjunctive therapy for ulcerative colitis. *Microb Biotechnol.* (2020) 13:2032–43. doi: 10.1111/1751-7915.13661
194. Yilmaz I, Dolar ME, Ozpinar H. Effect of administering kefir on the changes in fecal microbiota and symptoms of inflammatory bowel disease: a randomized controlled trial. *Turk J Gastroenterol.* (2019) 30:242–53. doi: 10.5152/tjg.2018.18227
195. Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, et al. Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis. *World J Gastroenterol.* (2015) 21:5985–94. doi: 10.3748/wjg.v21.i19.5985
196. Matsuoka K, Uemura Y, Kanai T, Kunisaki R, Suzuki Y, Yokoyama K, et al. Efficacy of bifidobacterium breve fermented milk in maintaining remission of ulcerative colitis. *Dig Dis Sci.* (2018) 63:1910–9. doi: 10.1007/s10620-018-4946-2
197. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol.* (2011) 9:356–68. doi: 10.1038/nrmicro2546
198. Chen F, Wen Q, Jiang J, Li HL, Tan YF, Li YH, et al. Could the gut microbiota reconcile the oral bioavailability conundrum of traditional herbs? *J Ethnopharmacol.* (2016) 179:253–64. doi: 10.1016/j.jep.2015.12.031
199. Swanson HI. Drug metabolism by the host and gut microbiota: a partnership or rivalry? *Drug Metab Dispos.* (2015) 43:1499–504. doi: 10.1124/dmd.115.065714
200. Xie W, Gu D, Li J, Cui K, Zhang Y. Effects and action mechanisms of berberine and rhizoma coptidis on gut microbes and obesity in high-fat diet-fed C57BL/6J mice. *Plos ONE.* (2011) 6:E24520. doi: 10.1371/journal.pone.0024520
201. Zhang X, Zhao Y, Zhang M, Pang X, Xu J, Kang C, et al. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. *Plos ONE.* (2012) 7:E42529. doi: 10.1371/journal.pone.0042529
202. Yang Y, Chen G, Yang Q, Ye J, Cai X, Tsering P, et al. Gut microbiota drives the attenuation of dextran sulphate sodium-induced colitis by Huangqin Decoction. *Oncotarget.* (2017) 8:48863–74. doi: 10.18632/oncotarget.16458
203. Sun Z, Li J, Dai Y, Wang W, Shi R, Wang Z, et al. Indigo naturalis alleviates dextran sulfate sodium-induced colitis in rats via altering gut microbiota. *Front Microbiol.* (2020) 11:731. doi: 10.3389/fmicb.2020.00731
204. Gao W, Guo Y, Wang C, Lin Y, Yu L, Sheng T, et al. Indirubin ameliorates dextran sulfate sodium-induced ulcerative colitis in mice through the inhibition of inflammation and the induction of Foxp3-Expressing Regulatory T Cells. *Acta Histochem.* (2016) 118:606–14. doi: 10.1016/j.acthis.2016.06.004
205. Liu Q, Zuo R, Wang K, Nong FF, Fu YJ, Huang SW, et al. Oroxindin Inhibits Macrophage NLRP3 Inflammasome Activation in DSS-Induced Ulcerative Colitis in Mice via Suppressing TXNIP-Dependent NF-KappaB Pathway. *Acta Pharmacol Sin.* (2020) 41:771–81. doi: 10.1038/s41401-019-0335-4
206. Gao Z, Yu C, Liang H, Wang X, Liu Y, Li X, et al. Andrographolide derivative CX-10 Ameliorates dextran sulphate sodium-induced ulcerative colitis in mice: involvement of NF-KappaB and MAPK Signalling Pathways. *Int Immunopharmacol.* (2018) 57:82–90. doi: 10.1016/j.intimp.2018.02.012
207. Li J, Zhang JL, Gong XP, Xiao M, Song YY, Pi HF, et al. Anti-inflammatory activity of Mollugin on DSS-induced colitis in mice. *Curr Med Sci.* (2020) 40:910–6. doi: 10.1007/s11596-020-2262-5
208. Wang X, Li D, Zhang Y, Wu S, Tang F. Costus root granules improve ulcerative colitis through regulation of TGF-Beta Mediation of the PI3K/AKT signaling pathway. *Exp Ther Med.* (2018) 15:4477–84. doi: 10.3892/etm.2018.5946
209. Liang J, Chen S, Chen J, Lin J, Xiong Q, Yang Y, et al. Therapeutic roles of polysaccharides from Dendrobium Officinaleon colitis and its underlying mechanisms. *Carbohydr Polym.* (2018) 185:159–68. doi: 10.1016/j.carbpol.2018.01.013
210. Ponnusamy K, Ramasamy M, Savarimuthu I, Paulraj MG. Indirubin potentiates ciprofloxacin activity in the nora efflux pump of Staphylococcus aureus. *Scand J Infect Dis.* (2010) 42:500–5. doi: 10.3109/00365541003713630

211. Yang YZ, Tang YZ, Liu YH. Wogonoside displays anti-inflammatory effects through modulating inflammatory mediator expression using RAW264.7 cells. *J Ethnopharmacol.* (2013) 148:271–6. doi: 10.1016/j.jep.2013.04.025
212. Moldoveanu AC, Diculescu M, Braticevici CF. Cytokines in inflammatory bowel disease. *Rom J Intern Med.* (2015) 53:118–27. doi: 10.1515/rjim-2015-0016
213. Thorsteinsdottir S, Gudjonsson T, Nielsen OH, Vainer B, Seidelin JB. Pathogenesis and biomarkers of carcinogenesis in ulcerative colitis. *Nat Rev Gastroenterol Hepatol.* (2011) 8:395–404. doi: 10.1038/nrgastro.2011.96
214. Pittayanon R, Lau JT, Leontiadis GI, Tse F, Yuan Y, Surette M, et al. Differences in gut microbiota in patients with vs without inflammatory bowel diseases: a systematic review. *Gastroenterology.* (2020) 158:930–46. doi: 10.1053/j.gastro.2019.11.294
215. Jia D, Dou Y, Li Z, Zhou X, Gao Y, Chen K, et al. Design, synthesis and evaluation of a baicalin and berberine hybrid compound as therapeutic agent for ulcerative colitis. *Bioorg Med Chem.* (2020) 28:115697. doi: 10.1016/j.bmc.2020.115697
216. Tang S, Liu W, Zhao Q, Li K, Zhu J, Yao W, et al. Combination of polysaccharides from astragalus membranaceus and codonopsis pilosula ameliorated mice colitis and underlying mechanisms. *J Ethnopharmacol.* (2021) 264:113280. doi: 10.1016/j.jep.2020.113280
217. Huang C, Dong J, Jin X, Ma H, Zhang D, Wang F, et al. Intestinal anti-inflammatory effects of Fuzi-Ganjiang herb pair against DSS-induced ulcerative colitis in mice. *J Ethnopharmacol.* (2020) 261:112951. doi: 10.1016/j.jep.2020.112951
218. Wang JR, Tanaka T, Zhang H, Kouno I, Jiang ZH. Formation and conformation of baicalin-berberine and wogonoside-berberine complexes. *Chem Pharm Bull.* (2012) 60:706–11. doi: 10.1248/cpb.60.706
219. Lu X, Zhang L, Li P, Wang J, Li R, Huang Y, et al. The protective effects of compatibility of Aconiti Lateralis Radix Praeparata and Zingiberis Rhizoma on rats with heart failure by enhancing mitochondrial biogenesis via Sirt1/PGC-1alpha pathway. *Biomed Pharmacother.* (2017) 92:651–60. doi: 10.1016/j.biopha.2017.05.117
220. Zong X, Yan X, Wu JL, Liu Z, Zhou H, Li N, et al. Potentially cardiotoxic diterpenoid alkaloids from the roots of aconitum carmichaelii. *J Nat Prod.* (2019) 82:980–9. doi: 10.1021/acs.jnatprod.8b01039
221. Chen G, Yang Y, Liu M, Teng Z, Ye J, Xu Y, et al. Banxia Xiexin decoction protects against dextran sulfate sodium-induced chronic ulcerative colitis in mice. *J Ethnopharmacol.* (2015) 166:149–56. doi: 10.1016/j.jep.2015.03.027
222. Chen YL, Zheng YY, Dai YC, Zhang YL, Tang ZP. Systems pharmacology approach reveals protective mechanisms of Jian-Pi Qing-Chang decoction on ulcerative colitis. *World J Gastroenterol.* (2019) 25:2603–22. doi: 10.3748/wjg.v25.i21.2603
223. Luo S, Wen R, Wang Q, Zhao Z, Nong F, Fu Y, et al. Rhubarb peony decoction ameliorates ulcerative colitis in mice by regulating gut microbiota to restoring Th17/Treg Balance. *J Ethnopharmacol.* (2019) 231:39–49. doi: 10.1016/j.jep.2018.08.033
224. Chao L, Li Z, Zhou J, Chen W, Li Y, Lv W, et al. Shen-Ling-Bai-Zhu-San Improves Dextran Sodium Sulfate-Induced Colitis by Inhibiting Caspase-1/Caspase-11-Mediated Pyroptosis. *Front Pharmacol.* (2020) 11:814. doi: 10.3389/fphar.2020.00814
225. Zhang Z, Cao H, Shen P, Liu J, Cao Y, Zhang N. Ping Weisan Alleviates Chronic Colitis in Mice by Regulating Intestinal Microbiota Composition. *J Ethnopharmacol.* (2020) 255:112715. doi: 10.1016/j.jep.2020.112715
226. Hu J, Huang H, Che Y, Ding C, Zhang L, Wang Y, et al. Qingchang Huashi Formula Attenuates DSS-induced colitis in mice by restoring gut microbiota-metabolism homeostasis and goblet cell function. *J Ethnopharmacol.* (2020) 266:113394. doi: 10.1016/j.jep.2020.113394
227. Mao TY, Shi R, Zhao WH, Guo Y, Gao KL, Chen C, et al. Qingchang Wenzhong Decoction ameliorates dextran sulphate sodium-induced ulcerative colitis in rats by downregulating the IP10/CXCR3 axis-mediated inflammatory response. *Evid Based Complement Alternat Med.* (2016) 2016:4312538. doi: 10.1155/2016/4312538
228. Wu ZC, Zhao ZL, Deng JP, Huang JT, Wang YF, Wang ZP. Sanhuang Shu'ai decoction alleviates DSS-induced ulcerative colitis via regulation of gut microbiota, inflammatory mediators and cytokines. *Biomed Pharmacother.* (2020) 125:109934. doi: 10.1016/j.biopha.2020.109934
229. Yuan Z, Yang L, Zhang X, Ji P, Hua Y, Wei Y. Huang-Lian-Jie-Du decoction ameliorates acute ulcerative colitis in mice via regulating NF-KappaB and Nrf2 signaling pathways and enhancing intestinal barrier function. *Front Pharmacol.* (2019) 10:1354. doi: 10.3389/fphar.2019.01354
230. Shao J, Liu Z, Wang L, Song Z, Chang H, Han N, et al. Screening of the optimized prescription from Suqingwan in terms of its therapeutic effect on DSS-induced ulcerative colitis by its regulation of inflammatory and oxidative mediators. *J Ethnopharmacol.* (2017) 202:54–62. doi: 10.1016/j.jep.2017.03.006
231. Munyaka PM, Rabbi MF, Khafipour E, Ghia JE. Acute dextran sulfate sodium (DSS)-induced colitis promotes gut microbial dysbiosis in mice. *J Basic Microbiol.* (2016) 56:986–98. doi: 10.1002/jobm.201500726
232. Wen J, Teng B, Yang P, Chen X, Li C, Jing Y, et al. The potential mechanism of Bawei Xileisan in the treatment of dextran sulfate sodium-induced ulcerative colitis in mice. *J Ethnopharmacol.* (2016) 188:31–8. doi: 10.1016/j.jep.2016.04.054
233. Sheng ZS, Gou LQ, Hong S, Kai Z, Bai Y. Consensus on diagnosis and treatment of ulcerative colitis by traditional Chinese medicine. *Chinese Journal of Integrated Traditional and Western Medicine.* (2010) 30:527–32.
234. Zhang YL, Cai LT, Qi JY, Lin YZ, Dai YC, Jiao N, et al. Gut microbiota contributes to the distinction between two traditional Chinese medicine syndromes of ulcerative colitis. *World J Gastroenterol.* (2019) 25:3242–55. doi: 10.3748/wjg.v25.i25.3242
235. JIA Yuan WZ, Zhen LX, Min MM, San. Data mining of drug use rules based on chinese medicine treatment of ulcerative colitis. *China Journal of Chinese Materia Medica.* (2020) 1–8. doi: 10.19540/J.CNKI.CJCMM.20200911.501
236. Ren H, Ting ZY, Jiang ZW, Fang WH, Sheng SX. Analysis of the medication rule and mechanism of patent-prescription of Chinese medicine for ulcerative colitis based on patent database. *Chin J Hosp Pharm.* (2020) 40:1811–5. doi: 10.13286/j.1001-5213.2020.17.04
237. Dai YC, Zhang YL, Wang LJ, Guo Q, Yang K, Ye RH, et al. Clinical presentation and treatment strategies for ulcerative colitis: a retrospective study of 247 inpatients. *Chin J Integr Med.* (2016) 22:811–6. doi: 10.1007/s11655-015-2118-1
238. Tang T, Targan SR, Li ZS, Xu C, Byers VS, Sandborn WJ. Randomised clinical trial: herbal extract HMPL-004 in active ulcerative colitis - a double-blind comparison with sustained release mesalazine. *Aliment Pharmacol Ther.* (2011) 33:194–202. doi: 10.1111/j.1365-2036.2010.04515.x
239. Gong Y, Zha Q, Li L, Liu Y, Yang B, Liu L, et al. Efficacy and safety of fufangkushen colon-coated capsule in the treatment of ulcerative colitis compared with mesalazine: a double-blinded and randomized study. *J Ethnopharmacol.* (2012) 141:592–8. doi: 10.1016/j.jep.2011.08.057
240. Sugimoto S, Naganuma M, Kiyohara H, Arai M, Ono K, Mori K, et al. Clinical efficacy and safety of Oral Qing-Dai in patients with ulcerative colitis: a single-center open-label prospective study. *Digestion.* (2016) 93:193–201. doi: 10.1159/000444217

241. Dai YC, Zheng L, Zhang YL, Chen X, Chen DL, Tang ZP. Effects of Jianpi Qingchang decoction on the quality of life of patients with ulcerative colitis: a randomized controlled trial. *Medicine. (Baltimore).* (2017) 96:E6651. doi: 10.1097/MD.0000000000006651

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.

frontiers | Frontiers in Medicine

CORRECTION
published: 10 May 2022
doi: 10.3389/fmed.2022.886105

Corrigendum: The Communication Between Intestinal Microbiota and Ulcerative Colitis: An Exploration of Pathogenesis, Animal Models, and Potential Therapeutic Strategies

Yu Hu[1†], Zhen Ye[1†], Mingquan Wu[2], Yingqi She[1], Linzhen Li[3], Yujie Xu[4], Kaihua Qin[5], Zhipeng Hu[3], Maoyi Yang[3], Fating Lu[1] and Qiaobo Ye[1]*

[1] School of Basic Medical Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2] Department of Pharmacy, Sichuan Provincial Orthopedic Hospital, Chengdu, China, [3] Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, China, [4] Guangzhou University of Chinese Medicine, Guangzhou, China, [5] Health Preservation and Rehabilitation College, Chengdu University of Traditional Chinese Medicine, Chengdu, China

Keywords: ulcerative colitis, pathogenesis, intestinal microbiota, animal model, treatment strategy, fecal microbiota transplantation, probiotic, traditional Chinese medicine

OPEN ACCESS

Approved by:
Frontiers Editorial Office, Frontiers Media SA, Switzerland

***Correspondence:**
Qiaobo Ye
yeqiaobo@cdutcm.edu.cn

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *28 February 2022*
Accepted: *28 April 2022*
Published: *10 May 2022*

Citation:
Hu Y, Ye Z, Wu M, She Y, Li L, Xu Y, Qin K, Hu Z, Yang M, Lu F and Ye Q (2022) Corrigendum: The Communication Between Intestinal Microbiota and Ulcerative Colitis: An Exploration of Pathogenesis, Animal Models, and Potential Therapeutic Strategies. Front. Med. 9:886105. doi: 10.3389/fmed.2022.886105

A Corrigendum on

The Communication Between Intestinal Microbiota and Ulcerative Colitis: An Exploration of Pathogenesis, Animal Models, and Potential Therapeutic Strategies
by Hu, Y., Ye, Z., Wu, M., She, Y., Li, L., Xu, Y., Qin, K., Hu, Z., Yang, M., Lu, F., and Ye, Q. (2021). Front. Med. 8:766126. doi: 10.3389/fmed.2021.766126

There is an error in the Funding statement. The Funding number for "the National Natural Science Foundation of China" was erroneously given as "8197151950." The correct number is "81973742".

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

frontiers in Medicine

ORIGINAL RESEARCH
published: 13 December 2021
doi: 10.3389/fmed.2021.789037

Nicotine Oral Administration Attenuates DSS-Induced Colitis Through Upregulation of Indole in the Distal Colon and Rectum in Mice

Akihito Nakajima[1], Tomoyoshi Shibuya[1], Takashi Sasaki[2], Yu Jie Lu[3], Dai Ishikawa[1], Keiichi Haga[1], Masahito Takahashi[1], Naoko Kaga[4], Taro Osada[1], Nobuhiro Sato[1] and Akihito Nagahara[1]*

[1] Department of Gastroenterology, Juntendo University, School of Medicine, Tokyo, Japan, [2] Animal Research Center, Sapporo Medical University School of Medicine, Sapporo, Japan, [3] Center of Excellence for Infection Control Science, Graduate School of Medicine, Juntendo University, Tokyo, Japan, [4] Laboratory of Proteomics and Biomolecular Science, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan

Nicotine affects the gastrointestinal environment and modulates ulcerative colitis (UC). However, the associations among nicotine, gut metabolites, and UC are still largely unknown. We investigated whether orally administered nicotine affected gut metabolites and dextran sodium sulfate (DSS)-induced colitis. C57BL/6 male mice were orally administered nicotine solution in drinking water prior to inducing DSS-induced colitis. Short-chain fatty acids (SCFAs) and indole in gut contents and fecal samples were measured by GC-MS and hydroxylamine-based indole assays, respectively. Oral administration of nicotine increased indole concentration in feces, but, in contrast, SCFA values did not differ with nicotine administration. Indole levels were increased in the distal colon and rectum but not in the cecum and proximal colon. DSS-induced colitis was less severe clinically and histological changes were minimal in the rectum of orally nicotine-administered mice compared to mice drinking only water. 16S rRNA microbiome on the feces revealed an increasing in *Clostridium* and *Porphyromonas* in nicotine-administered mice. In conclusion, nicotine administration was associated with increased indole levels in the distal colon and rectum and attenuated DSS-induced colitis. Oral administration of nicotine may play a potential role in indole upregulation and prevention of UC.

Keywords: nicotine, ulcerative colitis, indole, *Clostridium*, *Porphyromonas*, mice

OPEN ACCESS

Edited by:
Abbas Yadegar, Shahid Beheshti University of Medical Sciences, Iran

Reviewed by:
Atsushi Nakajima, Yokohama City University, Japan
Kan Uchiyama, Jikei University Kashiwa Hospital, Japan

***Correspondence:**
Tomoyoshi Shibuya
tomoyosi@juntendo.ac.jp

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *04 October 2021*
Accepted: *17 November 2021*
Published: *13 December 2021*

Citation:
Nakajima A, Shibuya T, Sasaki T, Lu YJ, Ishikawa D, Haga K, Takahashi M, Kaga N, Osada T, Sato N and Nagahara A (2021) Nicotine Oral Administration Attenuates DSS-Induced Colitis Through Upregulation of Indole in the Distal Colon and Rectum in Mice. Front. Med. 8:789037. doi: 10.3389/fmed.2021.789037

INTRODUCTION

That gut microbiota and its metabolites are involved in the immune response in the gut has been suggested (1, 2). Alterations of microbiota and metabolites are closely linked to many conditions including inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC). Dietary intake and cigarette smoking are major environmental factors that impact the gut microbiota and their metabolites (3–5). Cigarette smoking is considered a risk factor for many diseases such as lung cancer, heart diseases, and stroke. However, exceptionally, it has a protective effect against UC (6).

Nicotine is the main compound in cigarette products. Studies of nicotine and UC have suggested that nicotine has an anti-inflammatory effect in the gut (3, 7). However, why nicotine is protective

against UC is still largely unknown, and the effect of nicotine on gut metabolites remains elusive.

Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are end-products of microbial fermentation and influence host physiology (8). Previous studies demonstrated that a soluble high fiber diet increased SCFA levels in the gut and attenuated DSS-induced colitis in mice (9, 10). That SCFAs provide energy to intestinal epithelial cells and affect the gut's immune system by inducing regulatory T (T_{reg}) cells in the gut were shown (11, 12). In addition, we previously demonstrated that maternal SCFAs promoted the development of T_{reg} cells in offspring (13, 14). As SCFAs play many crucial roles in the immune system, it is important to study whether nicotine is involved in SCFA production and protects against colitis.

Indole is a gut bacterial metabolite derived from tryptophan (Trp). Indole is the most abundant Trp metabolite and plays a crucial role in the intestinal immune system (15, 16). Various levels of Trp metabolites are associated with UC disease activity (17). Additionally, indole compounds have been used for the treatment for UC (18). Although indole plays a pivotal role in the gut, it is not clear whether nicotine influences indole production in the gut. In this study, we aimed to investigate whether the oral administration of nicotine influenced gut metabolites such as SCFAs and indole and also examined if such administration had an influence on dextran sodium sulfate (DSS)-induced colitis.

RESULTS

Oral Administration of Nicotine Increased Fecal Indole Concentration and Attenuated DSS-Induced Colitis

To address the effect of nicotine on gut metabolites, wild-type (WT) mice were orally administered 20 μg/ml of nicotine solution dissolved in drinking water for 3 days, after which levels of indole and SCFAs in feces were analyzed. Previous studies demonstrated that low doses of oral nicotine prevented DSS-induced colitis (19, 20). Indole concentrations in feces were detected by the Hydroxylamine-based indole assay (HIA) method (21). The mean level of indole in feces of WT mice that were provided only deionized distilled water (DDW) was 1.16 mM and that in nicotine-administered mice was 1.77 mM (**Figure 1B**), suggesting that oral administration of nicotine significantly increased the fecal indole level. In contrast, fecal levels of SCFAs, such as acetate, propionate, and butyrate, did not differ between mice drinking only DDW and nicotine-administered mice (**Figures 1C–E**). This result suggested that oral administration of nicotine affected fecal levels of indole but not SCFAs. DSS-induced colitis is considered to be a mouse model of UC; thus, DSS was orally administered for 7 days to mice drinking only DDW and nicotine-administered mice subsequent to the oral administration of nicotine to examine the effect of nicotine on this mouse model of UC (**Figure 1A**). DSS-induced colitis in mice orally administered nicotine significantly decreased the percentage of body weight loss on Day 7 (**Figure 1F**). The mean intestinal length of the DDW group was significantly shorter than the mean intestinal length of the nicotine group (**Figure 1G**). Furthermore, clinical signs such as weight loss, stool consistency, and bleeding were scored as the disease activity index (DAI). DAI scores in nicotine-administered mice were significantly lower than in mice drinking only water at Day 7 (**Figure 1H**). Histological analysis of the rectum also showed that infiltration of lymphocytes and the damage to crypts in nicotine-administered mice were less than in mice drinking only DDW (**Figure 1I**).

Those results suggested that oral administration of nicotine attenuated DSS-induced colitis and that increased indole values might be associated with the protective effect of nicotine administration on DSS-induced colitis.

Levels of Indole Were Increased in the Distal Colon and Rectum

Next, we further analyzed levels of indole in the gut according to oral administration of nicotine. WT mice were orally administered DDW or nicotine solution dissolved in drinking water to nicotine-administered mice for 3 days (**Figure 2A**). Indole concentration in the cecum and the entire colon divided into the proximal colon, distal colon, and rectum was analyzed in both mice drinking only DDW and nicotine-administered mice (**Figure 2B**). Indole levels in the cecum and proximal colon did not differ between mice drinking only DDW and nicotine-administered mice (**Figures 2C,D**) but, interestingly, indole levels in the distal colon and rectum were significantly increased in nicotine-administered mice (**Figures 2E,F**). In the mice drinking only DDW group, mean indole concentrations in the cecum, proximal colon, distal colon, rectum, and feces varied from 0.77, 0.68, 0.90, 1.22, and 1.16 mM, respectively (**Figures 1B**, **2C–F**). Indole concentration in the rectum was almost twice as high as in the proximal colon in mice drinking only DDW. In the rectum, mean indole concentrations in mice drinking only DDW and nicotine-administered mice were 1.21 and 1.61 mM, respectively (**Figure 2F**). Thus, indole concentration was 1.5-fold higher in the rectum in the nicotine-administered mice than in mice drinking only DDW.

The Abundance of Clostridium and Porphyromonas Were Significantly Increased in the Nicotine-Administered Mice

Then, we analyzed the composition of the gut microbiota of feces by 16S rRNA microbiome method to reveal the association of nicotine, indole and the gut microbiota in mice drinking only DDW and nicotine-administered mice (**Figure 3A**). *Clostridium* and *Porphyromonas* were significantly increased ($p < 0.05$, a Wilcoxon rank sum test) in the nicotine-administered mice compared to DDW mice (**Figure 3B**).

Overall, production of indole was higher in the distal colon and rectum than in the cecum and proximal colon. Thus, oral administration of nicotine appeared to promote indole production in the distal colon and rectum. The indole level in the rectum and feces was associated with the severity of DSS-induced colitis.

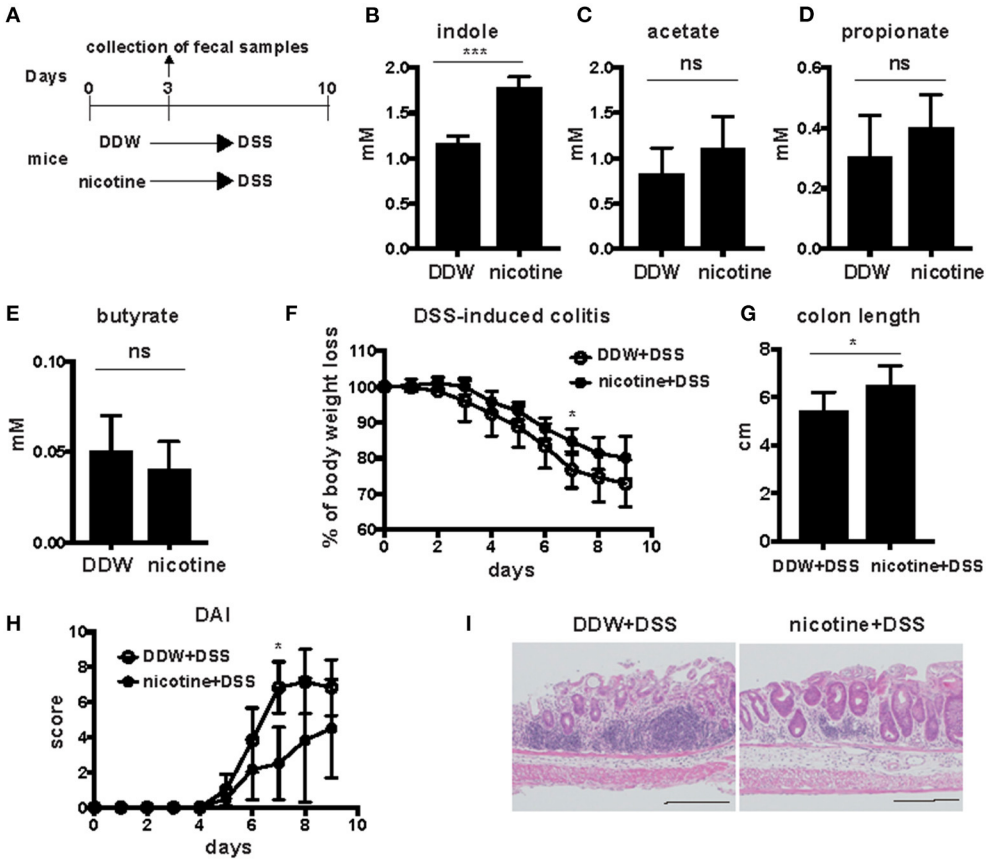


FIGURE 1 | Twenty microgram/milliliter of orally administered nicotine increased indole concentration and attenuated DSS-induced colitis. **(A)** Schema of the experiments in this figure. DDW or 20 μg/ml of nicotine dissolved in drinking water was orally administered for 3 days. Fecal samples were collected at day 3. Then, 2.5% DSS was orally administered in drinking water for 7 days to mice drinking only DDW and nicotine-administered mice. DSS drinking water was switched to DDW for the next 2 days. **(B–E)** Indole levels in feces from mice drinking only DDW and nicotine-administered mice were measured by the HIA method. $N = 5$ mice per group. Data are shown as mean ± SD. $^{***}P < 0.001$. Acetate **(C)**, propionate **(D)**, and butyrate **(E)** in feces of mice drinking only DDW and nicotine-administered mice were measured by GC-MS. Concentrations of SCFAs were the average of two runs. $N = 7$ mice per group. ns; not significant. **(F–I)** Body weight **(F)** was measured every day and colon length **(G)** was measured at Day 9. $N = 5$ mice per group. Data are presented as mean ± SD. $^{*}P < 0.05$. **(H)** The DAIs of mice drinking only DDW and nicotine-administered mice were scored in total for the following categories: weight loss, 0, no loss; 1, 5–10% loss; 2, 10–15% loss; 3, 15–20% loss; and 4, 20% weight loss; for stool consistency,: 0, normal; 1, mild loose stool; 2, moderate loose stool; and 3, diarrhea; for bleeding, 0, no blood; 1, presence of blood; and 2, gross blood. $N = 5$ mice per group. Data are presented as mean ± SD. $^{*}P < 0.05$. **(I)** Histological analysis of representative sections of the rectum with H&E staining from mice drinking only DDW and nicotine-administered mice after DSS-induced colitis. Black bars in lower right side indicated 200 μm.

DISCUSSION

In this study, we revealed an association of indole with the severity of DSS-induced colitis in mice orally administered nicotine. We detected major metabolites, SCFAs, and indole in gut contents and feces of orally administered nicotine mice and showed that indole levels in the rectum and feces were increased in nicotine-administered mice and that the severity of DSS-induced colitis was attenuated. Indole is the most abundant bacterial-derived Trp catabolite. It has been reported that low levels of Trp metabolites are a risk factor for and are associated with inflammatory bowel diseases (IBD) (17). In fact, serum levels of Trp were lower in IBD patients and Trp-deficient mice manifested severe colitis (17, 22). Trp catabolites, including indole and indole derivatives, mediate microbial signals (23). Indole contributed to epithelial barrier function by increasing certain molecules involved in tight junctions and adherence junctions (15, 16). It was reported that indole concentration was decreased in germ-free (GF) mice; however indole treatment enhanced associated molecules of tight junctions and adherence junctions, and attenuated DSS-induced colitis in GF mice (15). This indicated that upregulation of indole resulted in strengthening of epithelial barrier function and tight junctions in mice. In line with this result, we demonstrated here that upregulated indole in the distal colon and rectum through oral administration of nicotine strengthened barrier function. We observed attenuated DSS-induced colitis in the nicotine-administered mice (**Figures 1F–H**). Histological analysis revealed that colonic damage, such as loss of crypts and goblet cells and infiltration of inflammatory cells, was less severe in the rectum of such mice (**Figure 1I**). In the rectum, increased indole attenuated DSS-induced colitis, suggesting that

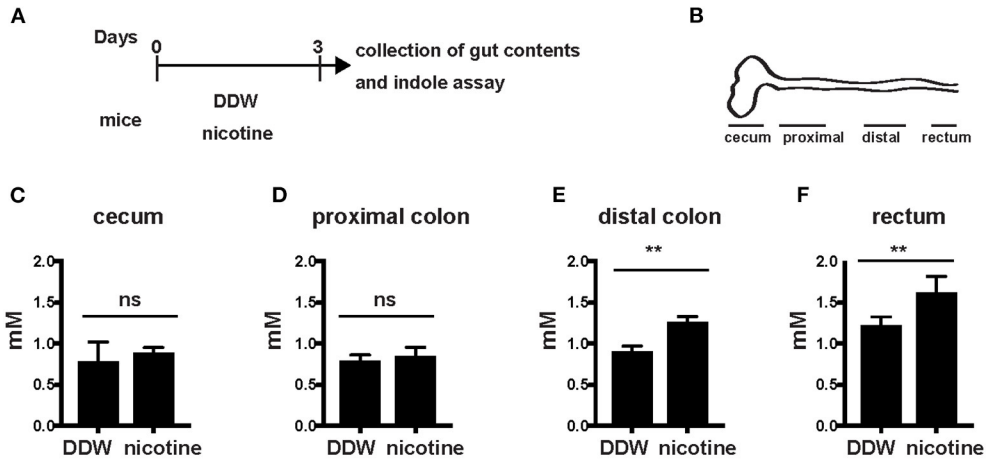


FIGURE 2 | Indole levels in the distal colon and rectum were increased in nicotine- administered mice. **(A)** Schema of the experiments in this figure. DDW or 20 μg/ml of nicotine was orally administered in drinking water for 3 days. Then, mice were sacrificed and the gut contents from the cecum, proximal colon, distal colon, and rectum were collected at day 3. **(B)** Schema of the colon divided into the cecum, proximal colon, distal colon, and rectum. **(C–F)** Gut contents of the cecum, proximal colon, distal colon, and rectum from mice drinking only DDW and nicotine-administered mice were collected and indole concentrations in the cecum **(B)**, proximal colon **(C)**, distal colon **(D)**, and rectum **(E)** were measured by the HIA method. $N = 5$ mice, per group. Data are shown as mean ± SD. $^{**}P < 0.01$. ns, not significant.

increased indole by nicotine administration attenuated UC through epithelial barrier function. As previously reported, oral administration of 40 kDa DSS solution, such as used in this study, was associated with more severe colitis in the distal colon and rectum than in the proximal colon (24). Upregulation of indole concentration in the distal colon and rectum may have a protective effect against colorectal damage by DSS-induced colitis.

Trp metabolites including indole and indole derivatives act as ligands of aryl hydrocarbon receptor (AhR). AhR is a transcriptional factor that regulate host immune system. For example, activation of AhR enhances Interleukin-22 production in type 3 innate lymphoid cells, which contributes to epithelial barrier function (25, 26). Based on these studies, AhR pathway may be involved in the attenuation of UC by nicotine administration.

The gut microbiota is closely linked to the gut metabolites. In this study, we showed increased the relative abundance of *Clostridium* and *Porphyromonas* by 16S rRNA microbiome method on fecal samples in the nicotine-administered mice. Previous studies have also demonstrated that cigarette smoking and nicotine treatment affect the composition of the gut microbiota in human and mice (4, 27–30). The relative abundance of *Bacteroides-Prevotella* and the Clostridia family *Veillonellaceae* were higher in current smokers (28, 31). In a mice model, it has been reported that the relative abundance of *Clostridium clostridiforme* was increased (29). According to the previous reports, indole is produced from Trp by tryptophanase encoded *tnaA* gene and some *Clostridium* and *Porphyromonas* species contain the *tnaA* gene and produce indole (23, 32). Increased *Clostridium* and *Porphyromonas* in nicotine-administered mice may contribute to promote the level of indole. Some species of *Bacteroides* and *Prevotella*, as well as *Clostridium* and *Porphyromonus* contained *tnaA* gene are thought to be involved in producing indole (23, 32).

Cigarette smoking is a major risk factor for Crohn's disease; however it is protective against the development and progression of UC (6, 33). UC is a chronic mucosal inflammation in the gut and is sub-classified into the following three types: ulcerative proctitis, left-sided UC, and extensive UC (34). Colonic mucosal inflammation of UC starts in the rectum and extends proximally in a continuous fashion (35). Therefore, to protect against rectal inflammation is a crucial strategy for UC treatment. Notably, we demonstrated here that nicotine affected indole concentrations in the distal colon and rectum rather than in the proximal colon. That orally administered nicotine increased indole in the rectum and attenuated DSS-induced colitis raised the therapeutic possibility of this strategy for UC patients although there might be a limitation to generalize the effect of nicotine.

In conclusion, we propose that increased indole by oral administration of nicotine has a potential role to protect the rectum, which is an important site for UC progression. Indole is a key metabolite and nicotine might have functions to protect against UC through upregulation of indole. Further studies should be required to clarify the mechanism by which nicotine increased indole.

MATERIALS AND METHODS

Mice

C57BL/6J male mice at 10 weeks old were purchased from Japan SLC (Shizuoka, Japan). Mice were housed at the animal facility of Juntendo University (Tokyo, Japan) and fed a normal diet, CRF-1 (Orietal Yeast, Tokyo, Japan). Five or seven mice were housed in each gauge under standard 12 h light/dark cycles, 22 ± 2 degrees, and 50 ± 5 percent humidity. All animal experiments

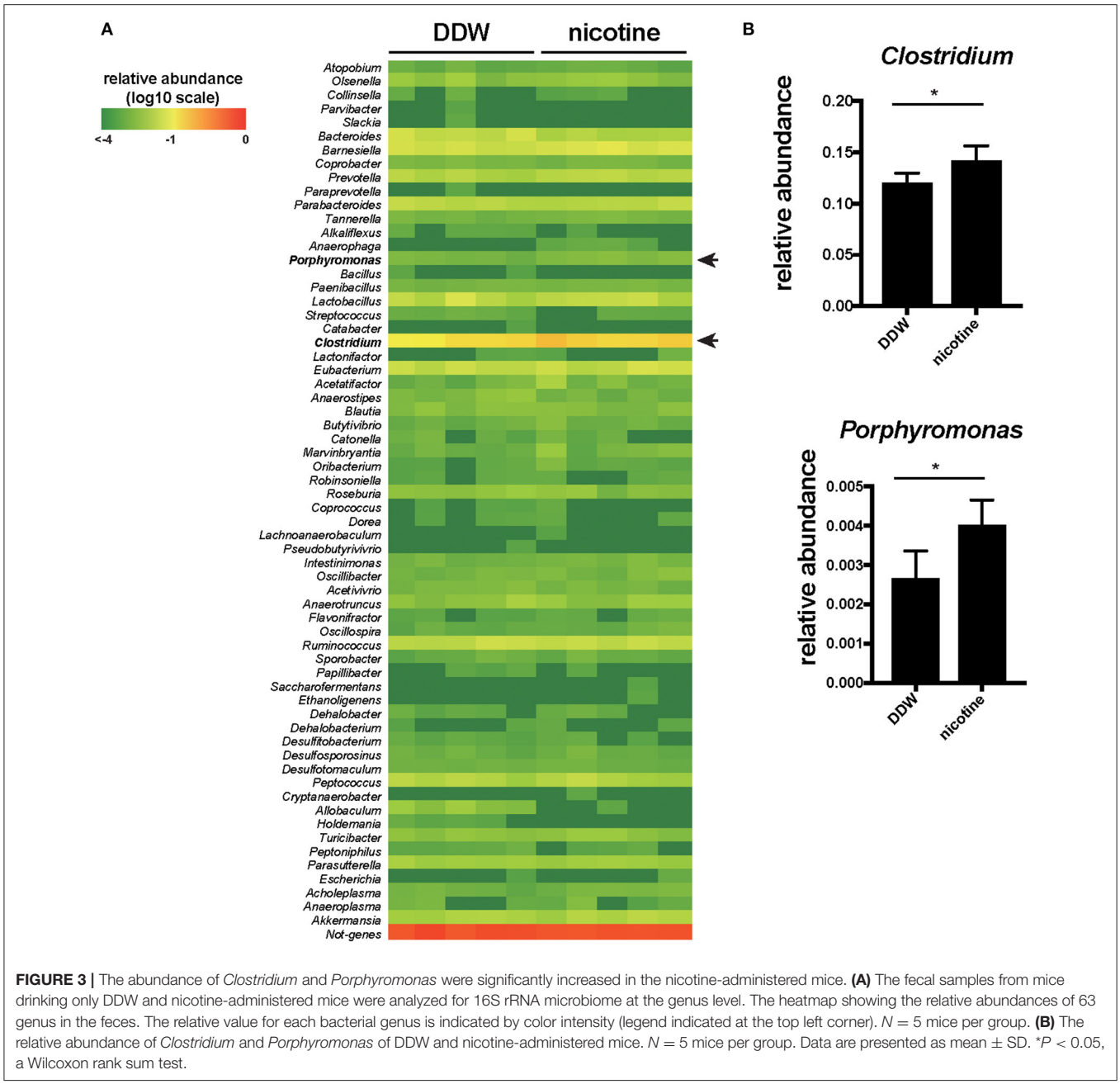


FIGURE 3 | The abundance of *Clostridium* and *Porphyromonas* were significantly increased in the nicotine-administered mice. **(A)** The fecal samples from mice drinking only DDW and nicotine-administered mice were analyzed for 16S rRNA microbiome at the genus level. The heatmap showing the relative abundances of 63 genus in the feces. The relative value for each bacterial genus is indicated by color intensity (legend indicated at the top left corner). $N = 5$ mice per group. **(B)** The relative abundance of *Clostridium* and *Porphyromonas* of DDW and nicotine-administered mice. $N = 5$ mice per group. Data are presented as mean ± SD. $^*P < 0.05$, a Wilcoxon rank sum test.

were approved by the Animal Experimentation Committee of Juntendo University (No. 2021122).

Reagents

(–)-Nicotine hydrogen tartrate salt was purchased from Sigma (St. Louis, MO, USA). Dextran sodium sulfate (M.W 36000-50000) was purchased from MP Biomedicals (Santa Ana, CA, USA).

Hydroxylamine-Based Indole Assay (HIA)

HIA was performed according to a previous paper describing the determination of indole concentration (21). Briefly, gut content samples were diluted with 70% ethanol to 100 mg/ml and disrupted by a Power Masher II (Nippi, Tokyo, Japan). After centrifugation, supernatants were filtered using a Millipore Ultrafree MC PLHCC centrifugal filter (Merck Millipore, Billerica, MA, USA). Samples were incubated for 15 min at room temperature with 5M NaOH (Wako, Osaka, Japan) and 50 μl of 0.3 M hydroxylamine hydrochloride (Wako) in a microtiter plate. Following incubation, 125 μl of 2.5 M H_2SO_4 (Wako) was added and incubated at room temperature for 30 min. The plates were immediately read at 530 nm using optical density readings by a microplate reader.

Measurement of SCFA Concentration in Feces

Hundred milligrams of fresh feces from C57BL/6 male mice were homogenized in 400 μl H_2O containing hexanoic acid (methyl-d_3) as an internal standard. Then 80 μl of 25% metaphosphoric acid was added to the homogenate and kept on ice for 30 min. Thereafter, samples were centrifuged at 17,500 × *g* for 15 min at 4°C. The supernatants were filtered using a Millipore Ultrafree MC PLHCC centrifugal filter (Merck Millipore) and analyzed by gas chromatography-mass spectrometry (GC-MS). One microliter of the sample was injected with a split mode (1:100) into a TRACE GC ULTRA gas chromatograph equipped with a TSQ QUANTUM GC mass spectrometer (ThermoFisher Scientific, Waltham, MA). A Nukol™ fused silica capillary column (0.25 mm ID × 30 m, 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) was used for separation. Column temperature was programmed for 150°C for 2 min, then increased to 200°C at a rate of 8°C/min and held at 200°C for 13 min. Helium was used as a carrier gas at a flow rate of 0.7 ml/min. Data were acquired by the electron impact ionization mode at 70 eV.

Dextran Sulfate Sodium (DSS)-Induced Colitis

C57BL/6J male mice at 10 weeks old were orally administered nicotine [(–)-Nicotine hydrogen tartrate salt, Sigma] in drinking water at 20 μg/ml for 3 days. Then, 2.5% DSS (M.W 36,000–50,000; MP Biomedicals) dissolved in sterile, distilled drinking water was orally administered for 7 days. On day 7, 2.5% was switched to regular drinking water for the next 2 days. Body weight was measured every day. On day 9, mice were sacrificed and colon lengths were measured.

Microbiome Analysis Based on 16S rRNA Amplicon Sequencing

The fecal samples 3 days after DDW and nicotine administration were diluted 10-fold in TE buffer were diluted 10-fold in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and frozen at −80°C until use. Five hundred microliter of each diluted sample was used for DNA extraction. After pretreatment in TE buffer with 50 U of achromopeptidase (Wako) at 50°C for 30 min, phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v) was used for DNA purification.

We performed an Illumina 16S metagenomic sequencing protocol, which targeted the V3 to V4 region of bacterial and archaeal 16S rRNA genes, for comprehensive analysis of the fecal microbiota, following the manufacturer's workflow of 16S Metagenomic Sequencing Library Preparation, recommended by Illumina. We used 16S universal primers without adapter sequences, 16S amplicon PCR forward primer (5′-TCGTCG GCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG-3′) and 16S amplicon PCR reverse primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC TACHVGGGTATCTAATCC-3′). The PCR reaction mixture consisted of two μL of DNA extract in a total volume of 25 μL containing 1× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Boston, MA, USA) and 10 pmol of each primer. Reaction mixtures were thermally cycled once at 95°C for 2 min; then 25–30 times at 95°C for 30 s, 65°C for 30 s, and 72°C for 90 s; and then once at 72°C for 2 min. DNA fragments were analyzed by electrophoresis in TAE buffer on a 1% agarose gel stained with ethidium bromide. The PCR products were then purified using AMPure beads (Beckman Coulter, Inc., CA), according to the manufacturer's protocol. PCR products were uniquely indexed using a Nextera XT Index Kit (Illumina, San Diego, CA, USA). NucleoMag NGS Cleanup and Size Select technology (Macherey-Nagel, D, Germany) was used twice for cleanup and size selection of NGS libraries, according to a protocol for removing adapter dimers. Sequencing was performed using a Miseq reagent kit v3 (600 Cycle) and a paired-end 2× 300-bp cycle run on an Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

MiSeq-read 1 and 2 reads were stitched by FLASH (36). The merged reads were filtered and trimmed by removing bases with quality value (QV) scores of 20 or less and read lengths shorter than 200 bases, and then converted from FASTQ to FASTA format using FASTX toolkit ver. 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit). Analyses of the trimmed sequencing reads were performed using blastn by blast 2.5.0+, with an e^{-10} e-value cutoff (37). Taxonomic classification was performed using MEGAN version 5 (38).

Histological Analysis

Entire colons were cut longitudinally and fixed in 10% formalin and embedded in paraffin. 3-μm sections of the rectum were stained with hematoxylin and eosin (H&E).

Statistical Analysis

Statistical analysis was performed using the Student's *t* test. GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) was used for all statistical calculations. We performed a Wilcoxon rank sum test against bacterial genus exhibiting increase or decrease in relative abundance. A *p*-value of <0.05 was considered statistically significant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ddbj.nig.ac.jp/, DRA012847.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experimentation Committee of Juntendo University (No. 2021122).

AUTHOR CONTRIBUTIONS

ANak and TSh conceived this project. ANak designed the study and performed most experiments. TSa and YL performed 16S rRNA microbiome and analysis of the data. NK performed measurement of SCFAs concentration in feces. TSh, TO, NS, and

ANag supervised the study. ANak and TSh wrote the manuscript. All authors interpreted the data and approved the final version of the manuscript.

FUNDING

This study received funding from Takeda Research Support (No. TKDS20180424018) and research grant from Mitsubishi Tanabe Pharma Corporation (No. MTPS20200527020 and MTPS20190518004). The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We thank the Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, for technical assistance.

REFERENCES

1. Lavelle A, Sokol H. Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol.* (2020) 17:223–7. doi: 10.1038/s41575-019-0258-z
2. Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol.* (2016) 16:341–52. doi: 10.1038/nri.2016.42
3. Berkowitz L, Schultz BM, Salazar GA, Pardo-Roa C, Sebastian VP, Alvarez-Lobos MM, et al. Impact of cigarette smoking on the gastrointestinal tract inflammation: opposing effects in Crohn's Disease and Ulcerative Colitis. *Front Immunol.* (2018) 9:74. doi: 10.3389/fimmu.2018.00074
4. Lee SH, Yun Y, Kim SJ, Lee EJ, Chang Y, Ryu S, et al. Association between cigarette smoking status and composition of gut microbiota: population-based cross-sectional study. *J Clin Med.* (2018) 7:282. doi: 10.3390/jcm7090282
5. Savin Z, Kivity S, Yonath H, Yehuda S. Smoking and the intestinal microbiome. *Arch Microbiol.* (2018) 200:677–84. doi: 10.1007/s00203-018-1506-2
6. Parkes GC, Whelan K, Lindsay JO. Smoking in inflammatory bowel disease: impact on disease course and insights into the aetiology of its effect. *J Crohns Colitis.* (2014) 8:717–25. doi: 10.1016/j.crohns.2014.02.002
7. Lakhan SE, Kirchgessner A. Anti-inflammatory effects of nicotine in obesity and ulcerative colitis. *J Transl Med.* (2011) 9:129. doi: 10.1186/1479-5876-9-129
8. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res.* (2013) 54:2325–40. doi: 10.1194/jlr.R036012
9. Nakajima A, Sasaki T, Itoh K, Kitahara T, Takema Y, Hiramatsu K, et al. A Soluble fiber diet increases bacteroides fragilis group abundance and immunoglobulin a production in the gut. *Appl Environ Microbiol.* (2020) 86:e00405–20. doi: 10.1128/AEM.00405-20
10. Makki K, Deehan EC, Walter J, Backhed F. The impact of dietary fiber on gut microbiota in host health and disease. *Cell Host Microbe.* (2018) 23:705–15. doi: 10.1016/j.chom.2018.05.012
11. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells [Research Support, Non-U.S. Gov't]. *Nature.* (2013) 504:446–50. doi: 10.1038/nature12721
12. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature.* (2013) 504:451–5. doi: 10.1038/nature12726
13. Nakajima A, Kaga N, Nakanishi Y, Ohno H, Miyamoto J, Kimura I, et al. Maternal high fiber diet during pregnancy and lactation influences regulatory T cell differentiation in offspring in mice. *J Immunol.* (2017) 199:3516–24. doi: 10.4049/jimmunol.1700248
14. Nakajima A, Habu S, Kasai M, Okumura K, Ishikawa D, Shibuya T, et al. Impact of maternal dietary gut microbial metabolites on an offspring's systemic immune response in mouse models. *Biosci Microbiota Food Health.* (2020) 39:33–8. doi: 10.12938/bmfh.19-013
15. Shimada Y, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, et al. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. *PLoS One.* (2013) 8:e80604. doi: 10.1371/journal.pone.0080604
16. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci U S A.* (2010) 107:228–33. doi: 10.1073/pnas.0906112107
17. Nikolaus S, Schulte B, Al-Massad N, Thieme F, Schulte DM, Bethge J, et al. Increased tryptophan metabolism is associated with activity of inflammatory bowel diseases. *Gastroenterology.* (2017) 153:1504–1516 e2. doi: 10.1053/j.gastro.2017.08.028
18. Sugimoto S, Naganuma M, Kanai T. Indole compounds may be promising medicines for ulcerative colitis. *J Gastroenterol.* (2016) 51:853–61. doi: 10.1007/s00535-016-1220-2
19. AlSharari SD, Akbarali HI, Abdullah RA, Shahab O, Auttachoat W, Ferreira GA, et al. Novel insights on the effect of nicotine in a murine colitis model. *J Pharmacol Exp Ther.* (2013) 344:207–17. doi: 10.1124/jpet.112.198796
20. Eliakim R, Karmeli F, Cohen P, Heyman SN, Rachmilewitz D. Dual effect of chronic nicotine administration: augmentation of jejunitis and amelioration of colitis induced by iodoacetamide in rats. *Int J Colorectal Dis.* (2001) 16:14–21. doi: 10.1007/s003840000262
21. Darkoh C, Chappell C, Gonzales C, Okhuysen P. A rapid and specific method for the detection of indole in complex biological samples. *Appl Environ Microbiol.* (2015) 81:8093–7. doi: 10.1128/AEM.02787-15
22. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, et al. ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. *Nature.* (2012) 487:477–81. doi: 10.1038/nature11228
23. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. *Nat Commun.* (2018) 9:3294. doi: 10.1038/s41467-018-05470-4
24. Kitajima S, Takuma S, Morimoto M. Histological analysis of murine colitis induced by dextran sulfate sodium of different molecular weights. *Exp Anim.* (2000) 49:9–15. doi: 10.1538/expanim.49.9
25. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity.* (2013) 39:372–85. doi: 10.1016/j.immuni.2013.08.003
26. Parks OB, Pociask DA, Hodzic Z, Kolls JK, Good M. Interleukin-22 signaling in the regulation of intestinal health and disease. *Front Cell Dev Biol.* (2015) 3:85. doi: 10.3389/fcell.2015.00085
27. Nolan-Kenney R, Wu F, Hu J, Yang L, Kelly D, Li H, et al. The association between smoking and gut microbiome in Bangladesh. *Nicotine Tob Res.* (2020) 22:1339–46. doi: 10.1093/ntr/ntz220
28. Prakash A, Peters BA, Cobbs E, Beggs D, Choi H, Li H, et al. Tobacco smoking and the fecal microbiome in a large, multi-ethnic cohort. *Cancer Epidemiol Biomarkers Prev.* (2021) 30:1328–35. doi: 10.1158/1055-9965.EPI-20-1417
29. Wang H, Zhao JX, Hu N, Ren J, Du M, Zhu MJ. Side-stream smoking reduces intestinal inflammation and increases expression of tight junction proteins. *World J Gastroenterol.* (2012) 18:2180–7. doi: 10.3748/wjg.v18.i18.2180
30. Kobayashi T, Fujiwara K. Identification of heavy smokers through their intestinal microbiota by data mining analysis. *Biosci Microbiota Food Health.* (2013) 32:77–80. doi: 10.12938/bmfh.32.77
31. Benjamin JL, Hedin CR, Koutsoumpas A, Ng SC, McCarthy NE, Prescott NJ, et al. Smokers with active Crohn's disease have a clinically relevant dysbiosis of the gastrointestinal microbiota. *Inflamm Bowel Dis.* (2012) 18:1092–100. doi: 10.1002/ibd.21864

32. Lee JH, Lee J. Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev.* (2010) 34:426–44. doi: 10.1111/j.1574-6976.2009.00204.x
33. Gao J, Xu K, Liu H, Liu G, Bai M, Peng C, et al. Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Front Cell Infect Microbiol.* (2018) 8:13. doi: 10.3389/fcimb.2018.00013
34. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut.* (2006) 55:749–53. doi: 10.1136/gut.2005.082909
35. Feuerstein JD, Moss AC, Farraye FA. Ulcerative Colitis. *Mayo Clin Proc.* (2019) 94:1357–73. doi: 10.1016/j.mayocp.2019.01.018
36. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics.* (2011) 27:2957–63. doi: 10.1093/bioinformatics/btr507
37. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. *BMC Bioinformatics.* (2009) 10:421. doi: 10.1186/1471-2105-10-421
38. Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC. Integrative analysis of environmental sequences using MEGAN4. *Genome Res.* (2011) 21:1552–60. doi: 10.1101/gr.120618.111

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 05 January 2022
doi: 10.3389/fmed.2021.755969

Ginger Extract Decreases Susceptibility to Dextran Sulfate Sodium-Induced Colitis in Mice Following Early Antibiotic Exposure

Xinghong Zhou[1†], Xiaoyu Liu[1†], Qiuxing He[1†], Ming Wang[1], Hanqi Lu[2], Yanting You[2], Liqian Chen[2], Jingru Cheng[3], Fei Li[4], Xiuqiong Fu[5], Hiu Yee Kwan[5], Lin Zhou[6] and Xiaoshan Zhao[1,2]*

[1] Department of Traditional Chinese Medicine, Zhujiang Hospital of Southern Medical University, Guangzhou, China, [2] Syndrome Laboratory of Integrated Chinese and Western Medicine, School of Chinese Medicine, Southern Medical University, Guangzhou, China, [3] Department of Nephrology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China, [4] Department of Traditional Chinese Medicine, The Affiliated Ganzhou Hospital of Nanchang University, Ganzhou, China, [5] School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China, [6] Department of Endocrinology, Nanfang Hospital, Southern Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Javier Ochoa-Repáraz, Eastern Washington University, United States

Reviewed by:
Jose Emilio Mesonero, University of Zaragoza, Spain
Ana Isabel Alvarez-Mercado, University of Granada, Spain

***Correspondence:**
Xiaoshan Zhao
zhaoxs0609@163.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *09 August 2021*
Accepted: *23 November 2021*
Published: *05 January 2022*

Citation:
Zhou X, Liu X, He Q, Wang M, Lu H, You Y, Chen L, Cheng J, Li F, Fu X, Kwan HY, Zhou L and Zhao X (2022) Ginger Extract Decreases Susceptibility to Dextran Sulfate Sodium-Induced Colitis in Mice Following Early Antibiotic Exposure. Front. Med. 8:755969. doi: 10.3389/fmed.2021.755969

Background: Intestinal microbial colonization in early life plays a crucial role in immune development and mucosal homeostasis in later years. Antibiotic exposure in early life increases the risk of inflammatory bowel disease (IBD). Ginger acts like a prebiotic and has been used in traditional Chinese medicine for colitis. We investigated the protective effect of ginger against dextran sulfate sodium (DSS)-induced colitis in mice exposed to antibiotic in their early years.

Methods: A weaned mouse model exposed to azithromycin (AZT) for 2 weeks was used to mimic antibiotic exposure in childhood among humans. A diet containing ginger extract was administered to mice for 4 weeks after antibiotic exposure. The susceptibility to DSS-induced colitis was evaluated in terms of weight loss, disease activity index (DAI) score, colon length, colitis biomarkers, and intestinal barrier function. The gut microbiota was analyzed in terms of 16S rRNA levels.

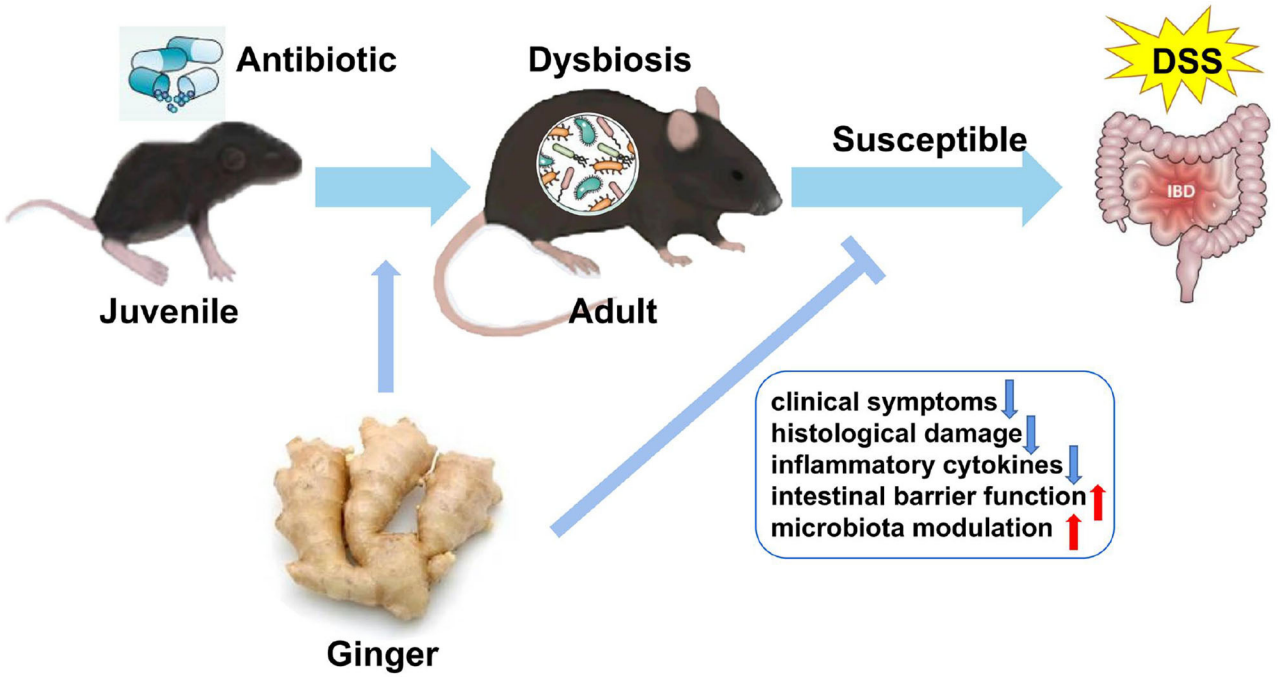
Results: Ginger extract prevented weight loss, colon shortening, inflammation, and intestinal barrier dysfunction in mice exposed to antibiotics in early life. Ginger increased the bacterial diversity and changed the abundance of bacterial belonging to family *Peptococcaceae* and *Helicobacter* species to modulate microbiota structure and composition adversely affected by early antibiotic exposure.

Conclusion: Ginger has a protective effect in potentially decreasing the susceptibility to colitis in mice exposed to antibiotics early in life.

Keywords: ginger, antibiotic, childhood, colitis, microbiota

INTRODUCTION

Inflammatory bowel disease (IBD), mainly including ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronic intestinal inflammation. The incidence and prevalence of IBD have increased worldwide in the recent decades, indicating its emergence as a global public health challenge (1). The lack of adequate and appropriate treatment prompted the search for alternative



GRAPHICAL ABSTRACT | Ginger extract decreases susceptibility to dextran sulfate sodium-induced colitis in mice following early antibiotic exposure.

therapeutic strategies. Although the etiology of IBD is not fully understood, current evidence indicates that a complex interaction among genetic susceptibility, environmental factors, intestinal microbiota, and immune response is involved in the pathogenesis of IBD (2). The role of intestinal microbiota in IBD pathogenesis has recently been highlighted (3).

Antibiotic exposure is one of the most common factors leading to intestinal microbiota dysbiosis. Antibiotic use accounts for a large proportion of children's prescriptions. In the United States, about 44.5 million courses were prescribed to children under the age of 10 years, with the highest prescription rate found in the first 2 years of life. Penicillins and macrolides were the most common antibiotic categories prescribed in children, whereas azithromycin (AZT), the typical drug belonging to the family of macrolides, was the most frequently prescribed antibiotic agent (4, 5).

Early childhood is considered a dynamic phase of the intestinal microbial ecosystem, which is easily shaped by environmental factors. Disruption of microbiota by antibiotic exposure in this sensitive period may be associated with long-term adverse effects (6, 7). Epidemiological and experimental studies have reported an association between early-life exposure to antibiotics and an increased risk of asthma, obesity, and impaired antibody response to vaccination (8–10). A strong association between antibiotic use and CD in childhood was found in a prospective study (11). Peripartum antibiotics promoted gut dysbiosis, immune dysfunction, and IBD in offspring in animal studies (12). Restoration of gut microbial levels reduced the risk of colitis associated with antibiotic-induced gut dysbiosis in mice (13).

Since early antibiotic exposure may increase the susceptibility to diseases later, it is meaningful to identify effective measures to prevent these changes. Ginger (*Zingiber officinale)* rhizomes are not only used as a food but are also a common traditional Chinese medicine administered to treat various diseases. Recent studies demonstrated that ginger and ginger extracts exhibit antiinflammatory effects and regulate intestinal microbiota (14, 15). Novel ginger-derived nanoparticles have been shown to reduce and prevent acute colitis and colitis-associated cancer and enhance intestinal repair (16, 17). Ginger exosome-like nanoparticles (ELNs) are preferentially taken up by specific bacteria, resulting in changes in bacterial composition and localization, and also host physiology, notably enhancing gut barrier function to alleviate colitis (18). These findings suggest the role of ginger in the modulation of microbiota and treatment of IBD. However, the anticolitis activity of ginger against intestinal microbiota in early antibiotic exposure mice has yet to be clearly established.

We hypothesized that early-life antibiotic exposure may have long-term effects in predisposing the host to IBD in later years by altering intestinal microbiota. Administration of ginger after early antibiotic exposure may decrease the susceptibility to IBD. To test this hypothesis, we analyzed the impact of early-life antibiotic exposure on the risk of IBD in later years and tested the regulatory effect of ginger using a juvenile mouse model mimicking childhood antibiotic exposure in humans.

MATERIALS AND METHODS

Chemicals and Materials

Dextran sulfate sodium (DSS, MW:36,000–50,000 Da) was purchased from MP Biomedicals (CA, USA). AZT was purchased from Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). An AZT dose of 50 mg/kg was used in this study to ensure therapeutic plasma levels in the mice; it is the highest dose administered to mice in a multidose regimen (19). Ginger extract (number 1708001W), which meets the quality criteria of granules used in Chinese herbal medicine prescriptions (T-SZ-PC-0390-003), was purchased from China Resources Sanjiu Medical and Pharmaceutical Company (Shenzhen, China). All feeds used in this study were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China). The feeds were sterilized with Co60 (25 kGy) radiation. A standard AIN-93G diet was used as a normal basic diet. Ginger diet was modified from AIN-93G by replacing 10-g corn starch in the original formula with equal amounts of ginger extract per 1 kg (**Table 1**). Fecal occult blood test kit was ordered from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits for assaying the contents of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10) in the colon were purchased from Dakewe Biotech Co., Ltd. (Shenzhen, China). All other reagents used were of analytical grade. Antibodies against zonula occludens 1 (ZO-1) (rabbit, AF5145), claudin-1 (rabbit, AF0127), and β-actin (mouse, BF0198) were purchased from Affinity Biosciences (OH, USA). CD-68 antibody (rat, ab53444) was purchased from Abcam (Cambridge, England).

UPLC-QTOF-MS Analysis of Ginger Extract

The ginger extract was analyzed with ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). The extract was separated on a HSS T3, 1.8 μm (2.1 mm ×100 mm) column and eluted with mobile phases of acetonitrile (A) and 0.25% formic acid in water (B) in gradient mode. The proportion of acetonitrile varied from 20 to 90% in 24 min (0–5 min, 20–30% A; 5–8 min, 30–40% A; 8–10 min, 40–45% A; 10–24 min, 45–90%) at a flow rate of 0.3 ml·min^{-1}, with each injection volume was set to 5 μl. The MS data were acquired on an AB SCIEX Triple TOF 5,600 (AB sciex Pte. Ltd., Singapore). The MS parameters were as follows: interface, negative electrospray ionization (ESI); gases one and two, nitrogen 55 psi; curtain gas, nitrogen 40 psi; source temperature, 400 C; ion spray voltage, 5,500 V; declustering potential, 100 V and collision energy, 45 eV. The Peakview (version 2.0, AB SCIEX) was employed for the analyses. A representative chromatogram of ginger is presented in **Supplementary Figure S1**. A total of 16 components were identified according to their retention times (**Supplementary Table S1**).

Animals

Three-week-old C57BL/6 male mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Mice were maintained under an automated 12-h light–dark cycle at a controlled temperature of 22°C ± 2°C, and a relative humidity of 50–60% with *ad libitum* access to a standard dry diet and tap water. The study was approved by the Standards for Animal Ethics in the Guangzhou Institute of Sport Science (GZTKSGNX-2016-4) and performed in accordance with the relevant experimental animal guidelines and regulations.

Experimental Design

Juvenile C57BL/6J male mice were randomly assigned to four treatment groups (8–10 mice/group) immediately after weaning (**Figure 1**). The control (CTR) group was vehicle-treated and the model (MOD) group was given drinking water containing 2.5% DSS only to induce colitis. The AZT group received 2.5% DSS after the daily administration of AZT (50 mg/kg/day, dissolved in drinking water) for 2 weeks. The ginger (GIN) group received a ginger diet *ad libitum* for 4 weeks after 14 days of AZT exposure and then treated with 2.5% DSS for 7 days to induce colitis. Fecal samples were collected from model, AZT, and GIN groups before the administration of DSS. The samples were stored at −80°C for further analyses. Body weight, stool consistency, and stool bleeding were recorded daily during DSS treatment period.

Evaluation of Disease Activity Index

Body weight, stool consistency, and stool bleeding were recorded daily. Disease activity index (DAI) was determined by dividing the combined scores of body weight loss, stool consistency, and

TABLE 1 | Composition of experimental diets (g).

Ingredient	Normal diet (AIN-93G)	Ginger diet
Corn starch	397.5	387.5
Casein	200	200
Dextrinized corn starch	132	132
Sucrose	100	100
Soybean oil	70	70
Fiber	50	50
AIN-93G-MX	35	35
AIN-93G-VM	10	10
Ginger	0	10
L-Cystine	3	3
Choline chloride	2.5	2.5

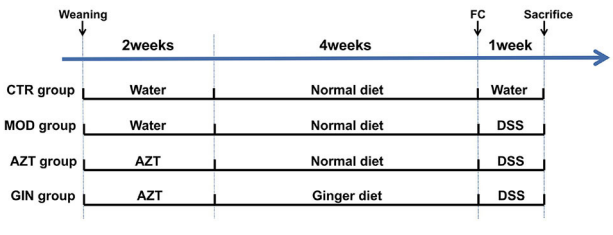


FIGURE 1 | Schematic diagram summarizing the timeline of experimental procedures. Three-week-old C57BL/6 male mice were randomly assigned to four groups (n = 8–10): control (CTR) group; model (MOD) group; AZT group; and ginger (GIN) group. FC, fecal collection.

stool bleeding, by three (**Supplementary Table S2**). Each score was determined as follows: change in body weight loss (0: none, 1: 1–5%, 2: 5–10%, 3: 10–15%, 4: > 15%), stool consistency (0: normal, 1–2: loose, 3–4: diarrhea), and stool bleeding (0: negative, 1: +, 2: ++, 3: + + +, 4: + + ++). Weight loss was calculated as the percent difference between the original body weight (day 0) and the body weight on any particular day. Blood in stool was detected using the fecal occult blood test kit according to the manufacturer's protocols. In addition, the colon length between the caecum and proximal rectum was measured.

Histologic Examination

Paraffin-embedded tissue sections of the colon were stained with H&E for light microscopic examination to assess colon injury and inflammation. A modified combined scoring system (**Supplementary Table S3**) including degree of inflammation (scale of 0–3) and crypt damage (0–4), percentage of inflammation (0–4), and depth of inflammation (0–3) was used to assess colitis induced by DSS. The total score ranged from 0 (normal) to 14 (severe colitis).

Electron Microscopy

Murine colon tissues were fixed in 3% glutaraldehyde, postfixed in 2% osmium tetroxide, and embedded in epoxy resin. Sections were stained with lead citrate and uranyl acetate and were viewed and photographed with an electron microscope (Hitachi, Japan).

Immunohistochemical Staining

Paraffin-embedded colon sections were deparaffinized. After unmasking antigens, colon sections were blocked with 5% bovine serum albumin (BSA) and immunostained with anti-ZO-1 antibody or CD68 overnight at 4°C. Following immunostaining, sections were washed three times with PBS and then incubated with Alexa Fluor 488 (1:200, Beyotime, China) or Alexa Fluor 568 (1:200, Abcam, England) conjugated secondary antibody for 2 h at room temperature in the dark. Sections were then mounted with a medium containing 4,6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining and observed by fluorescence microscopy.

Western Blot Analysis

Colons were excised and washed thoroughly with PBS, homogenized in RIPA buffer containing protease inhibitors, incubated for 20 min at 4°C, and centrifuged for 20 min, 14,000 rpm at 4°C. Protein extracts were isolated from colon tissue. Samples were separated on 10% acrylamide gels and transferred onto polyvinylidenedifluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) BSA in Tris-buffered saline/0.05% Tween-20 (TBST) at room temperature for 2 h in a covered container and incubated overnight at 4°C with primary antibodies in blocking buffer. The next day, membranes were washed with TBST (3*10 min) and incubated with a secondary goat antimouse or goat antirabbit IgG horseradish peroxidase (HRP) antibody (1: 10,000 dilution) diluted in 5% (w/v) dry nonfat milk in TBST for 1 h at room temperature. Finally, membranes were washed with TBST (3*10 min) and detected *via* electrochemiluminescence (ECL). .

Enzyme-Linked Immunosorbent Assays

The levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-10) in the mouse colon were determined with ELISA kits. The intestinal tissues were homogenized on ice in NP40 lysis buffer (Beyotime Biotechnology, China). The homogenates were quantified using the BCA assay (Beyotime Biotechnology, China). Tissue homogenates were collected for the determination of TNF-α, IL-1β, and IL-6 concentrations according to the manufacturers' instructions.

Fecal DNA Extraction and 16S rRNA Gene Amplicon Analysis

Fecal samples from all mice were collected before the administration of DSS and stored at −80°C. Bacterial DNA was extracted from mouse fecal samples using a QIAamp Fast DNA Stool Mini kit (Qiagen, California, USA) according to the manufacturer's protocols. Purity was determined and concentration was calculated. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the primers 338 F:5′- ACTCCTACGGGAGGCAGCAG-3′and 806 R:5′-GGACTACHVGGGTWTCTAAT-3′ Purified amplicons were pooled in equimolar ratios and paired-end sequenced on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database. Raw FASTQ files were demultiplexed, quality-filtered using QIIME (version 1.91). Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE, and chimeric sequences were identified and removed using UCHIME. Phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier against the Silva (SSU117/119)16S rRNA database using a confidence threshold of 70%. All the raw Illumina paired-end read data involved in this study were deposited in the NCBI SRA database under the accession number PRJNA751929. Analysis was performed at each taxonomical level (Phylum and Genus), separately. The Shannon index and Chao1 index were performed to analyze the alpha diversity. The principal coordinate analysis (PCoA) based on weighted UniFrac distance matrices was visualized for the beta diversity analysis. The dominant bacterial community difference between groups was detected using linear discriminant analysis effect size (LEfSe).

Statistical Analyses

Statistical analysis was performed using SPSS 22.0 software. Data are expressed as the mean ± S.D. For parametric variables, differences were analyzed *via* one-way ANOVA and least significant differences (LSD) *post hoc* tests for multiple group comparisons. For nonparametric variables, statistical significance of differences among the groups was tested using the nonparametric the Kruskal–Wallis test, followed by the Mann–Whitney *U* test when p-value is <0.05. A $p < 0.05$ was defined as statistically significant.

RESULTS

Ginger Reduced the Susceptibility to DSS-Induced Colitis in Mice With Early-Life AZT Exposure

To estimate the long-term effect of early-life AZT exposure on DSS-induced colitis in mice and the anticolitic effect of ginger, we assessed body weight loss, DAI scores, and colon length and performed histological analysis to determine susceptibility to colitis in the mouse models.

Compared with the control group, mice with DSS-induced colitis exhibited significant weight loss starting from day 5 until the end of DSS treatment period. Early-life AZT exposure accelerated the loss of body weight from days 5 to 7 when compared to the group treated with DSS only. However, ginger diet attenuated the progression of DSS-induced body weight loss in mice aggravated by early-life AZT exposure (**Figure 2A**).

Shortening of colon length in DSS-induced mice is one of the biological markers of the assessment of colonic inflammation. The colon length of the MOD group was shortened compared with that of the control group. Further, AZT treatment in early life exacerbated colonic shortening in DSS-induced colitis mice. However, ginger prevented DSS-induced colonic shortening aggravated by AZT exposure (**Figures 2B,C**).

The DAI scores of the MOD group were considerably increased when compared to the control group, whereas treatment with AZT in early life increased the DAI scores of DSS-induced colitis mice. In accordance with the weight loss, compared with the AZT group, mice treated with ginger after AZT exposure showed a lower DAI score (**Figure 2D**).

Histological characteristics of the colons were subsequently analyzed *via* H&E staining. DSS induced significant colon tissue injury, as demonstrated by loss of goblet cells, neutrophil infiltration, muscular layer thickening, goblet cell damage, and crypt distortion. AZT treatment aggravated the colon injury in DSS-induced colitis, as shown by severe transmural inflammation characterized by severe crypt damage, goblet cell loss and damage, superficial ulceration, and massive inflammatory cell infiltration and thus a significantly higher histological score than that of the model group. In turn, ginger treatment preserved the extension of crypt damage and ameliorated inflammatory reactions such as mucosal and submucosal infiltration and thus resulted in a lower histological score compared with the AZT group (**Figures 2E,F**).

These results indicate that early life AZT exposure promotes the development of DSS-induced colitis in adult mice. Ginger treatment decreased the susceptibility to DSS-induced colitis in mice exposed to AZT in early life.

Ginger Improved Intestinal Barrier Function in Mice With DSS-Induced Colitis Exposed to AZT in Early Life

Intestinal epithelial tight junctions (TJs) play a key role in protecting against inflammation, and disrupted TJs are the primary cause of intestinal barrier dysfunction and inflammation. To investigate the impact of early-life AZT exposure and ginger on intestinal barrier function, we used transmission electron microscopy and immunofluorescence to analyze changes in the morphology of TJs and TJ proteins in mice with DSS-induced colitis.

In control mice, the TJs appeared as typical membrane fusions with intact TJ structure and desmosomes. In contrast, the TJ ultrastructure was altered after DSS treatment. TJs were discontinuous with few membrane fusions apparent in colon tissues, which indicated disruption in TJ morphology. Compared with the model group, the AZT-treated mice showed altered TJ ultrastructure. The TJ showed few electron-dense materials and loss of desmosome. However, treatment with ginger restored the TJ ultrastructure disrupted by early-life AZT exposure (**Figure 3A**).

The distribution of tight junctional proteins, ZO-1 and Claudin-1, which are the markers of TJ structure, was analyzed *via* immunostaining and the Western blot. Compared with control mice, the expression of colonic ZO-1 and claudin-1 was significantly decreased in MOD group mice and further decreased in AZT group (**Figures 3B–E**). Ginger treatment upregulated the expression of TJ proteins in mice exposed to AZT in early life.

Ginger Inhibited Inflammatory Response in DSS-Induced Colitis of Mice With Early-Life AZT Exposure

Inflammatory bowel disease is characterized by infiltration of immune cells and elevated levels of proinflammatory factors. We first investigated the influence of early AZT exposure on colonic macrophages, since they were easily activated after disruption of intestinal epithelial barrier function. CD68 was used as a marker of macrophages in the colon of mice in this study. Immunofluorescence revealed increased infiltration of macrophages in colonic lamina propria of mice treated with DSS, and macrophages were further increased in AZT group. Treatment with ginger inhibited the infiltration of macrophages in DSS-induced colitis associated with early AZT exposure (**Figure 4A**).

Colonic inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-10, were measured using ELISA kits. The colitis model group expressed significantly higher levels of colonic TNF-α, IL-1β, and IL-6, and lower levels of IL-10 compared with the control group. The AZT group showed further increase of TNF-α, IL-1β, and IL-6 and a further decrease in IL-10 levels. Compared with AZT group, the group treated with ginger extract showed suppression of TNF-α and IL-1β levels and the expression of IL-10 (**Figures 4B–E**).

Ginger Modulated Intestinal Microbiota Dysbiosis Induced by Early-Life AZT Exposure

Gut microbiota has emerged as an important factor in the development and function of the immune system. As antibiotics directly influence gut microbiota, we performed 16S rRNA gene sequencing to determine the impact of early-life AZT exposure and ginger on gut microbiota in later life. Fecal samples derived

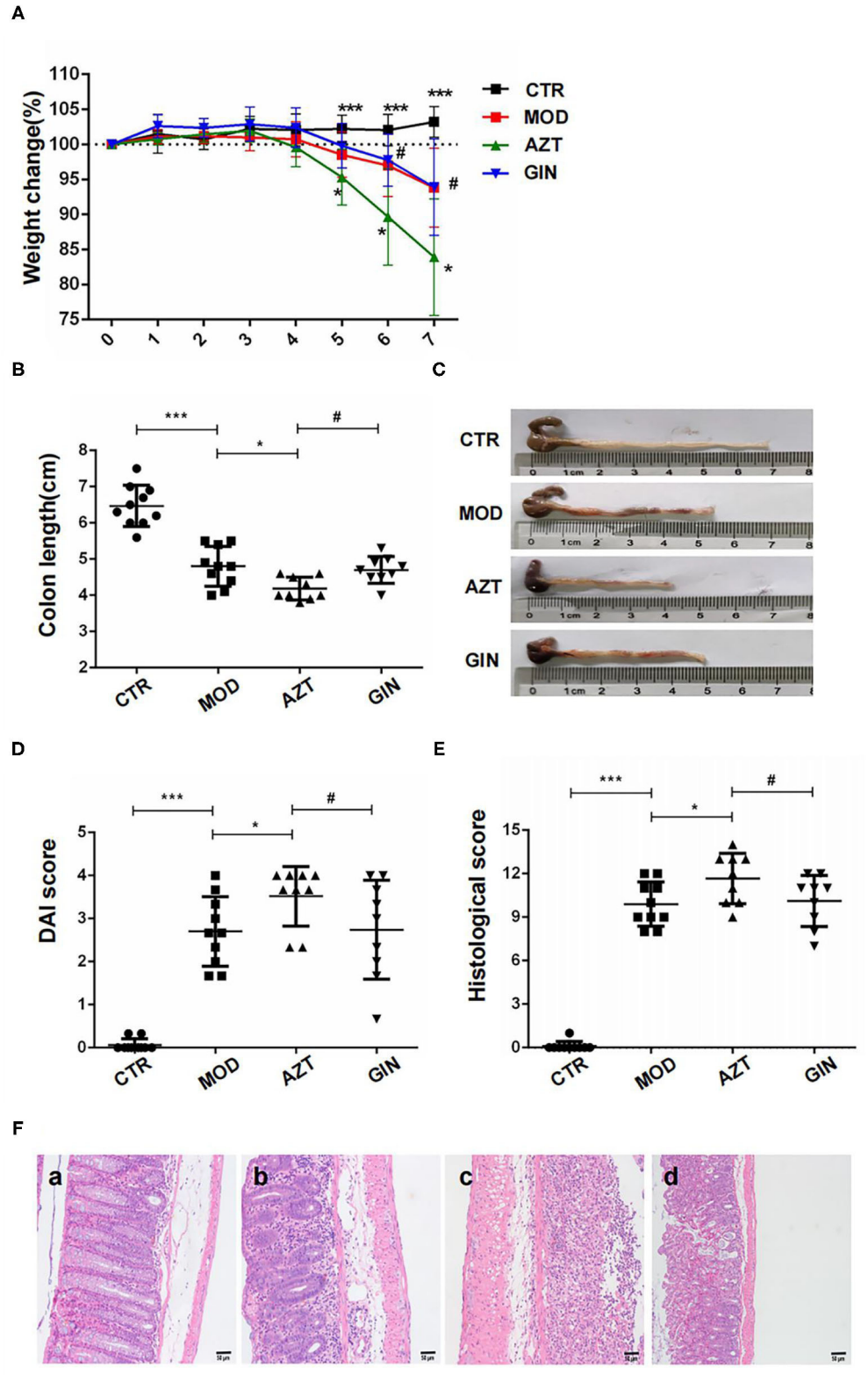


FIGURE 2 | Ginger reduces the susceptibility to DSS-induced colitis in mice exposed to AZT in early life. **(A)** Body weight loss, **(B)** and **(C)** colon length, **(D)** DAI score on day 6, **(E)** histological score, and **(F)** histological examination (magnification, ×200): (a) CTR group; (b) MOD group; (c) AZT group; and (d) GIN group. Data are represented as means ± SD ($n = 8$–10). $^{*}p < 0.05$ and $^{***}p < 0.01$ vs. model group; $^{\#}p < 0.05$ vs. AZT group.

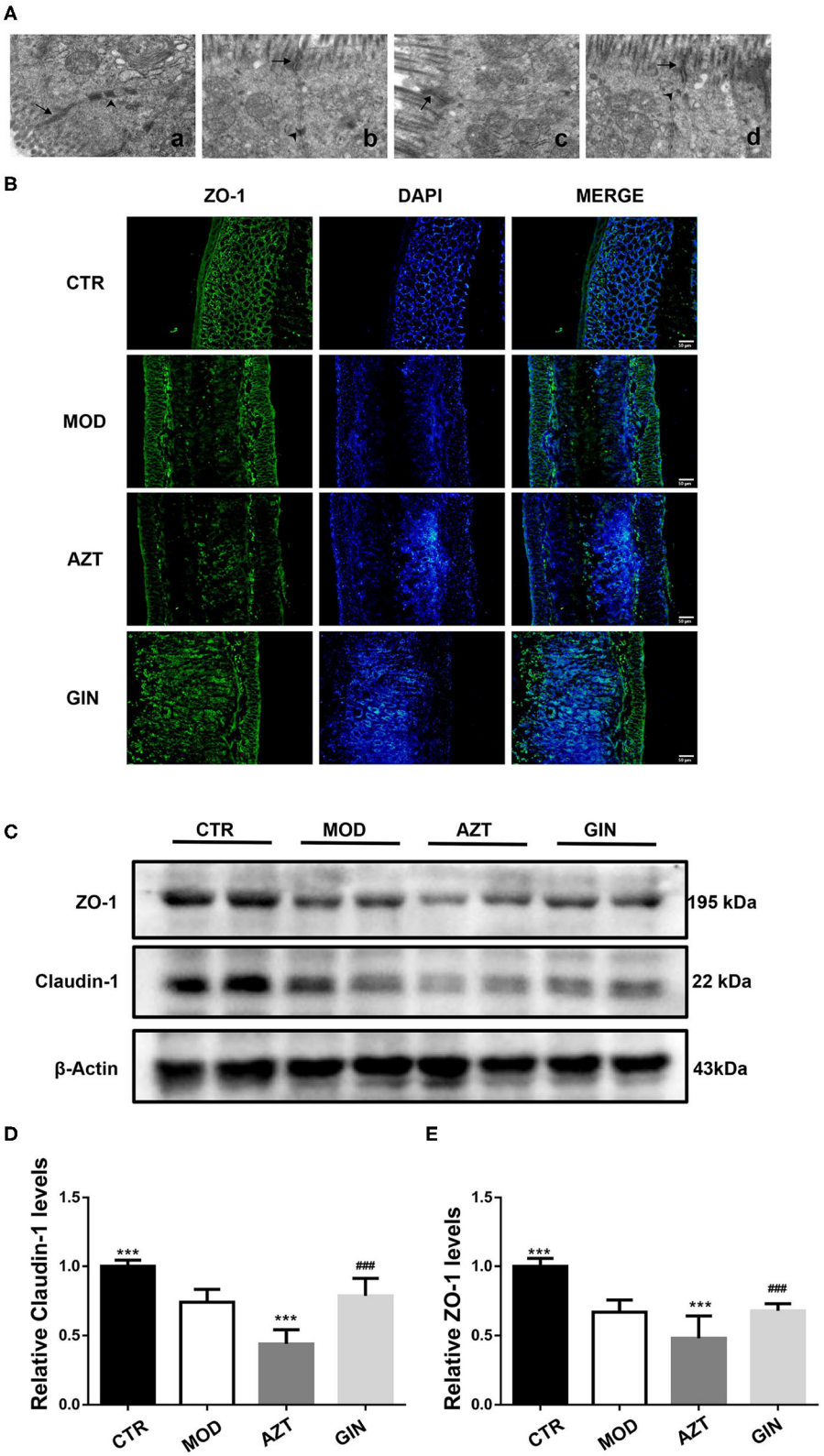


FIGURE 3 | Ginger improves the intestinal barrier function of mice with DSS-induced colitis exposed to AZT in early life. **(A)** TJ morphology (magnification, ×8000): (a) CTR group; (b) MOD group; (c) AZT group; and (d) GIN group. Arrows, TJ; arrow heads, desmosome. **(B)** Representative images of immunofluorescence of ZO-1 in colon sections. **(C)** Western blot analysis of claudin-1 and ZO-1 expression in colon tissue. **(D)** and **(E)** Relative levels of claudin-1 and ZO-1 ($n = 4$). $^{***}p < 0.01$ vs. MOD group; $^{\#\#\#}p < 0.01$ vs. AZT group.

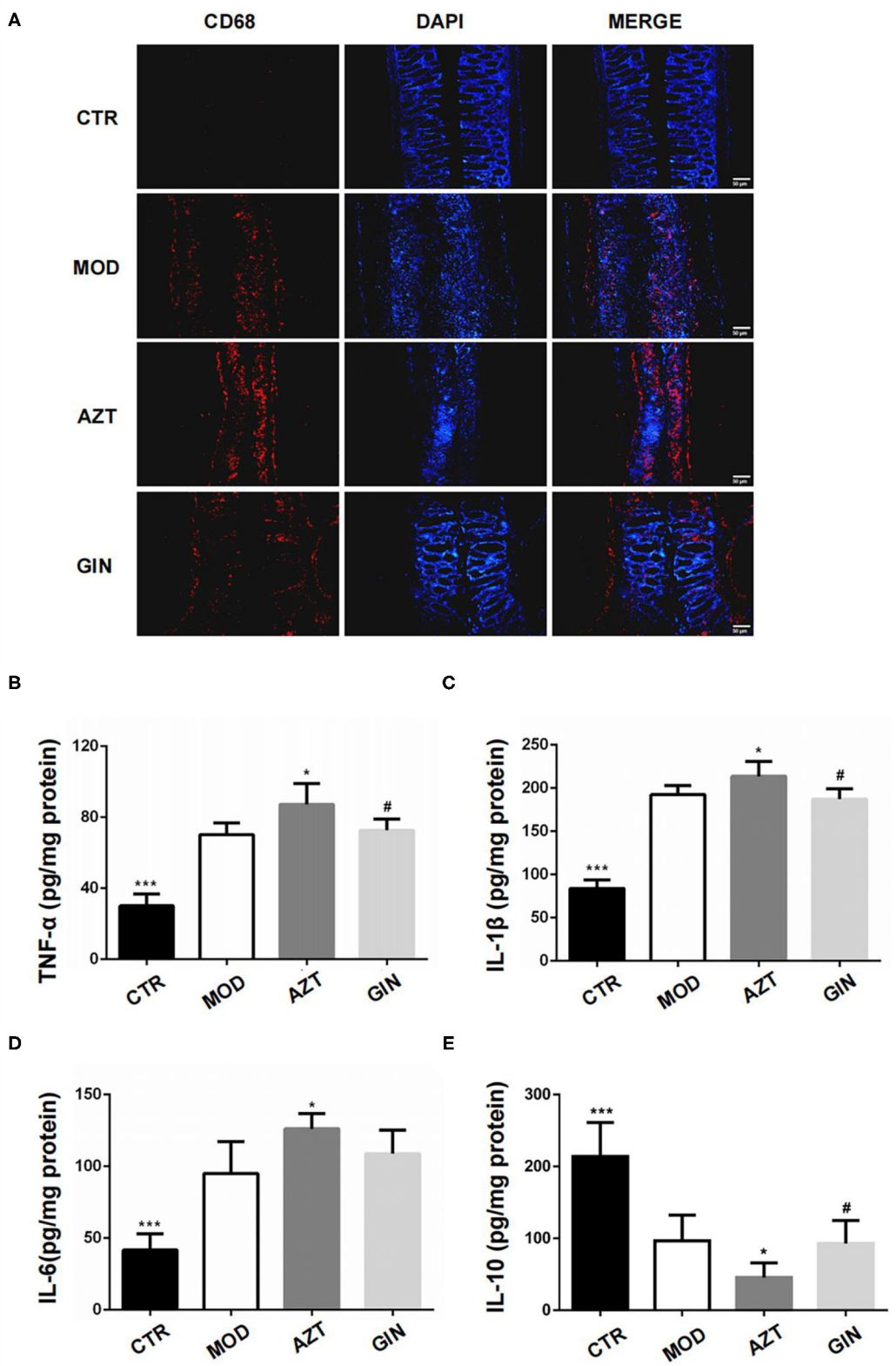


FIGURE 4 | Ginger inhibits inflammatory response in DSS-induced colitis mice exposed to AZT in early life. **(A)** Representative images of immunofluorescence of CD68-positive macrophages in colon sections. **(B–E)** Colonic cytokine levels of TNF-α, IL-1β, IL-6, and IL-10. Data represent means ± SD ($n = 5$). $^{*}p < 0.05$ and $^{***}p < 0.01$ vs. MOD group; $^{\#}p < 0.05$ vs. AZT group.

from the model group, AZT group, and GIN group were collected before DSS treatment. The control and model groups were exposed to similar treatment, and the model group alone was selected for the analysis.

Results showed that early-life AZT exposure significantly decreased the alpha diversity of bacteria, and treatment with ginger reversed it, based on Shannon index and Chao1 index (**Figures 5A,B**). Beta diversity represented by PCoA is shown in **Figure 5C**. The results revealed that the gut microbiota in the AZT group deviated from the baseline structure. Ginger modulated the effect on dysbiosis although it failed to completely restore the microbiota to normal status.

Further, early-life AZT exposure altered the composition of the microbiota, and ginger modulated the dysbiosis. Changes were observed at the levels of both phylum and genus. At the phylum level, a total of seven phyla were identified, including Bacteroidetes, Firmicutes, Verrucomicrobia, Actinobacteria, Proteobacteria, Tenericutes, and TM7 (**Figure 6A**). At the genus level, 27 genera (**Supplementary Table S4**) including *Akkermansia, Helicobacter, Peptococcus* rc4-4, *Lactobacillus,* and other genera were identified (**Figure 6B**). The relative abundance of Proteobacteria decreased and the relative abundance of Firmicutes and Verrucomicrobia increased in the AZT group compared with the model group. The changes in Firmicutes, Verrucomicrobia, and Proteobacteria induced by early AZT exposure were reversed by ginger treatment, although no statistically significant differences were detected in Firmicutes and Verrucomicrobia (**Figure 7B**) The changes in the main microbiota at the genus level were captured using a heatmap of 27 key species with varying degrees of abundance among the groups. Early AZT exposure not only eliminated harmful bacteria, including *Staphylococcus, Desulfovibrio,* and *Streptococcus*, but also decreased the abundance of beneficial bacteria, including *Lactobacillus, Bifidobacterium, and Parabacteroides* (**Figure 7A**). However, the levels of most of these reduced genera could not be restored to the normal level after ginger treatment. Notably, the relative abundance of *Helicobacter* decreased and the relative abundance of Peptococcaceae rc4-4 increased significantly in the AZT group compared with the model group. These changes induced by early AZT exposure were reversed by ginger treatment (**Figure 7B**). In addition, early antibiotic exposure also increased the relative abundance of *Akkermansia,* and ginger treatment had a modulatory effect without statistical significance.

A schematic diagram of evolutionary clustering analysis was generated taxonomically based on the LDA score to identify key microbiota responsible for the differences among groups. As shown in **Supplementary Figures S2A,B**, *Bacteroidia, Helicobacter,* and *Desulfovibrio* were the major microbiota in MOD group. Species belonging to Peptococcaceae and Turicibacter were identified as major microbiota in the AZT group. In GIN group, the predominant intestinal flora, including *Akkermansia muciniphila* and *Anaerostipes*, especially *A. muciniphila*, may play an important role in DSS-induced colitis of mice exposed to AZT in early life.

DISCUSSION

Mice exposed to AZT early in life were highly susceptible to DSS-induced colitis and were successfully treated with ginger in this study. A mouse model mimicking childhood antibiotic exposure in humans was used for the first time. DSS treatment induced clinical symptoms and pathological changes corresponding to those of human UC, including loss of body weight, diarrhea, bloody feces, and shortening of the colon. The measurement of body weight loss is a standard method used to evaluate disease progression in DSS-induced colitis. Weight loss and rectal bleeding are connected with colon shortening. Early AZT exposure significantly exacerbated DSS-induced colitis by decreasing weight loss, DAI scores, and colonic shortening. Treatment with ginger ameliorated these symptoms and histopathology effectively. In contrast, administration of ginger after early AZT exposure reduced all of the aforementioned inflammatory changes, suggesting that early AZT exposure exacerbated the inflammatory condition induced by DSS, which was ameliorated by treatment with ginger.

Intestinal epithelium constitutes a physical and functional barrier separating lamina propria from luminal pathogens and antigen exposure. It serves as a first line of defense for the mucosal immune system. Compromised intestinal barrier function is associated with the development of IBD. The TJ proteins are crucial for the maintenance of epithelial barrier integrity. ZO-1 and occludin represent TJ proteins playing a pivotal role in regulating paracellular permeability. In this study, compared with the MOD group, the AZT group decreased ZO-1 and occludin membrane localization in the colon. A previous study showed that ginger enhanced the expression of ZO-1 and occludin in the TJ of colonic mucosa in mice with colitis (20). Similarly, the expression of ZO-1 and occludin was recovered after ginger treatment. These results indicate the detrimental effects of early antibiotic exposure and the protective role of ginger in DSS-induced disruption of the intestinal epithelial barrier.

Disruption of intestinal epithelial barrier function may lead to translocation of intestinal bacteria and entry of gut-derived pathogens into portal circulation through the highly permeable intestinal barrier. The consequences include activated nonlymphoid cells, such as macrophages and increased proinflammatory cytokines, including TNF-α, IL-6, and IL-1β. Macrophages are the key regulators of immune response and critical for maintaining immunological homeostasis in the intestine. A previous study demonstrated that antibiotic-induced bacterial disturbances led to persistent changes in adaptive immunity in the intestine by interfering with microbiota-dependent regulation of intestinal macrophage function (21). Here, we also found that early antibiotic exposure increased infiltration of intestinal macrophages and proinflammatory cytokines in DSS-induced colitis of mice in later years. Consistent with previous studies (15, 20), ginger treatment after antibiotic exposure reduced the levels of TNF-α, IL-6, and IL-1β in the colon and decreased macrophage infiltration to inhibit inflammation.

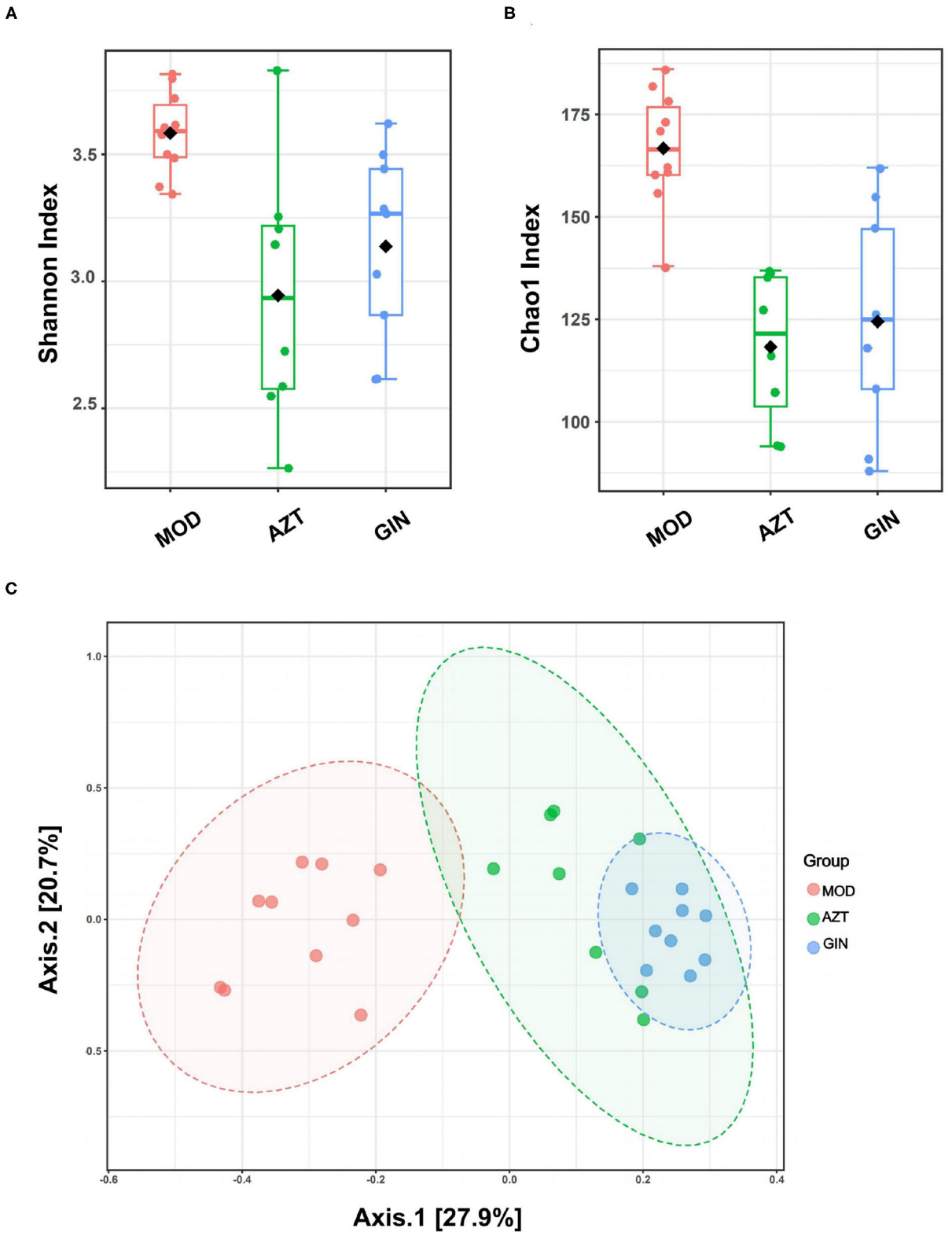


FIGURE 5 | Ginger modulates the diversity and structure of gut microbiota induced by early AZT exposure. Comparison of alpha diversity based on **(A)** Shannon index and **(B)** Chao1 index. **(C)** Multisample PCoA (n = 8–10).

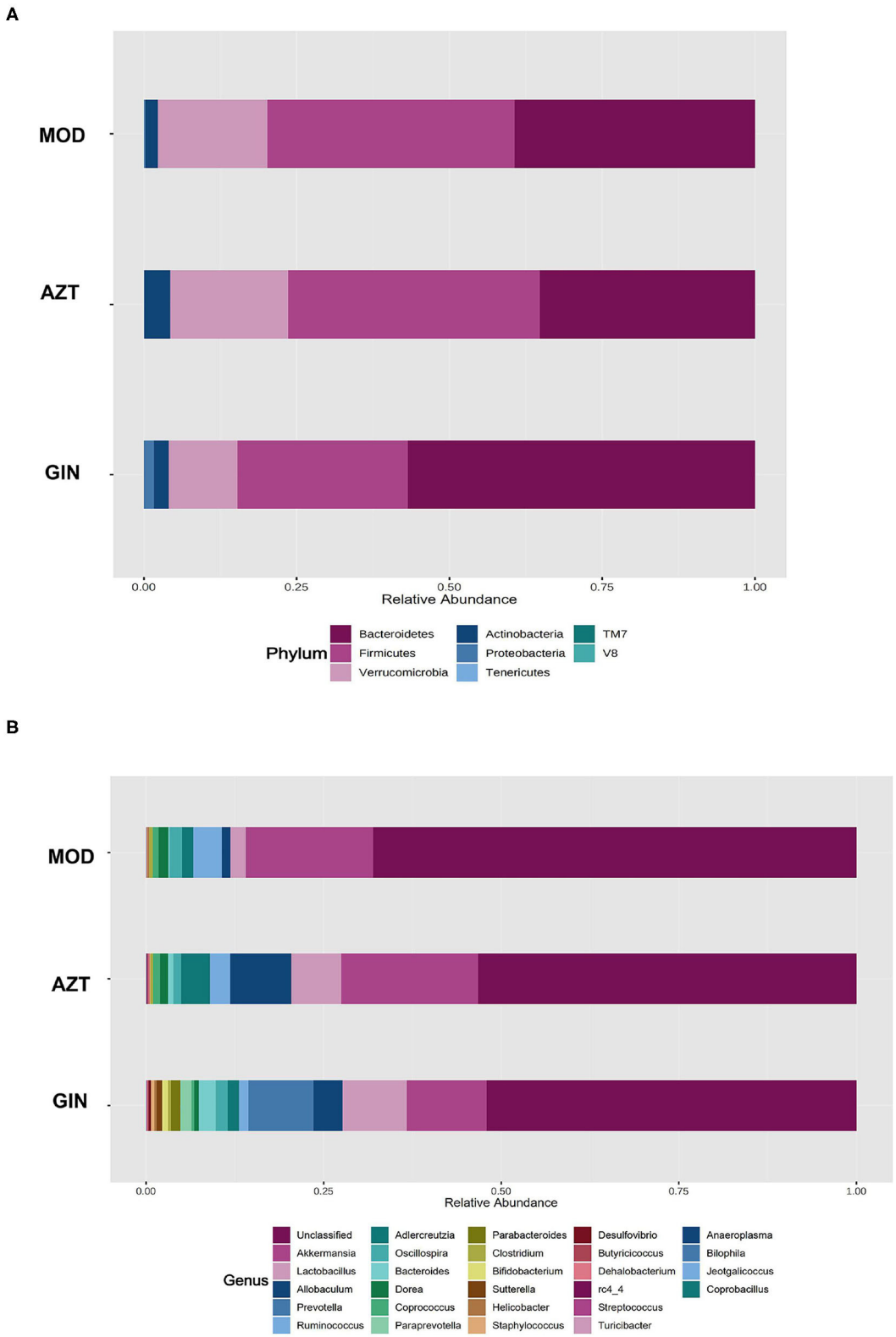


FIGURE 6 | Ginger modulates the composition of gut microbiota induced by early AZT exposure. **(A)** Microbial community bar plot by phylum. **(B)** Microbial community bar plot by genus.

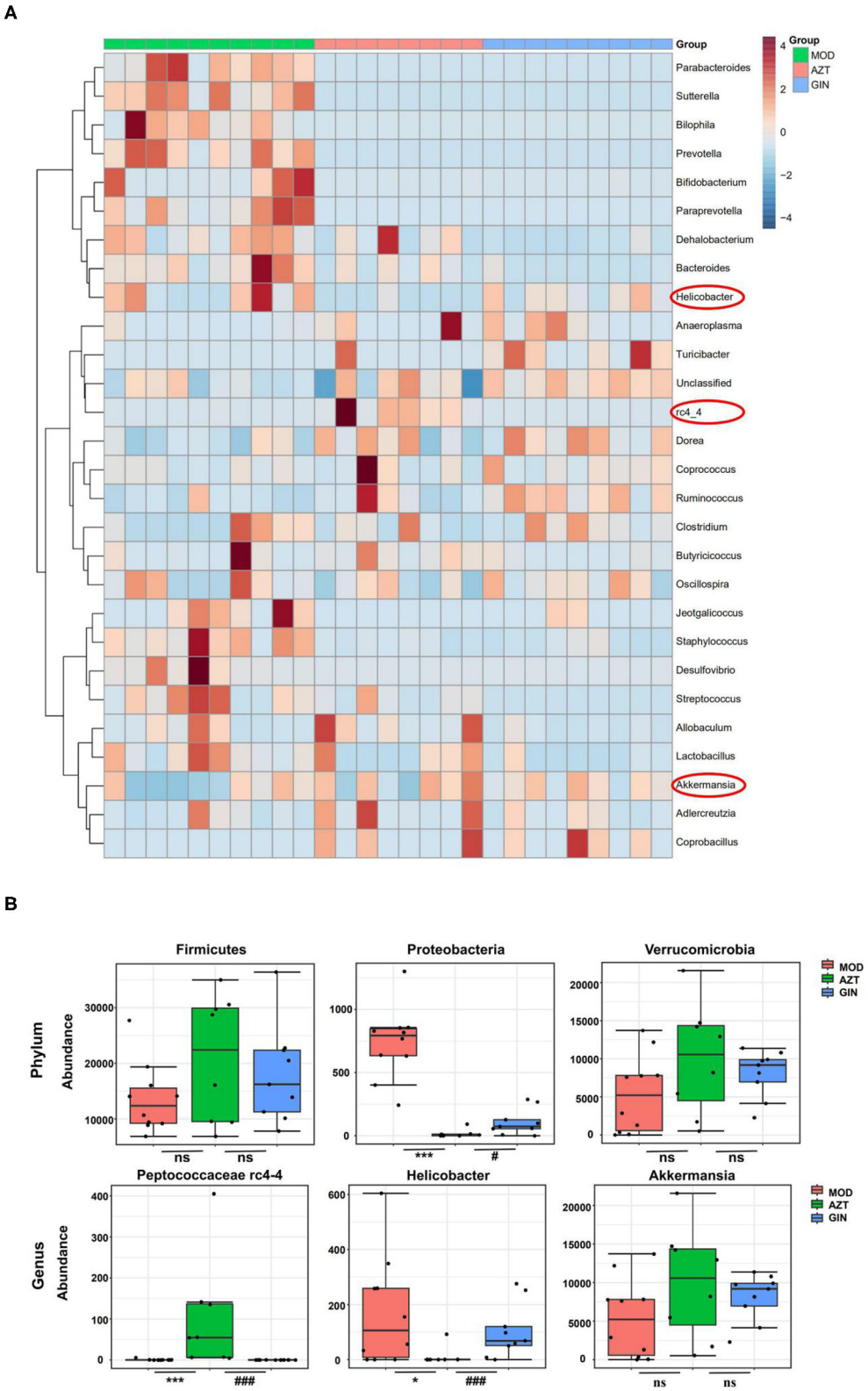


FIGURE 7 | Ginger modulates the composition of gut microbiota induced by early AZT exposure. **(A)** Heatmap of the relative abundance of key 27 OTUs altered by AZT and ginger at genus level. **(B)** The relative abundance of microbial species at phylum and genus level. $^{*}p < 0.05$ and $^{***}p < 0.01$ vs. MOD group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.01$ vs. AZT group.

Gut microbiota are the key factors in IBD pathogenesis, as colitis cannot be induced by DSS in germ-free animals and many IBD susceptibility genes are associated with host-microbe immune interactions (22). We then analyzed the impact of early antibiotic exposure on gut microbiota as a possible mechanism in aggravating colitis. The decline in microbial diversity was shown in AZT group, and the diversity was improved after ginger treatment. The PCoA showed the differential composition of bacterial communities in DSS and AZT groups. Ginger modulated the effect on dysbiosis, but still failed to restore microbiota to normal condition. The gut microbiota communities in all samples were evaluated based on phylum and genus. In terms of bacterial composition at the phylum level, early AZT exposure increased the relative abundance of Firmicutes and Verrucomicrobia and decreased the relative abundance of Proteobacteria. Treatment with ginger reversed the changes in Firmicutes, Proteobacteria, and Verrucomicrobia. At the genus level, early AZT exposure not only killed harmful bacteria, including *Staphylococcus, Desulfovibrio, and Streptococcus*, but also decreased the abundance of beneficial bacteria, including *Lactobacillus and Bifidobacterium*. The modulatory effect on bacteria mentioned above was weak, but ginger treatment reversed the increased abundance of Peptococcaceae rc4-4 and the decreased abundance of *Helicobacter* significantly induced by early AZT exposure.

Peptococcus, a type of intestinal sulfate-reducing bacteria (SRB), (23) was more prevalent in mice exposed to AZT early, but decreased significantly in the ginger-treated group. Although little is known about *Peptococcaceae*, SRB have been found to generate hydrogen sulfide (H2S), which is toxic to colonic epithelial cells by inhibiting butyrate metabolism in colonocytes and contributes to inflammation in experimental colitis (24). Previous studies also showed that SRB reduced the mucus barrier function and facilitated bacterial presence close to the colonic epithelium, leading to inflammation (25). These findings suggest that *Peptococcaceae* may be important for the development of colitis.

Helicobacter pylori has been characterized as the primary pathogenic factor in chronic gastritis and peptic ulcer. However, the relative abundance of *Helicobacter* was increased significantly in the AZT group but decreased in the ginger-treated group. Interestingly, numerous studies have reported a lower level of *Helicobacter pylori* infection rate in IBD patients than in controls (26, 27). Animal experiments also confirmed the inverse association between *Helicobacter pylori* and IBD. *H. pylori* DNA significantly ameliorated colitis and histopathological changes in a DSS-induced mouse colitis model (28). Epidemiological and basic experimental studies suggested that Helicobacter infection protects against IBD by inducing systematic immune tolerance and suppressing inflammatory responses (29). Further evidence indicates that children and youth with developing immune systems may benefit more than older individuals from immune tolerance induced by *Helicobacter* (30, 31). These studies underscore the need for caution in eradicating *H. pylori*, especially for children.

Interestingly, we also found that the relative abundance of *Akkermansia* in five of eight samples was increased after early AZT exposure, though there was no statistical difference. Mucin-degrading *A. muciniphila* is a commensal bacterium dwelling in the mammalian gastrointestinal tract adhering to the mucus layer and plays an essential role in maintaining gut barrier function. The presence of *Akkermansia* has also been reported in other antibiotic treatment models (32). Recent studies about *Akkermansia* indicate divergent host outcomes including improved metabolic phenotypes and enhanced lifespan (33, 34). However, the enrichment of *Akkermansia* has also been associated with increased susceptibility to colitis (35). *Akkermansia* is sufficient for promoting intestinal inflammation in both specific-pathogen-free and germ-free $IL10^{-/-}$ mice, representing models of spontaneous colitis (36). These findings suggested that *Akkermansia* may exhibit different or even contrasting physiological role in acute colitis models. In our mouse model, early antibiotic exposure induced a bloom of *Akkermansia*, which may have contributed to the aggravation of colitis. In turn, ginger treatment decreases the abundance of *Akkermansia*, indicating its modulation effect on microbiota of mice exposed to antibiotics early in life.

Therefore, early antibiotic exposure may lead to sustained dysbiosis that increases the susceptibility to colitis later, and ginger treatment modulates these changes effectively. This study extends our understanding of the impact of early-life antibiotic exposure with respect to microbial dysbiosis and colitis, and interventions *via* ginger targeting gut microbiota to ameliorate these changes.

However, this study still has certain limitations. First, the main active component in ginger extract that contributes to antiinflammatory effects and microbial regulation requires further investigation. Second, the specific bacterial species mediating susceptibility of murine colitis in mice exposed to AZT early in life remains to be verified in the future.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI SRA repository, accession number: PRJNA751929.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Guangzhou Institute of Sport Science (GZTKSGNX-2016-4). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

XZho, XL, and QH conceived the research idea. XZho, XL, QH, and HL performed the experiments. YY, LC, and MW provided technical and material support. XZho and QH analyzed the data. XZho prepared the manuscript. LZ, XF, and HK

revised the manuscript. LZ and XZha reviewed the manuscript. All authors contributed to the article and approved the final manuscript.

FUNDING

This work was supported by the National Key R&D Program of China (2020YFC2003100 and 2020YFC2003101), the Key Project of National Natural Science Foundation of China (81830117), the National Natural Science Foundation of China (81774212 and 81760821), the Natural Science Foundation of Guangdong Province, China (2019A1515010400), the Science and Technical Plan of Guangzhou, Guangdong, China (201903010069), and the Innovation Team and Talents Cultivation Program of the National Administration of Traditional Chinese Medicine (Number: ZYYCXTD-C-202001).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.755969/full#supplementary-material

REFERENCES

1. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Kaplan: worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet*. (2017) 390:2769–78. doi: 10.1016/S0140-6736(17)32448-0
2. Ramos GP, Papadakis AK. Mechanisms of disease: inflammatory bowel diseases. *Mayo Clin Proc.* (2019) 94:155–65. doi: 10.1016/j.mayocp.2018.09.013
3. Zhang M, Sun K, Wu Y, Yang Y, Tso P, Wu Z. Interactions between intestinal microbiota and host immune response in inflammatory bowel disease. *Front Immunol.* (2017) 8:942. doi: 10.3389/fimmu.2017.00942
4. Hicks LA, Bartoces MG, Roberts RM, Suda KJ, Hunkler RJ, Taylor TJ, et al. US outpatient antibiotic prescribing variation according to geography, patient population, and provider specialty in 2011. *Clin Infect Dis.* (2015) 60:1308–16. doi: 10.1093/cid/civ076
5. Hersh AL, Shapiro DJ, Pavia AT, Shah SS. Antibiotic prescribing in ambulatory pediatrics in the United States. *Pediatrics.* (2011) 128:1053–61. doi: 10.1542/peds.2011-1337
6. Roubaud-Baudron C, Ruiz VE, Swan AJ, Vallance BA, Ozkul C, Pei Z, et al. Long-term effects of early-life antibiotic exposure on resistance to subsequent bacterial infection. *mBio.* (2019) 10:e02820–19. doi: 10.1128/mBio.02820-19
7. Neuman H, Forsythe P, Uzan A, Avni O, Koren O. Antibiotics in early life: dysbiosis and the damage done. *FEMS Microbiol Rev.* (2018) 42:489–99. doi: 10.1093/femsre/fuy018
8. Ahmadizar F, Vijverberg SJH, Arets HGM, de Boer A, Turner S, Devereux G, et al. Early life antibiotic use and the risk of asthma and asthma exacerbations in children. *Pediatr Allergy Immunol.* (2017) 28:430–7. doi: 10.1111/pai.12725
9. Bailey LC, Forrest CB, Zhang P, Richards TM, Livshits A, DeRusso PA. Association of antibiotics in infancy with early childhood obesity. *JAMA Pediatr.* (2014) 168:1063–9. doi: 10.1001/jamapediatrics.2014.1539
10. Lynn MA, Tumes DJ, Choo JM, Sribnaia A, Blake SJ, Leong L, et al. Early-life antibiotic-driven dysbiosis leads to dysregulated vaccine immune responses in mice. *Cell Host Microbe.* (2018) 23:653–660.e5. doi: 10.1016/j.chom.2018.04.009
11. Hviid A, Svanstrom H, Frisch M. Antibiotic use and inflammatory bowel diseases in childhood. *Gut.* (2011) 60:49–54. doi: 10.1136/gut.2010.219683
12. Miyoshi J, Bobe AM, Miyoshi S, Huang Y, Hubert N, Delmont TO, et al. Peripartum antibiotics promote gut dysbiosis, loss of immune tolerance, and inflammatory bowel disease in genetically prone offspring. *Cell Rep.* (2017) 20:491–504. doi: 10.1016/j.celrep.2017.06.060
13. Miyoshi J, Miyoshi S, Delmont TO, Cham C, Lee S, Sakatani A, et al. Early-life microbial restitution reduces colitis risk promoted by antibiotic-induced gut dysbiosis in IL-10(-/-) mice. *Gastroenterology.* (2021). doi: 10.1101/2020.03.11.987412
14. Guo S, Geng W, Chen S, Wang L, Rong X, Wang S, et al. Ginger Alleviates DSS-induced ulcerative colitis severity by improving the diversity and function of gut microbiota. *Front Pharmacol.* (2021) 12:632569. doi: 10.3389/fphar.2021.632569
15. Zhang F, Ma N, Gao YF, Sun LL, Zhang JG. Therapeutic effects of 6-gingerol, 8-gingerol, and 10-gingerol on dextran sulfate sodium-induced acute ulcerative colitis in rats. *Phytother Res.* (2017) 31:1427–32. doi: 10.1002/ptr.5871
16. Zhang M, Viennois E, Prasad M, Zhang Y, Wang L, Zhang Z, et al. Edible ginger-derived nanoparticles: a novel therapeutic approach for the prevention and treatment of inflammatory bowel disease and colitis-associated cancer. *Biomaterials.* (2016) 101:321–40. doi: 10.1016/j.biomaterials.2016.06.018
17. Zhang M, Xu C, Liu D, Han MK, Wang L, Merlin D. Oral delivery of nanoparticles loaded with ginger active compound, 6-shogaol, attenuates ulcerative colitis and promotes wound healing in a murine model of ulcerative colitis. *J Crohns Colitis.* (2018) 12:217–29. doi: 10.1093/ecco-jcc/jjx115
18. Teng Y, Ren Y, Sayed M, Hu X, Lei C, Kumar A, et al. Plant-derived exosomal MicroRNAs shape the gut microbiota. *Cell Host Microbe.* (2018) 24:637–652.e8. doi: 10.1016/j.chom.2018.10.001
19. Girard D, Finegan SM, Dunne MW, Lame ME. Enhanced efficacy of single-dose versus multi-dose azithromycin regimens in preclinical infection models. *J Antimicrob Chemother.* (2005) 56:365–71. doi: 10.1093/jac/dki241
20. Kim MS, Kim JY. Ginger attenuates inflammation in a mouse model of dextran sulfate sodium-induced colitis. *Food Sci Biotechnol.* (2018) 27:1493–501. doi: 10.1007/s10068-018-0438-6
21. Scott NA, Andrusaite A, Andersen P, Lawson M, Alcon-Giner C, Leclaire C, et al. Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis. *Sci Transl Med.* (2018) 10:eaao4755. doi: 10.1126/scitranslmed.aao4755
22. McGovern DP, Kugathasan S, Cho JH. Genetics of inflammatory bowel diseases. *Gastroenterology.* (2015) 149:1163–76.e2. doi: 10.1053/j.gastro.2015.08.001
23. Figliuolo VR, Dos SL, Abalo A, Nanini H, Santos A, Brittes NM, et al. Sulfate-reducing bacteria stimulate gut immune responses and contribute to inflammation in experimental colitis. *Life Sci.* (2017) 189:29–38. doi: 10.1016/j.lfs.2017.09.014
24. Ijssennagger N, van der Meer R, van Mil SWC. Sulfide as a Mucus Barrier-Breaker in Inflammatory Bowel Disease? *Trends Mol Med.* (2016) 22:190–9. doi: 10.1016/j.molmed.2016.01.002
25. Bartels LE, Jepsen P, Christensen LA, Gerdes LU, Vilstrup H. Dahlerup JF. Diagnosis of helicobacter pylori infection is associated with lower prevalence and subsequent incidence of crohn's disease. *J Crohns Colitis.* (2016) 10:443–8. doi: 10.1093/ecco-jcc/jjv229
26. Roka K, Roubani A, Stefanaki K, Panayotou I, Roma E, Chouliaras G. The prevalence of Helicobacter pylori gastritis in newly diagnosed children with inflammatory bowel disease. *Helicobacter.* (2014) 19:400–5. doi: 10.1111/hel.12141
27. Harris PR, Wright SW, Serrano C, Riera F, Duarte I, Torres J, et al. Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. *Gastroenterology.* (2008) 134:491–9. doi: 10.1053/j.gastro.2007.11.006
28. Serrano C, Wright SW, Bimczok D, Shaffer CL, Cover TL, Venegas A, et al. Downregulated Th17 responses are associated with reduced gastritis in Helicobacter pylori-infected children. *Mucosal Immunol.* (2013) 6:950–9. doi: 10.1038/mi.2012.133

29. Nobel YR, Cox LM, Kirigin FF, Bokulich NA, Yamanishi S, Teitler I, et al. Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment. *Nat Commun.* (2015) 6:7486. doi: 10.1038/ncomms8486
30. Depommier C, Everard A, Druart C, Plovier H, Van Hul M, Vieira-Silva S, et al. Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat Med.* (2019) 25:1096–103. doi: 10.1038/s41591-019-0495-2
31. Barcena C, Valdes-Mas R, Mayoral P, Garabaya C, Durand S, Rodriguez F, et al. Healthspan and lifespan extension by fecal microbiota transplantation into progeroid mice. *Nat Med.* (2019) 25:1234–42. doi: 10.1038/s41591-019-0504-5
32. Ganesh BP, Klopfleisch R, Loh G, Blaut M. Commensal Akkermansia muciniphila exacerbates gut inflammation in Salmonella Typhimurium-infected gnotobiotic mice. *PLoS ONE.* (2013) 8:e74963. doi: 10.1371/journal.pone.0074963
33. Seregin SS, Golovchenko N, Schaf B, Chen J, Pudlo NA, Mitchell J, et al. NLRP6 Protects Il10(-/-) Mice from Colitis by Limiting Colonization of Akkermansia muciniphila. *Cell Rep.* (2017) 19:733–45. doi: 10.1016/j.celrep.2017.03.080
34. Yu Y, Zhu S, Li P, Min L, Zhang S. Helicobacter pylori infection and inflammatory bowel disease: a crosstalk between upper and lower digestive tract. *Cell Death Dis.* (2018) 9:961. doi: 10.1038/s41419-018-0982-2
35. Holmes DE, Giloteaux L, Williams KH, Wrighton KC, Wilkins MJ, Thompson CA, et al. Enrichment of specific protozoan populations during in situ bioremediation of uranium-contaminated groundwater. *ISME J.* (2013) 7:1286–98. doi: 10.1038/ismej.2013.20
36. Luther J, Owyang SY, Takeuchi T, Cole TS, Zhang M, Liu M, et al. Helicobacter pylori DNA decreases pro-inflammatory cytokine production by dendritic cells and attenuates dextran sodium sulphate-induced colitis. *Gut.* (2011) 60:1479–86. doi: 10.1136/gut.2010.220087

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.

frontiers in Medicine

OPINION
published: 12 January 2022
doi: 10.3389/fmed.2021.746230

How to Initiate Fecal Microbiota Transplantation in Developing Countries Using the Behavior Economics Concept of "Choice Architecture"

Chatuthanai Savigamin[1†], *Nuttida Mahakit*[2†], *Suchinda Stithit*[3] and *Chatpol Samuthpongtorn*[2*†]

[1] Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, [2] Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, [3] Century Pharmaceuticals, Inc., Indianapolis, IN, United States

OPEN ACCESS

Edited by:
Abbas Yadegar,
Shahid Beheshti University of Medical Sciences, Iran

Reviewed by:
Chenyang Wang,
Nanjing University, China

***Correspondence:**
Chatpol Samuthpongtorn
jamie15006@gmail.com

†ORCID:
Chatuthanai Savigamin
orcid.org/0000-0002-7423-4147
Nuttida Mahakit
orcid.org/0000-0002-9068-2636
Chatpol Samuthpongtorn
orcid.org/0000-0002-4135-3617

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 23 July 2021
Accepted: 20 December 2021
Published: 12 January 2022

Citation:
Savigamin C, Mahakit N, Stithit S and Samuthpongtorn C (2022) How to Initiate Fecal Microbiota Transplantation in Developing Countries Using the Behavior Economics Concept of "Choice Architecture". Front. Med. 8:746230. doi: 10.3389/fmed.2021.746230

Keywords: fecal microbiota transplantation, gastrointestinal diseases, stool bank, behavior economics, choice architecture, nudges

INTRODUCTION

Fecal Microbiota Transplantation (FMT) is a transfer of stool from a "healthy" donor to restore eubiosis (healthy microbiome) in a recipient believed to harbor an altered colonic microbiome (dysbiosis) resulting in disease development (1, 2). FMT is also referred to as stool transplantation, fecal transplantation, fecal flora reconstitution, or fecal bacteriotherapy. Though it is mostly employed in the treatment of *Clostridioides difficile* (formerly known as *Clostridium difficile*), FMT is now being investigated for its mechanism in the treatment of inflammatory bowel disease, irritable bowel syndrome, hepatic encephalopathy, and other conditions (2). Recently FMT has gained popularity in both the public media as well as peer-reviewed literature (1). However, one of the challenges obstructing a successful FMT program is a negative perception regarding FMT from both physicians and public citizens.

Nowadays, only *Clostridioides difficile* is approved for treatment using FMT. Fecal microbiota transplantation has the potential to cure *Clostridioides difficile* through a direct pathway (short-chain fatty acids and bile acids), nutrition competition, and bacteriophages that are unrelated to the host and are transferred directly following fecal microbiota transplantation. As a result, *Clostridioides difficile* can be cured through the use of a healthy donor who is not required to be specific to each patient (**Donor independent**) (2). Gastroenterologists can choose any healthy individual to donate stool. It is not necessary to have a program for donor recruitment for finding a perfect stool donor. However, many other diseases, such as irritable bowel syndrome, inflammatory bowel disease, and hepatic encephalopathy, require a specific, perfect donor. This is because FMT contributes to the development and treatment of these disorders in an indirect pathway (through bile acid and short-chain fatty acid metabolism) and is influenced by host-associated factors (**Donor dependent**) (2). Consequently, if we want to develop FMT as a successful novel therapeutic approach, we'll need a donor recruitment program to establish pools of perfect stool donor for research and treatment.

One of the main challenges facing public citizens is that feces is a "Yucks Term." Feces is associated with a dirty and unpleasant image. It's challenging to convince them that this is a novel treatment. We believe social media platforms should rename "feces" to more attractive words. This is what is referred to as "Punning." For example, Nerlich and Koteyko (3) redefined FMT as "vitalism," which is synonymous with probiotic (4). Furthermore, some asserted that FMT was a

supernatural power called "God's probiotic" (5). Thus, FMT was linked with the socially acceptable because they are familiar with probiotics in medicine (4).

We applied the Nudge principle to promote a positive perception regarding FMT to both physicians and public citizens. The Nudge principle was invented by Richard H Thaler since the early twentieth century, and he won a Nobel Prize in 2017 for his contribution in Nudge principle. Nudge is a behavior intervention designed to change people's behavior indirectly by changing the environment and situation to allow for a better choice and decision making (6). Nudge is a well-known principle employed in the business, economics, and healthcare domains. This principle is based on incentive, understanding mapping, default choice, feedback, relative comparison, expansion of the important outcome and structural complex choices (6). We believe the Nudge principle could lead to a structure that allows a successful establishment of FMT programs in the developing countries.

DISCUSSION AND SUGGESTIONS

We propose four essential steps for initiating fecal microbiota transplantation.

1. Finding a perfect stool donor.
2. Initiating a clinical trial.
3. Establishing a stool standard for use in other research trials.
4. Establishing a clinical center for the transplantation of fecal microbiota.

We focused on the first two steps in forming the fecal microbiota transplantation in this paper. We established nine interventions based on the Nudge principle which will be incorporated into each step of initiation of fecal microbiota transplantation.

1. Finding perfect stool donors—numerous steps should be taken to find perfect stool donors, such as completing a questionnaire to select an appropriate donor, collecting blood samples for further investigation, receiving stool specimens and investigating the stool specimens for proper criteria to be considered as a perfect donor (7). We propose that by incorporating Nudge theories and our suggestion, we can make these steps easier and more successful by nudging the stool donors.

Intervention 1: Certification

Openbiome (the world's largest non-profit stool bank) (8) as well as Asia Microbiota bank (Asia's largest stool bank) provide financial reward as an incentive to motivate fecal microbiota donors to continue their donation.

Apart from financial incentives, we propose a certificate of merits be given to fecal microbiota donors. This certificate will also indicate that the donor is healthy and passes the health examination screening. Additionally, this certificate can be used for a discount to future healthcare-related cost. This method was count as an incentive in nudge principle.

Intervention 2: Complimentary Breakfast

Most people defecate in the morning; therefore, fecal microbiota donation in the morning is usually preferred. A complimentary breakfast serves as both an incentive and is part of a pleasant environment prior to the donation. Additionally, it also triggers a gastrocolic reflex stimulating the defecation.

Intervention 3: Elegant Home-Like Toilet

Defecation can be challenging in an unfamiliar environment. Therefore, arranging a toilet that replicates an elegant home-like environment would be helpful to facilitate ease in defecation of fecal microbiota donors.

Intervention 4: Provide Essential Information

Information should contain pros and cons of the fecal microbiota donation along with the procedure in detail. Excessive information and text should be avoided. The infographic should attract attention and should be self-explanatory; the narration should be concise and easy to understand; and the presented data should be distinctive (9). We propose our infographic in **Figure 1**. By using this method, we incorporated the principle of understand mapping into our suggestion.

Intervention 5: Advertisement Imaging

If we want to make it easier to find perfect stool donors, one of the most essential aspects is to shift the imaging of fecal microbiota transplantation away from poop transfer and toward more sophisticated imaging such as a natural probiotic cocktail, God's probiotic, etc. This shift in perception should help persuade more stool donors to donate fecal microbiota.

Intervention 6: Online Community for Fecal Microbiota Transplantation

Social networking has become an essential part in this modern era. It can be a powerful tool to enhance the awareness of FMT and expand the donor pool. Multiple channels such as a Facebook page, an official Line group, or a webpage should be utilized to capture a larger interest group of donors. We had known from previous reports that people tend to conform to other people, and these effects especially increase when the size of other people increase. This online community should be a useful tool in finding perfect donors when everybody is talking about fecal donation. This type of intervention in nudge theories is called feedback (6).

2. Initiating clinical trial: After finding perfect stool donors, we must screen patients for a variety of diseases. Depending on our treatment objective, we can use selected stool from the perfect donor pool. In this section, we want to nudge the gastrointestinal medical doctors or referring physicians to pay attention or use this type of intervention. We suggest the following intervention for those processes.

Intervention 7: Comparison Between Our Country and Neighboring Countries

We propose using an infographic comparison between various core domains of each FMT program. This information can

FIGURE 1 | Infographic about stool donation created by our team. It contained not much excessive amounts of data and text, eye-catching picture and an organized story.

be utilized to continuously improve the program performance. However, given the difference in the culture and philosophy of each country, certain interventions may not always yield similar results. This intervention would nudge the GI doctors and referring doctors to believe that this FMT program has been widely used in their neighborhood. It will create the comparison which should nudge the doctors to use more FMT and do more research about them.

Intervention 8: Emphasize the Benefit of Fecal Microbiota Transplantation

We propose emphasizing how FMT could impact people's lives in a positive way and how fecal microbiota donor could have an enormous impact on helping others who suffer. For example, people with severe refractory *Clostridioides difficile* infection have a mortality rate of 43.2%. However, with FMT, the mortality rate is reduced to 12.1%. FMT can substantially decrease the requirement for colectomy in these patients from 31.8 to 7.6% (9, 10). We should simplify the essence of this intervention to nudge the doctors toward the belief that this FMT program is interesting and worth doing research on and worth referring patients to.

Because developing countries often lack resources and may encounter difficulties initiating FMT for only *Clostridioides difficile* infection, we should emphasize the impact and potential of FMT as a therapeutic target for various diseases. For example, FMT could be effective in curing inflammatory bowel disease, irritable bowel syndrome, and hepatic encephalopathy (2). Additionally, several studies have demonstrated that FMT may be beneficial in the treatment of metabolic syndrome, obesity, autism, Parkinson's disease, multidrug-resistant organism infections and autoimmune disorders (11–13). We should emphasize that, while initiating FMT in developing countries was challenging, if we succeed, we will have therapeutic targets for not only gastrointestinal disease but also for significant extra-gastrointestinal diseases that were the serious burden in developing countries.

Intervention 9: Default Choice of FMT in Comparison Between Developed Countries and Developing Countries

FMT has been included in several expert guidelines of standard practice for the treatment of recurrent and refractory *Clostridioides difficile* infection in developed countries in the recent years (14), and it is the indication approved by the United States (US) Food and Drug Administration (FDA) since 2013 (15). On the other hand, FMT was not included in clinical practice in developing countries, including Thailand (16). This feature obscured the application of FMT in clinical practice. We propose that incorporating FMT into clinical guidelines for *Clostridioides difficile* infection by adapting existing guidelines from developed countries in medical school and hospital training could benefit physicians to start FMT as standard practice. By adapting guidelines from developed countries in establishing FMT as a default choice for general physicians because physicians are more likely to follow the established guidelines.

3. Establishing a stool standard for use in other research trials.

4. Establishing a clinical center for the transplantation of fecal microbiota.

Steps 3 and 4 are the final steps, which require a multidisciplinary team comprised of dedicated medical doctors, scientists, pharmacists, and other associated staffs. It requires many complicated steps. However, if we can build a foundation in the first step, the subsequent steps will be a lot simpler.

We believe these interventions will promote the awareness and enable an establishment of a robust FMT program in Thailand and other developing countries.

AUTHOR CONTRIBUTIONS

CSav had the idea of writing the manuscript. CSav, NM, and CSam drafted the manuscript. SS revised the manuscript. CSam corrected and reviewed the manuscript. All authors approved the final version.

REFERENCES

1. Vindigni S, Surawicz C. Fecal microbiota transplantation. *Gastroenterol Clin North Am.* (2017) 46:171–85. doi: 10.1016/j.gtc.2016.09.012
2. Ooijevaar R, Terveer E, Verspaget H, Kuijper E, Keller J. Clinical application and potential of fecal microbiota transplantation. *Annu Rev Med.* (2019) 70:335–51. doi: 10.1146/annurev-med-111717-122956
3. Nerlich B, Koteyko N. Balancing food risks and food benefits: The coverage of probiotics in the UK National Press. *Sociol Res Online.* 13:1–14. doi: 10.5153/sro.1692
4. McLeod C, Nerlich B, Jaspal R. Fecal microbiota transplants: emerging social representations in the English-language print media. *New Genet Soc.* (2019) 38:331–51. doi: 10.1080/14636778.2019.1637721
5. The Hamilton Spectator. *Health News.* (2010). Available Online at: https://www.thespec.com (accessed August 4, 2021).
6. Thaler RH, Sunstein CR. *Nudge: Improving Decisions about Health, Wealth, and Happiness*. New Haven: Yale University Press (2008).
7. Cammarota G, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, et al. European consensus conference on faecal microbiota transplantation in clinical practice. *Gut.* (2017) 66:569–80. doi: 10.1136/gutjnl-2016-313017
8. OpenBiome. *Massachusetts, United States: openbiome*. (2020). Available online at: https://www.openbiome.org/home (accessed August 4, 2021).
9. Clua A. *The Anatomy of an Infographic: 6 Essential Elements*. (2017). Available online at: https://teamthunderfoot.com/ideas/the-anatomy-of-an-infographic/ (accessed August 6, 2021).
10. Cheng Y, Phelps E, Nemes S, Rogers N, Sagi S, Bohm M, et al. Fecal microbiota transplant decreases mortality in patients with refractory severe or fulminant *Clostridioides difficile* infection. *Clin. Gastroenterol. Hepatol.* (2020) 18:2234–43.e1. doi: 10.1016/j.cgh.2019.12.029
11. Wang J-W, Kuo C-H, Kuo F-C, Wang Y-K, Hsu W-H, Yu F-J, et al. Fecal microbiota transplantation: review and update. *J Formosan Med Assoc.* (2019) 118:S23–31. doi: 10.1016/j.jfma.2018.08.011
12. Vrieze A, Van Nood E, Holleman F, Salojärvi J, Kootte R, Bartelsman J, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. *Gastroenterology*. (2012) 143:913–6.e7. doi: 10.1053/j.gastro.2012.06.031
13. Aroniadis OC, Brandt LJ. Fecal microbiota transplantation: past, present and future. *Curr Opin Gastroenterol.* (2013) 29:79–84. doi: 10.1097/MOG.0b013e32835a4b3e

14. Surawicz C, Brandt L, Binion D, Ananthakrishnan A, Curry S, Gilligan P, et al. Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections. *Am J Gastroenterol*. (2013) 108:478–98; quiz 99. doi: 10.1038/ajg.2013.4
15. U.S. Food and Drug Administration. *Enforcement Policy Regarding Investigational New Drug Requirements for Use of Fecal Microbiota for Transplantation to Treat Clostridium difficile Infection Not Responsive to Standard Therapies*. White Oak, MD: U.S. Food and Drug Administration (2016).
16. Gastrothai.net. [Internet]. (2021). Available online at: https://www.gastrothai.net/source/content-file/100.vol83.pdf (accessed November 7, 2021)

Conflict of Interest: SS is employed by Century Pharmaceuticals, Inc.

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 08 February 2022
doi: 10.3389/fmed.2021.777961

Gut Metagenome as a Potential Diagnostic and Predictive Biomarker in Slow Transit Constipation

Hongliang Tian[1,2†], *Chen Ye*[1,2†], *Bo Yang*[1,2†], *Jiaqu Cui*[1,2], *Zhijun Zheng*[1,3], *Chunyan Wu*[1,3], *Shailan Zhou*[1,2], *Xiaoqiong Lv*[1,2], *Nan Qin*[1,3], *Huanlong Qin*[1,2], *Ning Li*[1,2] *and Qiyi Chen*[1,2*]

[1] *Intestinal Microenvironment Treatment Center of General Surgery, Tenth People's Hospital of Tongji University, Shanghai, China,* [2] *Clinical Research Center for Digestive Diseases of Tongji University, Shanghai, China,* [3] *Realbio Genomics Institute, Shanghai, China*

Slow transit constipation (STC) is one of the most frequent gastrointestinal diagnoses. In this study, we conducted a quantitative metagenomics study in 118 Chinese individuals. These participants were divided into the discovery cohort of 50 patients with STC and 40 healthy controls as well as a validation cohort of 16 patients and 12 healthy controls. We found that the intestinal microbiome of patients with STC was significantly different from that of healthy individuals at the phylum, genus, and species level. Patients with STC had markedly higher levels of Alistipes and Eubacterium and lower abundance of multiple species belonging to the *Roseburia* genus. Patients with STC gene expression levels and the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology pathway (such as fatty acid biosynthesis, butanoate metabolism, and methane metabolism pathways) enrichment were also substantially different from those of healthy controls. These microbiome and metabolite differences may be valuable biomarkers for STC. Our findings suggest that alteration of the microbiome may lead to constipation by changing the levels of microbial-derived metabolites in the gut. Above findings may help us in the development of microbial drugs.

Keywords: slow transit constipation, gut microbiome, metagenomic analysis, pathogenesis, diagnostic, biomarker

OPEN ACCESS

Edited by:
Yan-Dong Wang,
Beijing University of Chemical Technology, China

Reviewed by:
Fangyan Wang,
Wenzhou Medical University, China
Weiwei Ding,
Nanjing University, China

***Correspondence:**
Qiyi Chen
baiyi_nanjing@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *16 September 2021*
Accepted: *22 November 2021*
Published: *08 February 2022*

Citation:
Tian H, Ye C, Yang B, Cui J, Zheng Z, Wu C, Zhou S, Lv X, Qin N, Qin H, Li N and Chen Q (2022) Gut Metagenome as a Potential Diagnostic and Predictive Biomarker in Slow Transit Constipation. Front. Med. 8:777961. doi: 10.3389/fmed.2021.777961

INTRODUCTION

Chronic constipation is characterized by various symptoms such as straining, lumpy or hard stool, sensation of incomplete evacuation, sensation of anorectal obstruction, and infrequent defecation (<3 defecations/week) (1, 2). Slow transit constipation (STC) is the major category of chronic constipation (3). The characteristics of STC include slower colonic transit and fewer high-amplitude propagated contractions. It is a complex pathogenesis that involves the enteric nervous system, the interstitial cells of Cajal, and colonic smooth muscle, but it is still incompletely understood (4–7).

Decreased colonic motility is an important pathophysiological mechanism of STC. Recently, several studies have suggested that gut microbiota may be involved in the etiology of constipation. Disturbance of stool microbiota has been found in many patients with constipation (8, 9). Furthermore, Vandeputte et al. found that stool consistency is associated with the richness and composition of gut microbiota as well as enterotypes and bacterial growth rates (10). Recent

TABLE 1 | Characteristics of patients with slow transit constipation (STC) and healthy controls.

	STC patients ($n = 66$)	Healthy controls ($n = 52$)
Gender (M:F)	14:26	17:23
Age (y)	43.2 ± 2.6	45.2 ± 3.5
Weight (kg)	58.2 ± 3.2	59.7 ± 6.3
Height (cm)	166 ± 5.2	164 ± 7.2
BMI	20.4 ± 2.1	22.4 ± 1.6
Stools/day (week)	1.6 ± 0.8	5.4 ± 1.1
Stool consistency (1–7)	2.1 ± 0.8	4.2 ± 0.6
Medications history	Dietary modification, laxatives (including osmotic and stimulant laxatives), and biofeedback	None
Disease history (years)	6.1 ± 3.8	None
Colonic transit test (hours)	83.4 ± 12.6	None

advances in sequencing technology have profoundly affected the field of microbiology. Quantitative metagenomics analysis has been used successfully to study the pathogenesis of many chronic diseases (11, 12). However, to date, no study has used this approach to analyze STC.

In this study, to comprehensively catalog the gut microbiome features of constipation, we conducted a quantitative metagenomics study in 118 adults, including STC and healthy controls.

METHODS

Study Participants

A total of 66 patients with STC and 52 healthy controls from September 2015 to December 2019 were enrolled in this study from the Shanghai Tenth People's Hospital. Inclusion criteria: Ages eligible for study: 18 years and older; body mass index: 18–24 kg/m^2; and STC as defined by patients with a colonic transit test (CTT) of >48 h (13, 14). This clinical trial was registered at ClinicalTrials.gov (NCT02395484). The clinical diagnosis and fecal samples of all the individuals were obtained from the hospitals (**Table 1**).

Sample Collection and DNA Extraction

Fecal samples were collected from the recruited subjects from these 118 participants. All the samples were then frozen immediately and stored at −80°C until DNA extraction. DNA was extracted from each stool sample using the commercial QIAamp DNA Stool Mini Kit (Qiagen, Valencia, California, USA).

Deoxyribonucleic Acid Library Construction and Sequencing

The metagenomic DNA libraries were constructed with 2 μg genome DNA according to the instruction of the manufacturer (Illumina, California, USA), with an average of 350 bp insert size. The quality of all the libraries was evaluated using the Agilent 2100 Bioanalyzer with a DNA 1000 LabChip Kit.

Illumina Hiseq Sequencing

The Illumina Hiseq platform was employed to sequence the 118 samples, thereby obtaining a total of 1.6 Tbp sequencing data. Illumina raw reads were subjected to the following treatments: (1) reads with more than 3 ambiguous N bases were removed; (2) reads with less than 60% of high quality bases (Phred score ≥ 20) were deleted; and (3) 3′ end of reads were trimmed to the first high quality base. The subsequent high quality reads were further mapped to human genome by SOAPaligner (version 2.21) and any hit associated with the reads and their mated reads was removed. After QC, trimming, and remove host contaminate, 44.76 ± 0.86 million clean reads per sample on average are reserved. Sample statistic information is given in **Supplementary Table S1**.

De novo Assembly of the Illumina Short Reads

SOAPdenovo (version 2.04), which is based on De Bruijn graph construction, was employed to assemble short reads with parameters "-M 3 -u -L 100 -d 1 -F." k-mers, varying from 39 to 59 by 4, was tested for each sample (15). The resulting scaffolds were cut into contigs at ambiguous Ns and only contigs longer than 500 bp were saved. N50 was calculated for contigs of different k-mers and only the contigs of largest N50 assembly were attributed to a sample. All these contigs were applied for gene prediction by Meta-Gene Marker (version 3.25).

Taxonomic and Gene Profiling

Microbial composition at each taxonomic level was calculated using the MetaPhlAn2 program with default parameters. The program is available at https://bitbucket.org/biobakery/biobakery/wiki/humann2. Relative abundances of the genes were calculated with the procedure introduced in Qin et al. (12). When calculating the abundance of genes, the high quality reads from each sample were aligned against the gene catalog by using SOAPalign2.21 with parameters of "-r 2 -m 100 -× 1,000" and only the both paired-end reads, which could be mapped to a same gene, were accepted.

Gene Catalog, the Kyoto Encyclopedia of Genes and Genomes Database Annotation, and Pathway Profile

After removing redundancy by Collect DNA-HIT, genes were also annotated by the KEGG database. The KEGG Orthology (KO) profiling was calculated as the sum abundances of genes with the same KO number. The abundances of the KEGG pathway were calculated as the average abundances of all the KO under the pathway (16).

Statistical Methods

Characteristics of participant between two groups were compared by using the *t*-tests for continuous variables and the chi-squared tests for categorical variables. The richness

and the β-diversity of the microbiota dataset were analyzed using Quantitative Insights Into Microbial Ecology (17). The nonparametric Wilcoxon signed-rank test was employed to analyze the statistical significance of the gene, the KO, Ortholog Groups, enzyme, and different taxonomic (phylum, genus, and species) levels between the STC and healthy control group. The relative abundance of these features was subjected to statistical analyses. For categorical metadata and enterotype comparisons, samples were pooled into bins and significant features were identified using the Fisher's exact test with multiple testing correction of *p*-values. Statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, North Carolina, USA) and R software (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria) (18).

RESULTS

Enrollment of the Patient

Phenotype information was obtained from patients with STC and healthy controls during the discovery stage (90 samples) and the validation stage (28 samples), as shown in **Table 1**. The data obtained from these 118 samples are shown in **Supplementary Table S1**.

Difference in Gut Microbiota Composition

Our analysis revealed that STC displayed significant differences between two groups (**Figure 1**). At the phylum level, the intestinal flora in patients with STC was enriched in Firmicutes, Actinobacteria, and Verrucomicrobia and in healthy individuals was enriched in Bacteroidetes, Euryarchaeota, Fusobacteria, and Synergistetes (**Supplementary Table S2**). The genera enriched in patients with STC included Alistipes, Parabacteroides, Subdoligranulum, and Ruminococcus and the genera enriched in healthy individuals were Bacteroides, Roseburia, Haemophilus, and Klebsiella (**Supplementary Table S3**). At the species level, patients with STC were enriched in Alistipes putredinis, Parabacteroides merdae, Odoribacter splanchnicus, and Eubacterium eligens and healthy individuals were enriched in Roseburia intestinalis, Haemophilus parainfluenzae, Megamonas unclassified, and Klebsiella pneumoniae (**Supplementary Table S4**). Therefore, the intestinal microbiome of patients with STC is significantly different from that of healthy individuals at the phylum, genus, and species level.

Detection of Enterotypes in Patients With STC

Enterotype is a characteristic stratification of the intestinal microbiome population. To determine whether the patients with STC have a unique enterotype, we subjected 90 samples from cohort 1 to enterotype analysis, as described previously (19). The results showed that all the samples clustered into 1 of 3 enterotypes. The Bacteroides and Prevotella enterotypes were identical to those reported earlier (6) and the third enterotype was distinguished by high levels of Alistipes and Eubacterium (**Figure 2**; **Supplementary Figure S1**). The heatmap of top 30 most abundant genera for each of these 3 enterotypes is shown in **Supplementary Figure S2**.

Differences in Microbial Gene and Pathway Expression Between Two Groups

The gut microbiota of patients with STC and healthy individuals included in this study differed greatly, not only at the phylum, genus, and species level, but also in terms of gene expression. Genes corresponding to the 859 KO entries were differentially expressed in these 2 groups: 609 genes were enriched in patients with STC and 250 genes were enriched in healthy controls (**Supplementary Table S5**). To determine the functional impact of these differences, we performed a pathway analysis and found that the most abundant KEGG orthologs in both the groups were those associated with fatty acid biosynthesis (**Supplementary Figure S3**). In the fatty acid biosynthesis pathway, 3-oxoacyl-(acyl-carrier-protein) synthase II [EC:2.3.1.179] (fabF, K09458), 3-hydroxyacyl-(acyl-carrier-protein) dehydratase [EC:4.2.1.59] (fabZ, K02372), enoyl-(acyl-carrier protein) reductase I [EC:1.3.1.9 1.3.1.10] (fabI, K00208), and long-chain acyl-CoA synthetase [EC:6.2.1.3] (K01897) were enriched in healthy controls, whereas acetyl-CoA carboxylase/biotin carboxylase 1 [EC:6.4.1.2] (K11262) and fatty acid synthase, bacteria type [EC:2.3.1] (K11533) were enriched in patients with STC (**Supplementary Figure S4**).

In the butanoate metabolism (map00650) pathway, succinate dehydrogenase/fumarate reductase, flavoprotein subunit [EC:1.3.5.1] (K00239) was enriched in healthy controls, while (R,R)-butanediol dehydrogenase/meso-butanediol dehydrogenase/diacetyl reductase [EC:1.1.1.4 1.1.1.-1.1.1.303] (K00004), succinate-semialdehyde dehydrogenase [EC:1.2.1.76] (K18119), acetate CoA/acetoacetate CoA-transferase alpha subunit [EC:2.8.3.8 2.8.3.9] (K01034), acetaldehyde dehydrogenase (acetylating) [EC:1.2.1.10] (K00132), 4-hydroxybutyrate dehydrogenase [EC:1.1.1.61] (K00043), 4-hydroxybutyrate CoA-transferase [EC:2.8.3.-] (K18122), 4-hydroxybutyryl-CoA dehydratase/vinylacetyl-CoA delta-isomerase [EC:4.2.1.120 5.3.3.3] (K14534), butyryl-CoA dehydrogenase [EC:1.3.8.1] (K00248), enoyl-CoA hydratase [EC:4.2.1.17] (K01692), glutaconate CoA-transferase, subunit A [EC:2.8.3.12] (K01039), 3-hydroxybutyryl-CoA dehydrogenase [EC:1.1.1.157] (K00074), acetyl-CoA C-acetyltransferase [EC:2.3.1.9] (K00626), and hydroxymethylglutaryl-CoA lyase [EC:4.1.3.4] (K01640) were enriched in patients with STC (**Supplementary Figure S5**).

In the methane metabolism (map00680) pathway, acetyl-CoA synthetase [EC:6.2.1.1] (K01895), phosphate acetyltransferase [EC:2.3.1.8] (K00625), malate dehydrogenase [EC:1.1.1.37] (K00024), and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase [EC:5.4.2.12] (K15633) were enriched in healthy controls, whereas heterodisulfide reductase subunit A [EC:1.8.98.1] (K03388), 3-hexulose-6-phosphate synthase [EC:4.1.2.43] (K08093), (methyl-Co(III) methanol-specific corrinoid protein):coenzyme M methyltransferase [EC:2.1.1.246] (K14080), formylmethanofuran dehydrogenase subunit A [EC:1.2.7.12] (K00200), anaerobic carbon monoxide dehydrogenase, CODH/acetyl-CoA synthase (ACS) complex subunit alpha [EC:1.2.7.4] (K00192), acetyl-CoA decarbonylase/synthase, CODH/ACS complex subunit beta

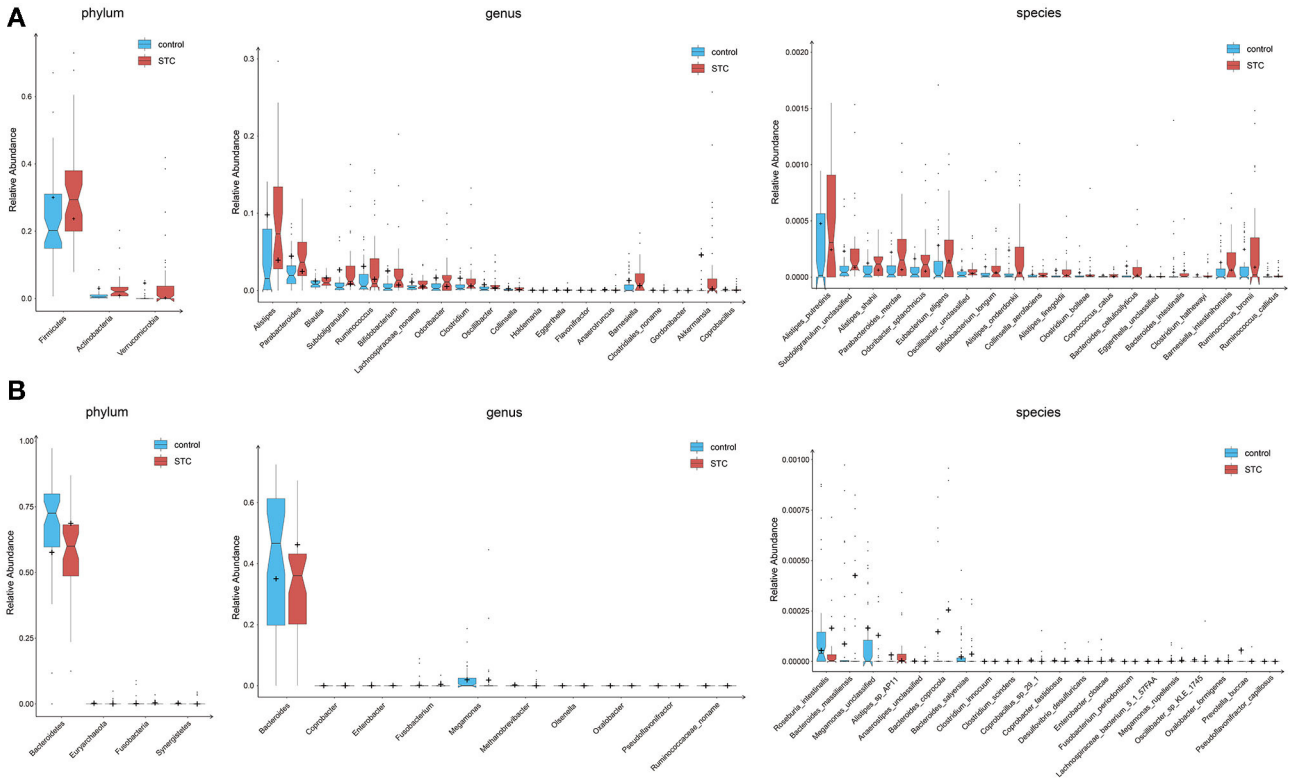


FIGURE 1 | Differences of phylogenetic abundance between patients with slow transit constipation (STC) and healthy controls. The phylotypes were increased **(A)** or decreased **(B)** in patients with STC at the phylum, genus, and species levels. Red and blue indicate patients with STC and healthy controls, respectively. The phylogenetic abundance of phyla that had mean values <1% and that of genera and species that were <0.01% was excluded. After exclusion, the Wilcoxon rank-sum tests were applied to identify the differentially abundant phyla, genera, and species. Among these, the highest medians of the phylogenetic abundance in the enriched cohort were drawn as boxplots.

[EC:2.3.1.169] (K00193), 2-phosphosulfolactate phosphatase [EC:3.1.3.71] (K05979), and methanogen homoaconitase large subunit [EC:4.2.1.114] (K16792) were enriched in patients with STC (**Supplementary Figure S6**).

Construction of a Model for Classifying Patients as Having STC Based on Intestinal Microbiome Biomarkers

On the basis of the microbial and gene expression characteristics identified as described above, we next sought to construct a model for distinguishing patients with STC from healthy individuals. First, we selected the 15 species with the most advantageous receiver operating characteristic (ROC) curve values from among the 59 species with differential abundance between the two groups (**Table 2**). The classification model constructed using these species as biomarkers had an Area Under Curve (AUC) of 88.65% in the discovery set and 78.65% in the validation set (**Figure 3**). Next, we constructed a classification model using the 10 differentially expressed KO markers with the most advantageous ROC curve values among the 859 differentially expressed KO biomarkers. This model had an AUC of 95.15% in the discovery set and 79.69% in the verification set (**Table 3**). Thus, the model constructed using KO biomarkers can adequately distinguish between patients with STC and healthy individuals.

DISCUSSION

In this study, we studied the gut microbiome in patients with STC using quantitative metagenomics. To the best of our knowledge, this is largest metagenomics study performed to date analyzing the characteristics of the gut microbiome in patients with STC. Our results show that the gut microbial characteristics of patients with STC differ from those of healthy controls, suggesting that gut microbiome composition may contribute to the development of STC.

We found that the intestinal flora of patients with constipation is more diverse than that of healthy individuals. In addition, we identified significant differences between the constipated and control groups at the phylum, genus, and species level. There was a statistically significant increase in the abundance of Actinobacteria, Firmicutes, and Verrucomicrobia and a statistically significant decrease in the abundance of Bacteroidetes, Euryarchaeota, Fusobacteria, and Synergistetes in the gut of patients with STC compared with the healthy control group.

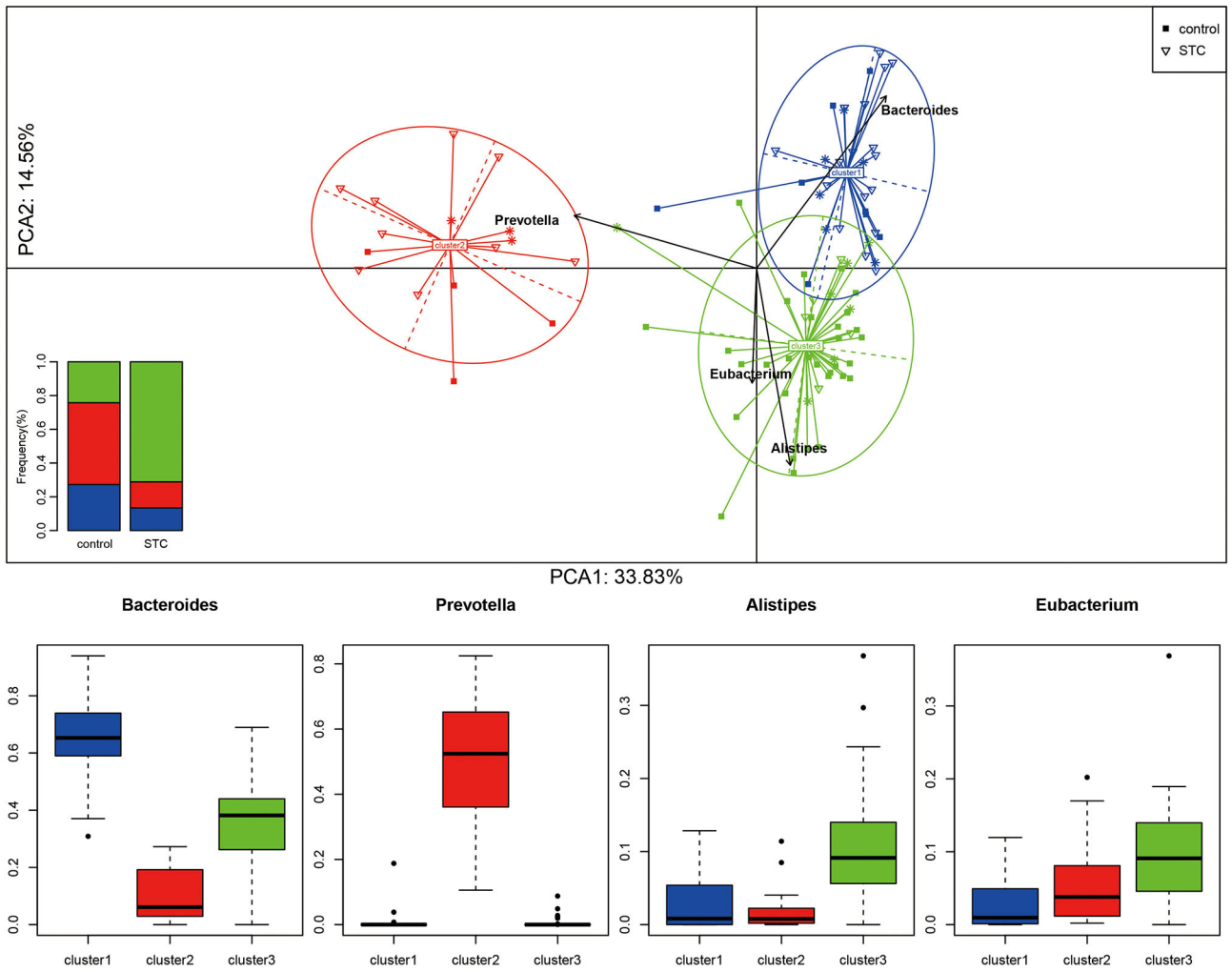


FIGURE 2 | Sample clustering and classification for Enterotype analysis. According to the Jensen–Shannon distance of genus level, all the samples are clustered into the 3 types by the method of Manimozhiyan. Each of these 3 clusters are identifiable by the variation in the levels of 1 of 3 genera: Bacteroides (cluster 1), Prevotella (cluster 2), Alistipes and Ruminococcus (cluster 3). The histogram shows the distribution ratio of the control and STC groups in the 3 clusters.

TABLE 2 | The best 10 KEGG Ontology (KO) markers in the receiver operating characteristic (ROC) curve picked from the 859 differential KO markers.

KO marker	Median (control)	Median (STC)	*P*-value	FDR	Enrichment in which group
K00087	0.000007	0.00001	0.0006	0.00004	STC
K00968	0.000006	0.00001	0.0006	0.002	STC
K03205	0.0003	0.0006	0.0006	0.0002	STC
K03696	0.0002	0.0002	0.0006	0.0002	STC
K07138	0.00009	0.0001	0.0006	0.000005	STC
K07444	0.0002	0.0002	0.0006	0.0001	Control
K07650	0.0000006	0.000001	0.004	0.027	STC
K11261	0.000001	0.000008	0.00005	0.00001	STC
K13787	0.000001	0.000003	0.00001	0.0005	STC
K17898	0.0000002	0.000001	0.000000001	0.0001	STC

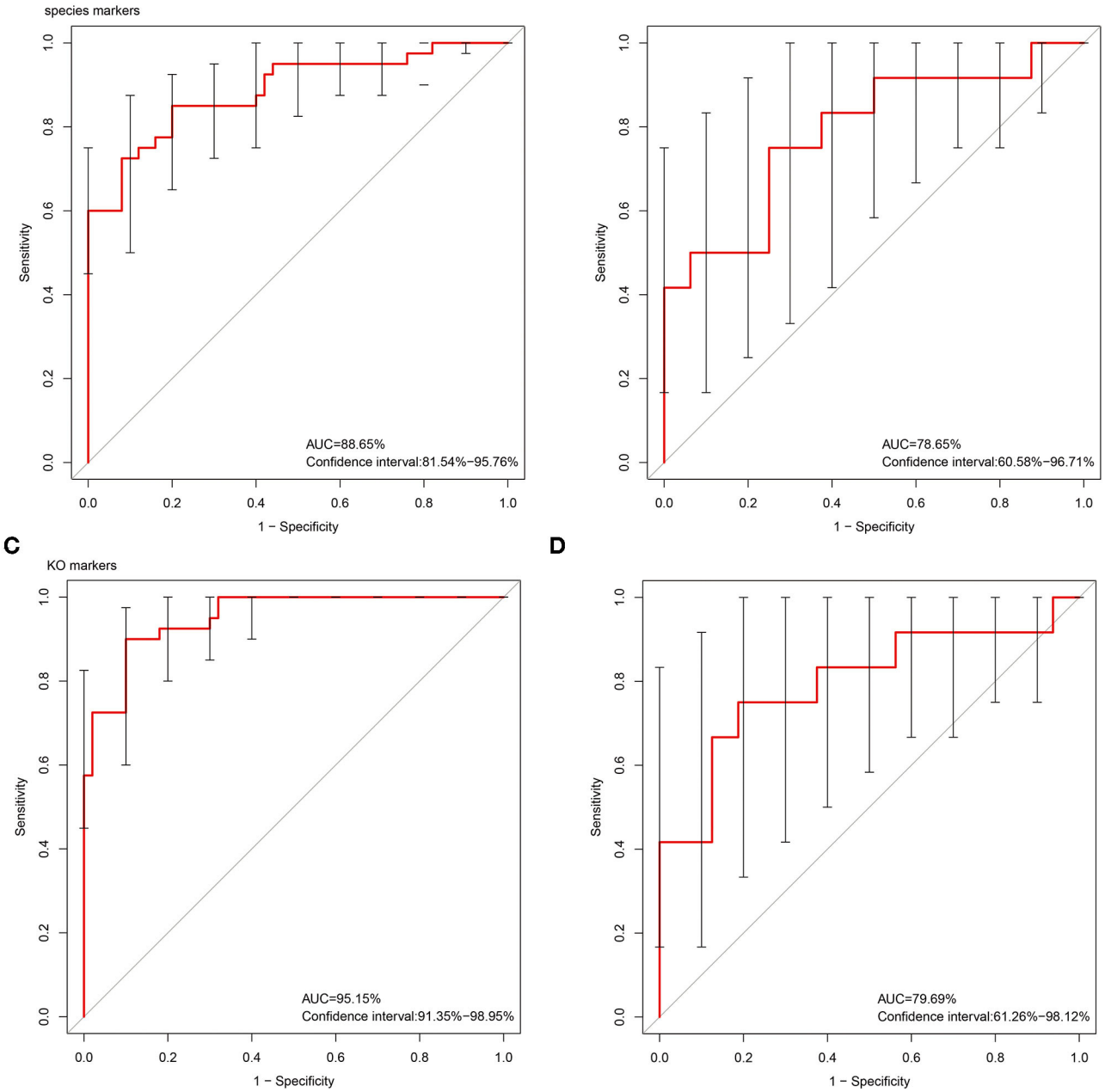


FIGURE 3 | The receiver operating characteristic (ROC) curves of the sequenced reference species markers and the KEEG Ontology (KO) markers. The classification model constructed using 15 species as biomarkers had an AUC of 88.65% in the discovery set **(A)** and 78.65% **(B)** in the validation set. We constructed a classification model using the 10 differentially expressed KO markers with the most advantageous ROC curve values among the 859 differentially expressed KO biomarkers. This model had an AUC of 95.15% **(C)** in the discovery set and 79.69% **(D)** in the verification set.

However, the abundance of *Roseburia intestinalis*, a prominent butyrate-producing Firmicute that is reported to be the primary degrader of dietary fiber (20), was increased in the healthy group compared with the STC group. This is the first study to robustly demonstrate that *Roseburia intestinalis* abundance is increased in the gut microbiome of patients with STC compared with healthy controls using shotgun sequencing. The altered abundances of this species are a cause or a consequence of constipation that warrant further investigation. In the recent years, the role of *Roseburia intestinalis* has been revealed in more and more studies (21, 22). The administration of *Roseburia intestinalis* contributes to restoration of the gut microbiota, promoting colon repair, and the recovery of gastrointestinal function.

TABLE 3 | The best 15 species markers in the ROC curve picked from the 59 differential species markers.

Species marker	Median (control)	Median (STC)	*P*-value	FDR	Enrichment in which group
Akkermansia_muciniphila	0	0.000001	0.0006	0.027	STC
Clostridium_hathewayi	0.000000006	0.000003	0.001	0.001	STC
Clostridium_symbiosum	0	0.000002	0.001	0.03	STC
Coprobacillus_sp_29_1	0	0	0.004	0.09	Control
CoprocoSTCus_catus	0.000002	0.00001	0.007	0.12	STC
Desulfovibrio_desulfuricans	0	0	0.007	0.12	control
Erysipelotrichaceae_bacterium_2_2_44A	0	0.0000001	0.0004	0.027	STC
Fusobacterium_periodonticum	0	0	0.04	0.34	Control
Gordonibacter_pamelaeae	0	0.0000007	0.0005	0.02	STC
Lachnospiraceae_bacterium_3_1_57FAA_CT1	0	0.00000003	0.00008	0.0099	STC
Oscillibacter_sp_KLE_1745	0	0	0.009	0.13	Control
Parabacteroides_merdae	0.00002	0.0001	0.002	0.05	STC
Roseburia_intestinalis	0.00004	0.000003	0.004	0.09	Control
Subdoligranulum_sp_4_3_54A2FAA	0	0	0.001	0.001	Control
Subdoligranulum_unclassified	0.00004	0.0001	0.0001	0.012	STC

The findings from this study may provide a rationale for microbiome-based therapies and patient stratification in the clinical management of constipation. The molecular Operational Taxonomic Units markers identified the current cohort that need to be validated in larger, independent studies. Although the alterations that we noted in the microbiota could serve as biomarkers for disease diagnosis or severity stratification, animal experiments and *in-vitro* studies are needed to elucidate whether these markers are cause of the disease or simply the consequence of increased CTT leading to stool retention and altered fermentation parameters (23, 24). Further investigation into possible mechanisms, microbial-assisted diagnosis, and therapeutics holds great potential for the effective management of constipation.

We constructed classification algorithms (classifiers) to identify patients with STC using 3 types of biomarkers. Each of these 3 enterotypes are identifiable by the variation in the levels of 1 of 3 genera: Bacteroides (enterotype 1), Prevotella (enterotype 2), and the 3rd enterotypes were marked by Alistipes and Eubacterium (**Figure 2**; **Supplementary Figure S1**). These phylogenetic and functional differences among enterotypes, thus, reflect different combinations of microbial trophic chains with a probable impact on synergistic interrelations with the human hosts. The robustness and predictability of the enterotypes in different cohorts and at multiple phylogenetic and functional levels indicate that they are the result of well-balanced, defined microbial community compositions of which only a limited number exist across individuals (25, 26).

The results showed that all the samples clustered into 1 of 3 enterotypes. The Bacteroides and Prevotella enterotypes were identical to those reported earlier in other diseases (27–29) and the 3rd enterotype was distinguished by high levels of Alistipes and Eubacterium, which have never been reported, may be unique to patients with STC, and were different from other types of diseases.

Some strong correlations occur between host properties and particular functions at the gene or module level (a module is a part of a pathway that is functionally tightly interconnected); we also attempted to identify bacterial biomarkers that may help in the diagnosis of STC by generating 10 different metagenomic shotgun markers. When using these biomarkers, we were able to distinguish patients with STC from normal patients with a moderate degree of certainty (AUC = 82.69). This is extremely important for the diagnosis and classification of constipation and serving the clinic through a cost-effective and time-saving technique is one of the purposes of this study.

The large sample size used in this study allowed us to focus on functional metagenomic data that are potentially related to prognosis and could be important for identifying adjuvant therapeutic targets that target functional pathways rather than specific species. Our analysis suggested that there are differences in the fatty acid biosynthesis, fatty acid degradation, butanoate metabolism, and methane metabolism pathways between STC and healthy microbiomes. Thus, modulating these metabolic pathways could influence the development of STC and related symptoms, providing new avenues for exploring the role of the gut microbiome in STC (30).

The most enriched orthologs in patients with STC were related to fatty acid, especially short-chain fatty acids (SCFAs), that play an important role in colonic motility. The intestinal microorganism ferment dietary fiber produces SCFAs, mainly acetic acid, propionic acid, and butyric acid. In the intestine, SCFAs not only act as nutrients for intestinal epithelial cells, but also serve to regulate intestinal pH, cell proliferation and differentiation, and gene expression (31, 32).

We also found that SCFAs have an important role in other diseases. Tan et al. reported low SCFAs in Parkinson's disease were significantly associated with poorer cognition. Lower butyrate levels correlated with worse postural instability-gait disorder scores (33). Liu et al. indicated that altered fecal microbiota might play vital roles in the pathogenesis of pediatric myasthenia gravis by reducing SCFAs. The microbial markers might serve as novel diagnostic methods for pediatric myasthenia gravis (34). Therefore, the intestinal microbiota in patients with STC may affect gut motility by altering host metabolism.

Metagenomics analysis showed that there was significant difference in the methane metabolism pathway between the two groups. This finding is particularly intriguing because one of the main clinical symptoms of patients with constipation is abdominal distension caused by excessive methane production. Compared with the healthy control group, methane metabolism pathways were significantly enriched in the STC group. Previous studies have shown that methane negatively affects intestinal motility, slowing colonic transit time (35, 36). This is the first study to focus on the correlation between methane and STC at the metagenomic level. On the basis of the above findings, we believe that methane quantification could be used to diagnose STC (37, 38).

There are several limitations of this study. First, we have collected for testing is natural feces, which are different from mucosal adherent bacteria. A cause–effect relationship between microbiome and constipation needs to be evaluated, especially for mucosal microbiome. Secondary, a thorough dietary survey was not performed prior to stool collection. Diet is a strong determinant of the microbial microenviroment. Constipated individuals are reported to consume fewer calories as well as lesser amount of protein and fat, but possibly increased amount of fibers and all of the above factors would affect the colonic microbiota and transit time. Thirdly, these diagnosis biomarkers should be tested through a larger clinical sample size and should be discussed to explore the application in future clinical diagnosis and treatment of STC. Fourth, we review the pros and cons of the current high-throughput methods. After isolating whole-genomic DNAs from a fecal sample, the high-throughput identification of 16S ribosomal DNA (rDNA) by Next Generation Sequencing (NGS) alone generates enough information for high-throughput identification of microbes constituting gut microbiota. NGS methods are the best approaches. However, this technology is far from becoming commonplace and affordable because of the high cost and infrastructure required for the analysis. In addition, this method cannot dynamically reflect the changes and effects of the intestinal flora and is gradually being replaced by metabolomics, transcriptomics, proteomics, bacterial culture, and fluorescence in situ hybridization (FISH), which facilitates the understanding the link between individual bacterial cells and their metabolic functions. Our results provide new avenues for the development of novel diagnostic tools and potential treatments. Furthermore, our findings suggest that alteration of the microbiome may lead to constipation by changing the levels of microbial-derived metabolites in the gut and that restoring balance to the disrupted microbiota could help to improve the clinical phenotype. Further experimental or preclinical studies are necessary to examine the role of the altered microbiota in the pathogenesis of colonic motility changes.

CONCLUSION

In this study, we have reviewed the association of gut microbiome composition with STC as well as its possible roles in the development of this disease. We revealed a novel link between the gut microbiome, host genome, and pathology of STC. This study will be a platform model of the microbiome studies to elucidate etiology of this disease, evidenced by the changes in genes, pathways, and various taxonomic levels. Discovery of the associated microbes of STC in the gut microbiome may help us to seek more intestinal microbial agents for this disease.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the: https://www.ncbi.nlm.nih.gov/bioproject/779475 repository, accession number PRJNA779475.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Tenth Hospital of Tongji University. 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

HT, JC, CW, SZ, and QC conceived the method. ZZ, NQ, XL, and HQ designed the experiments. XL, SZ, CY, JC, and NL performed the experiments and implemented the method. ZZ, CW, HT, NQ, NL, and HQ assessed the data and planned the statistical analyses. HT, BY, JC, CY, and QC wrote and edited the manuscript. All authors contributed to the article and approved the submitted version of the manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (No. 82100698) and the Clinical Research Plan of Shenkang Hospital Development Center (No. SHDC2020CR4026 and SHDC2020CR1030B).

ACKNOWLEDGMENTS

The authors would like to thank Chao Ding and Xiaolong Ge for their helpful advice and discussion.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.777961/full#supplementary-material

REFERENCES

1. Emmanuel A, Cools M, Vandeplassche L, Kerstens R. Prucalopride improves bowel function and colonic transit time in patients with chronic constipation: an integrated analysis. *Am J Gastroenterol*. (2014) 109:887–94. doi: 10.1038/ajg.2014.74
2. Bharucha AE, Pemberton JH, Locke GR III. American gastroenterological association technical review on constipation. *Gastroenterology*. (2013) 144:218–38. doi: 10.1053/j.gastro.2012.10.028
3. Wald A, Scarpignato C, Kamm MA, Mueller-Lissner S, Helfrich I, Schuijt C, et al. The burden of constipation on quality of life: results of a multinational survey. *Aliment Pharmacol Ther*. (2007) 26:227–36. doi: 10.1111/j.1365-2036.2007.03376.x
4. Zoppi G, Cinquetti M, Luciano A, Benini A, Muner A, Bertazzoni Minelli E. The intestinal ecosystem in chronic functional constipation. *Acta Paediatr*. (1998) 87:836–41. doi: 10.1111/j.1651-2227.1998.tb01547.x
5. Khalif IL, Quigley EM, Konovitch EA, Maximova ID. Alterations in the colonic flora and intestinal permeability and evidence of immune activation in chronic constipation. *Dig Liver Dis*. (2005) 37:838–49. doi: 10.1016/j.dld.2005.06.008
6. Zhu L, Liu W, Alkhouri R, Baker RD, Bard JE, Quigley EM, et al. Structural changes in the gut microbiome of constipated patients. *Physiol Genomics*. (2014) 46:679–86. doi: 10.1152/physiolgenomics.00082.2014
7. Kang DW, DiBaise JK, Ilhan ZE, Crowell MD, Rideout JR, Caporaso JG, et al. Gut microbial and short-chain fatty acid profiles in adults with chronic constipation before and after treatment with lubiprostone. *Anaerobe*. (2015) 33:33–41. doi: 10.1016/j.anaerobe.2015.01.005
8. Huang LS, Kong C, Gao RY, Yan X, Yu HJ, Wen B, et al. Analysis of fecal microbiota in patients with functional constipation undergoing treatment with synbiotics. *Eur J Clin Microbiol Infect Dis*. (2018) 37:555–63. doi: 10.1007/s10096-017-3149-7
9. Huang L, Zhu Q, Qu X, Qin H. Microbial treatment in chronic constipation. *Sci China Life Sci*. (2018) 61:744–52. doi: 10.1007/s11427-017-9220-7
10. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. *Gut*. (2016) 65:57–62. doi: 10.1136/gutjnl-2015-309618
11. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. *Nature*. (2013) 500:541–6. doi: 10.1038/nature12506
12. Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome in liver cirrhosis. *Nature*. (2014) 513:59–64. doi: 10.1038/nature13568
13. Metcalf AM, Phillips SF, Zinsmeister AR, MacCarty RL, Beart RW, Wolff BG. Simplified assessment of segmental colonic transit. *Gastroenterology*. (1987) 92:40–7. doi: 10.1016/0016-5085(87)90837-7
14. Southwell BR, Clarke MC, Sutcliffe J, Hutson JM. Colonic transit studies: normal values for adults and children with comparison of radiological and scintigraphic methods. *Pediatr Surg Int*. (2009) 25:559–72. doi: 10.1007/s00383-009-2387-x
15. Xie Y, Wu G, Tang J, Luo R, Patterson J, Liu S, et al. SOAPdenovo-Trans: de novo transcriptome assembly with short RNA-Seq reads. *Bioinformatics*. (2014) 30:1660–6. doi: 10.1093/bioinformatics/btu077
16. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*. (2006) 22:1658–9. doi: 10.1093/bioinformatics/btl158
17. Parameswaran P, Torres CI, Lee HS, Krajmalnik-Brown R, Rittmann BE. Syntrophic interactions among anode respiring bacteria (ARB) and Non-ARB in a biofilm anode: electron balances. *Biotechnol Bioeng*. (2009) 103:513–23. doi: 10.1002/bit.22267
18. Yu D, Yang Y, Long J, Xu W, Cai Q, Wu J, et al. Long-term diet quality and gut microbiome functionality: a prospective, shotgun metagenomic study among Urban Chinese Adults. *Curr Dev Nutr*. (2021) 5:nzab026. doi: 10.1093/cdn/nzab026
19. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. *Nature*. (2011) 473:174–80. doi: 10.1038/nature09944
20. Shahid S, Ramzan Z, Maurer AH, Parkman HP, Fisher RS. Chronic idiopathic constipation: more than a simple colonic transit disorder. *J Clin Gastroenterol*. (2012) 46:150–4. doi: 10.1097/MCG.0b013e318231fc64
21. Xu F, Cheng Y, Ruan G, Fan L, Tian Y, Xiao Z, et al. New pathway ameliorating ulcerative colitis: focus on Roseburia intestinalis and the gut-brain axis. *Therap Adv Gastroenterol*. (2021) 14:17562848211004469. doi: 10.1177/17562848211004469
22. Qin Q, Yan S, Yang Y, Chen J, Li T, Gao X, et al. A metagenome-wide association study of the gut microbiome and metabolic syndrome. *Front Microbiol*. (2021) 12:682721. doi: 10.3389/fmicb.2021.682721
23. Hadizadeh F, Walter S, Belheouane M, Bonfiglio F, Heinsen FA, Andreasson A, et al. Stool frequency is associated with gut microbiota composition. *Gut*. (2017) 66:559–60. doi: 10.1136/gutjnl-2016-311935
24. Barbara G, Feinle-Bisset C, Ghoshal UC, Quigley EM, Santos J, Vanner S, et al. The intestinal microenvironment and functional gastrointestinal disorders. *Gastroenterology*. (2016) 2016:S0016–5085. doi: 10.1053/j.gastro.2016.02.028
25. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora Science. (2005) 308:1635–8. doi: 10.1126/science.1110591
26. Hayashi H, Sakamoto M, Benno Y. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiol Immunol*. (2002) 46:535–48. doi: 10.1111/j.1348-0421.2002.tb02731.x
27. Brial F, Chilloux J, Nielsen T, Vieira-Silva S, Falony G, Andrikopoulos P, et al.Human and preclinical studies of the host-gut microbiome co-metabolite hippurate as a marker and mediator of metabolic health. *Gut*. (2021) 70:2105–14. doi: 10.1136/gutjnl-2020-323314
28. Lu J, Zhang L, Zhai Q, Zhao J, Zhang H, Lee YK, et al. Chinese gut microbiota and its associations with staple food type, ethnicity, and urbanization. *NPJ Biofilms Microbiomes*. (2021) 7:71. doi: 10.1038/s41522-021-00245-0
29. Seong E, Bose S, Han SY, Song EJ, Lee M, Nam YD, et al. Positive influence of gut microbiota on the effects of Korean red ginseng in metabolic syndrome: a randomized, double-blind, placebo-controlled clinical trial. *EPMA J*. (2021) 12:177–97. doi: 10.1007/s13167-021-00243-4
30. Vital M, Howe AC, Tiedje JM. Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *mBio*. (2014) 5:e00889. doi: 10.1128/mBio.00889-14
31. Bai J, Li Y, Zhang W, Fan M, Qian H, Zhang H, et al. Effects of cereal fibers on short-chain fatty acids in healthy subjects and patients: a meta-analysis of randomized clinical trials. *Food Funct*. (2021) 12:7040–53. doi: 10.1039/D1FO00858G
32. Kim MG, Jo K, Chang YB, Suh HJ, Hong KB. Changes in the gut microbiome after galacto-oligosaccharide administration in loperamide-induced constipation. *J Pers Med*. (2020) 10:161. doi: 10.3390/jpm10040161
33. Tan AH, Chong CW, Lim SY, Yap IKS, Teh CSJ, Loke MF, et al. Gut microbial ecosystem in parkinson disease: new clinicobiological insights from multi-omics. *Ann Neurol*. (2021) 89:546–59. doi: 10.1002/ana.25982
34. Liu P, Jiang Y, Gu S, Xue Y, Yang H, Li Y, et al. Metagenome-wide association study of gut microbiome revealed potential microbial marker set for diagnosis of pediatric myasthenia gravis. *BMC Med*. (2021) 19:159. doi: 10.1186/s12916-021-02034-0
35. Pimentel M, Saad RJ, Long MD, Rao SSC. ACG clinical guideline: small intestinal bacterial overgrowth. *Am J Gastroenterol*. (2020) 115:165–78. doi: 10.14309/ajg.0000000000000501
36. Attaluri A, Jackson M, Valestin J, Rao SS. Methanogenic flora is associated with altered colonic transit but not stool characteristics in constipation without IBS. *Am J Gastroenterol*. (2010) 105:1407–11. doi: 10.1038/ajg.2009.655
37. Pimentel M, Lin HC, Enayati P, van den Burg B, Lee HR, Chen JH, et al. Methane, a gas produced by enteric bacteria, slows intestinal transit and

augments small intestinal contractile activity. *Am J Physiol Gastrointest Liver Physiol*. (2006) 290:G1089–95. doi: 10.1152/ajpgi.00574.2004

38. Kunkel D, Basseri RJ, Makhani MD, Chong K, Chang C, Pimentel M. Methane on breath testing is associated with constipation: a systematic review and meta-analysis. *Dig Dis Sci*. (2011) 56:1612–8. doi: 10.1007/s10620-011-1590-5

Conflict of Interest: ZZ, CW, and NQ was employed by company Realbio Genomics Institute.

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.

frontiers in Medicine

STUDY PROTOCOL
published: 22 February 2022
doi: 10.3389/fmed.2022.779205

A Randomized Placebo-Controlled Trial of Combination Therapy With Post-triple-antibiotic-therapy Fecal Microbiota Transplantation and Alginate for Ulcerative Colitis: Protocol

Dai Ishikawa[1,2], Xiaochen Zhang[1], Kei Nomura[1], Natsumi Seki[3], Mayuko Haraikawa[1], Keiichi Haga[1], Tomoyoshi Shibuya[1], Yun-Gi Kim[3] and Akihito Nagahara[1,2]*

[1] Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan, [2] Department of Intestinal Microbiota Therapy, Juntendo University School of Medicine, Tokyo, Japan, [3] Research Center for Drug Discovery, Faculty of Pharmacy, Graduate School of Pharmaceutical Sciences, Keio University, Tokyo, Japan

OPEN ACCESS

Edited by:
Javier Ochoa-Repáraz,
Eastern Washington University,
United States

Reviewed by:
Taro Osada,
Juntendo University Urayasu Hospital, Japan
Shigeo Koido,
Jikei University Kashiwa Hospital, Japan
Qianqian Li,
Nanjing Medical University, China

***Correspondence:**
Dai Ishikawa
dai@juntendo.ac.jp
orcid.org/0000-0002-1130-2210

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 18 September 2021
Accepted: 19 January 2022
Published: 22 February 2022

Citation:
Ishikawa D, Zhang X, Nomura K, Seki N, Haraikawa M, Haga K, Shibuya T, Kim Y-G and Nagahara A (2022) A Randomized Placebo-Controlled Trial of Combination Therapy With Post-triple-antibiotic-therapy Fecal Microbiota Transplantation and Alginate for Ulcerative Colitis: Protocol. Front. Med. 9:779205. doi: 10.3389/fmed.2022.779205

Background: Fecal microbiota transplantation (FMT) has been widely performed for ulcerative colitis (UC) treatment at the clinical trial stage. Previous reports have used multiple FMT methods to enhance the colonization of healthy donor microbiota in the recipient's intestines. FMT following triple antibiotic therapy with amoxicillin, fosfomycin, and metronidazole (A-FMT) is not only effective but also requires only one FMT, which improves dysbiosis caused by reduced Bacteroidetes diversity in patients with UC. Alginate and its derivatives have the potential to induce the growth of intestinal bacteria including Bacteroides members and produce short-chain fatty acids (SCFAs), which are beneficial in regulating overactive autoimmunity. Our trial aims to investigate whether post-intervention with alginate, which can improve the intestinal environment, will enhance the therapeutic effect of A-FMT in UC and increase the long-term remission rate.

Methods and Analysis: This trial is a double-blinded, randomized, placebo-controlled, parallel assignment trial. Patients with UC and fecal donation candidates will undergo strict screening before being involved in the trial. Eligible patients are randomly divided into two groups: one group will drink one bottle of alginate twice a day for 8 consecutive weeks after A-FMT, while the other group will take a placebo instead of the alginate drink. The primary endpoints are the changes in the Total Mayo Score at 8 weeks after study initiation and A-FMT from baseline. The secondary endpoint is the comparison of clinical features, microbiota, and metabolomic analysis before and after 8 weeks of study food intake. Changes at 6, 12, 18, and 24 months after A-FMT will be assessed. Finally, a subpopulation analysis of the relationship between patients and donors is an exploratory endpoint.

Discussion: The FMT post-treatment used in this study is an oral alginate drink that is easily accepted by patients. If the regimen achieves the desired results, it

can further improve the A-FMT regimen and provide evidence for clinical practice guidelines for UC.

Clinical Trial Registration: https://jrct.niph.go.jp/latest-detail/jRCTs031200103, identifier: jRCTs031200103.

Keywords: ulcerative colitis, alginate, fecal microbiota transplantation (FMT), double-blind randomized controlled trial, antibiotic-FMT, placebo-controlled clinical study

INTRODUCTION

Although advances in ulcerative colitis (UC) drug treatment regimens have been made, 20–25% of patients choose a surgical intervention due to resistance or intolerance to drug treatment, which can cause pain and inconvenience to patients (1). To date, the pathogenesis of UC is not completely understood, and is only known to be the result of autoimmune processes and genetic and environmental factors; however, patients with UC commonly show decreased diversity and richness in the intestinal microbiota, resulting in dysbiosis (2, 3). Dysbiosis is thought to contribute to the development of aberrant immunological responses in inflammatory bowel disease (IBD) (4).

Fecal microbiota transplantation (FMT) is a minimally invasive therapeutic approach that involves transplanting intestinal microbiota from healthy donors to restore the normal intestinal microbiota functions in patients with disease-related dysbiosis. The implementation of FMT to treat UC is rapidly attracting the attention of the public (5, 6). Randomized clinical trials have assessed the use of multiple FMT operations for enhancing the colonization of the intestinal microbiota from healthy donors (7–10). The clinical remission rate of patients receiving FMT reached an average of 42.1% (11). However, the methods outlined in the above reports are difficult to manage and implement in actual clinical practice.

FMT following triple antibiotic therapy with amoxicillin, fosfomycin, and metronidazole (A-FMT), a method used by our team, is simple to operate because it only requires one FMT *via* colonoscopy followed by administration of antibiotics for 2 weeks. The combination antibiotic therapy consisting of amoxicillin, fosfomycin, and metronidazole (AFM) was modified for amoxicillin, tetracycline and metronidazole (ATM) therapy to reduce the adverse events caused by tetracycline. The efficacy of these combination antibiotic therapies (AFM, ATM) in treating patients with UC was previously reported (12, 13). We employed AFM as antibiotic pre-treatment to enhance reprogramming of the host intestinal microbiota by increasing the donor microbe colonization (14) and to achieve synergistic effects with FMT. We previously reported that A-FMT contributed to the recovery of the Bacteroidetes composition, which is associated with clinical responses and UC severity (15). It has also been observed that Bacteroidetes species components in clinical responders treated with A-FMT remarkably resembled those of their donors (16). These results indicate that A-FMT can effectively transplant the Bacteroidetes cells which is lost as UC activity progress (17) in fecal specimens from the donor to the intestinal environment of the recipient, which is parallel to the clinical improvement of UC. Furthermore, the high average clinical remission rate (43.8%) was observed in patients at four weeks after administering A-FMT comparing to previous reports with conventional FMT methods (42.1%) (15, 18). Although A-FMT exhibited obvious advantages compared to AFM monotherapy in our long-term clinical study, the remission rate gradually declined to 18.2% within 24 months (18). Wei et al. reported that intervention with pectin, a soluble dietary fiber extracted from apples, after FMT can preserve the diversity of the intestinal microbiota of patients with UC, providing results similar to those of donors (19). We speculate that FMT combined with post-interventions to maintain the diversity of the intestinal microbiota may improve the efficacy of FMT in UC treatment.

Alginic acid is a soluble dietary fiber polysaccharide that is widely distributed in the cell walls of brown algae in the form of alginate (20). Previous reports have found that alginate oligosaccharides have various activities, such as anti-inflammatory activity (21), antifungal activity (22) and immunomodulatory activity (23). Besides, alginate not only has a protective function on mucous membranes of the upper gastrointestinal tract (24), but also ameliorated the symptoms of experimental colitis and inflammatory responses (25). Mirshafiey et al. have also verified that alginate can be used as a potential treatment option for UC in both acute and chronic phase by rat models (26, 27). Furthermore, alginate and its derivatives can improve the growth of intestinal bacteria and can be fermented by human gut bacteria to produce short-chain fatty acids (SCFAs), which are beneficial to the intestine (28–30). A meta-analysis showed that clinical improvements in IBD are associated with the abundance of fecal microbiota and the enrichment of SCFA-producing anaerobes (31). Therefore, the aims of our study are to determine whether post-intervention with alginate can increase the diversity of microbiota to enhance the therapeutic effect of A-FMT in UC and delay the loss of microbiota diversity to improve the long-term remission rate. To our knowledge, this is the first randomized controlled study of the relationship between alginate and UC therapy.

METHODS AND ANALYSIS

Study Design and Patients

This double-blinded randomized, placebo-controlled, parallel assignment trial will be conducted at Juntendo University Hospital (Tokyo, Japan). The study will be conducted until

Abbreviations: UC, ulcerative colitis; IBD, inflammatory bowel disease; FMT, fecal microbiota transplantation; A-FMT, FMT following triple antibiotic therapy AFM (amoxicillin, fosfomycin, and metronidazole); FCSA, short-chain fatty acids; CDI, *Clostridium difficile* infection; HAMD, Hamilton Depression Scale; NPI, Neuropsychiatric Inventory; PEG, polyethylene glycol.

TABLE 1 | Exclusion criteria for patients with UC.

Informed consent not provided
Infectious enterocolitis
Receiving local therapy
Serious disease, such as liver disease, kidney disease, heart disease, or other serious complications
Autoimmune disease
Pregnant women and all cases with the possibility of pregnancy
Allergic diseases
Antibiotic therapy in the past 3 months
Any other cases judged inappropriate by the responsible researcher

UC, Ulcerative colitis.

December 31, 2024 and is expected to involve 60 patients with UC. Diagnosis of UC will be established based on standard clinical, endoscopic, and histological findings (32) and both hospitalized patients and outpatients will be included. To be eligible, patients must be diagnosed with active UC and meet the requirements of obtaining a Total Mayo Score of 3–10 (7, 9, 10) and a Sum Endoscopic Mayo Score of 2 or above. All patients will be over 20 years old and competent enough to provide informed consent. There are no restrictions on gender.

To ensure the safety of patients, rigorous screening of patients will be performed. If the patient has serious illness, pregnant women, or has received local therapy, etc. will not be included in our experiment. The detailed exclusion criteria for this study are listed in **Table 1**.

Donors

To understand the health status of the donors and reduce additional costs, we will conduct a preliminary medical inquiry and physical examination of the donor candidates. As dysbiosis is correlated with neuropsychiatric disorders including depression and autism (gut-brain interaction) (33, 34), the Hamilton Depression Scale (HAMD) and Neuropsychiatric Inventory (NPI) will be used to assess psychiatric symptoms (**Table 2A**). There are no restrictions on gender. In addition, to minimize the risk of infection transmission, the donor candidates who have passed the preliminary screening will undergo fecal and blood tests. Donor candidates who test positive for any of the items in **Tables 2B**, **2C** will be excluded. Candidates who pass the above rigorous screening will become eligible donors. Finally, eligible donors will confirm whether they have diarrhea, fever, or other uncomfortable symptoms when submitting stool, as well as whether any cohabitants have diarrhea symptoms. The donor candidates recommended by the patients themselves will also need to pass all the screening processes mentioned above. Approximately 150–200 g of fresh stool provided by the eligible donors will be dissolved in 500 ml of sterile normal saline. The sample will be processed to filter out crude fiber, and 100% glycerol will be added to make the final glycerol concentration 10%. The sample will then be divided into 200-ml bottles (Corning Co., Ltd., NY, USA). This procedure will be performed under an anaerobic environment by replacing air with nitrogen gas in a glove box (SANPLATEC Co., Ltd., Osaka, Japan). The fecal suspension will be cryopreserved at −60°C in advance.

TABLE 2A | Donor exclusion criteria.

Medical interview (exclusions)
Age <18 or >70
BMI <18 or >25 or metabolic syndrome
Informed consent not provided
International travel to area with high risk of traveler's diarrhea in the last 6 months
High-risk sex (unprotected sex outside of a monogamous relationship in last 3 months, or men who have sex with men, sex for drugs or money)
Tattoo, body piercing, or acupuncture in the last 6 months
Needle stick accident in the last 6 months
Household members with active gastrointestinal infection
History of vaccination with a live attenuated virus in the last 3 months
Incarceration or a history of incarceration
Medical history (exclusions)
History of major gastrointestinal surgery
Family history of colorectal carcinoma
Active medical illness or symptoms
Antimicrobials (antibiotics, antivirals, antifungals), probiotics, or PPIs in the last 3 months
Taking any medications
Acute diarrhea in the last 3 months
Irritable bowel syndrome, chronic constipation, Chronic diarrhea Other intrinsic gastrointestinal illness: Inflammatory bowel disease, Colonic polyps, Colon cancer
Autoimmune disease
Atopic disease (including atopic dermatitis)
Chronic fatigue syndrome
Psychiatric symptoms (exclusion)
Any psychiatric disorder assessed by HAMD or NPI

BMI, body mass index; PPIs, proton pump inhibitors; HAMD, Hamilton Depression Scale; NPI, Neuropsychiatric inventor.

Interventional Methods

First, eligible patients with UC will receive a combination antibiotic regimen called AFM comprising oral amoxicillin (1,500 mg/day), fosfomycin (3,000 mg/day), and metronidazole (750 mg/day). AFM will be administered to patients for 2 weeks until 2 days before the FMT (15). The fecal suspension (200 ml) will be thawed at 37°C using a water bath shaker on the day of the FMT operation. After bowel lavage using a standard polyethylene glycol (PEG) solution (Moviprep; EA Pharma, Tokyo, Japan), the patients will undergo total colonoscopy. A total of 200 mL of fecal suspension will be transferred to the patient's cecum. After the procedure, patients will receive scopolamine (10 mg) to slow intestinal transit and maintain a right lateral position. If the patient's drug dose for UC treatment has been stable for at least 12 weeks before the pre-registration, the patient will be permitted to continue ongoing treatment. However, changes in the dosage of drugs or initiation of new treatments will not be allowed during the intervention. After the A-FMT regimen described above, the patients will

TABLE 2B | Donor screening criteria: blood test.

Infections
Hepatitis A virus IgM, Hepatitis B virus surface antigen/antibody, Hepatitis B virus core antibody, Hepatitis C virus antibody, Hepatitis E virus IgA
HIV type 1 and type 2 antibody and antigen
Human T-cell lymphotropic virus-1 antibody
Syphilis (RPR/TP)
Parasite-specific antibody screening test
Epstein Barr virus IgM
Cytomegalovirus antigen pp65 * RT-PCR
Tuberculosis (IFN-γ)
COVID-19 antigen
Health condition
Complete blood count
Electrolytes (sodium, potassium, chlorine)
Renal function tests (blood urea nitrogen, creatinine)
Liver function tests (AST, ALT, ALP, γ-GT)
Albumin
C-reactive protein

Ig, immunoglobulin; HIV, human immunodeficiency virus; IFN, interferon; RPR, rapid plasma regain; TP, treponema pallidum; RT-PCR, reverse transcription-polymerase chain reaction; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; γ-GT, gamma-glutamyltransferase; SARS-CoV-2, severe acute respiratory syndrome corona virus 2.

TABLE 2C | Donor screening criteria: fecal test.

Fecal occult blood testing
Parasites, ova, cysts
Cryptosporidium
Giardia
Norovirus
Rotavirus
General bacterial culture for common enteric pathogens
Salmonella
Shigella
Yersinia
Campylobacter
Escherichia coli
Diarrheagenic *Escherichia coli*
Enterohemorrhagic *Escherichia coli*
Escherichia coli verotoxin
Clostridium difficile toxin/ *Clostridium difficile*-specific GDH

GDH, glutamate dehydrogenase.

be randomized into two groups for a double-blind FMT post-treatment. One group will consume one bottle of alginate drink twice a day for 8 consecutive weeks, while the other group will take a placebo. The alginate drink is a mixed tea drink provided by Kaigen Pharma Co., Ltd. (Osaka, Japan) containing 4 g of sodium alginate per bottle (150 g) and has been proven to be safe (https://www.h-food.or.jp/dbadm/media/231cec6ad1478e2d429f733d93a7b272.pdf). The placebo uses the same tea drink with the sodium alginate removed and does not have a different appearance or taste. Patients can adjust their medication during post-observation after the study food intervention. To compare the effects of treatment, all patients will undergo symptom checking and colonoscopy and have blood and fecal samples tested at the times indicated in **Table 3**. Colonoscopy founding at screening, A-FMT and 8 weeks after study food intake should be taken video and judged by a third-part expert to confirm the scope and severity of UC lesions. Biopsy of inflammatory sites will be used to evaluate the histological score. Blood samples will be used to test routine blood biochemistry, and plasma components will be separated by centrifugal force and cryopreserved at −60°C. Fecal samples will be collected to determine the occult blood condition and calprotectin levels and analyze the intestinal microbiota and metabolites.

Outcomes

The primary endpoint is to compare the changes in the Total Mayo Score at 8 weeks after study initiation and A-FMT from baseline between alginate intake group and placebo intake group. The secondary endpoints are the comparison of the following items at 8 weeks after study initiation and A-FMT from baseline:

- The change in the Sum Endoscopic Mayo Score.
- Analysis of intestinal microbiota by 16S rRNA gene amplicon sequencing and whole-genome sequencing.
- Metabolomic analysis: In addition to SCFAs in stool samples, the amount of lipids, free fatty acids, bile acids, and other clinical markers will also be measured by liquid chromatography tandem mass spectrometry (35).

We will also do same analysis as (1), (2), and (3) at 6, 12, 18, and 24 months after 8 weeks post-study initiation.

Subpopulation analysis of the relationship between patients and donors is considered an exploratory endpoint.

Clinical features will be judged by the Total Mayo Score, and the mucosal appearance of the Mayo Score will be evaluated by endoscopy. To evaluate the overall status of the intestine, endoscopic findings will be evaluated using the Sum Endoscopic Mayo Score, which refers to the sum of the scores of the seven segments (appendicular region, cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum). Intestinal microbial analysis will assess changes in composition and diversity. Metabolomic analysis will evaluate the metabolites of microbiota and elucidate the mechanism of action of alginate in the treatment of UC.

Adverse Events

Any adverse event caused by antibiotic (12), FMT therapy, study food intake, blood sampling, and endoscopy should be recorded in detail, including symptoms, time, duration, and follow-up until complete resolution or termination of treatment. If there is any serious adverse event that may lead to death, life-threatening, or intolerability, the study will be terminated and treatment will begin immediately.

TABLE 3 | Protocol for sequential therapy of A-FMT with alginate drink or placebo.

Item \ Period	Screening stage		Duration of administration				Post-observation
	Pre-registration (within 2 months)	Screening (within a month)	AFM	A-FMT	Alginate drink or Placebo		6, 12, 18, and 24 months after
			2 weeks		4 weeks after	8 weeks after	
Informed consent	•						
Check of patient background	•						
Administration of study food					•	•	
Check of symptoms	•	•		•	•	•	•
Endoscopy		•		•		•	
Blood samples		•		•		•	•
Fecal samples		•		•		•	•

A-FMT, FMT following triple antibiotic therapy AFM (amoxicillin, fosfomycin, and metronidazole).

TABLE 4 | The baseline characteristics of the study participants.

Items	All ($n = 33$)
Age	40.5 ± 12.0
Sex (M/F)	22/11
Duration of disease (years)	7.3 ± 5.4
Disease location	
Proctitis	4
Left-sided colitis	14
Extensive colitis	15
Total Mayo score	7.12 ± 1.9
Mild: 3–5	8
Moderate: 6–10	25
Sum endoscopic mayo score	6.5 ± 4.2
Ongoing treatment	
5-ASA	30
Corticosteroid	2
Azathioprine	4
Vedolizumab	1
Anti-TNF	3

5-ASA, 5-aminosalicylic acid; Anti-TNF, anti-tumor necrosis factor.

Progress and Estimated Study Period

This trial began on August 25, 2020. The first study participant was enrolled on September 28, 2020. At present, 33 eligible patients have been included in our study on December 8, 2021. We summarized the baseline characteristics of the 33 patients (**Table 4**). If the number of participants continues to increase at this pace, this trial is expected to end in October 2022.

Sample Size Estimation

In our previous clinical trials (15), ∼40 eligible patients with UC were collected in 1 year. Therefore, conservative estimates predict that 60 patients can be collected over 3 years. Eligible patients with UC will be randomized and assigned to two groups with a ratio of 1:1 in a double-blinded fashion. The estimated difference between each group in the Total Mayo Score as the primary endpoint from baseline is −2, and the standard deviation of each group is estimated to be ∼2.5. If a two-sample t-test is used to test the difference between groups, a power analysis predicts that statistical significance with a power of ∼86.1% can be reached.

Statistical Analysis

Relationships between Total Mayo Scores and Sum Endoscopic Mayo Scores will be assessed using a t-test. Furthermore, the correlation between the relative abundance of Bacteroidetes species using a Pearson's correlation coefficient will be evaluated. Differences will be considered significant at $P < 0.05$.

Randomization

Eligible patients will be randomized at a ratio of 1:1 using an Interactive Web Response System (REDCap: Research Electronic Data CAPture). The simple randomization is carried out by the staff in charge of the clinical research and trial Center of the Juntendo University Hospital to ensure concealment of allocation. The medical staff, assistants and patients of our study team did not know the condition of random allocation until the experiment was completely finished.

DISCUSSION

FMT is a well-established treatment regimen for recurrent *Clostridium difficile* infection (CDI). Clinical practice guidelines clearly state that FMT can be used as a treatment option for CDI (36, 37). However, using FMT for UC treatment remains in the clinical trial stage. Since 2014, we have been committed to studying how to enhance the therapeutic effect and operational feasibility of FMT on UC in clinical trials. The proposed A-FMT method has a significant effect on the treatment of UC in our previous studies. We hope to combine acceptable and convenient post-treatment methods based on A-FMT to further enhance the effect and prolong the remission period, including alginate supplements.

Alginate has the potential to increase the relative abundance of some beneficial Bacteroidetes members, resulting in an improved

intestinal environment (38–40). Therefore, we hypothesize that the diversity of microbiota and the proportion of Bacteroidetes in the group treated with A-FMT combined with alginate will be higher than that in the placebo group. An increased number of Bacteroidetes species can suppress the inflammatory response through zwitterionic capsular polysaccharides, which are bacterial products that modulate T cells to secrete the anti-inflammatory interleukin-10 (41). It has also been reported that alginate can be fermented by specific bacteria in feces, such as *Bacteroides ovatus*, *Bacteroides xylanisolvens*, and *Bacteroides theaiotaomicron* (30, 42), which can induce colonic regulatory T cells and promote anti-inflammatory effects (28, 43). Therefore, we believe that oral supplementation of alginate with exogenous sources can contribute to the intestinal anti-inflammatory response. Furthermore, we expect that the group treated with A-FMT combined with alginate will demonstrate better results in terms of clinical features and microbial analysis, and will retain its advantage over the placebo arm after 2 years' follow-up. The metabolites produced by microbiota can shape the colonic environment through a variety of activities, such as participating in signaling, immune system modulation, and antibiotic activity (44–46). However, it is not fully known how specific microbes and the small molecules they modulate interact to cause or control the inflammatory response. Therefore, in our study, a wide range of metabolomic analyses other than SCFAs will be included in the study.

Although this clinical study is limited to patients in single center, our method can also be applied to patients in other regions, then provide more evidence for the treatment of patients with UC.

ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of the Juntendo Institutional Review Board, Juntendo University School of Medicine, and the Clinical Study Committee of Juntendo University Hospital (Approval Number J20-011). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DI and Y-GK: conceptualization. DI: methodology, validation, funding acquisition, writing—review and editing, and project administration. DI and KN: formal analysis, data curation, and visualization. KH and TS: investigation. XZ and NS: writing—original draft preparation. AN: supervision. MH, KH, and TS: resources. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by the joint research course with Kirin Holdings Co., Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

REFERENCES

1. Kobayashi T, Siegmund B, Le Berre C, Wei SC, Ferrante M, Shen B, et al. Ulcerative colitis. *Nat Rev Dis Primers.* (2020) 6:74. doi: 10.1038/s41572-020-0205-x
2. Nemoto H, Kataoka K, Ishikawa H, Ikata K, Arimochi H, Iwasaki T, et al. Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis. *Dig Dis Sci.* (2012) 57:2955–64. doi: 10.1007/s10620-012-2236-y
3. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology.* (2014) 146:1489–99. doi: 10.1053/j.gastro.2014.02.009
4. Cammarota G, Ianiro G, Cianci R, Bibbò S, Gasbarrini A, Currò D. The involvement of gut microbiota in inflammatory bowel disease pathogenesis: potential for therapy. *Pharmacol Ther.* (2015) 149:191–212. doi: 10.1016/j.pharmthera.2014.12.006
5. Smits LP, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. *Gastroenterology.* (2013) 145:946–53. doi: 10.1053/j.gastro.2013.08.058
6. Wang JW, Kuo CH, Kuo FC, Wang YK, Hsu WH, Yu FJ, et al. Fecal microbiota transplantation: review and update. *J Formos Med Assoc.* (2019) 118(Suppl. 1):S23–31. doi: 10.1016/j.jfma.2018.08.011
7. Costello SP, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, et al. Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: a randomized clinical trial. *JAMA.* (2019) 321:156–64. doi: 10.1001/jama.2018.20046
8. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal microbiota transplantation induces remission in patients with active ulcerative colitis in a randomized controlled trial. *Gastroenterology.* (2015) 149:102–9.e6. doi: 10.1053/j.gastro.2015.04.001
9. Paramsothy S, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, et al. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. *Lancet.* (2017) 389:1218–28. doi: 10.1016/S0140-6736(17)30182-4
10. Rossen NG, Fuentes S, van der Spek MJ, Tijssen JG, Hartman JH, Duflou A, et al. Findings from a randomized controlled trial of fecal transplantation for patients with ulcerative colitis. *Gastroenterology.* (2015) 149:110–8.e4. doi: 10.1053/j.gastro.2015.03.045
11. Narula N, Kassam Z, Yuan Y, Colombel JF, Ponsioen C, Reinisch W, et al. Systematic review and meta-analysis: fecal microbiota transplantation for treatment of active ulcerative colitis. *Inflamm Bowel Dis.* (2017) 23:1702–9. doi: 10.1097/MIB.0000000000001228
12. Nishikawa Y, Sato N, Tsukinaga S, Uchiyama K, Koido S, Ishikawa D, et al. Long-term outcomes of antibiotic combination therapy for ulcerative colitis. *Ther Adv Chronic Dis.* (2021) 12:20406223211028790. doi: 10.1177/20406223211028790
13. Ohkusa T, Kato K, Terao S, Chiba T, Mabe K, Murakami K, et al. Newly developed antibiotic combination therapy for ulcerative colitis: a double-blind placebo-controlled multicenter trial. *Am J Gastroenterol.* (2010) 105:1820–9. doi: 10.1038/ajg.2010.84
14. Ji SK, Yan H, Jiang T, Guo CY, Liu JJ, Dong SZ, et al. Preparing the gut with antibiotics enhances gut microbiota reprogramming efficiency by promoting xenomicrobiota colonization. *Front Microbiol.* (2017) 8:1208. doi: 10.3389/fmicb.2017.01208
15. Ishikawa D, Sasaki T, Osada T, Kuwahara-Arai K, Haga K, Shibuya T, et al. Changes in intestinal microbiota following combination therapy with fecal microbial transplantation and antibiotics for ulcerative colitis. *Inflamm Bowel Dis.* (2017) 23:116–25. doi: 10.1097/MIB.0000000000000975
16. Ishikawa D, Sasaki T, Takahashi M, Kuwahara-Arai K, Haga K, Ito S, et al. The microbial composition of Bacteroidetes species in ulcerative colitis is effectively improved by combination therapy with fecal microbiota

transplantation and antibiotics. *Inflamm Bowel Dis.* (2018) 24:2590–8. doi: 10.1093/ibd/izy266
17. Nomura K, Ishikawa D, Okahara K, Ito S, Haga K, Takahashi M, et al. Bacteroidetes species are correlated with disease activity in ulcerative colitis. *J Clin Med*. (2021) 10:1749. doi: 10.3390/jcm10081749
18. Okahara K, Ishikawa D, Nomura K, Ito S, Haga K, Takahashi M, et al. Matching between donors and ulcerative colitis patients is important for long-term maintenance after fecal microbiota transplantation. *J Clin Med*. (2020) 9:1650. doi: 10.3390/jcm9061650
19. Wei Y, Gong J, Zhu W, Tian H, Ding C, Gu L, et al. Pectin enhances the effect of fecal microbiota transplantation in ulcerative colitis by delaying the loss of diversity of gut flora. *BMC Microbiol.* (2016) 16:255. doi: 10.1186/s12866-016-0869-2
20. Guo X, Wang Y, Qin Y, Shen P, Peng Q. Structures, properties and application of alginic acid: a review. *Int J Biol Macromol.* (2020) 162:618–28. doi: 10.1016/j.ijbiomac.2020.06.180
21. Zhou R, Shi X, Gao Y, Cai N, Jiang Z, Xu X. Anti-inflammatory activity of guluronate oligosaccharides obtained by oxidative degradation from alginate in lipopolysaccharide-activated murine macrophage RAW 2647 cells. *J Agric Food Chem*. (2015) 63:160–8. doi: 10.1021/jf503548a
22. Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Powell LC, Pritchard MF, et al. Alginate oligosaccharides inhibit fungal cell growth and potentiate the activity of antifungals against *Candida* and *Aspergillus* spp. *PLoS ONE.* (2014) 9:e112518. doi: 10.1371/journal.pone.0112518
23. Xu X, Wu X, Wang Q, Cai N, Zhang H, Jiang Z, et al. Immunomodulatory effects of alginate oligosaccharides on murine macrophage RAW2647 cells and their structure–activity relationships. *J Agric Food Chem.* (2014) 62:3168–76. doi: 10.1021/jf405633n
24. Daigo K, Wada Y, Yamada C, Yamaji M, Okuda S, Okada M, et al. Pharmacological studies of sodium alginate. 1 Protective effect of sodium alginate on mucous membranes of upper-gastrointestinal tract. *Yakugaku Zasshi.* (1981) 101:452–7. doi: 10.1248/yakushi1947.101.5_452
25. Yamamoto A, Itoh T, Nasu R, Nishida R. Effect of sodium alginate on dextran sulfate sodium- and 2,4,6-trinitrobenzene sulfonic acid-induced experimental colitis in mice. *Pharmacology.* (2013) 92:108–16. doi: 10.1159/000353192
26. Razavi A, Khodadadi A, Eslami MB, Eshraghi S, Mirshafiey A. Therapeutic effect of sodium alginate in experimental chronic ulcerative colitis. *Iran J Allergy Asthma Immunol.* (2008) 7:13–8.
27. Mirshafiey A, Khodadadi A, Rehm BH, Khorramizadeh MR, Eslami MB, Razavi A. Sodium alginate as a novel therapeutic option in experimental colitis. *Scand J Immunol.* (2005) 61:316–21. doi: 10.1111/j.1365-3083.2005.01571.x
28. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA. Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science.* (2013) 341:569–73. doi: 10.1126/science.1241165
29. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity.* (2014) 40:128–39. doi: 10.1016/j.immuni.2013.12.007
30. Li M, Li G, Shang Q, Chen X, Liu W, Pi X, et al. *In vitro* fermentation of alginate and its derivatives by human gut microbiota. *Anaerobe.* (2016) 39:19–25. doi: 10.1016/j.anaerobe.2016.02.003
31. Mocanu V, Rajaruban S, Dang J, Kung JY, Deehan EC, Madsen KL. Repeated fecal microbial transplantations and antibiotic pre-treatment are linked to improved clinical response and remission in inflammatory bowel disease: a systematic review and pooled proportion meta-analysis. *J Clin Med*. (2021) 10:959. doi: 10.3390/jcm10050959
32. Yoshino T, Nakase H, Ueno S, Uza N, Inoue S, Mikami S, et al. Usefulness of quantitative real-time PCR assay for early detection of cytomegalovirus infection in patients with ulcerative colitis refractory to immunosuppressive therapies. *Inflamm Bowel Dis.* (2007) 13:1516–21. doi: 10.1002/ibd.20253
33. Sanada K, Nakajima S, Kurokawa S, Barceló-Soler A, Ikuse D, Hirata A, et al. Gut microbiota and major depressive disorder: a systematic review and meta-analysis. *J Affect Disord.* (2020) 266:1–13. doi: 10.1016/j.jad.2020.01.102
34. Kang DW, Adams JB, Coleman DM, Pollard EL, Maldonado J, McDonough-Means S, et al. Long-term benefit of microbiota transfer therapy on autism symptoms and gut microbiota. *Sci Rep.* (2019) 9:5821. doi: 10.1038/s41598-019-42183-0
35. Franzosa EA, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. *Nat Microbiol.* (2019) 4:293–305. doi: 10.1038/s41564-018-0306-4
36. Cammarota G, Ianiro G, Tilg H, Rajilić-Stojanović M, Kump P, Satokari R, et al. European consensus conference on faecal microbiota transplantation in clinical practice. *Gut.* (2017) 66:569–80. doi: 10.1136/gutjnl-2016-313017
37. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, et al. 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.* (2018) 66:987–94. doi: 10.1093/cid/ciy149
38. Wang Y, Han F, Hu B, Li J, Yu W. *In vivo* prebiotic properties of alginate oligosaccharides prepared through enzymatic hydrolysis of alginate. *Nutr Res.* (2006) 26:597–603. doi: 10.1016/j.nutres.2006.09.015
39. Ai C, Jiang P, Liu Y, Duan M, Sun X, Luo T, et al. The specific use of alginate from Laminaria japonica by Bacteroides species determined its modulation of the Bacteroides community. *Food Funct.* (2019) 10:4304–14. doi: 10.1039/C9FO00289H
40. Wang Y, Li L, Ye C, Yuan J, Qin S. Alginate oligosaccharide improves lipid metabolism and inflammation by modulating gut microbiota in high-fat diet fed mice. *Appl Microbiol Biotechnol.* (2020) 104:3541–54. doi: 10.1007/s00253-020-10449-7
41. Neff CP, Rhodes ME, Arnolds KL, Collins CB, Donnelly J, Nusbacher N, et al. Diverse intestinal bacteria contain putative zwitterionic capsular polysaccharides with anti-inflammatory properties. *Cell Host Microbe.* (2016) 20:535–47. doi: 10.1016/j.chom.2016.09.002
42. Salyers AA, Vercellotti JR, West SE, Wilkins TD. Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. *Appl Environ Microbiol.* (1977) 33:319–22. doi: 10.1128/aem.33.2.319-322.1977
43. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J. deRoos P, Liu H, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature.* (2013) 504:451–5. doi: 10.1038/nature12726
44. Dorrestein PC, Mazmanian SK, Knight R. Finding the missing links among metabolites, microbes, and the host. *Immunity.* (2014) 40:824–32. doi: 10.1016/j.immuni.2014.05.015
45. McHardy IH, Goudarzi M, Tong M, Ruegger PM, Schwager E, Weger JR, et al. Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. *Microbiome.* (2013) 1:17. doi: 10.1186/2049-2618-1-17
46. Wu GD. Diet, the gut microbiome and the metabolome in IBD. *Nestlé Nutr Inst Workshop Ser.* (2014) 79:73–82. doi: 10.1159/000360686

Conflict of Interest: Kaigen Pharma Co., Ltd (Osaka, Japan) provided the alginate drink and placebo for this clinical study.

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.

The reviewer TO declared a shared affiliation with several of the authors, DI, XZ, KN, MH, KH, TS, and AN, to the handling editor at time of review.

frontiers in Medicine

REVIEW
published: 02 March 2022
doi: 10.3389/fmed.2022.822190

Oral and Gut Microbial Dysbiosis and Non-alcoholic Fatty Liver Disease: The Central Role of *Porphyromonas gingivalis*

Ting Wang[1]*, *Taichi Ishikawa*[2], *Minoru Sasaki*[2] and *Toshimi Chiba*[1]*

[1] Division of Internal Medicine, Department of Oral Medicine, Iwate Medical University, Morioka, Japan, [2] Division of Molecular Microbiology, Department of Microbiology, Iwate Medical University, Morioka, Japan

OPEN ACCESS

Edited by:
Toshifumi Ohkusa,
Juntendo University, Japan

Reviewed by:
Tomohisa Takagi,
Kyoto Prefectural University of Medicine, Japan
Raisa Catunda,
University of Alberta, Canada

***Correspondence:**
Toshimi Chiba
toschiba@iwate-med.ac.jp
Ting Wang
tingwang@iwate-med.ac.jp

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: 25 November 2021
Accepted: 19 January 2022
Published: 02 March 2022

Citation:
Wang T, Ishikawa T, Sasaki M and Chiba T (2022) Oral and Gut Microbial Dysbiosis and Non-alcoholic Fatty Liver Disease: The Central Role of Porphyromonas gingivalis. Front. Med. 9:822190. doi: 10.3389/fmed.2022.822190

Gut microbiota play many important roles, such as the regulation of immunity and barrier function in the intestine, and are crucial for maintaining homeostasis in living organisms. The disruption in microbiota is called dysbiosis, which has been associated with various chronic inflammatory conditions, food allergies, colorectal cancer, etc. The gut microbiota is also affected by several other factors such as diet, antibiotics and other medications, or bacterial and viral infections. Moreover, there are some reports on the oral-gut-liver axis indicating that the disruption of oral microbiota affects the intestinal biota. Non-alcoholic fatty liver disease (NAFLD) is one of the systemic diseases caused due to the dysregulation of the oral-gut-liver axis. NAFLD is the most common liver disease reported in the developed countries. It includes liver damage ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, and cancer. Recently, accumulating evidence supports an association between NAFLD and dysbiosis of oral and gut microbiota. Periodontopathic bacteria, especially *Porphyromonas gingivalis*, have been correlated with the pathogenesis and development of NAFLD based on the clinical and basic research, and immunology. *P. gingivalis* was detected in the liver, and lipopolysaccharide from this bacteria has been shown to be involved in the progression of NAFLD, thereby indicating a direct role of *P. gingivalis* in NAFLD. Moreover, *P. gingivalis* induces dysbiosis of gut microbiota, which promotes the progression of NAFLD, through disrupting both metabolic and immunologic pathways. Here, we review the roles of microbial dysbiosis in NAFLD. Focusing on *P. gingivalis*, we evaluate and summarize the most recent advances in our understanding of the relationship between oral-gut microbiome symbiosis and the pathogenesis and progression of non-alcoholic fatty liver disease, as well as discuss novel strategies targeting both *P. gingivalis* and microbial dysbiosis.

Keywords: oral microbiota, gut microbiota, microbial dysbiosis, NAFLD, *P. gingivalis*

INTRODUCTION

Alteration of the normal microbial composition, known as microbial dysbiosis, has been widely studied owing to its pathologic impacts on the body. Numerous studies have provided evidence that gut dysbiosis is closely related to systemic diseases centered in various organs (1). Moreover, recent studies have revealed that periodontal disease, which is a typical dysbiosis disease of the oral cavity, also contributes to the pathogenesis of systemic diseases, such as metabolic syndrome, cardiovascular disease, kidney disease, and brain disease, and may be related to cancer (2, 3). The pathogenic mechanisms underlying the effect of microbial dysbiosis beyond its original location are complicated, including direct effects of pathologic bacteria translocated by the blood to a specific organ, and effects of bacteria-derived endotoxins, metabolites, and inflammatory immune-mediators (4–6). Specifically, dysbiosis of the oral microbiota can be pathogenic to the internal organs *via* gut dysbiosis (7).

Non-alcoholic fatty liver disease (NAFLD) is of interest because, as is typical of metabolic diseases closely related to metabolic syndromes such as obesity and type 2 diabetes, it is also a systemic disorder affecting not only the liver but also various extrahepatic organs throughout the body and is linked to various systemic diseases, such as cardiovascular complications, kidney disease, and in particular the increase of extrahepatic malignancies (8, 9) (**Figure 1**). Studies on NAFLD have been limited because of its unclear origin, pathogenesis, and development, difficulties in early diagnosis, and a lack of effective therapeutic options. In this review, we focus on the relationship between NAFLD and *Porphyromonas gingivalis*, which is a typical oral pathogenic bacteria in periodontal disease (10, 11).

HUMAN-MICROORGANISM SYMBIOSIS

In recent years, PCR, quantitative PCR, and metagenomic analyses have been used to investigate indigenous bacterial populations more deeply than can conventional culture methods and microscopic observations, and have made great progress. As a result, it has been found that there are more than 1,000 types of microorganisms in the human body, and that they have, collectively, 3.3 million genes, far exceeding the 25,000 genes in the human genome (12). The total amount of genomic information possessed by microorganisms in a certain environment is called a microbiome; Lederberg has written

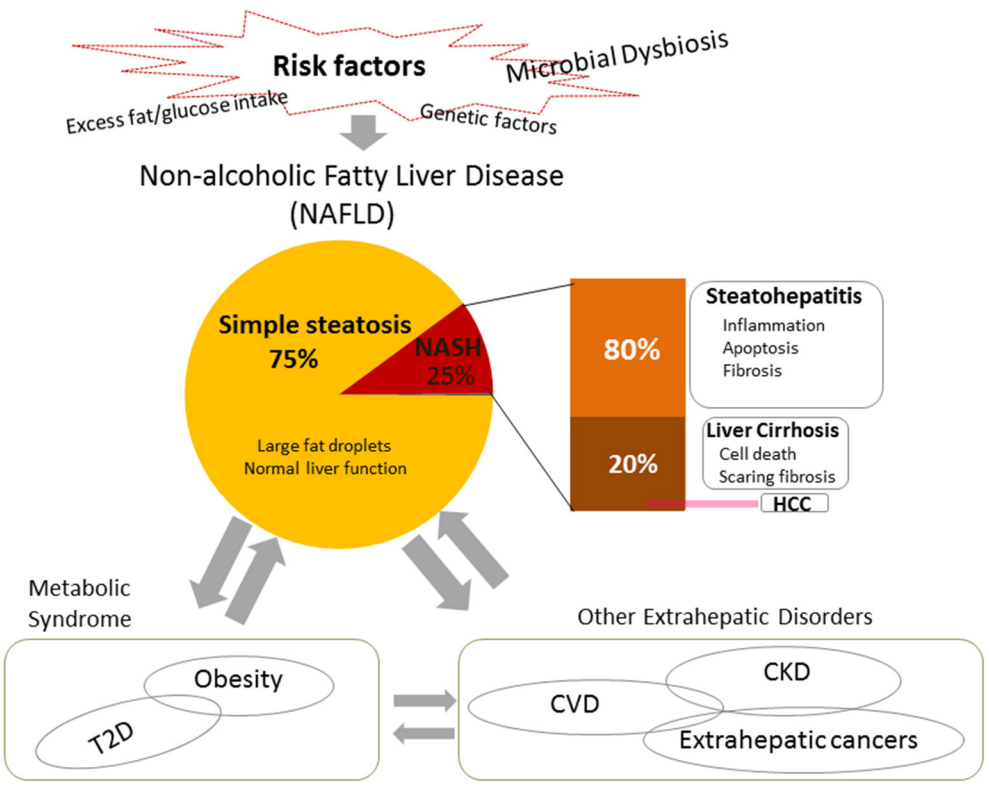


FIGURE 1 | Risk factors, pathogenesis, and systemic impacts of non-alcoholic fatty liver disease (NAFLD). NAFLD is the most common chronic liver disease worldwide, usually presenting as fatty hepatocytes (simple steatosis). Non-alcoholic steatohepatitis (NASH) is a severe type of NAFLD. It is usually characterized by inflammation, cell damage, and apoptosis, and may include fibrosis. In turn, it can progress to cirrhosis or end-stage liver disease, i.e., hepatocellular carcinoma (HCC). In addition to heredity, one of the most widely accepted risk factors for NAFLD is excessive fat/glucose intake. Recent studies indicate that microbial dysbiosis takes an important role in the pathogenesis and the development of NAFLD. NAFLD leads not only to the development of hepatic diseases, but also connects with and contributes to systemic diseases, such as metabolic syndrome and extrahepatic disorders. NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; CDVs, cardiovascular diseases; CKD, chronic kidney disease, T2D, type 2 diabetes.

that "human beings are super-living organisms composed of symbiotic microorganisms and human beings, and symbiotic microorganisms are extremely important to human beings" (13). Even sophisticated microbiota analysis can determine only the bacterial biota, whereas microbiome analysis can determine both the bacterial biota (number of bacteria, composition ratio) and its component functions and metabolism. Bacteria, fungi, and protozoa are among the microorganisms that are present throughout the human body. Typical indigenous fungi include *Candida albicans*, and protozoans include Trichomonas and amoeba*s* (14, 15). We focused on the bacteria that predominate among these indigenous microorganisms; they are present in healthy adults in or on the skin, nasopharynx, oral cavity, stomach, intestinal tract, vagina, etc. The number varies depending on the site, and the types vary from person to person (16–18). These bacteria are usually balanced to form a community (bacterial biota). Indigenous bacteria have beneficial effects on living organisms, such as acting against invasion and colonization by foreign pathogenic microorganisms, stimulating the immune system, enhancing host resistance and immune response, and synthesizing vitamins metabolites used by the living host body (19, 20). In contrast, such bacteria can also disadvantage the host by causing infectious disease in an easily infected host (opportunistic infection), or *via* pathogenic replacement, in which resident pathogenic microorganisms become predominant following reduction of the normal biota by antibacterial drugs. Moreover, resident bacteria that are not pathogenic in their original location can become pathogenic bacteria elsewhere (ectopic infection) (21). Indigenous bacteria are constantly exposed to the host's immune system, settle in the host tissues, and exist in a symbiotic, non-pathogenic state. It is thought that disruption of this interaction causes some infectious diseases (22).

THE INTESTINAL BIOTA

Indigenous bacterial communities in the intestinal tract are composed of various bacterial species, such as Firmicutes, Bacteroidetes, and Actinobacteria (23). These are thought to invade mainly through the oral cavity (24). To date, there have been numerous reports of microbiome analyses (**Table 1**).

Dysbiosis changes in the intestinal biota is closely related to various pathological conditions, including intestinal-related diseases and systemic diseases (25–28). For example, inflammatory bowel diseases, including Crohn's disease, have been associated with increased mucosa-associated facultive anaerobes, *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae*, *Fusobacteriaceae*, and decreased *Faecalibacterium prausitzii, Erysipelotrichales, Bacteroidales,* and *Clostridiales* (26). In obese mouse model, an increase in Firmicutes proportion and a decrease in Bacteroidetes proportion have been confirmed (29). Furthermore, the proportion of *Akkermansia muciniphila*, of the Verrucomicrobia, was reduced in diabetic patients (30), and the feces of children with autism spectrum disorders contained nine unknown species of *Clostridium* (28).

THE ORAL BIOTA

Previous reports suggest that the average adult has about 50–100 billion bacteria in the oral cavity, including as many as 500–700 bacterial species (31, 32). The oral biota varies across niches such as dental plaque, tongue, saliva, and gingival sulcus, and therefore exists as a wide variety of bacterial biota throughout the oral cavity (**Table 2**).

While these oral bacteria play a role as indigenous bacteria, some are thought to cause local infections such as tooth decay, periodontal disease, and endodontic infections (34).

TABLE 1 | Reports of the human intestinal microbiome by human genome analysis.

Published year	Participants	Country	The first author	PMID
2016	106	Japan	Nishijima S	26951067
	1,135	Netherlands	Zhernakova A	27126040
2017	59	USA	Galloway-Peña J	28245856
	405	China	Jie Z	29018189
2018	106	China	Ye Z	30077182
	54	Nepal	Jha A	30439937
2019	267 pairs	Norway	Iszatt N	30813950
	2,170	USA	Shama A	31046835
2020	147	Cameroun	Lokmer A	32071424
	1,475	China	Xu F	33032658
2021	758	USA	Baniel A	33485388
	65	USA	Thapa S	33956889

TABLE 2 | Flora site and bacterial distribution in the human oral cavity[a].

Bacterial group	Bacterial distribution			
	Tooth surface (plaque)	Tongue	Saliva	Gingival crevice
Gram-Positive facultative cocci	28.2	44.8	46.2	28.8
Streptococci	27.9	38.3	41.0	27.1
Staphylococci	0.3	6.5	4.0	1.7
Gram-Positive anaerobic cocci	12.6	4.2	13.0	7.4
Gram-Negative anaerobic cocci	6.4	16.0	15.9	10.7
Gram-Negative facultative cocci	0.4	3.4	1.2	0.4
Gram-Positive facultative rods	23.8	13.0	11.8	15.3
Gram-Positive anaerobic rods	18.4	8.2	4.8	20.2
Gram-Negative facultative rods	ND[b]	3.2	2.3	1.2
Gram-Negative anaerobic rods	10.4	8.2	4.8	16.1
Spirochetes	ND	ND	ND	1.0

[a] *Modified from Hamada and Slade (33).*
[b] *ND, Not detected.*

In addition, recent oral microbiome analysis shows that not only some pathogenic bacteria induce local disease, but also an abnormal bacterial biota (dysbiosis), in which the oral biota is disturbed by the growth of certain keystone species, may be the cause of various oral diseases (35). In addition, many studies have reported that dysbiosis of the oral bacteria is associated with many systemic diseases, such as aspiration pneumonia, bacterial endocarditis, preterm birth, diabetes, Alzheimer's disease, and atherosclerosis (36–38). Oral streptococci, including *Streptococcus anginosus* and *S. intermedius*, which are gram-positive facultative anaerobic cocci, and *Prevotella*, *Fusobacterium*, and *Bacteroides*, which are gram-negative obligate anaerobic cocci, are listed as the causative bacteria of aspiration pneumonia (39). It has been reported that bacterial endocarditis has a very high isolation frequency of oral streptococci and these are considered a causative agent (40). These are referred to as ectopic infections. In contrast, in preterm birth, diabetes, Alzheimer's disease, atherosclerosis, and one of the autoimmune diseases, rheumatism, the involvement of gram-negative obligate anaerobic bacilli, such as *P. gingivalis* and *Treponema denticola,* which are classified as periodontal pathogens, is strongly suspected; however, it is rare that the causative organism is specifically detected in the lesion. Various studies have been undertaken into the pathogenic mechanism of these bacteria, but there are many unclear points (41–43).

MECHANISM BY WHICH ORAL BIOTA INDUCES SYSTEMIC DISEASE

Separate mechanisms underlying systemic disease caused by oral bacteria, differing from those involved in ectopic infection, have been considered. Conventionally, inflammatory cytokines and enzymes, present because of bacterial cell components in local lesions of the oral cavity, are transferred from the bloodstream to the whole body. More recently, it has been suggested that oral dysbiosis induces systemic disease by inducing intestinal dysbiosis, affecting the immune system, metabolic system, and intestinal barrier function (44, 45). Concerning this induction of intestinal dysbiosis by pre-existing oral dysbiosis, it has been reported that oral administration of *Fusobacterium nucleatum*, a gram-negative obligate anaerobic bacillus, significantly reduces the activity of natural killer cells (46). Additionally, *P. gingivalis*, whose proportion increases in periodontitis, may pass through the acid stomach and reach the intestines (47). In mice administered *P. gingivalis* orally, *P. gingivalis* does not colonize or proliferate in the intestinal tract, but changes the intestinal bacterial biota (48). The inside of the stomach is usually pH 1–2, and *P. gingivalis* cannot grow there. However, reports that *P. gingivalis* forms a biofilm and has a survival rate of 50% or more at pH 3, suggest that it can pass through the stomach at pH 4–5 immediately after eating (49). Changes in the intestinal biota may also decrease the expression of tight junction proteins involved in intestinal barrier function, increase inflammatory cytokine gene expression, and increase blood endotoxin levels because of suppression of the small intestinal alkaline phosphatase gene (50). As mentioned above, there are various reports on the mechanisms by which the oral biota can induce systemic diseases; further analysis is expected to contribute further details of these relationships, aiding the elucidation of the currently unclear etiology of systemic diseases.

FATTY LIVER AND NON-ALCOHOLIC FATTY LIVER DISEASE

As blood circulation is the key pathway through which pathogenic bacteria and their toxic metabolites can reach distant locations across the whole body, it is reasonable that those organs with abundant blood supply are more vulnerable to the above substances, and more easily develop diseases related to oral and gut microbial dysbiosis. Among them, the liver is the largest solid internal organ and is unique in having two sources of blood supply: 80% from the portal vein and 20% from the hepatic artery. The liver plays critical roles in the body, such as regulating metabolic processes, including synthesizing glucose and lipids, maintaining homeostasis, and protecting against toxic substances by means of considerable detoxification abilities (9, 51).

The liver has the potential to regenerate following tissue damage, although chronic inflammatory damage or potent drug-induced toxicity may lead to various pathologies, including excessive fat accumulation (fatty liver), inflammation, fibrosis, and cirrhosis, or even to end-stage liver disease, hepatocellular carcinoma (HCC) (52, 53). Among these conditions, simple steatosis of hepatocytes only rarely leads to clinical symptoms; it is also difficult to make a diagnosis, as the only effective method of determination so far is biopsy, which is invasive and thus generally not acceptable to patients who are suspected of having such a disease. However, fatty liver may be a precursor to all of the other, more severe states of liver disease, even cancer (54, 55). Understanding the mechanisms underlying the etiology and pathology of fatty liver diseases therefore has definite clinical significance in prevention, early prognosis, and treatment. It is known that alcohol can lead to fatty liver (56), although fatty liver has also been found in non-drinkers. The latter is therefore called NAFLD.

NAFLD is currently regarded as the most common chronic liver disease worldwide (9). Data have shown that approximately one quarter of the world's population is affected by it (57). Approximately 25% of NAFLD patients develop a severe clinical phenotype called non-alcoholic steatohepatitis (NASH), which is characterized by inflammation, cell apoptosis, and fibrosis (58). The prevalence of NAFLD and NASH in adults in the United States ranges from 30–40% to 3–12%, respectively (59). Furthermore, ∼20% of NASH patients may progress to cirrhosis (60), and NASH-associated cirrhosis increases the risk of hepatocellular carcinoma (HCC) by 2.4–12.8% (61, 62). The pathogenesis of NAFLD has not yet been completely elucidated. A "two-hit" theory has been widely supported (63). Firstly, excess food intake causes metabolic disorders such as insulin resistance, which lead to excess fat accumulation, simple steatosis, in the liver. Secondly, lipotoxicity causes oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress, which induces cell injury, inflammation, and fibrosis. However, research over

the last decade supports a new "multiple-hits" hypothesis. In addition to various dietary components and genetic factors, microbial dysbiosis is a crucial pathogenic factor and plays important roles in the development of NAFLD (8, 58) (**Figure 1**).

GUT MICROBIAL DYSBIOSIS AND NAFLD: THE GUT-LIVER AXIS

Risk factors such as long-term consumption of a high-fat diet, antibacterial drug use, and intestinal inflammation, cause alterations in gut microbial composition and functions (64–67). As a result, the gut barrier function is impaired, facilitating the entry of pathogenic bacteria, bacterial endotoxins, and other inflammatory cells and mediators to the portal vein, and thereby reaching the liver (62).

Numerous clinical and experimental studies have been conducted to reveal the relationships between gut dysbiosis and NAFLD, and the impact of gut dysbiosis on the etiology and pathology of NAFLD (65, 68, 69). Firstly, gut microbial alterations were detected in patients with NAFLD. For example, in the gut of the patients with NASH or cirrhosis, there was a decrease in health-related bacteria such as *Bacteroidetes* and an increase of pathogenic bacteria *Proteobacteria* and *Enterobacteriaceae* specie (70–72); The abnormal changes in the abundance of some bacterial phyla, such as *Bacteroides*, *Prevotella*, Proteobacteria, and Firmicutes, correlate with disease severity (73–76).

Secondly, microbiome signature profiles and metabolomics analysis have recently promoted wide discussion of the pathologic roles of bacteria-derived metabolites and products (77, 78). Such factors are known to contribute to hepatic steatosis, insulin resistance, and fibrosis (79–81). For example, the microbiome signature in NAFLD indicates that 3-(4-hydroxyphenyl), a metabolic material from *Firmicutes, Bacteroidetes* and Proteobacteria, is associated with liver fibrosis (82, 83), and Glycocholate is positively associated with advanced liver fibrosis (84). Moreover, metabolic studies have clarified that microbiome-generated secondary bile acid triggers NAFLD (85), and the gut microbiome of NAFLD patients shows abnormalities in carbon and amino acid metabolism (86), choline depletion, and increased production of certain short-chain fatty acids and alcohols (71). This suggests that gut dysbiosis is related to NAFLD by metabolic pathways.

Thirdly, endotoxemia resulted from increased gut permeability is also associated with NAFLD pathogenesis, indicating the effects of gut dysbiosis on immune system (55). Bacteria-derived endotoxins such as lipopolysaccharide (LPS) are closely related with intestinal immune function (73). Most reports give function of LPS as stimulation of the Toll-like receptor 4 (TLR4) (87, 88), which belongs to the Toll-like family of receptors that is crucial in host defense against invading pathogens (89). The localization of LPS has been confirmed in the livers of NALFD patients (58, 90, 91). Blocking LPS receptors is related to improved NAFLD in *in-vivo* studies (89, 92). Recent clinical and animal studies provide evidence that elevated concentrations of LPS and endotoxin-producing bacterial strains are positively related to the progression of NAFLD, *via* inflammation and oxidative stress, which finally leads to chronic inflammation (93) and insulin resistance (88, 94). LPS also triggers lipid peroxidation in the liver (95, 96). We will discuss LPS in the later part of this review.

It should be noted that although the above evidence indicates a promoter role for gut dysbiosis in the pathogenesis of NAFLD, the bilateral relationship between the disease and gut dysbiosis remains to be completely understood.

P. gingivalis AND NAFLD

Recently, interest has been shown in the pathogenic role of oral, as well as gut, microbial dysbiosis. Chronic oral diseases such as periodontal disease typically involve such dysbiosis. Multiple reports from epidemiologic, *in-vivo*, and *in-vitro* studies indicate that periodontal disease is closely associated with NAFLD (93, 97). Oral pathologic bacteria related to oral diseases include *P. gingivalis, Treponema denticola, Prevotella intermedia, Aggregatibacter actinomycetemcomitans, Tannerella forsythia,* and *Campylobacter rectus*. Among them, *P. gingivalis* is regarded as a key pathogen leading to periodontitis and related systemic diseases (3, 10, 98). In this review, we emphasize the relationship between *P. gingivalis* and NAFLD, the effects of *P. gingivalis* on the pathogenesis of NAFLD, the mechanism underlying its pathogenic functions, and the therapeutic approaches targeting *P. gingivalis* and microbial dysbiosis.

P. gingivalis-Associated NAFLD

A potential link between *P. gingivalis* and NAFLD has been revealed. *P. gingivalis* and its DNA were detected in the oral cavities or livers of NAFLD patients at higher frequency than those of controls (99, 100). NASH patients with *P. gingivalis* infection had more severe fibrosis than those without infection (100). Furthermore, supportive data from various *in-vivo* studies showed that *P. gingivalis* infection can stimulate fat accumulation, increase the immune response, and result in insulin resistance, indicating the impact of *P. gingivalis* in NAFLD/NASH procession (97).

The spread of *P. gingivalis* to distant organs, such as the liver, occurs through two possible pathways. One is direct release into the blood circulation. During daily procedures such as brushing, or dental treatment, *P. gingivalis and* bacteria-associated factors such as LPS and cytokines spread into the blood *via* the microulceration in the periodontal pocket, and are transported to the liver through the hepatic artery. The indirect pathway involves swallowing *P. gingivalis*, which can translocate to the gastrointestinal tract and induce alteration of gut microbiota; this in turn negatively affects the liver through the portal vein system (93, 101). Therefore, the pathogenic action of the bacteria is related to both these pathways: directly through the oral-liver axis, and indirectly through the oral-gut-liver axis (**Figure 2**).

Direct Impacts of *P. gingivalis* in NAFLD: Structure-Related Functions

P. gingivalis is a gram-negative, obligate anaerobic bacteria. It is a common component of subgingival microbiomes that

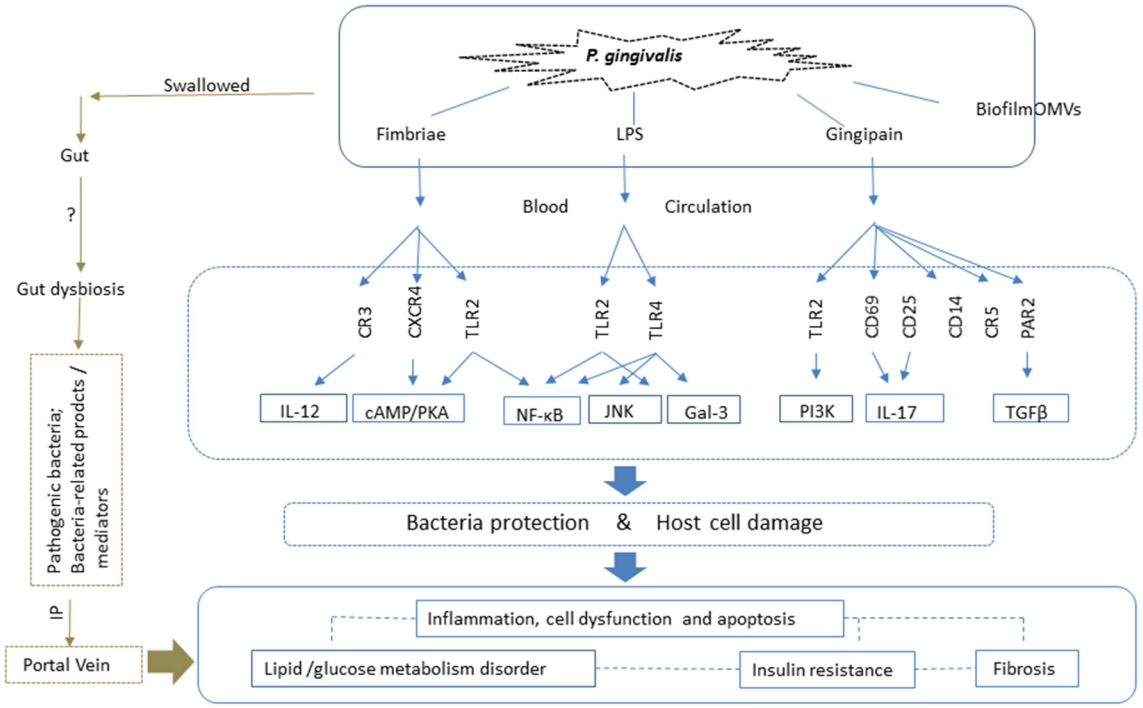


FIGURE 2 | *P. gingivalis* impacts NAFLD *via* both the oral-liver and oral-gut axes. With respect to the oral-liver axis, *P. gingivalis* and its virulence factors can reach the liver directly *via* the blood circulation. Virulence factors such as fimbriae proteins, lipopolysaccharide (LPS) and gingipains elicit inflammatory immune reactions and activate intracellular signaling pathways by binding to the corresponding receptors. This results in inflammation, cell death, and dysfunctions, all of which are closely related to glucose and lipid metabolic disorders, insulin resistance, and fibrosis, which contribute to the pathogenesis of NAFLD. Biofilm and outer membrane vesicles (OMVs) play roles in the transfer of bacteria and virulence factors, such as gingipains, to the liver, the site of pathogenic action. *P. gingivalis* also affects NAFLD *via* the oral-gut-liver axis. It can enter the gastrointestinal tract *via* swallowing, where it can induce dysbiosis of the gut microbiota and enhance intestinal permeability. Thus, both the pathogenic bacteria, and related toxic products and mediators, can reach the liver *via* the portal vein, and play a role in NAFLD. LPS, lipopolysaccharide; TLR, Toll-like receptor; IL, interleukin; OMVs, outer membrane vesicles; CXCR, CXC-chemokine receptor; CR, complement receptor; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; NF-κB, nuclear factor kappa B; TGF-β, transforming growth factor β; PAR, proteinase-activated receptor; JNK, c-Jun-NH2-terminal kinase; IP, intestinal permeability; Gal, galectin; PI3K, phosphatidylinositol-3 kinase.

can colonize oral epithelial cells (102). *P. gingivalis* has unique structural components, known as virulence factors. These include some bacteria's own structural components such as fimbriae and LPS, and secretory components such as gingipains, and play critical roles in the survival and spread of *P. gingivalis* through cellular colonization, along with its pathogenic functions, such as inducing host immune response and inflammatory reactions (98, 103).

P. gingivalis fimbriae are filamentous structures on the surface of the bacterium that enable bacterial binding to host cells and tissues, enhance bacterial motility and invasiveness, and contribute to biofilm formation (104). There are two forms of *P. gingivalis* fimbriae: FimA, and the less important minor fimbriae. Studies of NAFLD patients showed that the invasive type II FimA constituted half of the total fimbriae detected (99). Type IV FimA, however, is closely associated with advanced liver fibrosis (105).

The mechanism underlying the impact of FimA on host cells is primarily related to the activation of adhesion and immuno-inflammatory pathways *via* interactions with various host receptors, which facilitates bacterial colonization and leads to host cell inflammation (104, 106, 107). In addition, FimA can also help the bacteria survive in the host cell over extended periods by triggering the complement system to protect the bacteria from host immune clearance (104).

FimA acts through three major types of receptor. Regarding its inflammatory effects, FimA binds to Toll-like receptor (TLR) 2, which activates the nuclear factor kappa B (NF-κB) system to induce the production of various pro-inflammatory cytokines (108). TLR2 has been shown to be associated with the pathogenesis of NAFLD (109). FimA also triggers the innate immune system by binding to complement receptor (CR) 3 and stimulating macrophages/monocytes (10). As a result, FimA inhibits the production of interleukin (IL) (12), which is related to bacterial clearance (110), thus enhancing *P. gingivalis* survival. CR3 is expressed in the liver (111), and the activation level of the complement system is related to NAFLD severity (112), suggesting the involvement of *P. gingivalis* fimbriae in the pathogenesis of NAFLD *via* immune response induction.

Furthermore, FimA activates CXC-chemokine receptor 4 (CXCR4) and TLR2 to induce cyclic adenosine monophosphate-dependent protein kinase A signaling, which not only results in disruption of phagocytosis by macrophages (113), but

also inhibits host immune clearance of *P. gingivalis* (114). Dysfunctions of both macrophages and CXCR4 have been reported to be associated with NAFLD (115, 116).

Beyond FimA, *P. gingivalis* minor fimbriae can also induce inflammatory reactions in macrophages (117), and are reported to induce phosphorylation of FOXO1 (118), which plays a role in both lipid and glucose metabolism (119), indicating that both FimA and minor fimbriae in *P. gingivalis* may be factors leading to liver injury by this species.

LPS is a component of the outer cell membranes of gram-negative bacteria (120, 121). It critically contributes to the pathogenicity of microbes, mainly through lipid A, which is the biologically active region of LPS (122). LPS is structurally different in different bacterial species due to variations of fatty acid acyl chain composition of lipid A (10). *P. gingivalis* LPS contains multiple forms of lipid A, and the structural differences in lipid A may explain why *P. gingivalis* LPS can initiate differential signaling pathways and immune responses (10, 123). In addition to participating in biofilm formation (124), *P. gingivalis* LPS plays an important role in triggering host inflammatory responses *via* TLR activation; the specific type of TLR activation by LPS depends on the variation in the acylation of lipid A (10). However, it was recently reported that the activation of TLR2 by *P. gingivalis* is *via* other molecules on the surfaces of intact *P. gingivalis* cells rather than lipid A (125). Activation of TLR2 leads to an increase in the production of cytokines such as tumor necrosis factor α (TNF-α) and IL-6 by macrophages (126), while activation of TLR4 elevates the levels of IL-1β, IL-6, and IL-8 (127). In addition, NF-κB signaling pathway has also been reported to act downstream of TLRs, and to play a critical role in the inflammatory function of *P. gingivalis* LPS, including the production of cytokines (123). Several *in-vivo* and *in-vitro* studies have been performed to clarify the effects of *P. gingivalis* LPS on NAFLD. Injection of *P. gingivalis* LPS into the gingiva of animal models resulted in lipid deposition and inflammation in the liver (128–130), and *in-vitro* studies using HepG2 cells indicated that LPS may play a role in intracellular lipid accumulation and inflammation *via* both NF-κB and c-Jun-NH2-terminal kinase signaling (131). Moreover, *P. gingivalis* LPS can accelerate the progression of mild fatty liver to NASH (132), and studies using steatotic hepatocytes revealed that this activity may be related to increased TLR2 expression, inflammasome mRNA levels, and pro-inflammatory cytokines (100). Moreover, recent evidence shows that *P. gingivalis* LPS may contribute to hepatic fibrosis by activating hepatic stellate cells (HSCs); the mechanism involves triggering TLR4 to increase the production of galectin-3, which is critical for HSC activation (132). In addition, *P. gingivalis* LPS is possibly implicated in insulin resistance, either by stimulating the activation of pro-inflammatory cytokines such as TNF-α and IL-6, which play important roles in insulin resistance (133), or by directly inhibiting glucose incorporation into smooth muscle cells (134).

Kuraji et al. reported that *P. gingivalis* LPS accumulated predominantly in the liver, over other organs, and persisted in the livers of HFD-induced steatotic mice longer than in normal mouse liver. In addition, the diseased mice showed enhanced sensitivity to LPS and delayed clearance of LPS from the liver, indicating the potent role of *P. gingivalis* LPS in liver injury and NAFLD. The mechanism is possibly related to increased and activated hepatic macrophages (Kupffer cells) and TLR signaling (93).

As noted elsewhere in this review, gut bacteria-derived LPS also plays critical roles in the pathogenesis of NAFLD. The different functions and mechanisms involved in gut bacteria-derived LPS and *P. gingivalis* LPS have been investigated. For example, *P. gingivalis* LPS can activate both TLR2 and TLR4, whereas *Escherichia coli* LPS can only bind to TLR4 (135); although both LPS types activate TLR4, *P. gingivalis* LPS stimulates different pathways from those affected by *E. coli* LPS (136). Furthermore, *P. gingivalis* LPS shows a stronger ability to escape recognition by the host innate defense system than *E. coli* LPS (137), and *P. gingivalis* LPS induces more intracellular fat accumulation in HepG2 cells than *E. coli* LPS (131). In contrast to the substantial accumulated evidence that gut bacteria-derived LPS contributes to NAFLD by inducing oxidative stress (138), there is as yet little evidence (139) supporting direct oxidative stress stimulation by *P. gingivalis* LPS in NAFLD, which should be investigated further.

Gingipains are a family of secretory cysteine proteinases that are known to be the main virulence factors related to the pathogenicity of *P. gingivalis*. They consist of lysine and arginine gingipains (140, 141). As with fimbriae and LPS, gingipains also play roles in biofilm formation and produce immune-inflammatory responses by activating various immune cells (10, 142). Evidence shows that gingipains protect *P. gingivalis* from the host defense system in the following ways: they enhance the production of TNF-α by neutrophils by activating the TLR2/phosphatidylinositol-3 kinase (PI3K) pathway (143), thereby facilitating bacterial survival within host cells; they inhibit pathogen clearance by negatively regulating the production of neutrophil-derived molecules (144) and the macrophage immune receptor CD14 (145); and they promote the adaptability of *P. gingivalis* by triggering the complement system, for example by modulating the C5a receptor (C5aR) and its crosstalk with TLR2 signaling in macrophages (146).

Furthermore, gingipains may play a role in evading the host adaptive immune system by regulating T-cell immunity (147). Like LPS (148), they can induce the production by Th17 cells of the cell-specific cytokine, IL-17, by directly inducing the expression of CD69 and CD25 on T-cells (149). T-cell immunity, especially the Th17/IL-17 signaling pathway, plays a role in protecting pathogens and promoting inflammation, and was recently reported as important in the development of NAFLD (150–153). Moreover, recent work highlights a remarkable function of gingipains in NAFLD, revealing that they contribute to liver fibrosis by activating HSCs *via* the proteinase-activated receptor (PAR) 2 and TGF-β pathway (132). It should also be noted that gingipains can inactivate PI3K, protein kinase B (Akt), and Akt downstream proteins, including glycogen synthase kinase 3 (GSK3) and mammalian target of rapamycin (mTOR) (154). The PI3K/Akt signaling pathway plays multiple roles in various cell functions, including cell survival and glucose metabolism, and an *in-vitro* study has revealed that *P. gingivalis* suppresses glycogen synthesis in HepG2 cells by

inhibiting the insulin receptor substrate 1/Akt/GSK3β pathway (155). This indicates that gingipains possibly participate in glucose metabolism impairment/insulin resistance, which needs to be investigated further.

Biofilms and outer membrane vesicles (OMVs) are two important microorganism-produced structures responsible for the survival, spread, and pathogenicity of microbes. Biofilms are composed of water, bacterial cells, and extracellular polymeric substances, which are responsible for microorganism protection and resistance to clinical treatments, such as antibiotics (156–158). *P. gingivalis* virulence factors, such as fimbriae, LPS, and gingipains, have been reported to contribute to biofilm formation, as mentioned previously, and the pathogenicity of *P. gingivalis* is enhanced by biofilm (159, 160). In addition to being a reservoir of pathogenic bacteria, biofilms also contribute to inflammation in many diseases, including inflammatory bowel disease and hepatobiliary carcinomas (161); however, the pathogenic effect of *P. gingivalis* biofilm, such as direct reactions of biofilm in NAFLD, remains unclear.

OMVs are small, spherical, bilayered membrane structures that are constantly released from the bacterial surface during growth. Each vesicle is composed of outer membrane proteins, lipoproteins, LPS, and some periplasmic components (162, 163). Like other bacteria such as *Francisella* and *Pseudomonas putida*, *P. gingivalis* can produce OMVs (164). *P. gingivalis* OMVs can concentrate virulence factors such as gingipains and LPS in the form of OMVs and discharge them to the environment to participate in bacteria-associated disorders (165, 166). *P. gingivalis* OMVs play roles in biofilm formation by binding to other periodontopathogens (167, 168), and facilitate bacterial adhesion and invasion in host cells, adaption to stress, and immune defense evasion (168). In addition to contributing to the destruction of periodontal tissues, *P. gingivalis* OMVs can migrate to the blood and play an important role in the pathogenesis of various systemic diseases, such as cardiovascular disease, rheumatoid arthritis, Alzheimer's disease, and carcinoma; the mechanism involved has been recently reviewed by Zhang et al. (169). Although the direct impact of *P. gingivalis* OMVs in NAFLD has not been completely elucidated, *P. gingivalis* OMVs carrying gingipains can transfer to the liver and impair hepatic glycogen synthesis in a mouse model; they can also inhibit insulin-induced Akt/GSK-3β signaling in a gingipain-dependent manner in HepG2 cells (170), indicating a potential role of these gingipain-carrying OMVs in the development of NAFLD through negative regulation of glucose metabolism and insulin sensitivity.

In summary, these bacteria-associated structures play roles in bacterial protection, as well as affecting the virulence of *P. gingivalis*, by activating various risk factors for NAFLD, such as fat accumulation, inflammation, insulin resistance/disturbance of glucose metabolism, and fibrosis. The mechanism involves immune cell-related inflammatory reactions and various intracellular signaling pathways (**Figure 2**). The scope of this review includes only the reported virulence mechanisms of *P. gingivalis* in relation to the pathogenesis of NAFLD; the functioning of these mechanisms beyond NAFLD and in other diseases also requires consideration. Moreover, other virulence factors, such as heat shock protein 60 (171), have been mentioned in relation to liver diseases (172). However, the majority of *in-vitro* studies have focused on circulating immune cells, while few studies have investigated the role of *P. gingivalis* in hepatic cells such as Kupffer cells and hepatocytes. Further studies are needed to elucidate the roles of the pathogenic components of *P. gingivalis* in NAFLD.

Indirect Impacts of *P. gingivalis* in NAFLD: Bacteria-Derived Gut Dysbiosis

The effects of *P. gingivalis* on the modulation of gut microbiota have been investigated in many studies. Both intravenous injection and oral administration of the bacteria in a mouse model produced alterations in the gut microbiota (50, 173, 174). Clinical studies have detected remarkably higher proportions of *P. gingivalis* in the guts of NAFLD patients than in those of non-NAFLD controls 99; patients with chronic periodontitis tended to have less diversity in their gut microbiomes (175); the major changes in the gut microbiota of patients with liver cirrhosis result from invasion by oral bacterial species (176). These results indicate a possible relationship between the bacteria, gut, and NAFLD (**Figure 2**). Studies involving oral administration of *P. gingivalis* to mouse models show that, in contrast to the increased blood endotoxin levels that are typical of gut dysbiosis/barrier dysfunction and lead to systemic inflammation contributing to liver injury, no *P. gingivalis* was detected in the blood system (174), or outgrowth of the bacteria in the gut (80). This strongly supports the speculation that oral *P. gingivalis*-induced endotoxemia-related liver injury indirectly, by inducing gut dysbiosis and barrier dysfunction.

However, despite the evidence that oral *P. gingivalis* can survive in the acidic conditions induced by gastric juice (49), enabling it to enter the gastrointestinal tract, and that dead bacteria from the mouth may stimulate several gut pathogens, upregulate bacterial virulence genes, and thereby increase cytotoxicity (177), the mechanism underlying bacteria-induced gut dysbiosis remains unclear. For example, *P. gingivalis* must inhabit the host cells and trigger immune-inflammatory reactions to initiate subsequent actions, and *in-vitro* studies have shown that intestinal inflammation can be detected following oral administration of *P. gingivalis* (50), but there is as yet no evidence to show whether and how *P. gingivalis* can localize in the gastrointestinal tract; in addition, the bilateral interactions between oral bacteria and gut microbiota should be considered. Furthermore, the lack of large-scale epidemiologic studies and some inconsistent results in different animal models make it difficult to define a clear mechanism underlying the effects of *P. gingivalis* in NAFLD *via* modulation of gut microbiota (93).

On the other hand, *P. gingivalis* is not the only periodontopathogen that can contribute to the pathogenesis of NAFLD. *A. actinomycetemcomitans* can induce gut dysbiosis and impairing glucose metabolism (178); the detection frequency of *T. denticola* in the NAFLD patients was significantly higher than that in the control subjects (99); in patients with liver cirrhosis, more than half (54%) of the patient-enriched, taxonomically assigned bacterial species were of oral origin (mostly veillonella

and streptococci) (176, 179). Moreover, in comparison to growing evidence that supports *P. gingivalis* as a cause of gut dysbiosis, the precise mechanism through which *P. gingivalis* exerts its effects has yet to be determined. Further studies are needed to complete our knowledge of the roles of *P. gingivalis* in NAFLD *via* the oral-gut-liver axis.

THERAPEUTIC STRATEGIES IN NAFLD: TARGETING ORAL PATHOGENS AND MICROBIAL DYSBIOSIS

Dietary factors have been accepted for over a decade as a critical risk factor for NAFLD. Food intake control (caloric restriction) and methods to increase energy consumption, such as physical activity and sports, have therefore been the major treatment options for NAFLD. Vitamin reagents, PPAR agonists, and other treatments are in use or undergoing clinical trials (48). However, most of them are symptomatic treatments, of uncertain efficacy, especially in severe progressive forms such as NASH or HCC. The newly established theory of bacteria-induced NAFLD provides guidance for the prevention, prediction, and treatment of the disease. Attenuating oral bacteria and improving gut dysbiosis are the main therapeutic approaches being studied (**Figure 3**, **Table 3**).

Treatment Based on Controlling Oral Disease

Given the remarkable impacts of periodontal disease in NAFLD, improving oral hygiene, and other approaches targeting pathogenic oral bacteria, are regarded as effective treatment strategies for bacteria-associated NAFLD. Administration of

TABLE 3 | Therapeutic approaches in NAFLD targeting oral pathogens and oral/gut microbial dysbiosis.

Therapeutic approaches	Targets
Oral care/dental treatment	Oral pathogens & dysbiosis
Natural products	Oral pathogens & dysbiosis, gut dysbiosis
Probiotics/gut symbionts	Oral pathogens & dysbiosis, gut dysbiosis
Prebiotics	Gut dysbiosis
Synbiotics	Gut dysbiosis
FMT	Gut dysbiosis
Abs, CR3 blockers	Fimbriae of *P. gingivalis*
Inhibitors	Gingipains of *P. gingivalis*
RNA interference	Biofilm/OMVs of *P. gingivalis*
Antimicrobial agent-induced membrane vesicles	Biofilm/OMVs of *P. gingivalis*

Abs, antibodies; FMT, fecal microbiota transplantation.

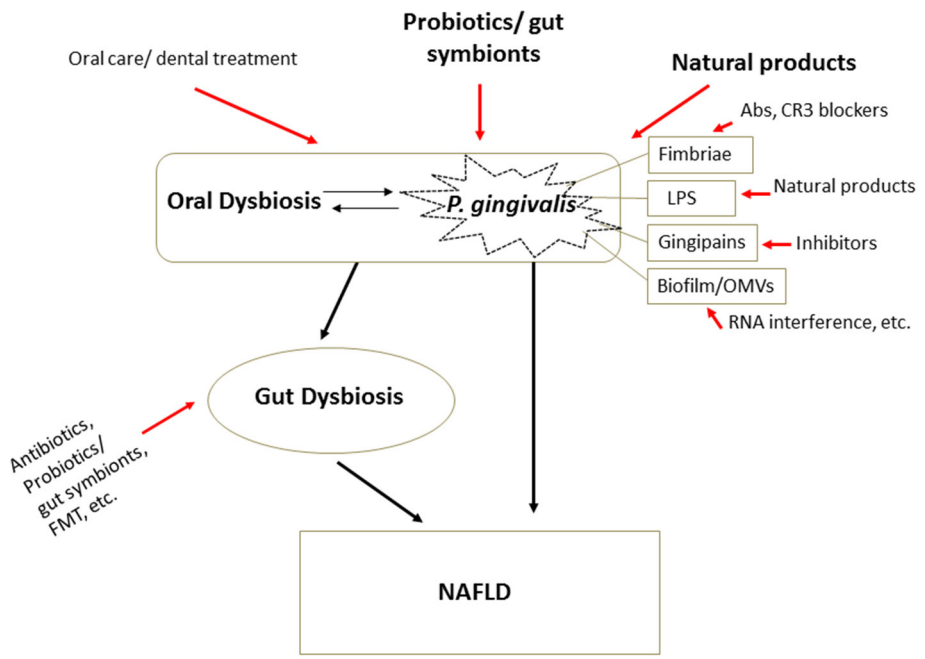


FIGURE 3 | Therapeutic strategies in microbial dysbiosis-related NAFLD. Understanding the mechanism underlying microbial dysbiosis and its impacts on the pathogenesis of NAFLD through the gut-liver, oral-liver, and oral-gut-liver axes may contribute to the development of new options for treatment of the disease. Some very safe options, such as some probiotics, gut symbionts, and natural products, aimed at attenuating oral bacteria and correcting dysbiosis of the oral and gut microbiota, have received substantial attention. Additionally, treatments targeting the structures and virulence factors of *P. gingivalis* offer new treatment options specifically targeting bacteria. Abs, antibodies; FMT, fecal microbiota transplantation.

broad-spectrum antibiotics can effectively protect against various liver diseases, and some specific antibiotic treatments have produced improvement in the clinical symptoms of NAFLD, by means of eliminating harmful microbes, lowering circulating endotoxin and transaminase levels, and preventing lipid accumulation in the liver (180). Notably, despite their powerful positive effects, long-term administration of antibiotics may cause toxic side effects, eliciting antibiotic-resistant bacterial strains and itself generating gut dysbiosis (181). There is therefore increasing interest in the safety and specificity of therapeutic approaches targeting pathogenic oral bacteria, such as *P. gingivalis*.

One of the treatment modalities is the administration of probiotics. Two lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus reuteri*, have millennia-long histories of use in the fermentation of foods and are generally accepted as safe for human consumption ("Generally Recognized As Safe," or GRAS, in US Federal Drug Administration terminology) (182). It has been reported that *L. lactis*, together with one of its derivatives, nisin, was effective in periodontal disease, with potent antibacterial ability but low side effects. Its mechanism involves modulation of the formation, composition, and survival of oral bacterial biofilms (82). Moreover, *L. lactis* treatment reduced hepatic fat accumulation and showed anti-inflammatory effects in the liver, indicating its potential in the treatment of NAFLD (82, 182). Furthermore, antibacterial effects on *P. gingivalis* and other oral pathogens were seen not only from live *L. reuteri* cells but also supernatants from cell-free cultures and heat-killed *L. reuteri* (183), indicating a potent yet safe antibacterial activity of this probiotic.

With regard to the therapeutic approaches specifically targeting the structure of *P. gingivalis,* further methods for inhibiting biofilm formation by modulating OMVs have been investigated, in addition to the biofilm modulation approach mentioned above. These include RNA interference technology, antimicrobial agent-induced membrane vesicles, and some natural products, which have all been reviewed recently (120). Plant-derived products have attracted considerable attention in chronic disease therapy, given their safety. Natural compounds have been demonstrated to inhibit the growth and virulence factor activity of bacteria (184–186). We have reported that carnosic acid, extracted from rosemary plants, exerts potent effects, inhibiting lipid accumulation in both the adipose tissue and liver, improving glucose metabolism and liver functions in the *ob-ob* obesity mouse model (187), and inhibiting lipid accumulation in HepG2 cells (188). Moreover, our studies have clarified that carnosic acid can protect normal hepatocytes from H2O2-induced oxidative stress (189). Interestingly, carnosic acid has been reported to have remarkable antibacterial activities (190, 191). The mechanism is related to its inhibition of biofilm information (192); carnosic acid can also protect against LPS-induced liver injury (193), and is thus a potential new therapeutic approach for treating NAFLD. In our laboratory, we are currently investigating the roles of carnosic acid in NAFLD associated with *P. gingivalis* inoculation in both mouse and cell models.

Anti-fimbrial Ab has also been reported as effective in inhibiting the adherence of *P. gingivalis* to its host cells (194). Inhibition of or pre-immunity against potent virulence factors of *P. gingivalis*, such as gingipains, was effective against periodontitis and systemic diseases (195, 196). Moreover, as the CR3 receptor plays an important role in the *P. gingivalis* fimbriae-stimulated adhesion signal, as described above, CR3 attenuation has been considered as an approach for treating periodontitis and *P. gingivalis*-related systemic diseases (197). However, little is known about the significance of the above materials for the management of NAFLD associated with infection by oral pathogenic bacteria such as *P. gingivalis*, which should be investigated further.

Treatments Targeting Dysbiosis of Microbiota

Since the gut microbiota is closely related to the pathogenesis of NAFLD, various therapeutic approaches targeting gut microbiota have been developed. They include antibiotics, probiotics, prebiotics, synbiotics, and fecal microbiota transplantation, which have recently been reviewed (76, 180). In particular, dietary fiber is cited as useful: it may improve early-stage NAFLD by reducing calorie absorption and correcting the imbalance of gut microbiota (198); *L.* reuteri not only improves the dysbiosis of oral microbiota by targeting biofilm, but also shows the ability to correct gut dysbiosis and protect the liver from inflammatory damage (199). Moreover, a gut symbiont, *Akkermansia muciniphila*, and one of its membrane proteins has produced anti-inflammatory activity and improved gut permeability in animal models (30, 200, 201). Furthermore, human studies have shown that administration of *A. muciniphila* leads to decreased body weight, and the markers associated with inflammation and liver dysfunction in obese objects (202). However, it should be noted that while microbiome-based therapeutic approaches are usually regarded as safe, they cannot be considered risk-free, as they are still bioactive. Therefore, large-scale studies are required to evaluate their safety.

CONCLUSIONS

The close relationship between systemic disease and dysbiosis of both the oral and gut microbiota has been supported by substantial evidence from both basic and clinical studies. In this review, we emphasize one of the most commonly diagnosed such diseases, NAFLD, using it as a typical example of systemic disease, and *P. gingivalis*, which is the major pathogen of periodontitis, and typical of the pathogenic bacteria involved in systemic diseases *via* the oral-liver and oral-gut-liver axes. We focused on the structure-derived functions of *P. gingivalis* in the progression of NAFLD, such as hepatic steatosis, inflammation, and fibrosis, which cause conversion to NASH. NASH is associated with the pathogenesis of HCC, and it is therefore reasonable to

suppose that *P. gingivalis* may contribute to HCC by promoting progression to NASH.

Currently, various therapeutic approaches are being developed, targeting *P. gingivalis* and dysbiosis of both oral and gut microbiota. Although most of these remain at the fundamental experimental level, limited to small-scale studies or experiencing difficulties in establishing ideal animal models, improving our understanding of the connections between NAFLD and oral/gut dysbiosis will definitely contribute to achieving the reasonable future goal of treating the disease successfully.

AUTHOR CONTRIBUTIONS

TC, TW, TI, and MS conceptualized the review. TW and TI drafted the manuscript. TC and MS edited the manuscript. All the authors read and approved the final manuscript.

FUNDING

This study was supported by Grants-in-Aid for Scientific Research (JP 19K07737) from the Japan Society for the Promotion of Science.

REFERENCES

1. Gomaa EZ. Human gut microbiota/microbiome in health and diseases: a review. *Antonie van Leeuwenhoek.* (2020) 113:2019–40. doi: 10.1007/s10482-020-01474-7
2. Fi C, Wo W. Periodontal disease and systemic diseases: an overview on recent progresses. *J Biol Regul Homeost Agents.* (2021) 35 (1 Suppl. 1):1–9.
3. Mei F, Xie M, Huang X, Long Y, Lu X, Wang X, et al. *Porphyromonas gingivalis* and its systemic impact: current status. *Pathogens.* (2020) 9:944. doi: 10.3390/pathogens9110944
4. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res.* (2020) 30:492–506. doi: 10.1038/s41422-020-0332-7
5. DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current understanding of dysbiosis in disease in human and animal models. *Inflamm Bowel Dis.* (2016) 22:1137–50. doi: 10.1097/MIB.0000000000000750
6. Singh R, Zogg H, Wei L, Bartlett A, Ghoshal UC, Rajender S, et al. Gut microbial dysbiosis in the pathogenesis of gastrointestinal dysmotility and metabolic disorders. *J Neurogastroenterol Motil.* (2021) 27:19–34. doi: 10.5056/jnm20149
7. Khor B, Snow M, Herrman E, Ray N, Mansukhani K, Patel KA, et al. Interconnections between the oral and gut microbiomes: reversal of microbial dysbiosis and the balance between systemic health and disease. *Microorganisms.* (2021) 9:496. doi: 10.3390/microorganisms9030496
8. Tilg H, Adolph TE, Moschen AR. Multiple parallel hits hypothesis in nonalcoholic fatty liver disease: revisited after a decade. *Hepatology.* (2021) 73:833–842. doi: 10.1002/hep.31518
9. Abdelmalek MF. Nonalcoholic fatty liver disease: another leap forward. *Nat Rev Gastroenterol Hepatol.* (2021) 18:85–6. doi: 10.1038/s41575-020-00406-0
10. Xu W, Zhou W, Wang H, Liang S. Roles of *Porphyromonas gingivalis* and its virulence factors in periodontitis. *Adv Prot Chem Struct Biol.* (2020) 120:45–84. doi: 10.1016/bs.apcsb.2019.12.001
11. Bregaint S, Boyer E, Fong SB, Meuric V, Bonnaure-Mallet M, Jolivet-Gougeon A. *Porphyromonas gingivalis* outside the oral cavity. *Odontology.* (2022) 110:1–19. doi: 10.1007/s10266-021-00647-8
12. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature.* (2010) 464:59–65. doi: 10.1038/nature08821
13. Lederberg J. Infectious history. *Science.* (2000) 288:287–93. doi: 10.1126/science.288.5464.287
14. Alaalm L, Crunden JL, Butcher M, Obst U, Whealy R, Williamson C, et al. Identification and phenotypic characterization of hsp90 phosphorylation sites that modulate virulence traits in the major human fungal pathogen *Candida albicans*. *Front Cell Infect Microbiol.* (2021) 11:637836. doi: 10.3389/fcimb.2021.637836
15. Mach J, Sutak R. Iron in parasitic protists - from uptake to storage and where we can interfere. *Metallomics.* (2020) 12:1335–47. doi: 10.1039/d0mt00125b
16. Fierer N, Hamady M, Lauber CL, Knight R. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc Natl Acad Sci USA.* (2008) 105:17994–9. doi: 10.1073/pnas.0807920105
17. Kim S, Jazwinski SM. The gut microbiota and healthy aging: a mini-review. *Gerontology.* (2018) 64:513–20. doi: 10.1159/000490615
18. Reid G. The need to focus on therapy instead of associations. *Front Cell Infect Microbiol.* (2019) 9:327. doi: 10.3389/fcimb.2019.00327
19. Suárez LJ, Arboleda S, Angelov N, Arce RM. Oral versus gastrointestinal mucosal immune niches in homeostasis and allostasis. *Front Immunol.* (2021) 12:705206. doi: 10.3389/fimmu.2021.705206
20. Ghattargi VC, Nimonkar YS, Burse SA, Davray D, Kumbhare SV, Shetty SA, et al. Genomic and physiological analyses of an indigenous strain, *Enterococcus faecium* 17OM39. *Funct Integr Genomics.* (2018) 18:385–99. doi: 10.1007/s10142-018-0596-x
21. Mu C, Zhu W. Antibiotic effects on gut microbiota, metabolism, and beyond. *Appl Microbiol Biotechnol.* (2019) 103:9277–85. doi: 10.1007/s00253-019-10165-x
22. Ballan R, Battistini C, Xavier-Santos D, Saad S. Interactions of probiotics and prebiotics with the gut microbiota. *Prog Mol Biol Transl Sci.* (2020) 171:265–300. doi: 10.1016/bs.pmbts.2020.03.008
23. Tap J, Mondot S, Levenez F, Pelletier E, Caron C, Furet JP, et al. Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol.* (2009) 11:2574–84. doi: 10.1111/j.1462-2920.2009.01982.x
24. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. *J Bacteriol.* (2010) 192:5002–17. doi: 10.1128/JB.00542-10
25. Kho ZY, Lal SK. The human gut microbiome - a potential controller of wellness and disease. *Front Microbiol.* (2018) 9:1835. doi: 10.3389/fmicb.2018.01835
26. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe.* (2014) 15:382–92. doi: 10.1016/j.chom.2014.02.005
27. Sharon G, Garg N, Debelius J, Knight R, Dorrestein PC, Mazmanian SK. Specialized metabolites from the microbiome in health and disease. *Cell Metab.* (2014) 20:719–30. doi: 10.1016/j.cmet.2014.10.016
28. Kushak RI, Sengupta A, Winter HS. Interactions between the intestinal microbiota and epigenome in individuals with autism spectrum disorder. *Dev Med Child Neurol.* (2021) 63:1204–12. doi: 10.1111/dmcn.15052
29. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. *Nature.* (2006) 444:1022–3. doi: 10.1038/4441022a
30. Plovier H, Everard A, Druart C, Depommier C, Van Hul M, Geurts L, et al. A purified membrane protein from *Akkermansia muciniphila* or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nat Med.* (2017) 23:107–13. doi: 10.1038/nm.4236
31. Krishnan K, Chen T, Paster BJ. A practical guide to the oral microbiome and its relation to health and disease. *Oral Dis.* (2017) 23:276–86. doi: 10.1111/odi.12509
32. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. *J Bacteriol.* (2001) 183:3770–83. doi: 10.1128/JB.183.12.3770-3783.2001
33. Hamada S, Slade HD. Biology, Immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev.* (1980) 44:331–84. doi: 10.1128/mr.44.2.331-384.1980
34. Shi C, Cai L, Xun Z, Zheng S, Shao F, Wang B, et al. Metagenomic analysis of the salivary microbiota in patients with caries, periodontitis and comorbid diseases. *J Dent Sci.* (2021) 16:1264–73. doi: 10.1016/j.jds.2020.12.002

35. Álvarez S, Leiva-Sabadini C, Schuh C, Aguayo S. Bacterial adhesion to collagens: implications for biofilm formation and disease progression in the oral cavity. *Crit Rev Microbiol.* (2021) 48:1–13. doi: 10.1080/1040841X.2021.1944054
36. Ryder MI, Xenoudi P. Alzheimer disease and the periodontal patient: new insights, connections, and therapies. *Periodontology.* (2021) 87:32–42. doi: 10.1111/prd.12389
37. Koga S, Takazono T, Serita T, Ashizawa N, Hirayama T, Tashiro M, et al. Assessment of oral health in elderly patients with dementia by measuring volatile sulfur compounds and its relationship with pneumonia development: a pilot study. *Respir Investig.* (2021) 60:162–6. doi: 10.1016/j.resinv.2021.08.006
38. Pavlic V, Peric D, Kalezic IS, Madi M, Bhat SG, Brkic Z, et al. Identification of periopathogens in atheromatous plaques obtained from carotid and coronary arteries. *BioMed Res Int.* (2021) 2021:9986375. doi: 10.1155/2021/9986375
39. Hirai J, Sakanashi D, Haranaga S, Kinjo T, Hagihara M, Kato H, et al. Case-control study of pneumonia patients with *Streptococcus anginosus* group bacteria in their sputum. *J Infect Chemother.* (2016) 22:794–9. doi: 10.1016/j.jiac.2016.08.014
40. Sasaki M, Shimoyama Y, Kodama Y, Ishikawa T. *Abiotrophia defectiva*DnaK promotes fibronectin-mediated adherence to HUVECs and induces a proinflammatory response. *Int J Mol Sci.* (2021) 22:8528. doi: 10.3390/ijms22168528
41. Mahendra J, Mahendra L, Mugri MH, Sayed ME, Bhandi S, Alshahrani RT, et al. Role of periodontal bacteria, viruses, and placental *mir155* in chronic periodontitis and preeclampsia-a genetic microbiological study. *Curr Issues Mol Biol.* (2021) 43:831–44. doi: 10.3390/cimb43020060
42. Gorabi AM, Kiaie N, Khosrojerdi A, Jamialahmadi T, Al-Rasadi K, Johnston TP, et al. Implications for the role of lipopolysaccharide in the development of atherosclerosis. *Trends Cardiovasc Med.* (2021) S1050-1738(21)00099-2. doi: 10.1016/j.tcm.2021.08.015. [Epub ahead of print].
43. Esberg A, Johansson L, Johansson I, Dahlqvist SR. Oral microbiota identifies patients in early onset rheumatoid arthritis. *Microorganisms.* (2021) 9:1657. doi: 10.3390/microorganisms9081657
44. Park SY, Hwang BO, Lim M, Ok SH, Lee SK, Chun KS, et al. Oral-Gut microbiome axis in gastrointestinal disease and cancer. *Cancers.* (2021) 13:2124. doi: 10.3390/cancers13092124
45. Fiorillo L, Cervino G, Laino L, D'Amico C, Mauceri R, Tozum TF, et al. *Porphyromonas gingivalis*, periodontal and systemic implications: a systematic review. *Dent J.* (2019) 7:114. doi: 10.3390/dj7040114
46. Kim YJ, Kim BK, Park SJ, Kim JH. Impact of *Fusobacterium nucleatum* in the gastrointestinal tract on natural killer cells. *World J Gastroenterol.* (2021) 27:4879–89. doi: 10.3748/wjg.v27.i29.4879
47. Tsuzuno T, Takahashi N, Yamada-Hara M, Yokoji-Takeuchi M, Sulijaya B, Aoki-Nonaka Y, et al. Ingestion of *Porphyromonas gingivalis* exacerbates colitis via intestinal epithelial barrier disruption in mice. *J Periodontal Res.* (2021) 56:275–88. doi: 10.1111/jre.12816
48. Kashiwagi Y, Aburaya S, Sugiyama N, Narukawa Y, Sakamoto Y, Takahashi M, et al. Porphyromonas gingivalis induces entero-hepatic metabolic derangements with alteration of gut microbiota in a type 2 diabetes mouse model. *Sci Rep.* (2021) 11:18398. doi: 10.1038/s41598-021-97868-2
49. Sato K, Takahashi N, Kato T, Matsuda Y, Yokoji M, Yamada M, et al. Aggravation of collagen-induced arthritis by orally administered *Porphyromonas gingivalis* through modulation of the gut microbiota and gut immune system. *Sci Rep.* (2017) 7:6955. doi: 10.1038/s41598-017-07196-7
50. Nakajima M, Arimatsu K, Kato T, Matsuda Y, Minagawa T, Takahashi N, et al. Oral Administration of P. *gingivalis* induces dysbiosis of gut microbiota and impaired barrier function leading to dissemination of enterobacteria to the liver. *PLoS ONE.* (2015) 10:e0134234. doi: 10.1371/journal.pone.0134234
51. Gao B, Jeong WI, Tian Z. Liver: an organ with predominant innate immunity. *Hepatology.* (2008) 47:729–36. doi: 10.1002/hep.22034
52. Shojaie L, Iorga A, Dara L. Cell death in liver diseases: a review. *Int J Mol Sci.* (2020) 21:9682. doi: 10.3390/ijms21249682
53. Michalopoulos GK, Bhushan B. Liver regeneration: biological and pathological mechanisms and implications. *Nat Rev Gastroenterol Hepatol.* (2021) 18:40–55. doi: 10.1038/s41575-020-0342-4
54. Antunes C, Azadfard M, Hoilat GJ, Gupta M. *Fatty Liver*. Treasure Island, FL: StatPearls Publishing (2021).
55. Kim JY, He F, Karin M. From liver fat to cancer: perils of the western diet. *Cancers.* (2021) 13:1095. doi: 10.3390/cancers13051095
56. Patel R, Mueller M. *Alcoholic Liver Disease*. Treasure Island, FL: StatPearls Publishing (2021).
57. Cotter TG, Rinella M. Nonalcoholic fatty liver disease 2020: the state of the disease. *Gastroenterology.* (2020) 158:1851–64. doi: 10.1053/j.gastro.2020.01.052
58. Peng C, Stewart AG, Woodman OL, Ritchie RH, Qin CX. Non-Alcoholic steatohepatitis: a review of its mechanism, models and medical treatments. *Front Pharmacol.* (2020) 11:603926. doi: 10.3389/fphar.2020.603926
59. Sharma B, John S. *Nonalcoholic Steatohepatitis (NASH)*. Treasure Island, FL: StatPearls Publishing (2021).
60. Khan F.Z, Perumpail RB, Wong RJ, Ahmed A. Advances in hepatocellular carcinoma: nonalcoholic steatohepatitis-related hepatocellular carcinoma. *World J Hepatol.* (2015) 7:2155–61. doi: 10.4254/wjh.v7.i18.2155
61. White DL, Kanwal F, El-Serag HB. Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. *Clin Gastroenterol Hepatol.* (2012) 10:1342–59.e2. doi: 10.1016/j.cgh.2012.10.001
62. Renne SL, Sarcognato S, Sacchi D, Guido M, Roncalli M, Terracciano L, et al. Hepatocellular carcinoma: a clinical and pathological overview. *Pathologica.* (2021) 113:203–17. doi: 10.32074/1591-951X-295
63. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology.* (1998) 114:842–5. doi: 10.1016/S0016-5085(98)70599-2
64. Ragonnaud E, Biragyn A. Gut microbiota as the key controllers of "healthy" aging of elderly people. *Immunity Ageing.* (2021) 18:2. doi: 10.1186/s12979-020-00213-w
65. Hrncir T, Hrncirova L, Kverka M, Hromadka R, Machova V, Trckova E, et al. Gut microbiota and NAFLD: pathogenetic mechanisms, microbiota signatures, and therapeutic interventions. *Microorganisms.* (2021) 9:957. doi: 10.3390/microorganisms9050957
66. Wieland A, Frank DN, Harnke B, Bambha K. Systematic review: microbial dysbiosis and nonalcoholic fatty liver disease. *Aliment Pharmacol Ther.* (2015) 42:1051–63. doi: 10.1111/apt.13376
67. Zhang YJ, Li S, Gan RY, Zhou T, Xu DP, Li HB. Impacts of gut bacteria on human health and diseases. *Int J Mol Sci.* (2015) 16:7493–519. doi: 10.3390/ijms16047493
68. Tilg H, Cani PD, Mayer EA. Gut microbiome and liver diseases. *Gut.* (2016) 65:2035–44. doi: 10.1136/gutjnl-2016-312729
69. Milosevic I, Vujovic A, Barac A, Djelic M, Korac M, Radovanovic Spurnic A, et al. Gut-Liver axis, gut microbiota, and its modulation in the management of liver diseases: a review of the literature. *Int J Mol Sci.* (2019) 20:395. doi: 10.3390/ijms20020395
70. Bashiardes S, Shapiro H, Rozin S, Shibolet O, Elinav E. Non-alcoholic fatty liver and the gut microbiota. *Mol Metab.* (2016) 5:782–94. doi: 10.1016/j.molmet.2016.06.003
71. Leung C, Rivera L, Furness JB, Angus PW. The role of the gut microbiota in NAFLD. *Nat Rev Gastroenterol Hepatol.* (2016) 13:412–25. doi: 10.1038/nrgastro.2016.85
72. Qi X, Yang M, Stenberg J, Dey R, Fogwe L, Alam MS, et al. Gut microbiota mediated molecular events and therapy in liver diseases. *World J Gastroenterol.* (2020) 26:7603–18. doi: 10.3748/wjg.v26.i48.7603
73. Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, et al. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. *Sci Rep.* (2015) 3:8096. doi: 10.1038/srep08096
74. Oikonomou T, Papatheodoridis GV, Samarkos M, Goulis I, Cholongitas E. Clinical impact of microbiome in patients with decompensated cirrhosis. *World J Gastroenterol.* (2018) 24:3813–20. doi: 10.3748/wjg.v24.i34.3813
75. Caussy C, Tripathi A, Humphrey G, Bassirian S, Singh S, Faulkner C, et al. A gut microbiome signature for cirrhosis due to nonalcoholic fatty liver disease. *Nat Commun.* (2019) 10:1406. doi: 10.1038/s41467-019-09455-9
76. Boursier J, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, et al. The severity of nonalcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. *Hepatology.* (2016) 63:764–75. doi: 10.1002/hep.28356

77. Ji Y, Yin Y, Li Z, Zhang W. Gut microbiota-derived components and metabolites in the progression of non-alcoholic fatty liver disease (NAFLD). *Nutrients.* (2019) 11:1712. doi: 10.3390/nu11081712
78. Zhou D, Fan JG. Microbial metabolites in non-alcoholic fatty liver disease. *World J Gastroenterol.* (2019) 25:2019–28. doi: 10.3748/wjg.v25.i17.2019
79. He FF, Li YM. Role of gut microbiota in the development of insulin resistance and the mechanism underlying polycystic ovary syndrome: a review. *J Ovarian Res.* (2020) 13:73. doi: 10.1186/s13048-020-00670-3
80. Wu J, Wang K, Wang X, Pang Y, Jiang C. The role of the gut microbiome and its metabolites in metabolic diseases. *Protein Cell.* (2021) 12:360–373. doi: 10.1007/s13238-020-00814-7
81. Delzenne NM, Bindels LB. Microbiome metabolomics reveals new drivers of human liver steatosis. *Nat Med.* (2018) 24:906–7. doi: 10.1038/s41591-018-0126-3
82. Sookoian S, Salatino A, Castaño GO, Landa MS, Fijalkowky C, Garaycoechea M, et al. Intrahepatic bacterial metataxonomic signature in non-alcoholic fatty liver disease. *Gut.* (2020) 69:1483–91. doi: 10.1136/gutjnl-2019-318811
83. Caussy C, Hsu C, Lo MT, Liu A, Bettencourt R, Ajmera VH, et al. Genetics of NAFLD in twins consortium. Link between gut-microbiome derived metabolite and shared gene-effects with hepatic steatosis and fibrosis in NAFLD. *Hepatology.* (2018) 68:918–32. doi: 10.1002/hep.29892
84. Caussy C, Ajmera VH, Puri P, Hsu CL, Bassirian S, Mgdsyan M, et al. Serum metabolites detect the presence of advanced fibrosis in derivation and validation cohorts of patients with non-alcoholic fatty liver disease. *Gut.* (2019) 68:1884–92. doi: 10.1136/gutjnl-2018-317584
85. Zhou J, Tripathi M, Sinha RA, Singh BK, Yen PM. Gut microbiota and their metabolites in the progression of non-alcoholic fatty liver disease. *Hepatoma Res.* (2021) 7:11. doi: 10.20517/2394-5079.2020.134
86. Tripathi A, Debelius J, Brenner DA, Karin M, Loomba R, Schnabl B, et al. The gut-liver axis and the intersection with the microbiome. *Nat Rev Gastroenterol Hepatol.* (2018) 15:397–411. doi: 10.1038/s41575-018-0011-z
87. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology.* (2009) 137:1716–24.e242. doi: 10.1053/j.gastro.2009.08.042
88. Ogawa Y, Imajo K, Honda Y, Kessoku T, Tomeno W, Kato S, et al. Palmitate-induced lipotoxicity is crucial for the pathogenesis of nonalcoholic fatty liver disease in cooperation with gut-derived endotoxin. *Sci Rep.* (2018) 8:11365. doi: 10.1038/s41598-018-29735-6
89. Spruss A, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Bergheim I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. *Hepatology.* (2009) 50:1094–104. doi: 10.1002/hep.23122
90. Dietrich P, Hellerbrand C. Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. *Best Pract Res Clin Gastroenterol.* (2014) 28:637–53. doi: 10.1016/j.bpg.2014.07.008
91. Bertani B, Ruiz N. Function and biogenesis of lipopolysaccharides. *EcoSal Plus.* (2018) 8:1–19. doi: 10.1128/ecosalplus.ESP-0001-2018
92. Kuraji R, Sekino S, Kapila Y, Numabe Y. Periodontal disease-related nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: an emerging concept of oral-liver axis. *Periodontology.* (2021) 87:204–40. doi: 10.1111/prd.12387
93. Hussain T, Tan B, Yin Y, Blachier F, Tossou MC, Rahu N. Oxidative stress and inflammation: what polyphenols can do for us? *Oxid Med Cell Longev.* (2016) 2016:7432797. doi: 10.1155/2016/7432797
94. Fei N, Bruneau A, Zhang X, Wang R, Wang J, Rabot S, et al. Endotoxin producers overgrowing in human gut microbiota as the causative agents for nonalcoholic fatty liver disease. *mBio.* (2020) 11:e03263–19. doi: 10.1128/mBio.03263-19
95. Xu DX, Chen YH, Wang JP, Sun MF, Wang H, Wei LZ., et al. Perinatal lipopolysaccharide exposure downregulates pregnane X receptor and Cyp3a11 expression in fetal mouse liver. *Toxicol Sci.* (2005) 87:38–45. doi: 10.1093/toxsci/kfi239
96. Abdel-Salam OM, Youness ER, Mohammed NA, Morsy SM, Omara EA, Sleem AA. Citric acid effects on brain and liver oxidative stress in lipopolysaccharide-treated mice. *J Med Food.* (2014) 17:588–98. doi: 10.1089/jmf.2013.0065
97. Hatasa M, Yoshida S, Takahashi H, Tanaka K, Kubotsu Y, Ohsugi Y, et al. Relationship between NAFLD and periodontal disease from the view of clinical and basic research, and immunological response. *Int J Mol Sci.* (2021) 22:3728. doi: 10.3390/ijms22073728
98. How KY, Song KP, Chan KG. Porphyromonas gingivalis: an overview of periodontopathic pathogen below the gum line. *Front Microbiol.* (2016) 7:53. doi: 10.3389/fmicb.2016.00053
99. Yoneda M, Naka S, Nakano K, Wada K, Endo H, Mawatari H, et al. Involvement of a periodontal pathogen, *Porphyromonas gingivalis* on the pathogenesis of non-alcoholic fatty liver disease. *BMC Gastroenterol.* (2012) 12:16. doi: 10.1186/1471-230X-12-16
100. Furusho H, Miyauchi M, Hyogo H, Inubushi T, Ao M, Ouhara K, et al. Dental infection of *Porphyromonas gingivalis* exacerbates high fat diet-induced steatohepatitis in mice. *J Gastroenterol.* (2013) 48:1259–70. doi: 10.1007/s00535-012-0738-1
101. Mulhall H, Huck O, Amar S. *Porphyromonas gingivalis*, a long-range pathogen: systemic impact and therapeutic implications. *Microorganisms.* (2020) 8:869. doi: 10.3390/microorganisms8060869
102. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol.* (2018) 16:745–59. doi: 10.1038/s41579-018-0089-x
103. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis. Periodontology.* (1999) 20:168–238. doi: 10.1111/j.1600-0757.1999.tb00162.x
104. Enersen M, Nakano K, Amano A. *Porphyromonas gingivalis* fimbriae. *J Oral Microbiology.* (2013) 5:1–10. doi: 10.3402/jom.v5i0.20265
105. Nakahara T, Hyogo H, Ono A, Nagaoki Y, Kawaoka T, Miki D, et al. Involvement of *Porphyromonas gingivalis* in the progression of non-alcoholic fatty liver disease. *J Gastroenterol.* (2018) 53:269–80. doi: 10.1007/s00535-017-1368-4
106. Pathirana RD, O'Brien-Simpson NM, Reynolds EC. Host immune responses to *Porphyromonas gingivalis* antigens. *Periodontology.* (2010) 52:218–37. doi: 10.1111/j.1600-0757.2009.00330.x
107. Dashper SG, Mitchell HL, Seers CA, Gladman SL, Seemann T, Bulach DM, et al. *Porphyromonas gingivalis* uses specific domain rearrangements and allelic exchange to generate diversity in surface virulence factors. *Front Microbiol.* (2017) 8:48. doi: 10.3389/fmicb.2017.00048
108. Hajishengallis G, Tapping RI, Harokopakis E, Nishiyama S, Ratti P, Schifferle RE, et al. Differential interactions of fimbriae and lipopolysaccharide from *Porphyromonas gingivalis* with the toll-like receptor 2-centred pattern recognition apparatus. *Cell Microbiol.* (2006) 8:1557–1570. doi: 10.1111/j.1462-5822.2006.00730.x
109. Miura K, Ohnishi H. Role of gut microbiota and toll-like receptors in nonalcoholic fatty liver disease. *World J Gastroenterol.* (2014) 20:7381–91. doi: 10.3748/wjg.v20.i23.7381
110. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol.* (2003) 3:133–46. doi: 10.1038/nri1001
111. Lima WG, Oliveira PS, Caliari MV, Gonçalves R, Michalick MS, Melo MN, et al. Histopathological and immunohistochemical study of type 3 complement receptors (CD11b/CD18) in livers and spleens of asymptomatic and symptomatic dogs naturally infected with leishmania (leishmania) chagasi. *Vet Immunol Immunopathol.* (2007) 117:129–36. doi: 10.1016/j.vetimm.2007.02.012
112. Rensen SS, Slaats Y, Driessen A, Peutz-Kootstra CJ, Nijhuis J, Steffensen R, et al. Activation of the complement system in human nonalcoholic fatty liver disease. *Hepatology.* (2009) 50:1809–17. doi: 10.1002/hep.23228
113. Li L, Zhang YL, Liu XY, Meng X, Zhao RQ, Ou LL, et al. Periodontitis exacerbates and promotes the progression of chronic kidney disease through oral flora, cytokines, and oxidative stress. *Front Microbiol.* (2021) 12:656372. doi: 10.3389/fmicb.2021.656372
114. Hajishengallis G, Wang M, Liang S, Triantafilou M, Triantafilou K. Pathogen induction of CXCR4/TLR2 cross-talk impairs host defense function. *Proc Natl Acad Sci USA.* (2008) 105:13532–7. doi: 10.1073/pnas.0803852105
115. Zhang N, Qu Y, Qin B. Sodium butyrate ameliorates non-alcoholic fatty liver disease by upregulating miR-150 to suppress CXCR4 expression. *Clin Exp Pharmacol Physiol.* (2021) 48:1125–36. doi: 10.1111/1440-1681.13497
116. Boujedidi H, Robert O, Bignon A, Cassard-Doulcier AM, Renoud ML, Gary-Gouy H, et al. CXCR4 dysfunction in non-alcoholic steatohepatitis in mice and patients. *Clin Sci.* (2015) 128:257–67. doi: 10.1042/CS20130833

117. Amano A, Nakagawa I, Okahashi N, Hamada N. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. *J Periodontal Res.* (2004) 39:136–42. doi: 10.1111/j.1600-0765.2004.00719.x
118. Meghil MM, Tawfik OK, Elashiry M, Rajendran M, Arce RM, Fulton DJ, et al. Disruption of immune homeostasis in human dendritic cells via regulation of autophagy and apoptosis by *Porphyromonas gingivalis*. *Front Immunol.* (2019) 10:2286. doi: 10.3389/fimmu.2019.02286
119. Gross DN, van den Heuvel AP, Birnbaum MJ. The role of FoxO in the regulation of metabolism. *Oncogene.* (2008) 27:2320–36. doi: 10.1038/onc.2008.25
120. Płóciennikowska A, Hromada-Judycka A, Borzecka K, Kwiatkowska K. Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci.* (2015) 72:557–81. doi: 10.1007/s00018-014-1762-5
121. Darveau RP, Arbabi S, Garcia I, Bainbridge B, Maier RV. Porphyromonas gingivalis lipopolysaccharide is both agonist and antagonist for p38 mitogen-activated protein kinase activation. *Infect Immunity.* (2002) 70:1867–73. doi: 10.1128/IAI.70.4.1867-1873.2002
122. Marcano R, Rojo MÁ, Cordoba-Diaz D, Garrosa M. Pathological and therapeutic approach to endotoxin-secreting bacteria involved in periodontal disease. *Toxins.* (2021) 13:533. doi: 10.3390/toxins13080533
123. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immunity.* (2004) 72:5041–51. doi: 10.1128/IAI.72.9.5041-5051.2004
124. Li Y, Shi Z, Radauer-Preiml I, Andosch A, Casals E, Luetz-Meindl U, et al. Bacterial endotoxin (lipopolysaccharide) binds to the surface of gold nanoparticles, interferes with biocorona formation and induces human monocyte inflammatory activation. *Nanotoxicology.* (2017) 11:1157–75. doi: 10.1080/17435390.2017.1401142
125. Jain S, Coats SR, Chang AM, Darveau RP. A novel class of lipoprotein lipase-sensitive molecules mediates toll-like receptor 2 activation by *Porphyromonas gingivalis*. *Infect Immunity.* (2013) 81:1277–86. doi: 10.1128/IAI.01036-12
126. Holden JA, Attard TJ, Laughton KM, Mansell A, O'Brien-Simpson NM, Reynolds EC. *Porphyromonas gingivalis* lipopolysaccharide weakly activates M1 and M2 polarized mouse macrophages but induces inflammatory cytokines. *Infect Immunity.* (2014) 82:4190–203. doi: 10.1128/IAI.02325-14
127. Dahlen G, Basic A, Bylund J. Importance of virulence factors for the persistence of oral bacteria in the inflamed gingival crevice and in the pathogenesis of periodontal disease. *J Clin Med.* (2019) 8:1339. doi: 10.3390/jcm8091339
128. Fujita M, Kuraji R, Ito H, Hashimoto S, Toen T, Fukada T, et al. Histological effects and pharmacokinetics of lipopolysaccharide derived from *Porphyromonas gingivalis* on rat maxilla and liver concerning with progression into non-alcoholic steatohepatitis. *J Periodontol.* (2018) 89:1101–11. doi: 10.1002/JPER.17-0678
129. Kuraji R, Ito H, Fujita M, Ishiguro H, Hashimoto S, Numabe Y. *Porphyromonas gingivalis* induced periodontitis exacerbates progression of non-alcoholic steatohepatitis in rats. *Clin Exp Dent Res.* (2016) 2:216–25. doi: 10.1002/cre2.41
130. Varela-López A, Bullón P, Ramírez-Tortosa CL, Navarro-Hortal MD, Robles-Almazán M, Bullón B, et al. A diet rich in saturated fat and cholesterol aggravates the effect of bacterial lipopolysaccharide on alveolar bone loss in a rabbit model of periodontal disease. *Nutrients.* (2020) 12:1405. doi: 10.3390/nu12051405
131. Ding LY, Liang LZ, Zhao YX, Yang YN, Liu F, Ding QR, et al. *Porphyromonas gingivalis*-derived lipopolysaccharide causes excessive hepatic lipid accumulation via activating NF-κB and JNK signaling pathways. *Oral Dis.* (2019) 25:1789–97. doi: 10.1111/odi.13153
132. Nagasaki A, Sakamoto S, Chea C, Ishida E, Furusho H, Fujii M, et al. Odontogenic infection by *Porphyromonas gingivalis* exacerbates fibrosis in NASH via hepatic stellate cell activation. *Sci Rep.* (2020) 10:4134. doi: 10.1038/s41598-020-60904-8
133. Tilg H, Moschen AR. Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med.* (2008) 14:222–31. doi: 10.2119/2007-00119.Tilg
134. Nagata T. [Relationhip between diabetes and periodontal disease]. *Clin Calcium.* (2009) 19:1291–8.
135. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* (1999) 11:443–51. doi: 10.1016/S1074-7613(00)80119-3
136. Ding PH, Wang CY, Darveau RP, Jin L. Porphyromonas gingivalis LPS stimulates the expression of LPS-binding protein in human oral keratinocytes *in vitro*. *Innate Immunity.* (2013) 19:66–75. doi: 10.1177/1753425912450348
137. Liu R, Desta T, Raptis M, Darveau RP, Graves DT. P. *gingivalis* and *E. coli* lipopolysaccharides exhibit different systemic but similar local induction of inflammatory markers. *J Periodontol.* (2008) 79:1241–7. doi: 10.1902/jop.2008.070575
138. Arroyave-Ospina JC, Wu Z, Geng Y, Moshage H. Role of oxidative stress in the pathogenesis of non-alcoholic fatty liver disease: implications for prevention and therapy. *Antioxidants.* (2021) 10:174. doi: 10.3390/antiox10020174
139. Le Sage F, Meilhac O, Gonthier MP. *Porphyromonas gingivalis* lipopolysaccharide induces pro-inflammatory adipokine secretion and oxidative stress by regulating toll-like receptor-mediated signaling pathways and redox enzymes in adipocytes. *Mol Cell Endocrinol.* (2017) 446:102–10. doi: 10.1016/j.mce.2017.02.022
140. Guo Y, Nguyen KA, Potempa J. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontology.* (2010) 54:15–44. doi: 10.1111/j.1600-0757.2010.00377.x
141. Bostanci N, Belibasakis GN. Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen. *FEMS Microbiol Lett.* (2012) 333:1–9. doi: 10.1111/j.1574-6968.2012.02579.x
142. Grenier D, Tanabe S. Porphyromonas gingivalis gingipains trigger a proinflammatory response in human monocyte-derived macrophages through the p38α mitogen-activated protein kinase signal transduction pathway. *Toxins.* (2010) 2:341–52. doi: 10.3390/toxins2030341
143. Makkawi H, Hoch S, Burns E, Hosur K, Hajishengallis G, Kirschning CJ, et al. *Porphyromonas gingivalis* stimulates TLR2-PI3K signaling to escape immune clearance and induce bone resorption independently of MyD88. *Front Cell Infect Microbiol.* (2017) 7:359. doi: 10.3389/fcimb.2017.00359
144. Maisetta G, Brancatisano FL, Esin S, Campa M, Batoni G. Gingipains produced by *Porphyromonas gingivalis* ATCC49417 degrade human-β-defensin 3 and affect peptide's antibacterial activity *in vitro*. *Peptides.* (2011) 32:1073–7. doi: 10.1016/j.peptides.2011.02.003
145. Wilensky A, Tzach-Nahman R, Potempa J, Shapira L, Nussbaum G. *Porphyromonas gingivalis* gingipains selectively reduce CD14 expression, leading to macrophage hyporesponsiveness to bacterial infection. *J Innate Immunity.* (2015) 7:127–35. doi: 10.1159/000365970
146. Wang M, Krauss JL, Domon H, Hosur KB, Liang S, Magotti P, et al. Microbial hijacking of complement-toll-like receptor crosstalk. *Sci Signal.* (2010) 3:ra11. doi: 10.1126/scisignal.2000697
147. Khalaf H, Bengtsson T. Altered T-cell responses by the periodontal pathogen *Porphyromonas gingivalis*. *PLoS ONE.* (2012) 7:e45192. doi: 10.1371/journal.pone.0045192
148. Park YD, Kim YS, Jung YM, Lee SI, Lee YM, Bang JB, et al. *Porphyromonas gingivalis* lipopolysaccharide regulates interleukin (IL)-17 and IL-23 expression via SIRT1 modulation in human periodontal ligament cells. *Cytokine.* (2012) 60:284–93. doi: 10.1016/j.cyto.2012.05.021
149. Yun LW, Decarlo AA, Hunter N. Blockade of protease-activated receptors on T cells correlates with altered proteolysis of CD27 by gingipains of *Porphyromonas gingivalis*. *Clin Exp Immunol.* (2007) 150:217–29. doi: 10.1111/j.1365-2249.2007.03488.x
150. Fabbrini E, Cella M, McCartney SA, Fuchs A, Abumrad NA, Pietka TA, et al. Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. *Gastroenterology.* (2013) 145:366–74.e743. doi: 10.1053/j.gastro.2013.04.010

151. Rolla S, Alchera E, Imarisio C, Bardina V, Valente G, Cappello P, et al. The balance between IL-17 and IL-22 produced by liver-infiltrating T-helper cells critically controls NASH development in mice. *Clin Sci.* (2016) 130:193–203. doi: 10.1042/CS20150405
152. Van Herck MA, Weyler J, Kwanten WJ, Dirinck EL, De Winter BY, Francque SM, et al. The differential roles of t cells in non-alcoholic fatty liver disease and obesity. *Front Immunol.* (2019) 10:82. doi: 10.3389/fimmu.2019.00082
153. Tang Y, Bian Z, Zhao L, Liu Y, Liang S, Wang Q, et al. Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease. *Clin Exp Immunol.* (2011) 166:281–90. doi: 10.1111/j.1365-2249.2011.04471.x
154. Nakayama M, Ohara N. Molecular mechanisms of *Porphyromonas gingivalis*-host cell interaction on periodontal diseases. *Jpn Dent Sci Rev.* (2017) 53:134–40. doi: 10.1016/j.jdsr.2017.06.001
155. Ishikawa M, Yoshida K, Okamura H, Ochiai K, Takamura H, Fujiwara N, et al. Oral *Porphyromonas gingivalis* translocates to the liver and regulates hepatic glycogen synthesis through the Akt/GSK-3β signaling pathway. *Biochim Biophys Acta.* (2013) 1832:2035–43. doi: 10.1016/j.bbadis.2013.07.012
156. Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. *Sci Am.* (1978) 238:86–95. doi: 10.1038/scientificamerican0178-86
157. Yang L, Liu Y, Wu H, Song Z, Høiby N, Molin S, et al. Combating biofilms. *FEMS Immunol Med Microbiol.* (2012) 65:146–57. doi: 10.1111/j.1574-695X.2011.00858.x
158. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobiol Resist Infect Control.* (2019) 8:76. doi: 10.1186/s13756-019-0533-3
159. Kuboniwa M, Tribble GD, Hendrickson EL, Amano A, Lamont RJ, Hackett M. Insights into the virulence of oral biofilms: discoveries from proteomics. *Exp Rev Proteomics.* (2012) 9:311–23. doi: 10.1586/epr.12.16
160. Sakanaka A, Takeuchi H, Kuboniwa M, Amano A. Dual lifestyle of *Porphyromonas gingivalis* in biofilm and gingival cells. *Microbiol Pathog.* (2016) 94:42–7. doi: 10.1016/j.micpath.2015.10.003
161. Vestby LK, Grønseth T, Simm R, Nesse LL. Bacterial biofilm and its role in the pathogenesis of disease. *Antibiotics.* (2020) 9:59. doi: 10.3390/antibiotics9020059
162. Wang W, Chanda W, Zhong M. The relationship between biofilm and outer membrane vesicles: a novel therapy overview. *FEMS Microbiol Lett.* (2015) 362:fnv117. doi: 10.1093/femsle/fnv117
163. Pérez-Cruz C, Delgado L, López-Iglesias C, Mercade E. Outer-inner membrane vesicles naturally secreted by gram-negative pathogenic bacteria. *PLoS ONE.* (2015) 10:e0116896. doi: 10.1371/journal.pone.0116896
164. Williams GD, Holt SC. Characteristics of the outer membrane of selected oral bacteroides species. *Can J Microbiol.* (1985) 31:238–50. doi: 10.1139/m85-046
165. Nakao R, Takashiba S, Kosono S, Yoshida M, Watanabe H, Ohnishi M, et al. Effect of *Porphyromonas gingivalis* outer membrane vesicles on gingipain-mediated detachment of cultured oral epithelial cells and immune responses. *Microbes Infect.* (2014) 16:6–16. doi: 10.1016/j.micinf.2013.10.005
166. Bai D, Nakao R, Ito A, Uematsu H, Senpuku H. Immunoreactive antigens recognized in serum samples from mice intranasally immunized with *Porphyromonas gingivalis* outer membrane vesicles. *Pathog Dis.* (2015) 73:ftu006. doi: 10.1093/femspd/ftu006
167. Ho MH, Chen CH, Goodwin JS, Wang BY, Xie H. Functional advantages of Porphyromonas gingivalis vesicles. *PLoS ONE.* (2015) 10:e0123448. doi: 10.1371/journal.pone.0123448
168. Gui MJ, Dashper SG, Slakeski N, Chen YY, Reynolds EC. Spheres of influence: *Porphyromonas gingivalis* outer membrane vesicles. *Mol Oral Microbiol.* (2016) 31:365–78. doi: 10.1111/omi.12134
169. Zhang Z, Liu D, Liu S, Zhang S, Pan Y. The role of *Porphyromonas gingivalis* outer membrane vesicles in periodontal disease and related systemic diseases. *Front Cell Infect Microbiol.* (2021) 10:585917. doi: 10.3389/fcimb.2020.585917
170. Seyama M, Yoshida K, Yoshida K, Fujiwara N, Ono K, Eguchi T, et al. Outer membrane vesicles of *Porphyromonas gingivalis* attenuate insulin sensitivity by delivering gingipains to the liver. *Biochim Biophys Acta Mol Basis Dis.* (2020) 1866:165731. doi: 10.1016/j.bbadis.2020.165731
171. Rajaiah R, Moudgil KD. Heat-shock proteins can promote as well as regulate autoimmunity. *Autoimmun Rev.* (2009) 8:388–93. doi: 10.1016/j.autrev.2008.12.004
172. Han P, Sun D, Yang J. Interaction between periodontitis and liver diseases. *Biomed Rep.* (2016) 5:267–76. doi: 10.3892/br.2016.718
173. Sasaki N, Katagiri S, Komazaki R, Watanabe K, Maekawa S, Shiba T, et al. Endotoxemia by *Porphyromonas gingivalis* injection aggravates non-alcoholic fatty liver disease, disrupts glucose/lipid metabolism, and alters gut microbiota in mice. *Front Microbiol.* (2018) 9:2470. doi: 10.3389/fmicb.2018.02470
174. Arimatsu K, Yamada H, Miyazawa H, Minagawa T, Nakajima M, Ryder MI., et al. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep.* (2014) 4:4828. doi: 10.1038/srep04828
175. Lourenço T, Spencer SJ, Alm EJ, Colombo A. Defining the gut microbiota in individuals with periodontal diseases: an exploratory study. *J Oral Microbiol.* (2018) 10:1487741. doi: 10.1080/20002297.2018.1487741
176. Qin N, Yang F, Li A, Prifti E, Chen Y, Shao L, et al. Alterations of the human gut microbiome in liver cirrhosis. *Nature.* (2014) 513:59–64. doi: 10.1038/nature13568
177. Rodriguez Herrero E, Boon N, Pauwels M, Bernaerts K, Slomka V, Quirynen M, et al. Necrotrophic growth of periodontopathogens is a novel virulence factor in oral biofilms. *Sci Rep.* (2017) 7:1107. doi: 10.1038/s41598-017-01239-9
178. Komazaki R, Katagiri S, Takahashi H, Maekawa S, Shiba T, Takeuchi Y, et al. Periodontal pathogenic bacteria, *Aggregatibacter actinomycete*mcomitans affect non-alcoholic fatty liver disease by altering gut microbiota and glucose metabolism. *Sci Rep*. (2017) 7:13950. doi: 10.1038/s41598-017-14260-9
179. Olsen I, Yamazaki K. Can oral bacteria affect the microbiome of the gut?. *J Oral Microbiol.* (2019) 11:1586422. doi: 10.1080/20002297.2019.1586422
180. Chen HT, Huang HL, Li YQ, Xu HM, Zhou YJ. Therapeutic advances in non-alcoholic fatty liver disease: a microbiota-centered view. *World J Gastroenterol.* (2020) 26:1901–11. doi: 10.3748/wjg.v26.i16.1901
181. Shekhar S, Petersen FC. The dark side of antibiotics: adverse effects on the infant immune defense against infection. *Front Pediatr.* (2020) 8:544460. doi: 10.3389/fped.2020.544460
182. Van Pijkeren JP, Neoh KM, Sirias D, Findley AS, Britton RA. Exploring optimization parameters to increase ssDNA recombineering in *Lactococcus lactis* and *Lactobacillus reuteri*. *Bioengineered.* (2012) 3:209–17. doi: 10.4161/bioe.21049
183. Yang KM, Kim JS, Kim HS, et al. Lactobacillus reuteri AN417 cell-free culture supernatant as a novel antibacterial agent targeting oral pathogenic bacteria. *Sci Rep.* (2021) 11:1631. doi: 10.1038/s41598-020-80921-x
184. Murakami Y, Kawata A, Ito S, Katayama T, Fujisawa S. The radical scavenging activity and cytotoxicity of resveratrol, orcinol and 4-allylphenol and their inhibitory effects on cox-2 gene expression and Nf-κb activation in RAW264.7 cells stimulated with *Porphyromonas gingivalis*-FIMBRIAE. *In Vivo.* (2015) 29:341–349.
185. Choi GE, Hyun KY. Inhibitory effect of acer tegmentosum maxim extracts on *P. gingivalis* LPS-induced periodontitis. *Arch Oral Biol.* (2020) 109:104529. doi: 10.1016/j.archoralbio.2019.104529
186. Kariu T, Nakao R, Ikeda T, Nakashima K, Potempa J, Imamura T. Inhibition of gingipains and *Porphyromonas gingivalis* growth and biofilm formation by prenyl flavonoids. *J Periodontal Res.* (2017) 52:89–96. doi: 10.1111/jre.12372
187. Wang T, Takikawa Y, Satoh T, Yoshioka Y, Kosaka K, Tatemichi Y, et al. Carnosic acid prevents obesity and hepatic steatosis in ob/ob mice. *Hepatol Res.* (2011) 41:87–92. doi: 10.1111/j.1872-034X.2010.00747.x
188. Wang T, Takikawa Y, Tabuchi T, Satoh T, Kosaka K, Suzuki K. Carnosic acid (CA) prevents lipid accumulation in hepatocytes through the EGFR/MAPK pathway. *J Gastroenterol.* (2012) 47:805–13. doi: 10.1007/s00535-012-0546-7
189. Wang T, Takikawa Y. Carnosic acid protects normal mouse hepatocytes against H2 O2 -induced cytotoxicity via sirtuin 1-mediated signaling. *Hepatol Res.* (2016) 46:239–46. doi: 10.1111/hepr.12563
190. Del Campo J, Amiot MJ, Nguyen-The C. Antimicrobial effect of rosemary extracts. *J Food Prot.* (2000) 63:1359–68. doi: 10.4315/0362-028X-63.10.1359
191. Bernardes WA, Lucarini R, Tozatti MG, Souza MG, Silva ML, Filho AA, et al. Antimicrobial activity of *Rosmarinus officinalis* against oral pathogens:

relevance of carnosic acid and carnosol. *Chem Biodivers.* (2010) 7:1835–40. doi: 10.1002/cbdv.200900301
192. Tsukatani T, Sakata F, Kuroda R, Akao T. Biofilm eradication activity of herb and spice extracts alone and in combination against oral and food-borne pathogenic bacteria. *Curr Microbiol.* (2020) 77:2486–95. doi: 10.1007/s00284-020-02017-z
193. Xiang Q, Liu Z, Wang Y, Xiao H, Wu W, Xiao C, et al. Carnosic acid attenuates lipopolysaccharide-induced liver injury in rats via fortifying cellular antioxidant defense system. *Food Chem Toxicol.* (2013) 53:1–9. doi: 10.1016/j.fct.2012.11.001
194. J Jeong SH, Nam Y, Jung H, Kim J, Rim YA, Park N, et al. Interrupting oral infection of Porphyromonas gingivalis with anti-FimA antibody attenuates bacterial dissemination to the arthritic joint and improves experimental arthritis. *Exp Mol Med*. (2018) 50:e460. doi: 10.1038/emm.2017.301
195. Dominy SS, Lynch C, Ermini F, Benedyk M, Marczyk A, Konradi A, et al. *Porphyromonas gingivalis* in Alzheimer's disease brains: evidence for disease causation and treatment with small-molecule inhibitors. *Sci Adv.* (2019) 5:eaau3333. doi: 10.1126/sciadv.aau3333
196. Peng HY, Chen SY, Siao SH, Chang JT, Xue TY, Lee YH, et al. Targeting a cysteine protease from a pathobiont alleviates experimental arthritis. *Arthritis Res Ther.* (2020) 22:114. doi: 10.1186/s13075-020-02205-z
197. Cai J, Chen J, Guo H, Pan Y, Zhang Y, Zhao W, et al. Recombinant fimbriae protein of *Porphyromonas gingivalis* induces an inflammatory response via the TLR4/NF-κB signaling pathway in human peripheral blood mononuclear cells. *Int J Mol Med.* (2019) 43:1430–40. doi: 10.3892/ijmm.2019.4069
198. Pérez-Montes de Oca A, Julián MT, Ramos A, Puig-Domingo M, Alonso N. Microbiota, fiber, and NAFLD: is there any connection?. *Nutrients.* (2020) 12:3100. doi: 10.3390/nu12103100
199. Mu Q, Tavella VJ, Luo XM. Role of *Lactobacillus reuteri* in human health and diseases. *Front Microbiol.* (2018) 9:757. doi: 10.3389/fmicb.2018.00757
200. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci USA*. (2013) 110:9066–71. doi: 10.1073/pnas.1219451110
201. Ottman N, Reunanen J, Meijerink M, Pietilä TE, Kainulainen V, Klievink J, et al. Pili-like proteins of *Akkermansia muciniphila* modulate host immune responses and gut barrier function. *PLoS ONE.* (2017) 12:e0173004. doi: 10.1371/journal.pone.0173004
202. Depommier C, Everard A, Druart C, Plovier H, Van Hul M, Vieira-Silva S, et al. Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat Med.* (2019) 25:1096–03. doi: 10.1038/s41591-019-0495-2

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.

frontiers | Frontiers in Medicine

ORIGINAL RESEARCH
published: 19 July 2022
doi: 10.3389/fmed.2022.788992

The Interplay Between Use of Biological Therapies, Psychological State, and the Microbiome in IBD

Paris Tavakoli[1], Ute Vollmer-Conna[2], Dusan Hadzi-Pavlovic[2], Xabier Vázquez-Campos[3] and Michael Carl Grimm[1]*

[1] St. George and Sutherland Clinical School, University of New South Wales, Sydney, NSW, Australia, [2] School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia, [3] School of Biotechnology and Biomolecular Sciences, Faculty of Science, University of New South Wales, Sydney, NSW, Australia

Background: This study examines longitudinal bio-psychological dynamics and their interplay in IBD patients undergoing conventional and biological therapies.

Methods: Fifty IBD participants (24 UC, 26 CD) in clinical remission were followed for 12 months. Complete longitudinal datasets, biological samples, validated scores of psychological status were collected monthly for analysis of association. Microbiome analysis was performed to identify microbial dynamics and signatures. Patients were grouped on disease phenotype (CD, UC) and mode of treatment (biological therapies, non-biological treatment). General linear models, mixed models, cluster analysis, and analyses of variance were used to examine the longitudinal trends of the variables and their associations over time. Results were corrected for multiple testing.

Results: Results substantiated different interactions between biological therapy and longitudinal trends of inflammatory biomarkers in remission CD and UC patients as well as significant differences between CD and UC patients in their psychological measures during clinical remission, with UC patients having inferior condition compared to CD. A significant reduction in microbial diversity in CD patients compared to UC was identified. Results characterized considerable differences in longitudinal microbial profile between those taking and not taking biological treatment in UC patients, but not in CD patients.

Conclusion: A different trajectory of interdependence was identified between psychological state, sleep, and microbial dynamics with mode of treatment when compared between CD and UC patients. Further studies should investigate the causal relationships between bio-psychological factors for improved treatment purposes.

Keywords: Crohn's disease, ulcerative colitis, inflammatory bowel disease, gut microbiome, psychological state

OPEN ACCESS

Edited by:
Javier Ochoa-Repáraz, Eastern Washington University, United States

Reviewed by:
Rinaldo Pellicano, Molinette Hospital, Italy
Abbas Yadegar, Shahid Beheshti University of Medical Sciences, Iran

****Correspondence:***
Paris Tavakoli
paris858@gmail.com

Specialty section:
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

Received: *04 October 2021*
Accepted: *06 June 2022*
Published: *19 July 2022*

Citation:
Tavakoli P, Vollmer-Conna U, Hadzi-Pavlovic D, Vázquez-Campos X and Grimm MC (2022) The Interplay Between Use of Biological Therapies, Psychological State, and the Microbiome in IBD. Front. Med. 9:788992. doi: 10.3389/fmed.2022.788992

INTRODUCTION

Study of chronic inflammatory diseases such as inflammatory bowel diseases (IBDs), generates numerous challenges including assessing inflammatory pathways that might be common between different chronic inflammatory disorders with either shared or disease-specific mechanisms. One approach is to examine the relationship between major contributing

factors over time to identify key drivers and their interplay. Several chronic conditions including IBDs have arisen and increased in incidence during the past century points to powerful environmental influences, perhaps as a product of industrialization and modernization (1, 2). The more recent exploration of IBD genetics has shown genetic variances selectively associated with IBD (3, 4), but also made clear that no single or combination of genetic variation can fully explain Crohn's disease (CD) or ulcerative colitis (UC) (5, 6). Since the early study of the IBDs, immunological mechanisms were the dominant area of research. Further, microbiome and genetic studies indeed supported the role of immune cells (mainly type 1, type 2 and type 17 T cells) and cytokines (7). These findings were incentives to tackle and block principal mediators in immune responses with the therapeutic aim to control inflammation and potentially alter the natural history of disease. It has long been proposed that gut bacteria play an important role in the pathogenesis of IBD through their direct interaction with the intestinal mucosa. IBDs are characterized by immune dysregulation in genetically susceptible patients and it seems that gut microbiota are the target of this inapt immune response either due to loss of tolerance toward commensal bacteria or secondary to an altered microbial diversity and/or function (8, 9). Many subsequent studies present convincing evidence confirming the involvement of the enteric bacteria in pathogenesis of IBD. A range of bacteria is stated to have aggressive or protective functions in intestinal inflammatory disorders such as Crohn's disease; for example, phlogistic effects of adherent-invasive *Escherichia coli* (10) and protective effects of *Faecalibacterium prausnitzii* (11). To examine the integrated impact of gut microbiota in the pathogenesis of IBDs, it is important to incorporate microbiome data with other data related to immune modulation, genetics, psychological and physiological factors.

IBDs are chronic debilitating disorders which may affect many aspects of the sufferer's life. They can add to the psychological burden including high levels of perceived stress (12), negative mood and depression (13), and anxiety compared to the healthy population (14). The prevalence estimate of both depression and anxiety were higher in IBD patients—even among patients in remission—than in the general population (15–17).

These many factors suggest that disease mechanisms in IBDs are multifaceted and gut inflammation is the product of complex pathways in addition to known immune response types, notwithstanding the direction of newer targeted therapies (6, 18). Longitudinal assessments of biological and psychological factors and understanding their temporal trajectories in the course of the disease are essential to clarifying vulnerabilities and individual differences in IBD patients. Previous published studies were limited in the number of risk factors examined or they lacked time series analysis of such disease contributing factors (17, 19–21), therefore this study has been designed to examine how multiple contributing factors and their interrelationships have influenced the disease's course over time and how different interactions are represented in IBD patients who received biological treatment compared to those on conventional treatment.

Abbreviations: CD, Crohn's disease; CRP, C-reactive protein; DASS, Depression Anxiety Stress Scale; DMI, depression in medically ill; DS-NA, Type D personality scale of negative affectivity; DS-SI, Type D personality scale of social inhibition; FC, Fecal calprotectin; IBD, Inflammatory Bowel Disease; PSQI, Pittsburgh sleep quality index.

METHODS

Cohort Demographics

Patients with confirmed diagnoses of IBD who met all the inclusion criteria and none of the exclusion criteria (see **Supplementary Information**) were recruited from gastroenterology departments, IBD clinics and endoscopy units based at two tertiary referral hospitals in Sydney, Australia, between Oct 2015 and August 2017. Study participants were in complete clinical remission based on their disease activity indices; partial Mayo score for UC <2 (22), Crohn's disease activity index (CDAI) for CD <150 (23) and/or Harvey Bradshaw Index for CD <5 (23–25) confirmed by their gastroenterologists, and supported by endoscopic and histological results, if available. Baseline data were collected, and longitudinal data accumulated monthly. Data comprised scores related to symptoms of psychological state including perceived stress (PSQ) (26, 27), depression- anxiety and stress (DASS) (28, 29), depression in medically ill (DMI) (30, 31), personality characteristics, i.e., negative affectivity (NA) and social inhibition (SI) traits (32), wellbeing scores (33–35) and sleep quality (PSQI) (36) with clinical course and disease activity as a measure of outcome. To build the outcome, all variables including severe disease symptom/s and flare events were considered through formalized follow up assessments by study investigators. We studied the longitudinal dynamics of multiple contributing factors to disease activity from a cohort of 50 IBD patients (CD, $n = 26$; UC, $n = 24$; **Tables 1**, **2**). IBD participants were grouped in disease types (CD or UC) and subdivided based on use or not of biological treatments. Monthly blood and stool samples were collected for assessment of serum C-reactive protein (CRP), fecal calprotectin (FC) levels and microbiome dynamics. The microbiome composition in each sample was determined by sequencing the V4 region of the 16S rRNA gene and a total of 3.3 million contigs were retained after quality control and subsampling. To determine the links between the gut microbiome and clinical components, we collected clinical data including inflammatory biomarkers, clinical indices for disease activity, and self-administered validated questionnaires to quantify psychological state on a monthly basis for a period of 12 months while participants still met the inclusion criteria for the study. Data related to routine medications, use of antibiotics and probiotics and disease activity scores were registered at monthly follow up visits. The end point was at 12 months follow-up or at confirmation of the onset of relapse; in the latter case the last assessment reflected the state at the time of disease relapse. The study was approved by the Human Research Ethics Committee of South Eastern Sydney Local Health District (Ref: 15/094 HREC/15/POWH 245−20 Aug).

Sample Collection

Fecal samples were self-collected by participants and were aliquoted and stored at −80°C together with original collection pots for DNA extraction and quantifying of FC. The concentration of FC was assessed by commercially available enzyme-linked immunosorbent assay (ELISA; Calprotectin Elisa Buhlmann Laboratories, S100A8 and S100A9), according to the manufacturer's protocol. Twenty ml peripheral blood samples were drawn and centrifuged (at 2,000 g for 15 min). Serum samples were aliquoted and stored at −80°C to be used for CRP quantitation by high sensitivity ELISA (37). For microbiome assessment, stool samples were taken from collections at months 1, 4, 8, and 12; where there were any missing samples, the sample from the preceding available month was used.

DNA Extraction and Amplification

Genomic DNA was extracted from 0.11 to 0.12 g of fecal material of each sample using the Allprep Power Viral DNA/RNA Kit (Qiagen) DNA extraction protocol. Briefly, the concentration of the extracted DNAs was quantified by Nanodrop mass spectrophotometry (38) and Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's instructions before dilution to 20 ng/μl. For DNA concentrations of <5 ng/μl (39 extractions), SYBR-Green-based qPCR assay was performed to quantify the absolute amount of a target sequence, to compare relative amounts of a target sequence between samples and to analyse whether they were amplifiable (**Supplementary Figure 1**; **Supplementary Table 1** qPCR plot and table). DNA was submitted for sequencing at the Ramaciotti Center for Genomics (Australia).

TABLE 1 | Participants' distribution in each group of diagnosis and treatment options.

IBD phenotypes	*F*	*M*	On-biologic therapy	Non-biologic therapy	Total
CD	12	14	10	16	26
UC	9	15	5	19	24
Total	21	29	15	35	**50**

An IBD cohort of 50 (CD, n = 26; UC, n = 24) were enrolled and followed up for the period of 12 months. One UC participant withdrew after 2 months of follow up.

Library Preparation

Barcoding PCR for bacterial and archaeal 16S rRNA genes was carried out using a mix of 10 μL of HotMasterMix (5 PRIME), 0.2 μM of each primer and 1 μL of DNA template. Barcoded PCR primers based on 515F, and 806R (39). Reactions were kept at 94°C for 3 min for denaturing, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min and elongation at 72°C for 1 min 30s, ending with a final elongation at 72°C for 10 min and final hold at 4°C. All PCRs were carried out in 25 μL volumes. PCR concentrations were normalized and pooled using SequalPrep™ Normalization Plate Kit (ThermoFisher) according to the manufacturer's instructions. The library was purified using Axygen® AxyPrep™ Mag PCR Clean-Up Kit (Fisher Biotec) as per the manufacturer's instructions. Concentration and quality of the pooled library were checked with Qubit® and the library size on an Agilent 2,200 TapeStation instrument.

Community Analysis

Raw sequencing data were processed with the OTUreporter pipeline (40), based on mothur v1.39.5 (41) and according to the MiSeq SOP. Samples with a length between 228 and 278 bp were retained and those with homopolymers longer than 8 bp were removed. Sequences were grouped into OTUs based on 97% similarity using the OptiClust algorithm. From each patient, quarterly microbiome samples with matched FC concentrations were sub-selected for use in downstream analysis.

Statistical Analysis

Following the power analysis estimation (80%) to detect significant (two sided p-values $\leq$ 0.05) correlations (42) cluster analysis was used to examine whether categories of respondents (IBD patients) share common characteristics within

TABLE 2 | Sample distribution in IBD groups.

IBD phenotypes	Number of samples—Blood	Number of serum samples/on-biologic therapy	Number of serum samples/non-biologic therapy	Number of samples—Stool	Number of stool samples included in microbial analysis	Number of stool samples/on-biologic therapy	Number of stool samples/non-biologic therapy
CD	273	104*	169	273	98**	30*	68
UC	235	53	182	235	85***	19	66
Total	508	157	351	508	183****	49	134

*One hundred eighty-eight samples from 50 IBD patients were included in the analysis. An additional 5 samples were removed from calculation at the sub-sampling level after sequencing and filtering as they did not have a minimum of 10,000 sequences. *One CD participant was on biologic on 1st month and on non-biologic therapy for the rest of assessment period, so added to 508 total samples in both groups of treatment for rest of assessment period. Samples from this participant were 1 (bio) + 10 (non-bio). **3 CD stool samples did not qualify for microbiome results and were excluded (only 98 stool samples were included in the results therefore the numbers are 98 instead of 101 in this group). ***2 UC stool samples did not qualify for microbiome results and were excluded (only 85 stool samples were included in the results instead of 87). ****5 stool samples (3 CD and 2 UC) were removed as non-qualified therefore the total was 183 (49 + 134) instead of 188.*

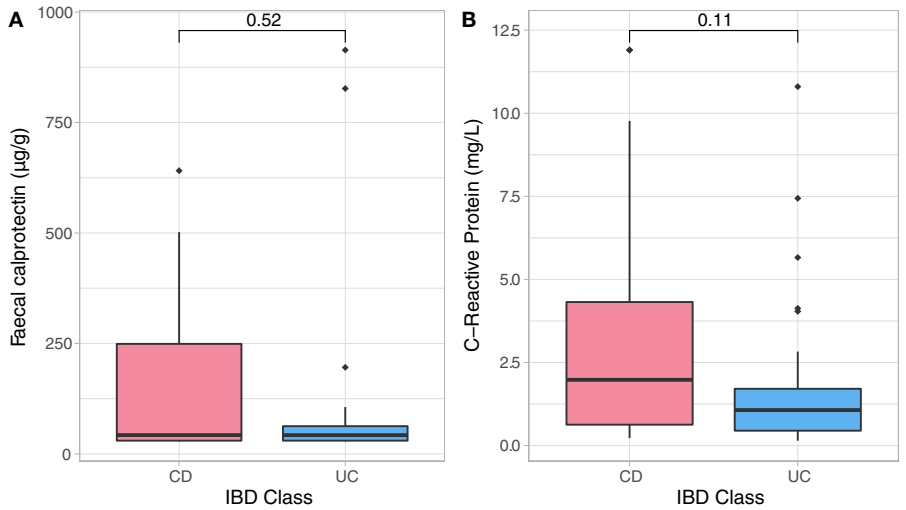


FIGURE 1 | Non-parametric analysis quantifying F-calprotectin **(A)** and C-reactive protein (CRP) **(B)** at baseline and across diagnosis. Analysis did not show significant differences in measures of both CRP and FC in CD and UC participants at baseline assessment.

clusters at the baseline and maintain the same properties over time (43). Utilizing SPSS 25 (SPSS Inc), general linear models, non-parametric methods, time series analysis and mixed model analysis were used to examine longitudinal data related to psychological and biological measures, their linear/quadratic trends and differences between two groups of treatment options over time (44). Furthermore, a regression analysis using panel data was conducted applying Stata (Stata V16; Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC. 2019) to apply corrections for multiple analysis. Microbial profile analysis was carried out using the phyloseq (45) and microbiome (https://github.com/microbiome/microbiome) R packages to import and graph data, and vegan was used to perform differential abundance testing (46–48). Wilcoxon signed-rank test, and Permutational multivariate analysis of variance (PERMANOVA) test, were applied to examine statistical significance of differences in some bacteria abundances at family level and to evaluate compositional differences between the CD and UC groups, and within treatment modalities (49, 50). Bray Curtis dissimilarity matrices were used as input for PERMANOVA to evaluate compositional differences between the CD and UC and treatment modalities. Analysis of Variance (ANOVA) was used onto beta dispersion test output to validate significant result obtained by PERMANOVA. Community and species diversity were estimated using the Shannon diversity index (51) while species richness estimates were generated using Chao1 (52). Pielou's evenness index was used to examine species evenness (53). Categorical variables were used in subgroup analysis (alpha diversity, abundance testing with LEfSe) (54) (detailed information on statistical methods can be found in **Supplementary Table 2**).

RESULTS

Study participants from different ethnic groups and diverse cultural backgrounds together provided 508 stool and 508 blood samples during the assessment period. To control for sampling bias, we restricted our microbial and statistical analysis of volatility to a subset of the cohort that had sequence data from quarterly time points with matched FC concentration which yielded 188 samples from 50 IBD patients (an additional 5 samples were removed from calculation at the sub-sampling level after sequencing and filtering as they did not have a minimum of 10,000 sequences) (**Table 2**).

Baseline Assessment

The entry disease remission data related to bio-psychological state of all IBD patients were employed as a benchmark for assessment of their longitudinal dynamics. Sample analysis included 50 stool and 50 blood samples from CD ($n = 26$) and UC ($n = 24$) individuals who were in clinical remission. As expected, CD and UC participants had similar baseline distributions of inflammatory biomarkers (**Figure 1**) which were strongly and positively associated with one another, but none were significantly related to any of the psychological measures and sleep quality at study entry. There was an analogous baseline distribution for most psychological factors, while there were significant differences in baseline scores related to depression (DASS, Dep, $p = 0.026$) and negative affectivity (DS-NA, $p = 0.001$) between CD and UC patients at reference point with UC patients showing higher baseline depression and negative affectivity scores compared to CD patients (**Supplementary Table 2**). Baseline anxiety scores were

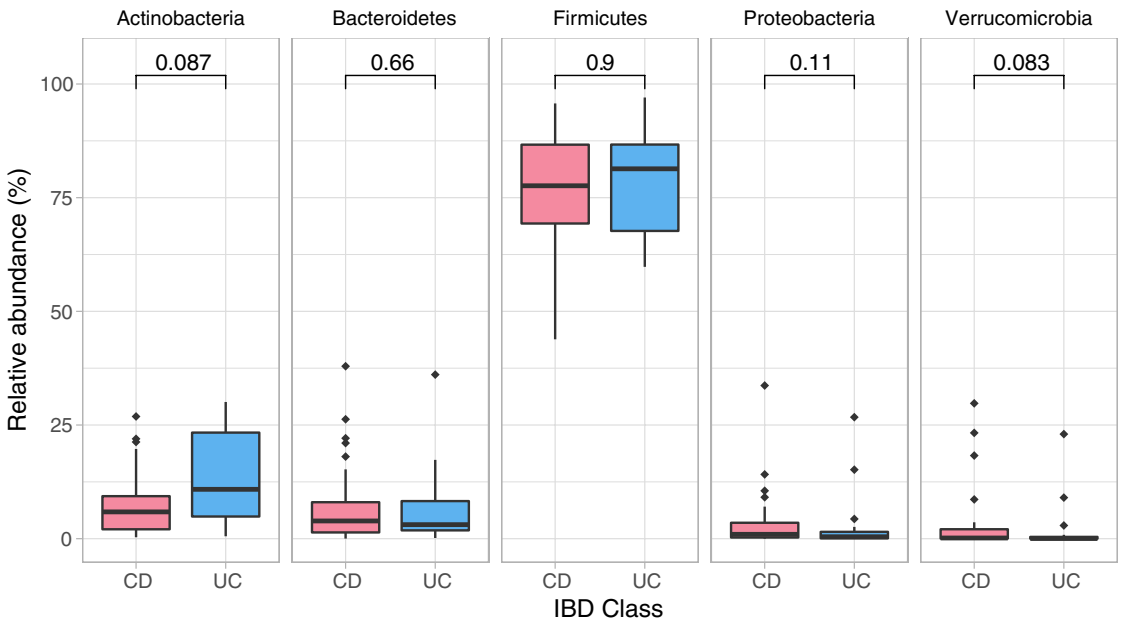


FIGURE 2 | Phylum composition in baseline analysis (CD, UC). Phylum composition relative abundance for baseline analysis in all CD, UC participants. Only the top 5 phyla are shown. Numbers indicate the *p*-value from the Wilcoxon tests.

TABLE 3 | Grouped summary IBD class: detailed comparison of the baseline phyla abundance in CD and UC participants.

IBD_ Class	Phylum	Mean	SD	*n*
CD	*Actinobacteria*	8.01	7.72	25
CD	*Bacteroidetes*	8.10	9.81	25
CD	*Firmicutes*	75.7	13.5	25
CD	*Proteobacteria*	4.22	7.35	24
CD	*Verrucomicrobia*	4.03	8.23	23
UC	*Actinobacteria*	12.3	9.59	24
UC	*Bacteroidetes*	6.24	7.92	24
UC	*Firmicutes*	77.4	11.8	24
UC	*Proteobacteria*	2.55	6.15	23
UC	*Verrucomicrobia*	2.25	5.78	17

Verrucomicrobia is absent in 2 CDs and 7 UCs.

TABLE 4 | Cluster membership based on similarities found within IBD participants.

Number of clusters			**Freq. of IBD patients in each cluster**
(A) Based on their baseline psychological state.			
1			9
2			22
3			18
Clusters			**Total**
(B) Based on gender, IBD disease phenotype and treatment modalities for consistency			
Gender	F	M	
1	5	4	9
2	9	13	22
3	7	11	18
IBD phenotype	UC	CD	
1	3	6	9
2	13	9	22
3	9	9	18
Treatment modes	On-biologic	Non-biologic	
1	8	1	9
2	12	10	22
3	15	3	18

significantly associated with sleep disturbances ($p < 0.001$, $r^2 = 0.52$) and baseline stress scores were strongly related to sleep duration ($p < 0.001$, $r^2 = 0.46$). Both outcomes suggest strong relationships between anxiety and stress, and sleep — **Supplementary Table 2**.

Treatment Modalities at Baseline

Both biologic and non-biologic treatment groups were similar in most baseline psychological scores with the exception of higher baseline stress scores ($p = 0.004$) and negative affectivity scores ($p = 0.004$) in non-biological group. At baseline, UC patients had higher mean psychological scores compared to CD patients in both treatment groups (except for DS-SI score which was higher in CD group who received biological treatment). These findings overall were not surprising given the mandated clinical remission at baseline—**Supplementary Table 2**.

Baseline Microbiome Assessment

Data showed no significant baseline differences in the Shannon index ($p = 0.67$), Pielou's evenness ($p = 0.80$) and Chao1 (p

= 0.429) between CD and UC patients. Comparison of the baseline phyla abundance is shown in **Figure 2**. Results showed a strong negative baseline relationship between sleep latency and Shannon index ($p = 0.001$, $r^2 = -0.425$), Pielou's evenness ($p = 0.002$, $r^2 = -0.401$) and Chao1 index ($p < 0.001$, $r^2 = -0.455$), indicating that lower intestinal microbial diversity, richness, and uneven microbial composition was associated with longer time to fall asleep.

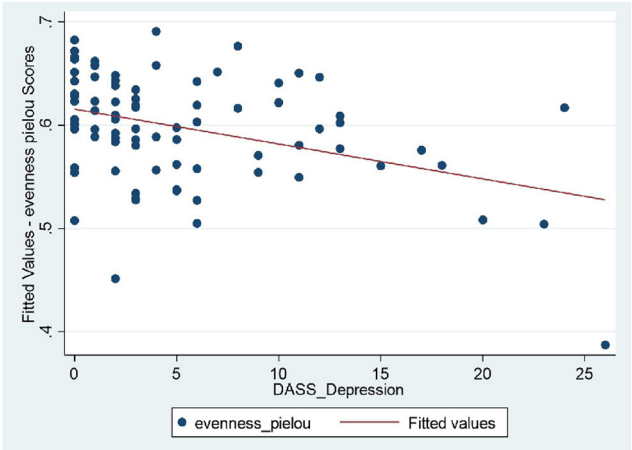


FIGURE 3 | UC patients: strong negative relationship between microbial evenness and depression scores ($p < 0.001$), meaning that lower microbial evenness was strongly associated with higher depression scores in UC cohort.

Detailed comparison of the baseline phyla abundance showed that *Verrucomicrobia* was present in 23/25 CD participants (4.0% ± 8.2), but only in 17/24 UC participants (2.3% ± 5.8). CD participants had more baseline abundance of some disease associated bacteria when compared to UC including *Bacteroidetes* (CD = 8.1% ± 9.8, UC = 6.2% ± 7.9) and *Proteobacteria* (CD = 4.2% ± 7.4, UC = 2.6% ± 6.2), but UC participants had more *Firmicutes* compared to CD at baseline assessment (CD = 75.7% ± 13.5, UC = 77.4% ± 11.8). Notwithstanding these data, none of the baseline differences were statistically significant between the CD and UC (Wilcoxon test, **Figure 2**; **Table 3**).

Longitudinal Assessment

We used cluster analysis to study the bio-psychological behavior of IBD participants over time. Here clusters were identified based on systematic relationships found in psycho-biological and microbiome dynamics over time and across all study participants. Three stable clusters were identified which persisted

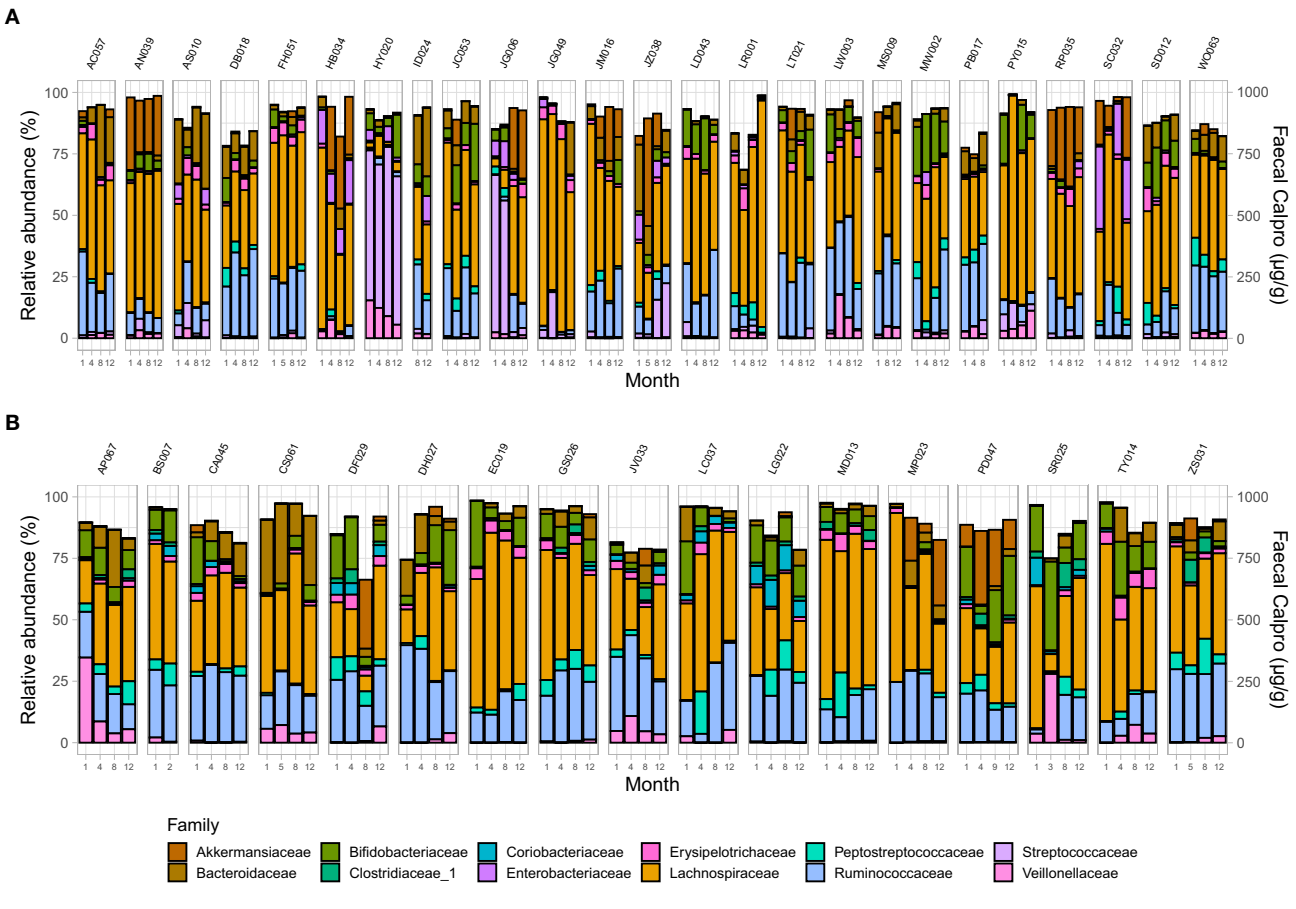


FIGURE 4 | Microbial community composition at family level in patients in remission. **(A)** CD, and **(B)** UC. Only families with a relative abundance ≥2% in any sample are shown.

in their baseline group categories during follow-up. One potential explanation is most participants remained in clinical remission during the 12 months follow up period (**Table 4A**). The first cluster (No 1) was the youngest IBD cluster which earned the highest anxiety, depression, and stress scores over time and revealed worst sleep quality sores, higher measures related to depression in medically ill state during follow up (**Supplementary Tables 3, 4**). This cluster also had lowest number of IBD patients on biological therapy. Second cluster (No. 2) is the oldest cluster with higher number of participants in this group (both male and female and mostly UC, **Table 4B**—detailed information on cluster analysis is included in **Supplementary Tables 20**, **21**).

Cluster Memberships

Longitudinal bio-psychological data of all remission IBD patients (CD, $n = 25$; UC, $n = 17$) were analyzed (460 blood and 460 stool samples) using mixed models. Results did not show significant shift in measures of inflammatory biomarkers within IBD cohort which was expected due to persisting remission. Results identified significant longitudinal coefficient of change in psychological scores (linear, quadratic or both; **Supplementary Table 2**) including negative affectivity showing greater magnitude of change in the remission UC group and sleep quality showing larger magnitude of change in the remission CD group. Microbial diversity and richness displayed larger magnitude of linear coefficient of change in the remission UC group. Further examination of complete study cohort including independent variables (psychobiological factors) with outcome variables (wellbeing scores and inflammatory biomarkers) was applied. In CD patients, results suggested a statistically significant negative relationship between wellbeing scores with depressive scores ($p = 0.018$) and positive relation with sleep quality ($p = 0.027$) over time. Outcomes did not suggest any significant interdependence between longitudinal psychological scores, sleep, mode of treatment and microbial indices in CD patients. In UC patients, longitudinal wellbeing scores retained a significant and positive relationship with sleep quality ($p = 0.040$), and a significant and unexpectedly negative relationship with stress (DASS stress, $p = 0.009$). FC had a positive and significant association with sleep quality (PSQI, $p = 0.006$) and a strongly negative relationship with stress (DASS stress, $p = 0.023$) (**Supplementary Table 17**). Assessment of longitudinal psycho-microbial dynamics in UC cohort did not suggest any significant interdependence between longitudinal psychological scores, sleep, and mode of treatment with microbial dynamics in UC patients except for a strong negative association between microbial diversity and depression (DASS depression, $p = 0.011$), and a negative association between microbial evenness and depression ($p < 0.001$, **Figure 3**) but not with microbial richness, **Supplementary Table 19**.

Longitudinal Microbiome Assessment

Analysis demonstrated a significant interplay between wellbeing scores with Shannon index and with Pielou's evenness but not with Chao1 (**Supplementary Table 7**). To examine microbial composition shift over time, microbial dynamics

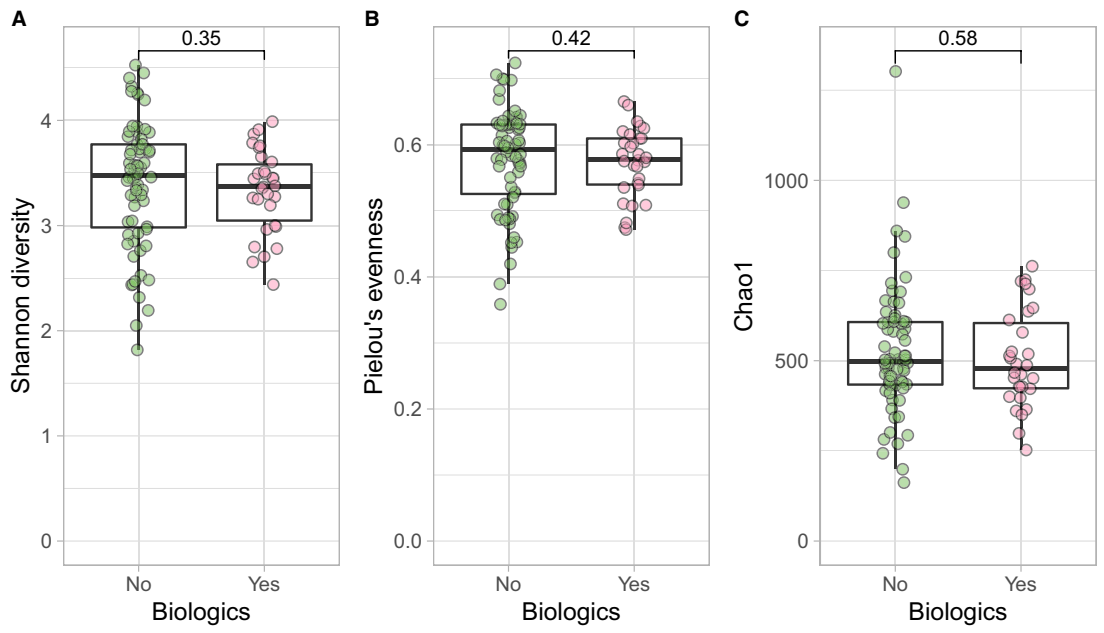


FIGURE 5 | CD participants- microbial α diversity and treatment modalities. Measures of microbial α diversity between treatment options in CD group (Comprising remission and relapse) including: **(A)** Shannon's index (diversity); **(B)** Pielou's evenness; and **(C)** Chao1 index (richness). Analysis did not show any significant differences between the two groups over time.

were explored in remission samples of IBD subtypes in addition to changes in FC concentration longitudinally. CD cohort in clinical remission showed greater microbiome fluctuations mainly by trading off different microbial families as *well* as in relative abundance of existing microbial profile (**Figures 4**, **5**).

Treatment Options

Longitudinal trends of psycho-biological factors and their interactions with modes of treatment (biological vs. no biological) were examined using mixed models of analysis. In CD and UC cohorts such factors were similarly distributed in both treatment groups over time (**Supplementary Table 2**). At the baseline there were similar ecological indices between the two treatment options in both disease classes which was also suggested by previous study (55) (Shannon, $p = 0.380$; Pielou's, $p = 0.246$; Chao1, $p = 0.934$), although the age effect was significant between the two groups for all three ecological indices (56) (information related to sample demographics for microbial analysis and output of PERMANOVA for both biologics and bio-flare variables in CD and UC cohorts are in **Supplementary Table 9**).

At the family level (**Figure 6A**), the CD group showed significant differences (Wilcoxon, $p \leq 0.05$) between the treatment received (biologics vs. non-biologic) in the abundance of *Barnesiellaceae*, *Bifidobacteriaceae*, unclassified *Clostridia*, and *Clostridiaceae* (**Figure 7**). Results showed similar ecological index values in samples from the two treatment options. Linear discriminant analysis (LDA) with LEfSe was performed to characterize the differences between two treatment options in CD group (both in remission and during relapse). A total of 12 microbial biomarkers (OTUs) characteristic of CD under biological treatment, and 22 in CD with non-biological treatments, were identified (**Figure 8**).

Samples in UC groups on biological and non-biologic treatment modes (**Figure 6B**) were found to have significantly different microbial communities based on Bray-Curtis dissimilarity (PERMANOVA, $p = 0.0031$). Microbial family composition in UC group and on two treatment modalities showed a total of 12 families that were significantly different (Wilcoxon, $p \leq 0.05$): *Acidaminococcaceae*, unclassified *Bacteroidales*, *Barnesiellaceae*, *Christensenellaceae*, *Clostridiales* vadinBB60 group, *Coriobacteriaceae*, *Defluvitaleaceae*, *Eggerthelaceae*, *Fusobacteriaceae*, *Muribaculaceae*, *Prevotellaceae*,

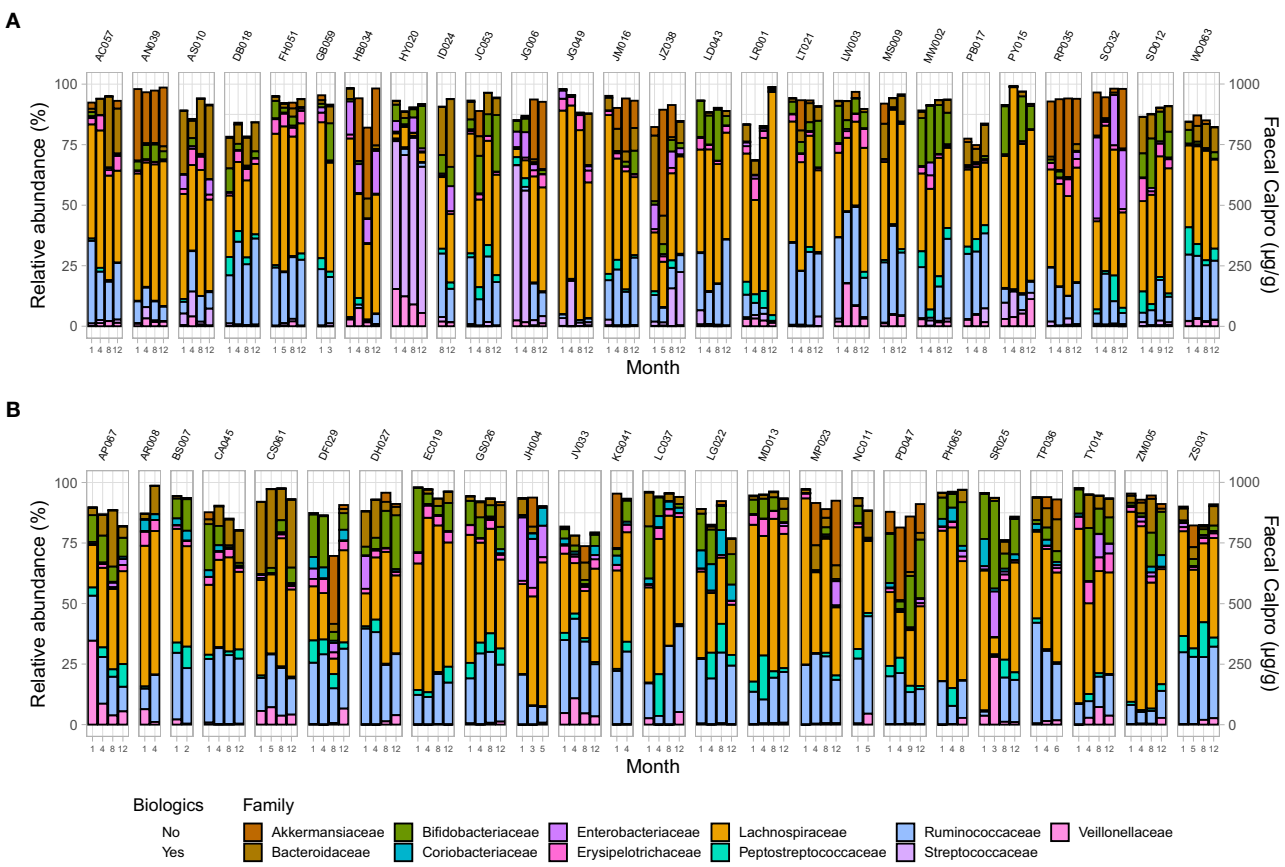


FIGURE 6 | Microbial community composition at family level in IBD patients across two treatment options including changes in FC concentration over time in **(A)** CD, and **(B)** UC. Only families with a relative abundance ≥2% in any sample are shown.

and *Streptococcaceae* (**Figure 9**). Results identified significant differences in microbial diversity (Shannon index, $p = 0.041$), and evenness (Pielou's evenness, $p = 0.045$) between the two treatment modes (**Figure 10**) (see **Supplementary Table 22** for microbial dynamics across the three clusters). No differences were identified in richness (Chao1) in UC participants (**Figure 10**) (57). LDA of UC group between two treatment options identified 11 microbial biomarkers (OTUs) which were more abundant in UC group with no-biological treatments and 35 microbial biomarkers were identified in UC group under biological treatments (**Figure 11**).

DISCUSSION

Inflammatory Bowel Diseases are chronic and complex gut inflammatory condition, both associated with significant morbidity. IBD is correlated with a highly relevant and significant psychosocial burden (58). Early studies suggested that both CD and UC are associated with high incidence of psychological manifestation (59). A large Canadian population-based study reported 3 times higher depressive rates in IBD patients compared to healthy population, with significant higher scores during active phase of the disease (60). Greater anxiety and stress scores have been reported in patients with more sever IBD symptoms and lower compliance with treatment (61). Nevertheless, similar studies have concluded that significant number of CD patients present with depressive or anxiety symptoms, despite clinical remission, can therefore benefit from psychological support (62).

It is widely accepted that DSS-induced colitis in mice results in anxiety-like behavior that increases with and can be controlled by managing the inflammation. The degree of the DDS-induced inflammation can also be regulated by manipulation of the gut microbiota prior to DDS initiation (i.e., administration of pre and/or probiotics), which consequently prevents the behavioral deficits provoked by DSS application (63–66).

Gut microbiota dysbiosis is considered as a novel factor in the pathogenesis of IBD. Gut microbiome and its products foster a distinct effect on host immune system and promote intestinal homeostasis and healthy state. Once the symbiotic interplay between gut microbiome and its profile is disturbed, its various physiological functions will then be impaired (67–69). Yet the role and dynamics of gut microbiota in IBD development and whether the gut microbiota alteration is the cause of the intestinal inflammation or simply a product of the IBD, is not clear (70, 71).

Four major bacterial phyla: *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*, constitute more than 90% of healthy human gut bacterial species (72–74) with substantial inter-individual microbial diversity within these major phylotypes (75). In IBD patients, the dysbiosis was mostly associated with reduced bacterial diversity (predominantly in *Firmicutes* and *Bacteroidetes*) and increased bacterial species belonging to *Enterobacteriaceae* (76–78). More in-depth studies

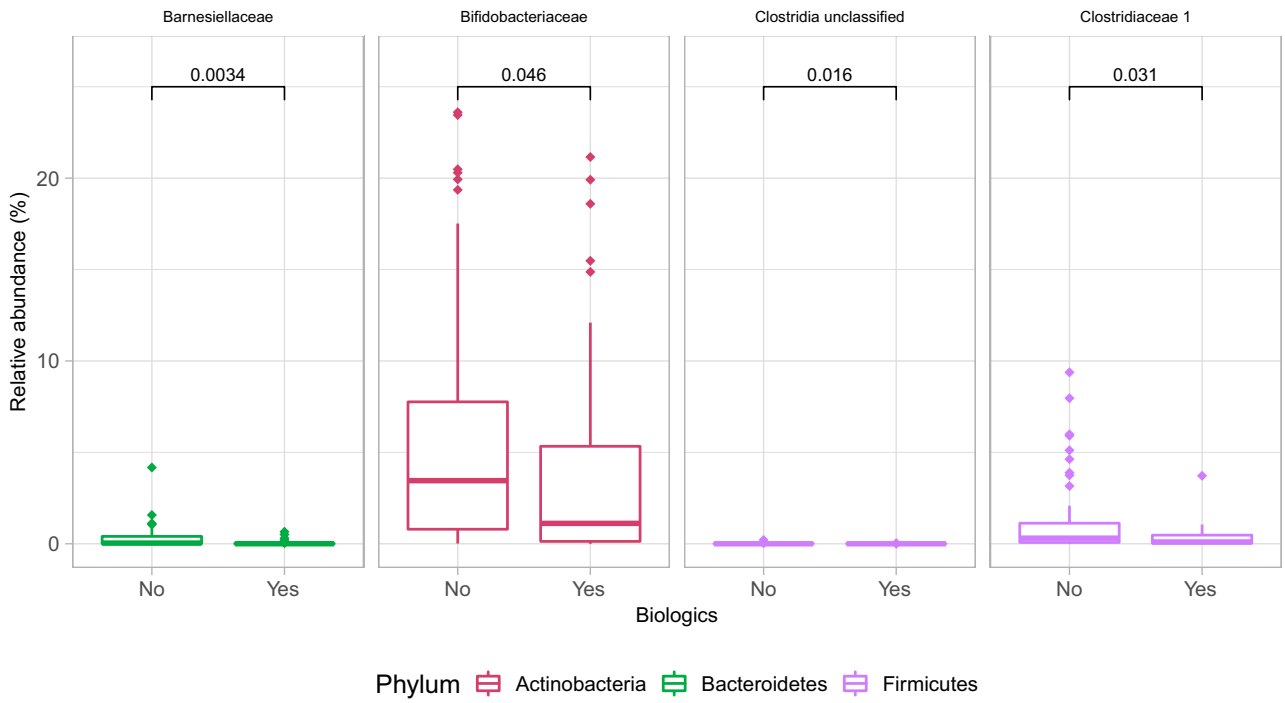


FIGURE 7 | Differences in some microbial family abundances between the two treatment options in CD group (biologics vs. non-biologic). Only significantly different families are shown (Wilcoxon, $p \leq 0.05$).

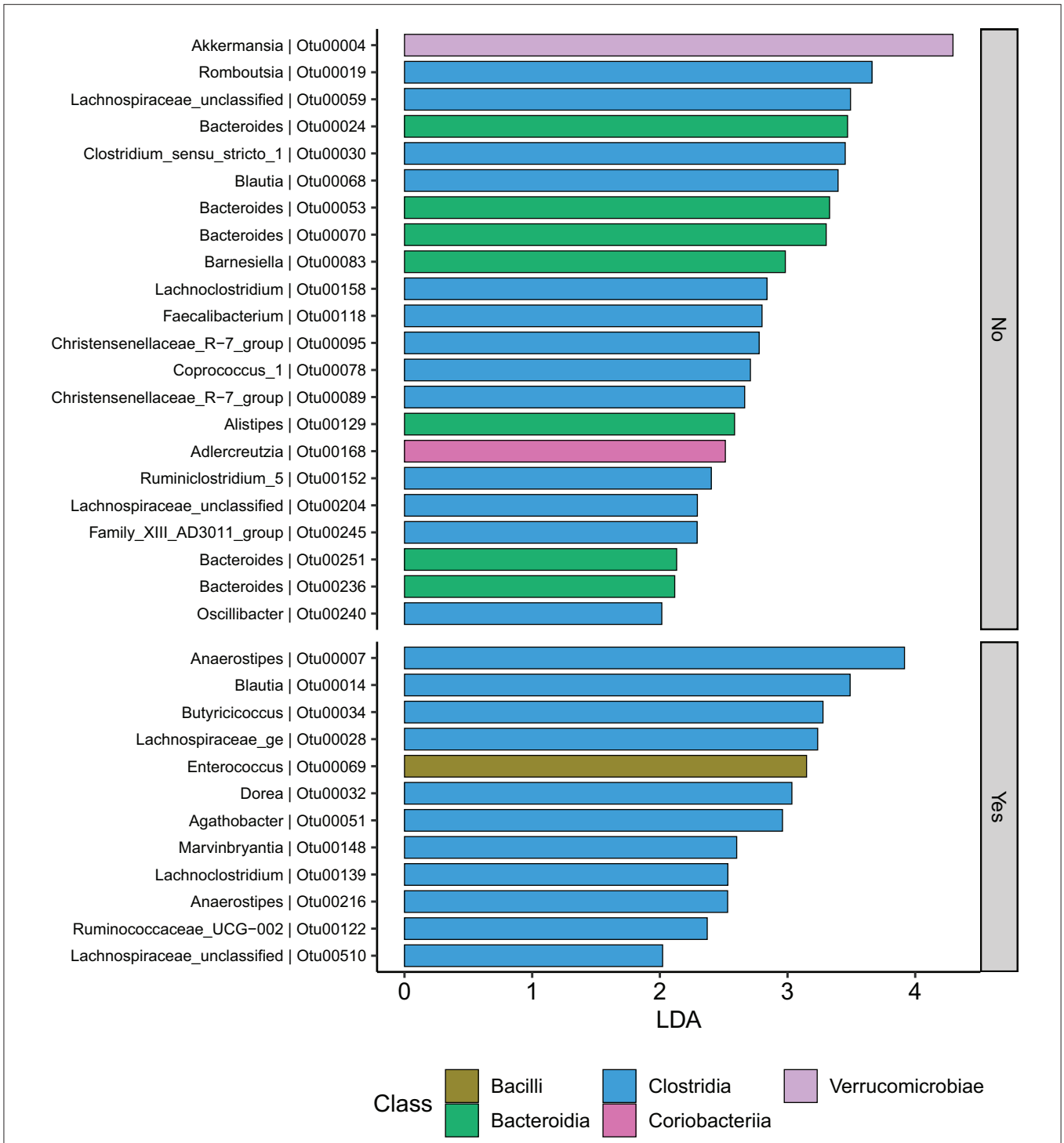


FIGURE 8 | CD participants-Microbial biomarkers. OTUs microbial biomarkers for CD groups with two treatment options including biologic treatment (Yes) and non-biologic treatments (No) (OTUs and Genus information on **Supplementary Table 14**).

have shown a clear reduction in *Firmicutes* and significant decrease of many other beneficial bacterial species from the genera *Lactobacillus*, *Eubacterium* and *Bacteriodes* (79–82). Other literature addressed the association between relative abundance of a specific gut microbiome species with the mode of treatment (83–86). Such studies also examined the gut microbiome profile before and after therapeutic intervention and the duration to relapse after withdrawal. Examples would be

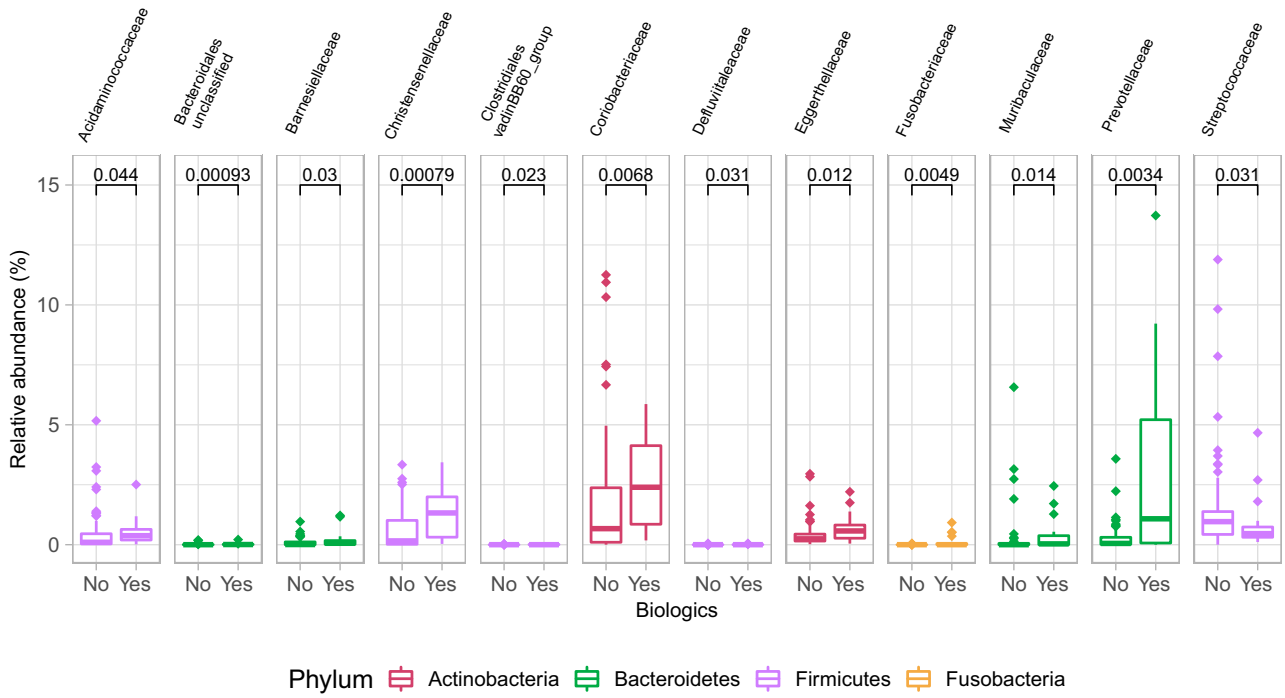


FIGURE 9 | Differences in some microbial family abundances between the two treatment options in UC group. Only significant results are shown (Wilcoxon, $p \leq 0.05$).

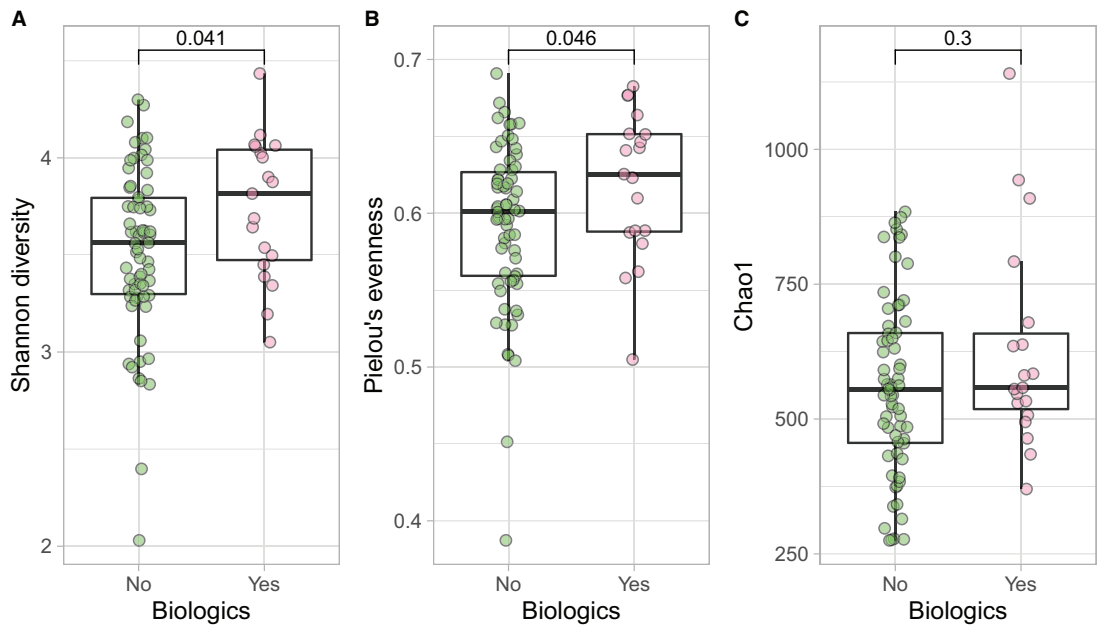


FIGURE 10 | UC participants- microbial a diversity and treatment modalities. Measures of microbial alpha diversity between treatment options in UC group (comprising remission and relapse) including: **(A)** Shannon's index (diversity); **(B)** Pielou's evenness; and **(C)** Chao1 index (richness).

the reduction of *Proteobacteria* in CD patients with anti TNF-α therapy (86) and increase of the abundance of *Faecalibacterium prausnitzii* in responders during the induction of anti-TNF-α antibody therapy. Another example would be the inhibitory effect of thiopurines on growth of *Mycobacterium avium* subspecies *paratuberculosis in vitro* (84). Studies also shown the

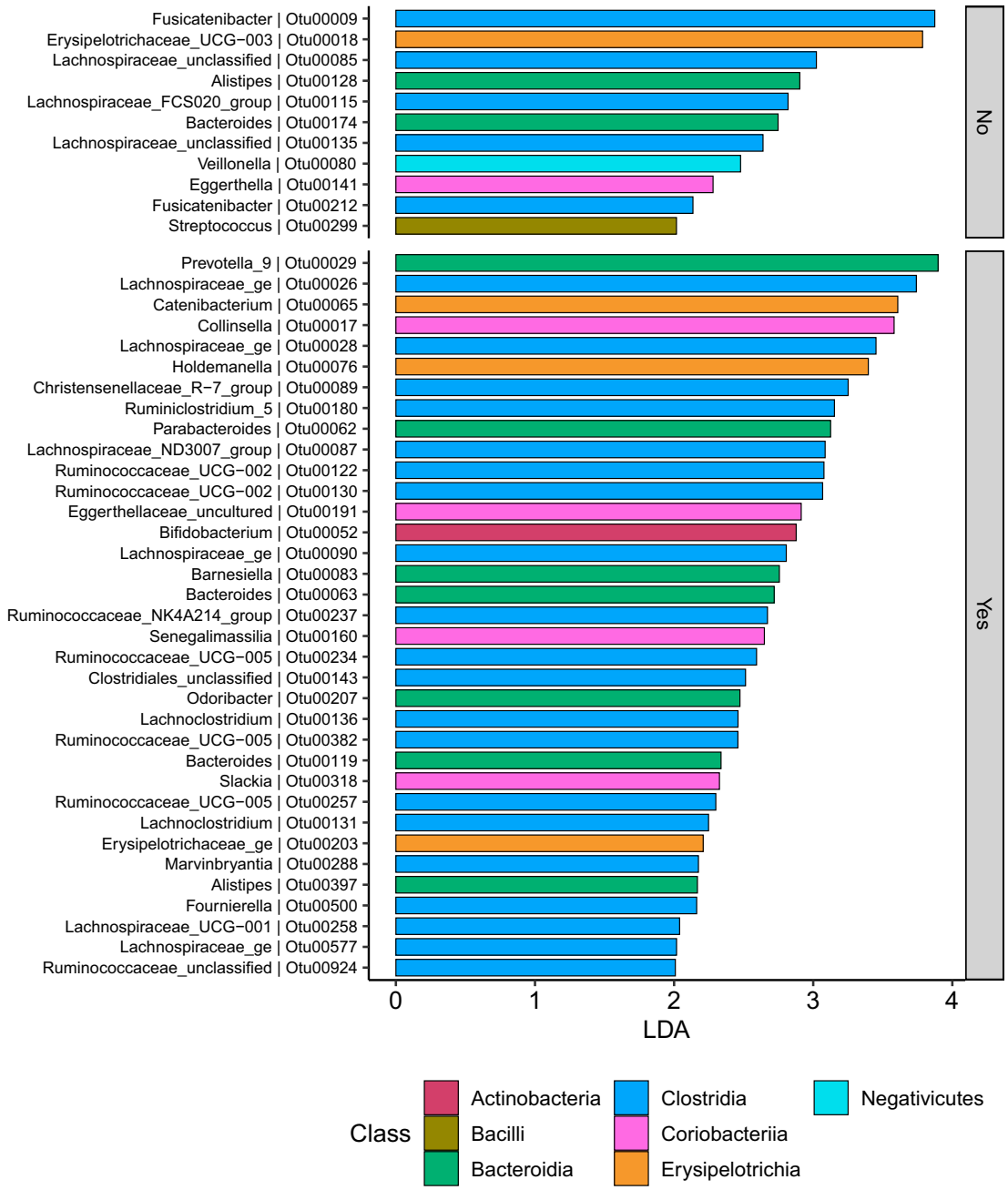


FIGURE 11 | UC participants-Microbial biomarkers. OTUs microbial biomarkers for UC groups with two treatment options (biologic as Yes and non-biologic treatments as No) (OTUs and Genus information on **Supplementary Information** No. 14).

correlation between reduced *Firmicutes* abundance and shorter time to relapse after Infliximab withdrawal in pediatric IBD (86).

Thus, gut microbiota may be used as a potential biomarker in respond to the treatment of IBD or can be employed to modify the host's environment and enhance the intestinal dysbiosis. Examples of the latter could be the complementary and alternative medicine (CAM), including pre and probiotics, antibiotics, fecal microbiota transplant (FMT) and nutraceuticals (87, 88).

This study was designed to characterize the longitudinal temporal trajectories of biological and psychological factors and their interdependence with disease activity and symptom manifestations in IBD patients. Assessment was made during clinical remission and at the time of relapse. At baseline analysis, all IBD patients were in clinical remission, therefore similar bio-psychological and microbiome dynamics across all IBD patients and between the disease phenotypes, were not surprising.

Results suggested a higher anxiety level was associated with greater sleep disturbances and increased stress was correlated significantly with longer sleep time in IBD participants at baseline assessment. This outcome is not surprising considering the state of clinical remission at baseline and the evidence suggested by clinical studies on the negative impact by anxiety (89) and depression (90) on sleep quality in general population. Remission CD and UC patients demonstrated significant differences in their baseline depression and negative affectivity of personality type, which entails further investigation.

Assessment of baseline microbial profile indicated that lower intestinal microbial diversity and richness, and uneven microbial composition were associated with longer time to fall asleep in both CD and UC patients. Microbial phyla abundance analysis showed that CD patients had higher abundance of some disease associated bacteria (including *Bacteroidetes* and *Proteobacteria*) compared to UC at baseline. Like previous studies (55), the baseline microbial state was similar between the two treatment modalities in CD and UC cohorts.

Longitudinal assessment of psychological factors in IBD patients who maintained clinical remission revealed strong coefficient of change although the magnitude of this longitudinal shift was not similar between remissive CD and UC patients over time. In the remission CD group, greater microbial fluctuations at the family level were observed as well as alteration in relative abundance of existing microbial profile compared to remissive UC patients over time. This outcome was also suggested by previous studies (55, 91) and might be explained by fundamental differences in the nature of the two disease phenotypes. CD patients who received biological therapy revealed similar psychobiological state to those who received non-biological treatments although there was either marginal or significant interaction between biological therapies and longitudinal state of depression and stress in this group. Comparable outcomes were detected in UC patients over time, but the significant interaction constituted by biological therapy was mainly registered on measures of inflammatory biomarkers and quality of life of these patients. The nature and mechanism of such interactions was not investigated in this study but could imply that biological therapy and their immune-physiological pathways might have play a role in sustaining the biopsychological interplay.

Longitudinal analysis on mode of treatment and gut microbiome did not identify significant differences in microbial profile in two CD treatment groups, whereas in UC group, there were significant differences in microbial diversity and evenness between the biologic and non-biologic interventions, with UC patients on biologics benefiting from more diverse and more even bacterial dynamics, but no richness differences were identified between the two. This outcome suggests that although majority of bio-psychological factors remained relentless, biological therapies potentially influenced such factors while maintaining clinical remission and might be the product of effective therapy choice in controlling the disease activity. Conceivably, this result could be related to the mechanism of action enforced by biological therapies in controlling the disease activities/maintaining remission or microbial function in responding to the therapy and requires further investigations and inclusion of multiomic analyses as well as assessment of microbial function. The differences might also reflect distinctions between clinical and deeper (e.g., endoscopic) remission, as the latter was not routinely assessed in this patient cohort. This study reinforced that lower ecological indices are significantly correlated with depression in UC patients, a feature previously identified (92, 93) and higher disease activity measures were negatively related to quality of life and sleep quality of both CD and UC patients, as previously shown (94).

Strengths and Limitations of the Current Study and Future Directions

This study was the first prospective longitudinal study designed to evaluate bio-psychological interdependence, their complex orchestrated interplay, and their influence on the clinical course of IBDs including the treatment modalities. Time series analysis was used to examine bidirectional and longitudinal interplay between multiple disease contributing factors to measure the rebound effect. A limitation of this study was lack of healthy controls. Diet is a very significant factor affecting gut health and microbial profile (95, 96), but dietary intake was not assessed in this study. None of the participants were exmained for potential pre-existing psychological conditions. Clearly, replication of the current study is needed to further test the conceptual framework with a larger sample size, a better sample representation and by including a large cohort of healthy controls.

CONCLUSION

This study indicates temporal and close interplay between psychological, immune system and microbiome dynamics in IBD patients. The mechanism of such interactions and directional sequence of such interplay remained unexplained and rather speculative, therefore requiring further investigations.

DATA AVAILABILITY STATEMENT

Raw amplicon data is available at ENA under project PRJEB43193 (https://www.ebi.ac.uk/ena/browser/view/PRJEB43193).

ETHICS STATEMENT

This study was approved by the Human Research Ethics Committee of South Eastern Sydney Local Health District (Ref: 15/094 HREC/15/POWH 245—20 Aug). Site Specific Approval was obtained for St. George Hospital (SSA ref: 15/G/150 – 31 Aug 2015) and for St. Vincent's Hospital (Ref: 15/207 – 2 Nov 2015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PT: conceptualization, methodology, project administration, analysis, writing—original draft, preparation, investigation, data

curation and validation, resources, and formal analysis. UV-C: supervision, writing—review, and editing. DH-P: supervision, software, formal analysis, and validation. XV-C: software, analysis, and producing results related to microbiome section. MG: supervision, conceptualization, writing—review and editing, and validation. All authors contributed to the article and approved the submitted version.

FUNDING

This research was partly funded by a grant from the St George and Sutherland Medical Research Foundation. XV-C acknowledges support from the New South Wales State Government RAAP scheme and the National Collaborative Research Infrastructure Strategy.

ACKNOWLEDGMENTS

The authors acknowledge the support provided by Marc Wilkins.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.788992/full#supplementary-material

REFERENCES

1. Rogler G, Vavricka S. Exposome in IBD: recent insights in environmental factors that influence the onset and course of IBD. *Inflamm Bowel Dis.* (2014) 21:400–8. doi: 10.1097/MIB.0000000000000229
2. Saidel-Odes L, Odes S. Hygiene hypothesis in inflammatory bowel disease. *Ann Gastroenterol.* (2014) 27:189. doi: 10.3748/wjg.14.165
3. Cleynen I, Vermeire S. The genetic architecture of inflammatory bowel disease: past, present and future. *Curr Opin Gastroenterol.* (2015) 31:456–63. doi: 10.1097/MOG.0000000000000215
4. McGovern DP, Kugathasan S, Cho JH. Genetics of inflammatory bowel diseases. *Gastroenterology.* (2015) 149:1163–76. e2. doi: 10.1053/j.gastro.2015.08.001
5. Wang M-H, Fiocchi C, Ripke S, Zhu X, Duerr RH, Achkar J-P. A novel approach to detect cumulative genetic effects and genetic interactions in Crohn's disease. *Inflam Bowel Dis.* (2013) 19:1799–808. doi: 10.1097/MIB.0b013e31828706a0
6. De Souza HS, Fiocchi C, Iliopoulos D. The IBD interactome: an integrated view of aetiology, pathogenesis and therapy. *Nat Rev Gastroenterol Hepatol.* (2017) 14:739. doi: 10.1038/nrgastro.2017.110
7. Neurath MF. Cytokines in inflammatory bowel disease. *Nat Rev Immunol.* (2014) 14:329. doi: 10.1038/nri3661
8. Actis GC, Pellicano R, Fagoonee S, Ribaldone DG. History of inflammatory bowel diseases. *J Clin Med.* (2019) 8:1970. doi: 10.3390/jcm8111970
9. De Souza HS, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol.* (2016) 13:13–27. doi: 10.1038/nrgastro.2015.186
10. Darfeuille-Michaud A, Boudeau J, Bulois P, Neut C, Glasser A-L, Barnich N, et al. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology.* (2004) 127:412–21. doi: 10.1053/j.gastro.2004.04.061
11. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, et al. A decrease of the butyrate-producing species *Roseburia hominis and Faecalibacterium prausnitzii* defines dysbiosis in patients with ulcerative colitis. *Gut*. (2013) 2013:304833. doi: 10.1136/gutjnl-2013-304833
12. Rogala L, Miller N, Graff LA, Rawsthorne P, Clara I, Walker JR, et al. Population-based controlled study of social support, self-perceived stress, activity and work issues, and access to health care in inflammatory bowel disease. *Inflam Bowel Dis.* (2008) 14:526–35. doi: 10.1002/ibd.20353
13. Miehsler W, Weichselberger M, Öfferlbauer-Ernst A, Dejaco C, Reinisch W, Vogelsang H, et al. Which patients with IBD need psychological interventions? A controlled study. *Inflam Bowel Dis.* (2008) 14:1273–80. doi: 10.1002/ibd.20462
14. Graff LA, Vincent N, Walker JR, Clara I, Carr R, Ediger J, et al. A population-based study of fatigue and sleep difficulties in inflammatory bowel disease. *Inflam Bowel Dis.* (2010) 17:1882–9. doi: 10.1002/ibd.21580
15. Ferrari AJ, Charlson FJ, Norman RE, Patten SB, Freedman G, Murray CJ, et al. Burden of depressive disorders by country, sex, age, and year: findings from the global burden of disease study 2010. *PLoS Med.* (2013) 10. doi: 10.1371/journal.pmed.1001547
16. Baxter A, Scott K, Vos T, Whiteford H. Global prevalence of anxiety disorders: a systematic review and meta-regression. *Psychol Med.* (2013) 43:897–910. doi: 10.1017/S003329171200147X
17. Mikocka-Walus A, Knowles SR, Keefer L, Graff L. Controversies revisited: a systematic review of the comorbidity of depression and anxiety with inflammatory bowel diseases. *Inflam Bowel Dis.* (2016) 22:752–62. doi: 10.1097/MIB.0000000000000620
18. Wang P, Han W, Ma D. Electronic sorting of immune cell subpopulations based on highly plastic genes. *J Immunol.* (2016) 197:665–73. doi: 10.4049/jimmunol.1502552
19. Gracie DJ, Guthrie EA, Hamlin PJ, Ford AC. Bi-directionality of brain–gut interactions in patients with inflammatory bowel disease. *Gastroenterology.* (2018) 154:1635–46. e3. doi: 10.1053/j.gastro.2018.01.027
20. Lakatos PL, Burisch J. Environment and invironment in IBDs: partners in crime. *Gut.* (2015) 64:1009–10. doi: 10.1136/gutjnl-2014-308460
21. Zhang Y-Z, Li Y-Y. Inflammatory bowel disease: pathogenesis. *World J Gastroenterol.* (2014) 20:91. doi: 10.3748/wjg.v20.i1.91
22. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. *N Engl J Med.* (1987) 317:1625–9. doi: 10.1056/NEJM198712243172603
23. Best WR, Becktel JM, Singleton JW, Kern F. Development of a Crohn's disease activity index. *Gastroenterology.* (1976) 70:439–44. doi: 10.1016/S0016-5085(76)80163-1
24. Vermeire S, Schreiber S, Sandborn WJ, Dubois C, Rutgeerts P. Correlation between the Crohn's disease activity and Harvey–Bradshaw indices in assessing Crohn's disease severity. *Clin Gastroenterol Hepatol.* (2010) 8:357–63. doi: 10.1016/j.cgh.2010.01.001
25. Harvey R, Bradshaw J. A simple index of Crohn's-disease activity. *Lancet.* (1980) 315:514. doi: 10.1016/S0140-6736(80)92767-1
26. Levenstein S, Prantera C, Varvo V, Scribano ML, Berto E, Luzi C, et al. Development of the perceived stress questionnaire: a new tool for psychosomatic research. *J Psycho Res.* (1993) 37:19–32. doi: 10.1016/0022-3999(93)90120-5
27. Cohen S, Kamarck T, Mermelstein R. Perceived stress scale. *Meas Stress.* (1994) 10:1–2.
28. Antony MM, Bieling PJ, Cox BJ, Enns MW, Swinson RP. Psychometric properties of the 42-item and 21-item versions of the depression anxiety stress scales in clinical groups and a community sample. *Psychol Assess.* (1998) 10:176. doi: 10.1037/1040-3590.10.2.176
29. Brown TA, Chorpita BF, Korotitsch W, Barlow DH. Psychometric properties of the depression anxiety stress scales (DASS) in clinical samples. *Behav Res Ther.* (1997) 35:79–89. doi: 10.1016/S0005-7967(96)00068-X
30. Parker G, Hilton T, Bains J, Hadzi-Pavlovic D. Cognitive-based measures screening for depression in the medically ill: the DMI-10 and the DMI-18. *Acta Psychiatrica Scandinavica.* (2002) 105:419–26. doi: 10.1034/j.1600-0447.2002.01248.x
31. Orive M, Padierna JA, Quintana JM, Las-Hayas C, Vrotsou K, Aguirre U. Detecting depression in medically ill patients: comparative accuracy of four screening questionnaires and physicians' diagnoses in Spanish population. *J Psychos Res.* (2010) 69:399–406. doi: 10.1016/j.jpsychores.2010.04.007

32. Denollet J. DS14: standard assessment of negative affectivity, social inhibition, and type D personality. *Psychos Med.* (2005) 67:89–97. doi: 10.1097/01.psy.0000149256.81953.49
33. Greene BR, Blanchard EB, Wan CK. Long-term monitoring of psychosocial stress and symptomatology in inflammatory bowel disease. *Behav Res Ther.* (1994) 32:217–26. doi: 10.1016/0005-7967(94)90114-7
34. Shochet BR. Gastrointestinal disorders: behavioral and physiological basis for treatment. *J Nerv Mental Dis.* (1986) 174:441. doi: 10.1097/00005053-198607000-00018
35. Kirschner BS, Voinchet O, Rosenberg IH. Growth retardation in inflammatory bowel disease. *Gastroenterology.* (1978) 75:504–11. doi: 10.1016/0016-5085(78)90859-4
36. Buysse DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ. The pittsburgh sleep quality index: a new instrument for psychiatric practice and research. *Psychiatry Res.* (1989) 28:193–213. doi: 10.1016/0165-1781(89)90047-4
37. Aziz N, Fahey JL, Detels R, Butch AW. Analytical performance of a highly sensitive C-reactive protein-based immunoassay and the effects of laboratory variables on levels of protein in blood. *Clin Diagn Lab Immunol.* (2003) 10:652–7. doi: 10.1128/CDLI.10.4.652-657.2003
38. Desjardins P, Conklin D. NanoDrop microvolume quantitation of nucleic acids. *J Vis Exp*. (2010) 45:e2565. doi: 10.3791/2565
39. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA*. (2010) 2010:201000080. doi: 10.1073/pnas.1000080107
40. Vázquez-Campos X, McFarlane D, Chilton AM, Koval JR, Wilkins MR. OTUreporter: Amplicon to report in a single command. In: *Joint 30th International Conference on Genome Informatics (GIW) & Australian Bioinformatics and Computational Biology Society (ABACBS) Annual Conference (GIW/ABACBS 2019)*. Sydney, NSW (2019). Available online at: https://bitbucket.org/xvazquezc/otureporter
41. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* (2009) 75:7537–41. doi: 10.1128/AEM.01541-09
42. Vollmer-Conna U, Chen M, Lloyd A, Donovan B. Neuropsychiatric symptoms and immune activation in patients with genital herpes. *Acta Neurops.* (2008) 20:145–51. doi: 10.1111/j.1601-5215.2008.00281.x
43. Fraley C, Raftery AE. How many clusters? Which clustering method? Answers via model-based cluster analysis. *Comp J.* (1998) 41:586–8. doi: 10.1093/comjnl/41.8.578
44. McArdle JJ. Latent variable modeling of differences and changes with longitudinal data. *Ann Rev Psychol.* (2009) 60:577–605. doi: 10.1146/annurev.psych.60.110707.163612
45. Team RC. R: a language and environment for statistical computing. (2013).
46. Ihaka R, Gentleman R. R: a language for data analysis and graphics. *J Comput Graph Stat.* (1996) 5:299–314. doi: 10.1080/10618600.1996.10474713
47. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE.* (2013) 8:e61217. doi: 10.1371/journal.pone.0061217
48. Liaw A, Wiener M. Classification and regression by randomForest. *R News.* (2002) 2:18–22. doi: 10.1057/9780230509993
49. Wilcoxon F, Katti S, Wilcox RA. Critical values and probability levels for the Wilcoxon rank sum test and the Wilcoxon signed rank test. *Selec Tables Mathem Stat.* (1970) 1:171–259.
50. Kruskal WH, Wallis WA. Use of ranks in one-criterion variance analysis. *J Am stat Assoc.* (1952) 47:583–621. doi: 10.1080/01621459.1952.10483441
51. Shannon CE. A mathematical theory of communication. *Bell System Tech J.* (1948) 27:379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x
52. Chao A, Shen TJ, Hwang WH. Application of Laplace's boundary-mode approximations to estimate species and shared species richness. *Austr N Zeal J Stat.* (2006) 48:117–28. doi: 10.1111/j.1467-842X.2006.00430.x
53. Troussellier M, Legendre P. A functional evenness index for microbial ecology. *Microbial Ecol.* (1981) 7:283–96. doi: 10.1007/BF023 41423
54. Hagen S. *How the World Can be the Way it Is: An Inquiry for the New Millennium into Science, Philosophy, and Perception*. 1st ed. Wheaton, IL: Quest Books (1995). p. 343.
55. Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol.* (2017) 2:17004. doi: 10.1038/nmicrobiol.2017.4
56. Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao J-z, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol.* (2016) 16:90. doi: 10.1186/s12866-016-0708-5
57. Chao A. Nonparametric estimation of the number of classes in a population. *Scand J Stat*. (1984) 1984:265–70.
58. Taft TH, Keefer L. A systematic review of disease-related stigmatization in patients living with inflammatory bowel disease. *Clin Exp Gastroenterol.* (2016) 9:49. doi: 10.2147/CEG.S83533
59. Abautret-Daly Á, Dempsey E, Parra-Blanco A, Medina C, Harkin A. Gut–brain actions underlying comorbid anxiety and depression associated with inflammatory bowel disease. *Acta Neurops.* (2018) 30:275–96. doi: 10.1017/neu.2017.3
60. Fuller-Thomson E, Sulman J. Depression and inflammatory bowel disease: findings from two nationally representative Canadian surveys. *Inflamm Bowel Dis.* (2006) 12:697–707. doi: 10.1097/00054725-200608000-00005
61. Mikocka-Walus A, Pittet V, Rossel J-B, von Känel R, Anderegg C, Bauerfeind P, et al. Symptoms of depression and anxiety are independently associated with clinical recurrence of inflammatory bowel disease. *Clin Gastroenterol Hepatol.* (2016) 14:829–35. e1. doi: 10.1016/j.cgh.2015.12.045
62. Iglesias M, De Acosta MB, Vázquez I, Figueiras A, Nieto L, Lorenzo A, et al. Psychological impact of Crohn's disease on patients in remission: anxiety and depression risks. *Rev Español Enfermedades Digestivas.* (2009) 101:249. doi: 10.4321/S1130-01082009000400003
63. Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, et al. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. *Gastroenterology.* (2010) 139:2102–12. e1. doi: 10.1053/j.gastro.2010.06.063
64. Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology.* (2011) 141:599–609. e3. doi: 10.1053/j.gastro.2011.04.052
65. Romijn AR, Rucklidge JJ, Kuijer RG, Frampton C. A double-blind, randomized, placebo-controlled trial of Lactobacillus helveticus and Bifidobacterium longum for the symptoms of depression. *Austr N Zealand J Psychiatry.* (2017) 51:810–21. doi: 10.1177/0004867416686694
66. Liu W-H, Chuang H-L, Huang Y-T, Wu C-C, Chou G-T, Wang S, et al. Alteration of behavior and monoamine levels attributable to lactobacillus plantarum PS128 in germ-free mice. *Behav Brain Res.* (2016) 298:202–9. doi: 10.1016/j.bbr.2015.10.046
67. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol.* (2010) 10:159–69. doi: 10.1038/nri2710
68. Arpaia N, Campbell C, Fan X, Dikiy S, Van Der Veeken J, Deroos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature.* (2013) 504:451–5. doi: 10.1038/nature12726
69. Asquith M, Powrie F. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. *J Exp Med.* (2010) 207:1573–7. doi: 10.1084/jem.20101330
70. Rosen CE, Palm NW. Navigating the microbiota seas: triangulation finds a way forward. *Cell Host Microbe.* (2018) 23:1–3. doi: 10.1016/j.chom.2017.12.015
71. Gkouskou K, Deligianni C, Tsatsanis C, Eliopoulos AG. The gut microbiota in mouse models of inflammatory bowel disease. *Front Cell Infect Microbiol.* (2014) 4:28. doi: 10.3389/fcimb.2014.00028
72. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell.* (2012) 148:1258–70. doi: 10.1016/j.cell.2012.01.035
73. Tlaskalová-Hogenová H, Štěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, et al. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cell Mol Immunol.* (2011) 8:110–20. doi: 10.1038/cmi.2010.67

74. Larussa T, Abenavoli L, Fabiano G, Mancuso MA, Polimeni N, Dumitrascu DL, et al. Gut microbiota in inflammatory bowel disease: a target for therapy not to be missed. *Minerva Gastroenterol.* (2021) 67:357–68. doi: 10.23736/S2724-5985.21.02907-7
75. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature.* (2012) 489:220–30. doi: 10.1038/nature11550
76. Hedin CR, McCarthy NE, Louis P, Farquharson FM, McCartney S, Taylor K, et al. Altered intestinal microbiota and blood T cell phenotype are shared by patients with Crohn's disease and their unaffected siblings. *Gut.* (2014) 63:1578–86. doi: 10.1136/gutjnl-2013-306226
77. Li J, Butcher J, Mack D, Stintzi A. Functional impacts of the intestinal microbiome in the pathogenesis of inflammatory bowel disease. *Inflamm Bowel Dis.* (2015) 21:139–53. doi: 10.1097/MIB.0000000000000215
78. Mondot S, Kang S, Furet J-P, Aguirre de Cárcer D, McSweeney C, Morrison M, et al. Highlighting new phylogenetic specificities of Crohn's disease microbiota. *Inflamm Bowel Dis.* (2011) 17:185–92. doi: 10.1002/ibd.21436
79. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe.* (2014) 15:382–92. doi: 10.1016/j.chom.2014.02.005
80. Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroenterology.* (2008) 134:577–94. doi: 10.1053/j.gastro.2007.11.059
81. Frank DN, Amand ALS, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci.* (2007) 104:13780–5. doi: 10.1073/pnas.0706625104
82. Khan I, Ullah N, Zha L, Bai Y, Khan A, Zhao T, et al. Alteration of gut microbiota in inflammatory bowel disease (IBD): cause or consequence? IBD treatment targeting the gut microbiome. *Pathogens.* (2019) 8:126. doi: 10.3390/pathogens8030126
83. Busquets D, Mas-de-Xaxars T, López-Siles M, Martínez-Medina M, Bahí A, Sàbat M, et al. Anti-tumour necrosis factor treatment with adalimumab induces changes in the microbiota of Crohn's disease. *J Crohn Col.* (2015) 9:899–906. doi: 10.1093/ecco-jcc/jjv119
84. Shin SJ, Collins MT. Thiopurine drugs azathioprine and 6-mercaptopurine inhibit mycobacterium paratuberculosis growth in vitro. *Antimicro Agents Chemother.* (2008) 52:418–26. doi: 10.1128/AAC.00678-07
85. Ananthakrishnan AN, Luo C, Yajnik V, Khalili H, Garber JJ, Stevens BW, et al. Gut microbiome function predicts response to anti-integrin biologic therapy in inflammatory bowel diseases. *Cell Host Microbe.* (2017) 21:603–10. e3. doi: 10.1016/j.chom.2017.04.010
86. Kolho K-L, Korpela K, Jaakkola T, Pichai MV, Zoetendal EG, Salonen A, et al. Fecal microbiota in pediatric inflammatory bowel disease and its relation to inflammation. *Offic J Am Coll Gastroenterol.* (2015) 110:921–30. doi: 10.1038/ajg.2015.149
87. Abenavoli L, Scarpellini E, Colica C, Boccuto L, Salehi B, Sharifi-Rad J, et al. Gut microbiota and obesity: a role for probiotics. *Nutrients.* (2019) 11:2690. doi: 10.3390/nu11112690
88. Basson AR, Lam M, Cominelli F. Complementary and alternative medicine strategies for therapeutic gut microbiota modulation in inflammatory bowel disease and their next-generation approaches. *Gastroenterol Clin.* (2017) 46:689–729. doi: 10.1016/j.gtc.2017.08.002
89. Fehr KK, Chambers DE, Ramasami J. The impact of anxiety on behavioral sleep difficulties and treatment in young children: a review of the literature. *J Clin Psychol Med Sett.* (2021) 28:102–12. doi: 10.1007/s10880-020-09703-x
90. Roberts RE, Duong HT. The prospective association between sleep deprivation and depression among adolescents. *Sleep.* (2014) 37:239–44. doi: 10.5665/sleep.3388
91. Alam MT, Amos GC, Murphy AR, Murch S, Wellington EM, Arasaradnam RP. Microbial imbalance in inflammatory bowel disease patients at different taxonomic levels. *Gut Pathog.* (2020) 12:1–8. doi: 10.1186/s13099-019-0341-6
92. Maes M, Kubera M, Leunis J-C, Berk M. Increased IgA and IgM responses against gut commensals in chronic depression: further evidence for increased bacterial translocation or leaky gut. *J Affect Dis.* (2012) 141:55–62. doi: 10.1016/j.jad.2012.02.023
93. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci.* (2012) 13:701–12. doi: 10.1038/nrn3346
94. Lix LM, Graff LA, Walker JR, Clara I, Rawsthorne P, Rogala L, et al. Longitudinal study of quality of life and psychological functioning for active, fluctuating, and inactive disease patterns in inflammatory bowel disease. *Inflamm Bowel Dis.* (2008) 14:1575–84. doi: 10.1002/ibd.20511
95. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature.* (2014) 505:559. doi: 10.1038/nature12820
96. Lewis JD, Chen EZ, Baldassano RN, Otley AR, Griffiths AM, Lee D, et al. Inflammation, antibiotics, and diet as environmental stressors of the gut microbiome in pediatric Crohn's disease. *Cell Host Microbe.* (2015) 18:489–500. doi: 10.1016/j.chom.2015.09.008

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.

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 27 September 2022
DOI 10.3389/fmed.2022.868812

OPEN ACCESS

EDITED BY
Abbas Yadegar,
Shahid Beheshti University of Medical Sciences, Iran

REVIEWED BY
Marcela A. Hermoso,
University Medical Center Groningen, Netherlands
Yuji Naito,
Kyoto Prefectural University of Medicine, Japan

*CORRESPONDENCE
Reidar Fossmark
reidar.fossmark@ntnu.no

†These authors have contributed equally to this work and share first authorship

SPECIALTY SECTION
This article was submitted to Gastroenterology, a section of the journal Frontiers in Medicine

RECEIVED 03 February 2022
ACCEPTED 08 September 2022
PUBLISHED 27 September 2022

CITATION
Olaisen M, Richard ML, Beisvåg V, Granlund AvB, Røyset ES, Rué O, Martinsen TC, Sandvik AK, Sokol H and Fossmark R (2022) The ileal fungal microbiota is altered in Crohn's disease and is associated with the disease course. *Front. Med.* 9:868812. doi: 10.3389/fmed.2022.868812

The ileal fungal microbiota is altered in Crohn's disease and is associated with the disease course

Maya Olaisen[1,2†], Mathias L. Richard[3,4†], Vidar Beisvåg[1,5], Atle van Beelen Granlund[1,6], Elin S. Røyset[1,6,7], Olivier Rué[8,9], Tom Christian Martinsen[1,2], Arne Kristian Sandvik[1,2,6], Harry Sokol[3,4,10] and Reidar Fossmark[1,2*]

[1]Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway, [2]Department of Gastroenterology and Hepatology, St. Olav's Hospital - Trondheim University Hospital, Trondheim, Norway, [3]INRAE, AgroParisTech, Micalis Institute, Université Paris-Saclay, Jouy-en-Josas, France, [4]Paris Center for Microbiome Medicine, Fédération Hospitalo-Universitaire, Paris, France, [5]Central Administration, St. Olav's Hospital - Trondheim University Hospital, Trondheim, Norway, [6]Centre of Molecular Inflammation Research, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway, [7]Department of Pathology, St. Olav's Hospital - Trondheim University Hospital, Trondheim, Norway, [8]INRAE, MaIAGE, Université Paris-Saclay, Jouy-en-Josas, France, [9]INRAE, BioinfOmics, MIGALE Bioinformatics Facility, Université Paris-Saclay, Jouy-en-Josas, France, [10]Gastroenterology Department, INSERM, Centre de Recherche Saint-Antoine, CRSA, AP-HP, Saint Antoine Hospital, Sorbonne Université, Paris, France

Introduction: Fungal microbiota's involvement in the pathogenesis of Crohn's disease (CD) is incompletely understood. The terminal ileum is a predilection site both for primary involvement and recurrences of CD. We, therefore, assessed the mucosa-associated mycobiota in the inflamed and non-inflamed ileum in patients with CD.

Methods: The mucosa-associated mycobiota was assessed by ITS2 sequencing in a total of 168 biopsies sampled 5 and 15 cm proximal of the ileocecal valve or ileocolic anastomosis in 44 CD patients and 40 healthy controls (HC). CD patients with terminal ileitis, with endoscopic inflammation at 5 cm and normal mucosa at 15 cm and no history of upper CD involvement, were analyzed separately. The need for additional CD treatment the year following biopsy collection was recorded.

Results: CD patients had reduced mycobiota evenness, increased Basidiomycota/Ascomycota ratio, and reduced abundance of Chytridiomycota compared to HC. The mycobiota of CD patients were characterized by an expansion of *Malassezia* and a depletion of *Saccharomyces*, along with increased abundances of *Candida albicans* and *Malassezia restricta*. *Malassezia* was associated with the need for treatment escalation during follow-up. Current anti-TNF treatment was associated with lower abundances of Basidiomycota. The alpha diversity of the inflamed and proximal non-inflamed mucosa within the same patients was similar. However, the inflamed mucosa had a more dysbiotic composition with increased abundances of *Candida sake* and reduced abundances of *Exophiala equina* and *Debaryomyces hansenii*.

Conclusions: The ileal mucosa-associated mycobiota in CD patients is altered compared to HC. The mycobiota in the inflamed and proximal non-inflamed ileum within the same patients harbor structural differences which may play a role in the CD pathogenesis. Increased abundance of *Malassezia* was associated with an unfavorable disease course.

KEYWORDS

Crohn's disease, inflammatory bowel disease, mycobiota, fungal microbiota, fungi

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) characterized by transmural and segmental inflammation of the gastrointestinal tract. Currently, CD is thought to develop in genetically susceptible individuals exposed to environmental factors and gut microbiota, causing an aberrant immune response that leads to inflammation and subsequent tissue damage (1). The presence of a luminal factor causing inflammation was early demonstrated as an ileostomy diverting the intestinal contents has a well-known protective effect, whereas reestablishment of bowel continuity or infusion of fecal content triggers recurrence (2, 3). Disease recurrence typically manifests at and immediately proximal to an anastomosis (4, 5) and studies of the terminal and neo-terminal ileum are therefore of particular interest to understand the pathogenesis of CD. The ileal bacterial mucosa-associated microbiota at the time of ileocecal resection (ICR) and postoperatively has been associated with the risk of disease recurrence (6–8).

Several findings also suggest that the mycobiota is involved in CD pathogenesis (9). Anti-*Saccharomyces cerevisiae* antibodies (ASCA) were early proposed as a diagnostic biomarker of CD (10). Genome-wide association studies (GWAS) have later identified *CARD9* single-nucleotide polymorphism (rs4077515 creating substitution p.S12N) to be associated with CD (11, 12). Identification of intestinal fungi through C-lectin receptors depends on *CARD9* in the signaling pathway to stimulate a pro-inflammatory response to commensal fungi (13, 14), and a defect in *CARD9* is associated with susceptibility to fungal infections and a lower number of Th-17 cells in humans (13). In addition, Toll-like receptor 4 polymorphisms associated with both CD and UC also predispose to systemic *Candida* infections in humans (15). A proportion of IBD patients have genetic polymorphisms that increase susceptibility to fungal infections, also the fungal load and richness are elevated in CD patients (16, 17).

Although the majority of studies have analyzed the fecal mycobiota, the mucosa-associated and fecal bacterial microbiotas are different (18–20), and the mucosa-associated microbiota is by many considered more relevant to the pathogenesis of CD (21). Only a few studies have described the mucosa-associated mycobiota in CD patients (14, 16, 22). The mucosa-associated mycobiota in CD is characterized by a skewed Ascomycota to Basidiomycota ratio, increased abundances of Basidiomycota, and decreased abundances of Ascomycota phyla in CD compared to controls (14, 22). Inflamed tissue in CD patients has a 40-fold higher load of fungi compared to healthy controls (HC) and increased abundances of Xylariales order and Sordariomycetes class (16). Water-lavage samples obtained from CD patients during colonoscopy have increased abundances of *Malassezia*, *Cladosporium,* and *Aureobasidium* and decreased abundances of *Fusarium* compared to HC (14). Notably, *Malassezia* was found to be overrepresented in patients carrying a CARD9 allele which is associated with an increased risk of CD (14). CD has also been associated with increased abundance of Psathyrellaceae and Cortinariaceae families and *Psathyrella* and *Gymnopilus* genera (22) in a cohort of teenagers in Saudi Arabia. More recently, *Debaromyces* have been reported to be abundant in CD ulcerations and could be of importance in the pathogenesis of CD (23). However, the prognostic value of the mycobiota has to the best of our knowledge not been evaluated before.

In the current study, we have assessed the mycobiota of patients with CD and HC, focusing on differences between inflamed and proximal non-inflamed ileal mucosa within CD patients and the association between mycobiota and the clinical course during follow-up.

Materials and methods

Patients and control subjects

We have previously assessed the bacterial ileal microbiota of the same patient cohort (24). Study participants were recruited from the Department of Gastroenterology, St. Olav's Hospital, Trondheim, Norway between 2017 and 2019. Patients 18–70 years of age with Norwegian ethnicity and referred to ileocolonoscopy were invited to participate if they were eligible. Inclusion criteria were an established diagnosis of CD based on clinical, endoscopic, and histological criteria or patients with CD symptoms where the diagnosis was

confirmed after both endoscopic and histologic evaluation. CD characteristics were registered according to the Montreal classification (25). Age- and sex-matched subjects referred to colonoscopy due to rectal bleeding or screening for the disease were included as healthy controls (HC) if the ileocolonoscopy and histologic evaluation of biopsies were normal. Exclusion criteria were as described by Olaisen et al. (24), that is, use of antibacterial or antifungal treatment for the past 2 months or comorbidity with diabetes mellitus, celiac disease, or liver diseases including primary sclerosing cholangitis and primary biliary cholangitis. Additional exclusion criteria for HCs were previous gastrointestinal surgery, gastrointestinal polyps, cancer, diverticulitis, or irritable bowel disease fulfilling the ROME IV criteria (26). Information about the initiation of treatment escalation against CD the following year after biopsy collection was extracted from the medical records and electronic registry of prescriptions. CD treatment escalation was defined as the initiation of new medication, change within drug class or increased dose of systemic glucocorticoids (including budesonide), immunomodulators (azathioprine and methotrexate), biologics, or surgery, whichever occurred first.

Endoscopic procedure

The ileum was reached during endoscopy using either a colonoscope (Olympus Exera II GIF HQ190 or PH190L, Olympus Europa GmbH, Hamburg, Germany) or a single-balloon enteroscope (Olympus SIF-Q180). A total of six ileal pinch biopsies were collected from each study participant, three biopsies from approximately 5 and 15 cm proximal of the ileocecal valve or ileocolic anastomosis, respectively. In CD patients with terminal ileitis, the 5-cm samples were taken from an endoscopically inflamed area and 15-cm samples from normal-appearing mucosa. In CD patients categorized as having active disease, both biopsy locations (5 and 15 cm) were endoscopically inflamed. For CD patients in remission and the HC group, both biopsy locations (5 and 15 cm) appeared endoscopically normal. Endoscopic inflammation was evaluated using Rutgeerts score (27), whether the patients had been operated on by ICR or not, with inflammation defined as Rutgeerts score $\geq$1. One pair of mucosal pinch biopsies from the 5- and 15-cm locations were put on formalin for histological grading of inflammation. The two remaining biopsy pairs were put directly on liquid N_2 and stored on N_2 until subsequent bacterial or fungal DNA isolation and sequencing of the bacterial (24) and fungal microbiota, respectively.

Histological evaluation of biopsies

Formalin-fixed biopsies were stained with hematoxylin and eosin (H&E). Histological examination was performed blinded for phenotype by an experienced pathologist and scored according to the Global Histologic Disease Activity Score (GHAS) and Robarts score (3, 28, 29). A validated histological scoring index for the evaluation of disease activity in CD is lacking, and the reciprocity between histological scoring and disease activity measures is poor (29, 30). However, histological evaluation blinded for phenotype verified all biopsies from HC as histologically normal.

DNA isolation

The fungal cell wall is particularly robust and is known to be hard to lyse (31, 32). A DNA isolation protocol specially designed to lyse the fungal cell wall, with both a chemical and mechanical lysis step, was therefore chosen. DNA from two mucosal biopsies (at 5 and 15 cm locations) was isolated according to a previously described protocol (33) with the following adjustments; bead beating was performed with Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6,500 rpm for 60 s two times. Centrifugation steps were performed at 21,000 g, otherwise, the original protocol was followed (33). The DNA samples were quantified using Qubit (Thermo Fisher Scientific, Waltham, MA).

ITS2 sequencing

ITS2 metagenomic sequencing libraries were prepared according to the "Illumina Metagenomics Sequencing Demonstrated Protocol" (34) with minor adjustments. In brief, 200 ng genomic DNA (extracted from biopsy samples) was used as a template for PCR amplification of the ITS2 region (98°C at 30 s, followed by 34 cycles with 15 s at 98°C, 53°C for 30 s, and 72°C for 45 s, followed by 7 min at 72°C). The ITS2 PCR primers were based on sequences first published by Liguori et al. (16). Illumina adaptor-compatible overhang nucleotide sequences were added to the gene/locus-specific sequences (ITS2 Amplicon PCR Forward Primer = 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCGAATCTTT and ITS2 Amplicon PCR Reverse Primer = 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATATGCTTAAGTTCAGCGGGT). The PCR products were then cleaned up by using AMPure XP beads (Beckman Coulter, Woerden, Netherlands) to purify ITS2 amplicons away from free primers and primer dimer species. In a second PCR amplification step (9 cycles), dual indices and Illumina sequencing adaptors were added by using the Nextera XT indexing kit (Illumina Inc., San Diego, CA) according to the manufacturer's instructions. A second PCR clean-up step was performed using AMPure XP beads (Beckman Coulter), before the validation of the library by a LabChip GX DNA

high sensitivity assay (PerkinElmer, Inc., Waltham, MA). Libraries were normalized and pooled to 12 pM and subjected to clustering on two MiSeq V3 flowcells. Finally, paired-end read sequencing was performed for 2 x 300 cycles on a MiSeq instrument (Illumina, Inc.), according to the manufacturer's instructions. Base calling was done on the MiSeq instrument by RTA v1.18.54. FASTQ files were generated using bcl2fastq2 conversion software v2.17 (Illumina, Inc.).

Bioinformatics

Sequencing data were processed using the FROGS pipeline (35, 36) for sequence quality control, filtering, and affiliation of taxa with the UNITE ITS database (version 8_2) (37), using the FROGS guidelines for ITS data (http://frogs.toulouse.inra.fr/). Five biopsy samples were removed from the study due to a low number of sequences. This included two 5-cm samples from HC and three 5-cm samples from CD patients. Phyloseq Package for R analysis was used for alpha and beta diversity analyses as well as illustration. Deseq2 package for R analysis was used for differential analysis of OTUs with respect to the different phenotypes (38). The linear discriminant analysis (LDA) effect size (LEfSe) algorithm (39) was used to identify taxa that were specific to phenotype or inflamed vs. proximal non-inflamed mucosa.

Statistics

IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY) was used for statistical analysis apart from analyses of sequencing data. Demographic and clinical characteristics are presented as % (n) for categorical variables, median [interquartile range (IQR)] for skewed distributed variables, and mean value [standard deviation (SD)] for normally distributed variables. Accordingly, the chi-squared test, Mann–Whitney *U* test, or independent *t*-test were used for comparing CD patients with HC. For all statistical analyses, a p-value < 0.05 was considered statistically significant.

Ethical considerations

The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference, 2016/2164). All study participants provided written informed consent.

TABLE 1 Demographic and clinical characteristics of Crohn's disease (CD) patients and healthy controls (HC).

	CD	HC	p-value[a]
Number of patients, *n*	44	40	
Male gender, *n* (%)	24 (54.5%)	19 (47.5%)	0.52
Age, *years*, mean (SD)	42.2 (14.4)	36.6 (12.9)	0.07
BMI, mean (SD)	25.8 (4.8)	26.6 (4.7)	0.40
Acid reflux medication, *n* (%)			0.72
PPI	5 (11.4%)	2 (5%)	
H_2 blockers	0	0	
PPI on demand	0	0	
H_2 blockers on demand	1 (2.3%)	1 (2.5%)	
Smoking, *n* (%)			0.57
Never smoker	23 (52.3%)	25 (62.5%)	
Active smoker	5 (11.4%)	5 (12.5%)	
Snuff	10 (22.7%)	8 (20%)	
Ex-smoker	6 (13.6%) %)	2 (5%)	
Laboratory values			
Hb (g/dL), mean (SD)	14.1 (1.5)	14.5 (1.7)	0.197
Leukocytes ($x10^9$/L), median (IQR)	6.4 (2.3)	6.5 (2.3)	0.50
CRP (mg/L), median (IQR)	<5 (4)	<5 (0)	**0.017**

[a]Comparing CD ($n = 44$) with HC ($n = 40$) using Mann–Whitney *U*-test for skewed distributed continuous variables, independent *t*-test for normal distributed continuous variables, and Chi-square/Fisher exact test for categorical variables.

Results

Patients

Forty-four CD patients and 40 HC were included. Demographic and clinical characteristics are presented in Table 1. CD patients had higher CRP levels compared to HC ($p = 0.017$). The groups were otherwise similar. The bacterial microbiota characteristics in this cohort have been described previously (24). CD characteristics are provided in Table 2. Twenty-two CD patients had terminal ileitis with endoscopic inflammation at the 5-cm location and normal mucosa at the 15-cm location, of which 20 had no history of upper gastrointestinal CD involvement. Of the remaining CD patients, 10 had active disease and 12 were in remission.

Ileal mycobiota in CD patients vs. HC

CD patients had a lower fungal alpha diversity compared to HC based on the Simpson diversity index ($p = 0.025$), whereas the observed numbers of operational taxonomic units (OTUs) were similar ($p = 0.21$). This implies that the fungal species richness was similar, but that the evenness

10.3389/fmed.2022.868812

TABLE 2 Crohn's disease (CD) characteristics, medical treatment, endoscopic evaluation, and surgical history.

CD characteristics	CD ($n = 44$)
Disease duration, *years (median, IQR)*	10.0 (19.8)
Subclassification of patients, *n* (%)[a]	
Terminal ileitis (Inflamed 5-cm + normal 15-cm)	22 (50.0%)
Active disease (Inflamed 5-cm + 15-cm)	10 (22.7%)
Remission (Normal 5 + 15 cm)	12 (27.3%)
Montreal location, *n* (%)	
Terminal ileum (L1)	23 (52.3%)
Ileocolonic (L3)	16 (36.4%)
Ileocolonic + Upper GI (L3 + L4)	5 (11.4%)
Montreal behavior, *n* (%)	
Non-stricturing, non-penetrating (B1)	8 (18.2%)
Non-stricturing, non-penetrating + perianal (B1p)	2 (4.5%)
Stricturing (B2)	15 (34.1%)
Stricturing + perianal (B2p)	6 (13.6%)
Penetrating (B3)	11 (25%)
Penetrating + perianal (B3p)	2 (4.5%)
Montreal age (age at diagnosis), *n* (%)	
16 years or younger (A1)	12 (27.3%)
17–40 years (A2)	22 (50%)
Over 40 years (A3)	10 (22.7%)
CD-medication, *n* (%)[b]	
No medical therapy for CD	18 (40.9%)
Budesonide	7 (15.9%)
Prednisolone	4 (9.1%)
5-ASA	3 (6.8%)
Azathioprine	6 (13.6%)
Methotrexate	3 (6.8%)
Adalimumab	4 (9.1%)
Infliximab	7 (15.9%)
Vedolizumab	1 (2.3%)
Treatment naïve, *n (%)*	6 (13.6%)
Anti-TNF treatment naïve, *n (%)*	23 (52.3%)
Rutgeerts score, *n* (%)	
i0	12 (27.3%)
i1	12 (27.3%)
i2	5 (11.4%)
i3	6 (13.6%)
i4	9 (20.5%)
Ileocecal resection	28 (63.6%)

[a]Based on endoscopic evaluation of inflammation.
[b]Co-medication: $n = 8$ (18.2) used two CD medications, $n = 1$ (2.3%) used three CD medications.

of fungi was reduced within the CD group compared to HC (Figure 1A). The most prevalent phyla in the samples overall were Ascomycota, Basidiomycota, and Chytridiomycota, and some Rozellomycota were also detected (Figure 1C). In CD patients, the Basidiomycota-to-Ascomycota ratio was increased compared to HC (Supplementary Figure 1A). CD patients also had lower abundances of Chytridiomycota phyla (Supplementary Figure 1A). Beta diversity analysis assessed by Bray–Curtis dissimilarity showed a clustering of the samples according to the disease status ($p < 0.001$), confirming structural differences in the mycobiota composition between CD patients and HC (Figure 1B). Using LEfSe (39), fungal composition in CD patients and HC were compared and differentially abundant fungi were identified (Figure 2). *Malassezia* and *Vishniacozyma* genera were increased in CD patients, while *Saccharomyces*, *Paludomyces,* and *Oculimacula* were depleted in comparison to HC (Figure 2A and Supplementary Figure 1B). When the comparison was performed at the species level, CD patients had increased abundances of *Malassezia restricta* as well as *Malassezia sympodialis* and two other *Malassezia* species (Figure 2B). *Candida albicans and Vishniacozyma victoriae* were also increased in CD patients (Figure 2B). In HC, *Trichosporon asahii*, *Paludomyces mangrovei,* and a species from the Chaetomiaceae family were overrepresented compared to CD patients.

Mycobiota in the inflamed and proximal non-inflamed ileum in CD patients ($n = 20$)

Twenty CD patients had terminal ileitis with an inflamed 5-cm location and a non-inflamed 15-cm location, and no history of upper CD involvement. These patients were analyzed separately. Fungal alpha diversity did not differ between the distal inflamed 5-cm and proximal non-inflamed 15-cm locations in CD patients with terminal ileitis, based on observed OTUs and Simpson index (Figure 3A). Interestingly, on the beta diversity plot assessed by the Jaccard index, which focuses more on low abundant OTUs in comparison to Bray–Curtis dissimilarity, inflamed 5-cm samples clustered furthest away from HC with non-inflamed CD 15-cm samples in an intermediate location (Figure 3B), suggesting a more dysbiotic fungal composition in the distal inflamed ileum. In a beta diversity plot including only CD patients with terminal ileitis, 5- and 15-cm samples were separated clearly ($p < 0.05$) according to the Jaccard index (Figure 3C). When we compared the fungal composition in inflamed 5-cm samples with non-inflamed 15-cm samples using LEfSe, we identified six taxa that were increased at the 5-cm location and four taxa that were increased at the 15-cm location (Figure 4). Cordycipitaceae and Sporidiobolaceae families and *Lecanicillium* genus were overrepresented at the inflamed 5-cm location, whereas *Exophiala* and *Debaryomyces* genera were overrepresented at the non-inflamed 15-cm location. Differentially abundant species were identified using LEfSe

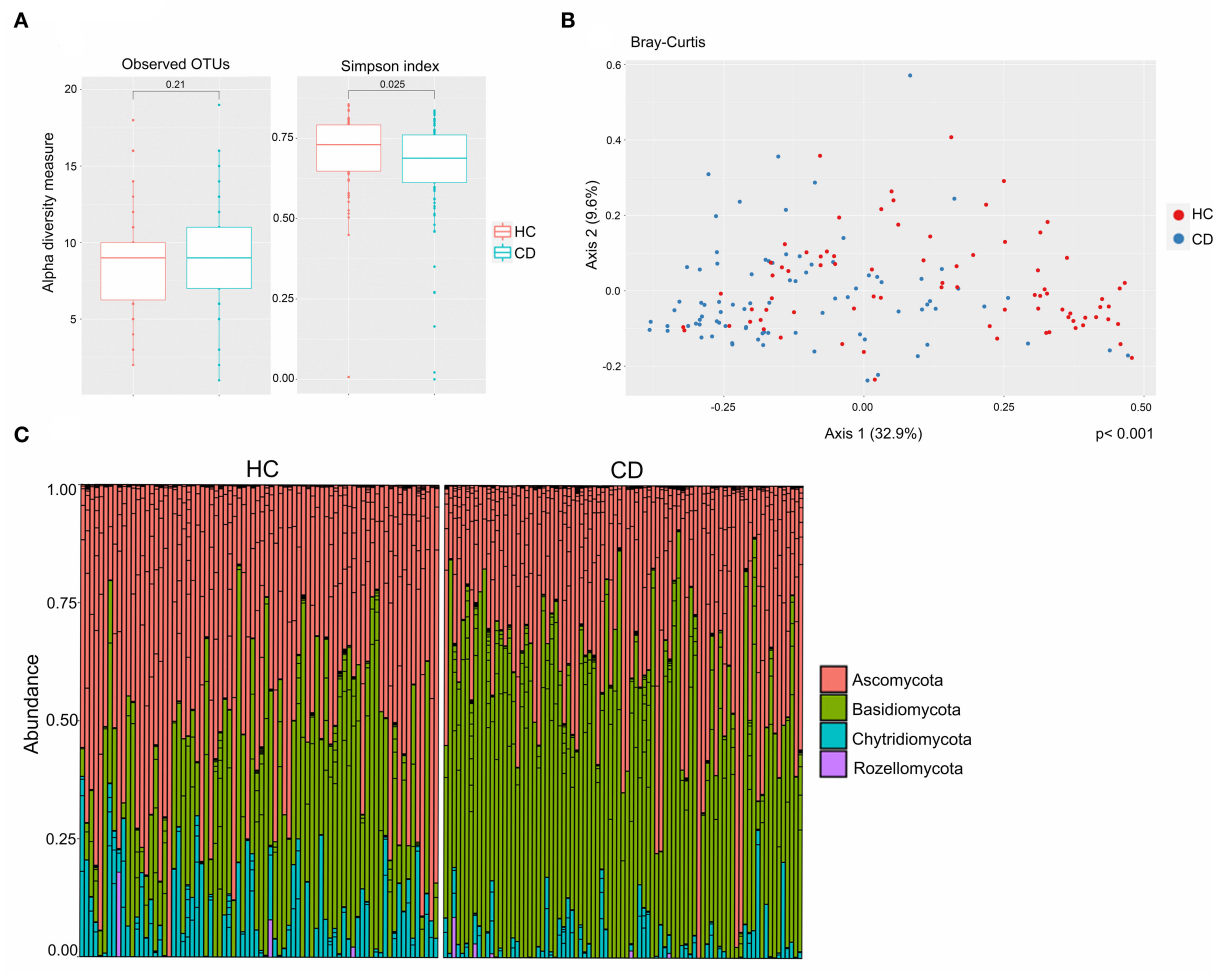


FIGURE 1
The mucosa-associated mycobiota in Crohn's disease (CD) patients ($n = 44$) was altered in comparison to healthy controls (HC) ($n = 40$). **(A)** Alpha-diversity, according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots colored according to disease phenotype (HC = red, CD = blue). **(B)** Beta-diversity. Principal coordinates analysis of Bray–Curtis dissimilarity with samples colored according to study group, (CD = blue and HC = red). The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups were compared using the Permanova method. **(C)** Relative abundance of fungal phyla in HC and CD patients.

are presented in a heatmap (Figure 4B). *Candida sake* was overrepresented at the inflamed 5-cm location. The *Exophiala* and *Debaryomyces* genera, which were increased at the non-inflamed 15-cm location, were identified as *Exophiala equina and Debaryomyces hansenii* (Figure 4B).

Mycobiota associated with the need for escalation of CD treatment

CD patients were stratified by their need for treatment escalation within the first year after biopsy collection and the mycobiota was compared across this variable. Seventeen of 44 patients received additional anti-inflammatory treatment. Of those 17 patients, 15 received escalation of anti-inflammatory medical treatment, while two patients underwent surgery (Supplementary Table 1). There was no difference in alpha diversity based on observed OTUs and Simpson index (Figure 5A). Beta diversity assessed by Bray–Curtis dissimilarity (p =0.082) and Jaccard index (p =0.051) did not differ significantly between CD patients with and without the need for treatment escalation (Figures 5B,C). In a differential analysis using LefSE, we found fungi at several taxonomic levels to be more abundant in CD patients needing treatment escalation within the first year after sampling (Figure 5D). These fungal taxa can possibly be predictive of poor prognosis in CD patients. In particular, the Malasseziaceae family and

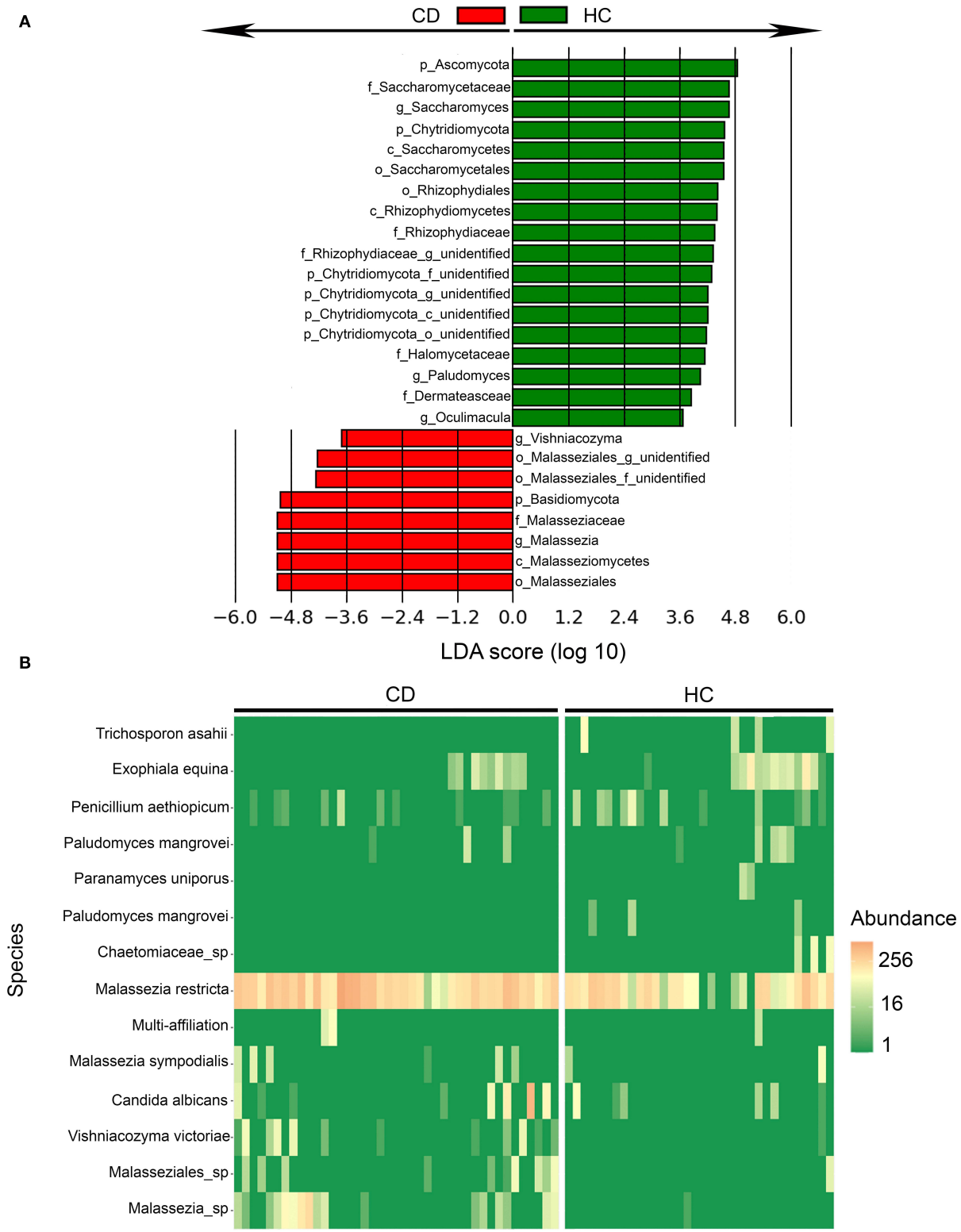


FIGURE 2
Fungal taxa were differentially abundant in Crohn's disease (CD) patients ($n = 44$) in comparison to healthy controls (HC) ($n = 40$) identified by linear discriminant analysis effect size (LEfSe). **(A)** Fungal taxa overrepresented in CD patients (red) and HC (green) with Linear Discriminant Analysis (LDA) score for differentially abundant fungal taxa. **(B)** Heatmap of differentially abundant fungal species between CD and HC mucosal pinch biopsies sampled 5-cm proximal of the ileocecal valve or ileocolic anastomosis.

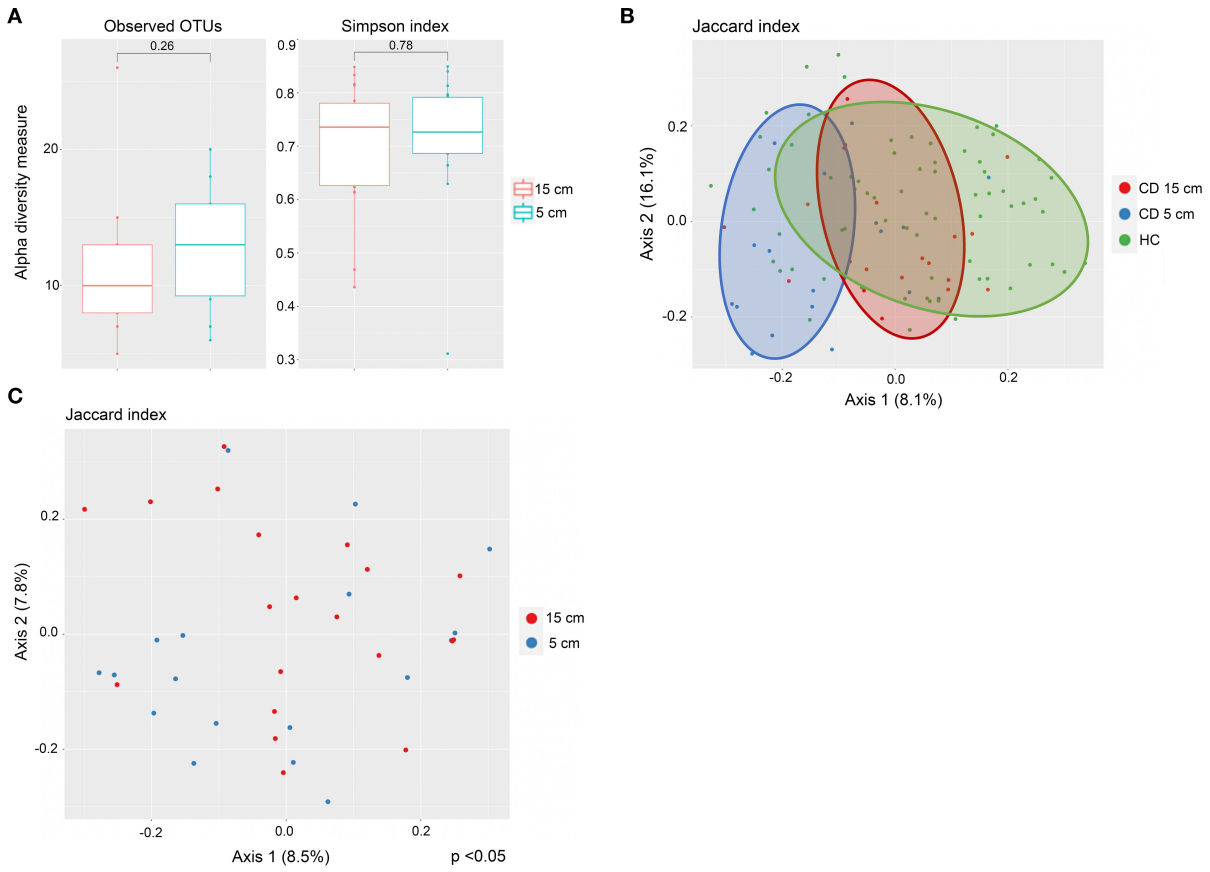


FIGURE 3
Fungal mycobiota in inflamed and proximally non-inflamed ileal mucosa in Crohn's disease (CD) patients without upper CD involvement (n = 20). Biopsies sampled at inflamed 5-cm and non-inflamed 15-cm proximal to the ileocecal valve or ileocolic anastomosis. **(A)** No differences in alpha diversity, according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots colored according to biopsy location (inflamed 5 cm = blue, non-inflamed 15 cm = red). **(B)** Mycobiota composition in inflamed terminal ileum (blue) and proximally non-inflamed ileum (red) of CD patients (n = 20) and in healthy controls (HC) (green) (n = 40). Principal coordinates analysis of Jaccard index with samples colored according to disease status (CD and HC) and ileal location. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. **(C)** Different mycobiota composition in inflamed (blue) and proximally non-inflamed ileum (red) according to beta diversity. Principal coordinates analysis of Jaccard index with samples colored according to ileal location. The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups were compared using the Permanova method.

Malassezia genus were more abundant in CD patients needing treatment escalation.

Effect of anti-TNF treatment on mycobiota

Eleven patients under current treatment with anti-TNF agents were compared to 18 patients with no current medical treatment for CD (Table 2). We found no differences in alpha- or beta diversities between these groups (Supplementary Figure 2). Interestingly, differential analysis with LefSE identified anti-TNF users to have higher abundances of Ascomycota and correspondingly lower abundances of Basidiomycota (Figure 6A), whereas in CD patients with no current treatment, the abundances of Basidiomycota (Figures 6A,B) was high. Correspondingly, the whole CD cohort had increased levels of Basidiomycota and reduced levels of Ascomycota compared to HC, as described earlier. When comparing anti-TNF naïve patients (never-users), n = 23, with ever-users (historically) of anti-TNF treatment, n = 21 (Table 2), we found a similar mycobiota composition according to both alpha diversity and beta diversity (Supplementary Figures 3A–C).

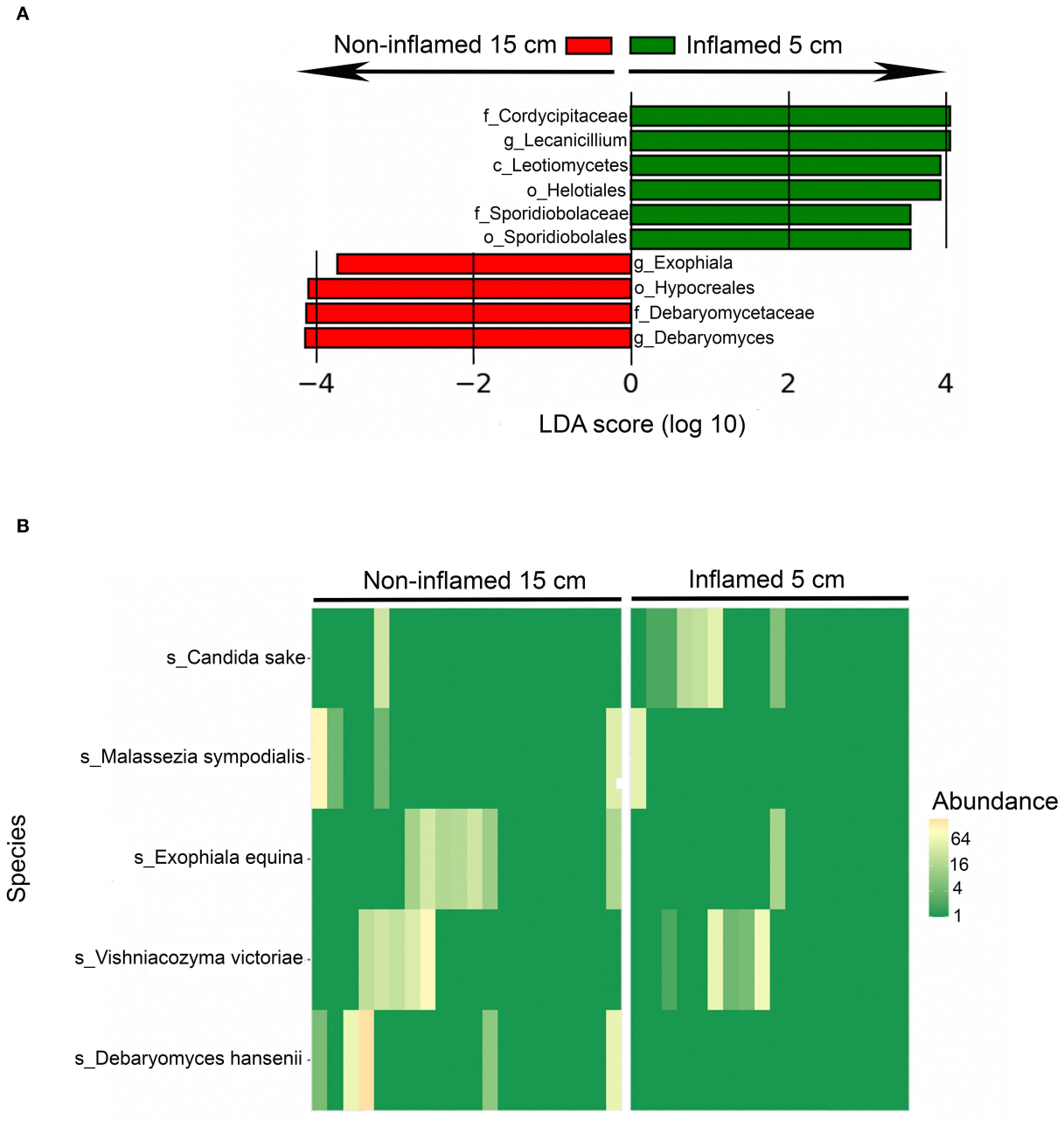


FIGURE 4
Differentially abundant fungal taxa between inflamed 5-cm vs. proximal non-inflamed 15-cm ileum of Crohn's disease (CD) patients ($n = 20$) with terminal ileitis and no history of upper CD involvement. Biopsies sampled from 5- and 15 cm proximal of the ileocecal valve or ileocolic anastomosis within the same patients. **(A)** Fungal taxa overrepresented in inflamed 5-cm biopsies (green) and non-inflamed 15-cm biopsies (red) in a histogram with Linear Discriminant Analysis (LDA) score computed using linear discriminant analysis effect size (LEfSe). **(B)** Heatmap showing the distribution of differentially abundant fungal species in inflamed 5-cm samples (right) and non-inflamed 15-cm samples (left) identified using LEfSe.

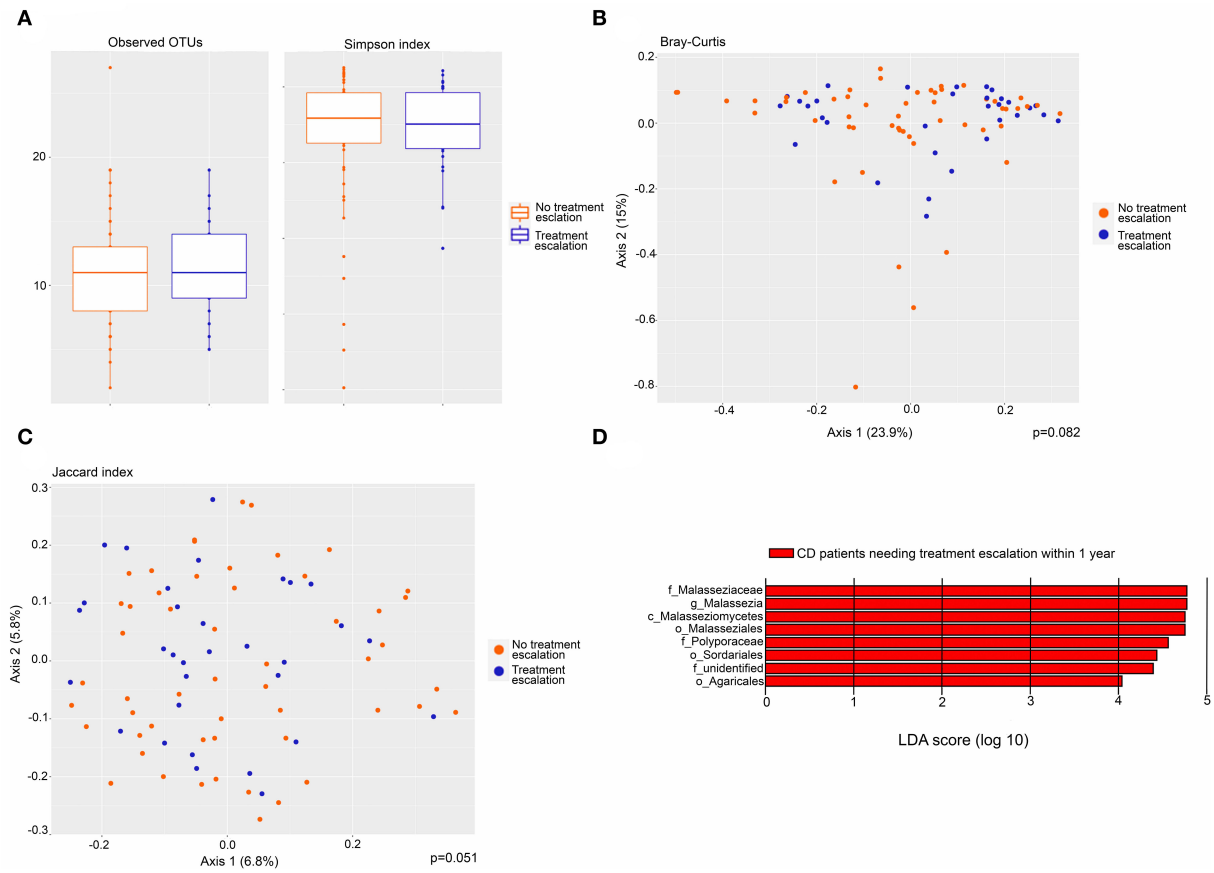


FIGURE 5
Fungal microbiota in CD patients needing treatment escalation ($n = 17$) within the first year after biopsy sampling compared to CD patients not needing treatment escalation ($n = 27$). **(A)** Fungal alpha-diversity, according to observed operational taxonomic units (OTUs) (left) and Simpson index (right), boxplots colored according to need for treatment escalation (blue) and no need for treatment escalation (red). **(B)** Beta-diversity. Principal coordinates analysis of Bray–Curtis dissimilarity with samples colored according to the need for treatment escalation (blue) and no need for treatment escalation (red). The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups were compared using the Permanova method. **(C)** Principal coordinates analysis of Jaccard index with samples colored according to need for treatment escalation (yes = blue, no = red). The fraction of diversity captured by the coordinate is given in percentage on axes 1 and 2. Groups were compared using the Permanova method. **(D)** Fungal taxa overrepresented in CD patients needing treatment escalation within 1 year compared to CD patients not needing treatment escalation, illustrated in a histogram with Linear Discriminant Analysis (LDA) score computed using linear discriminant analysis effect size (LEfSe).

Mycobiota according to ileal inflammation and sub-location in CD patients overall

Within the whole CD cohort ($n = 44$), inflammation (both endoscopic and histologic) was not associated with an altered mycobiota diversity or composition, based on observed OTUs, Simpson index, and Bray–Curtis dissimilarity (Supplementary Figure 4). This argues that endoscopic and histologic inflammation *per se* does not dominantly alter the fungal microbiota. Similarly, ileal location (5 vs. 15 cm) was not associated with an altered fungal microbiota diversity or composition in the CD cohort ($n = 44$) (Supplementary Figure 5). These findings imply that the altered fungal microbiota in CD terminal ileitis could not be explained by the effect of location or inflammation alone and is in accordance with our previous findings suggesting that mucosa-associated bacterial alterations in CD are also present across locations and independent of inflammation (24).

Discussion

This study investigated the mucosa-associated fungal microbiota in CD and compared the inflamed and proximal non-inflamed ileum within CD patients. Previous reports have found altered fungal composition in CD compared to HC (14, 16, 22, 23, 40). We found that the mucosa-associated mycobiota in the ileum of CD patients had reduced alpha diversity based on the Simpson index, but a similar number of observed OTUs compared to HC, implicating similar species

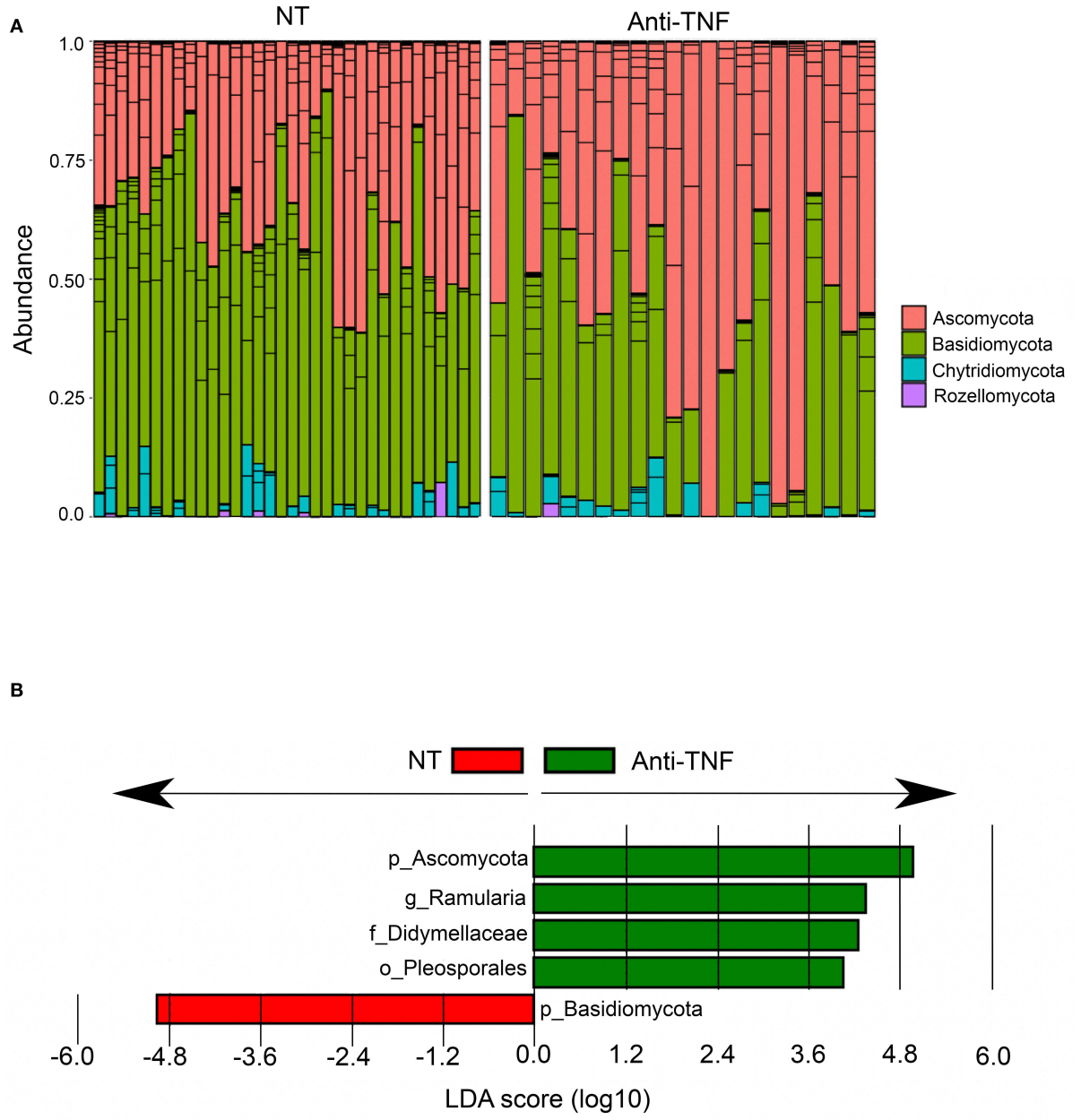


FIGURE 6
Differentially abundant fungal taxa between Crohn's disease (CD) patients currently using anti-TNF agents (Anti-TNF) ($n = 11$) compared to CD patients with no medical treatment (NT) ($n = 18$). **(A)** Relative abundance of fungal phyla in CD patients with no medical treatment (NT) and anti-TNF treated CD patients. **(B)** Fungal taxa overrepresented in anti-TNF-treated CD patients (green) and in CD patients with no medical treatment (NT) (red) illustrated in a histogram with Linear Discriminant Analysis (LDA) score computed using linear discriminant analysis effect size (LEfSe).

richness, but reduced evenness in CD patients. CD patients had an increased Basidiomycota-to-Ascomycota ratio as reported by others (14, 40), but also an altered mycobiota composition characterized by a significant gain of *Malassezia* and loss of *Saccharomyces*. At species level, *Malassezia* was identified as *Malassezia restricta* and *Malassezia sympodialis*. The expansion of *Malassezia restricta* in CD has been reported previously (14, 41), particularly in CD patients carrying the *CARD9* risk allele (14). Depletion of *Saccharomyces* has been described in feces from IBD patients, where *Saccharomyces* was positively

correlated with abundances of bacteria depleted in IBD, such as the butyrate-producing *Roseburia*, *Blautia,* and *Ruminococcus* genera (40, 42). Several *Saccharomyces* spp. have been suggested to have anti-inflammatory effects (40, 43–45).

We found increased abundance of *C. albicans* in the ileal mucosa of CD patients. *C. albicans* has been proposed to promote IBD by increasing the inflammatory response, and due to its increased abundance during inflammation, a vicious circle is created (45, 46). The fecal abundance of *Candida* before fecal microbiota transplantation (FMT) in UC patients has been associated with therapeutic response, and effects of FMT may be mediated by a reduction in *Candida* abundance (47). Increased abundances of *Candida albicans* and *Candida glabrata* have previously been found in fecal and colonic samples, respectively, from CD patients (16, 40, 48). To the best of our knowledge, this is the first study to confirm increased *Candida* in the ileal mucosa, thus supporting a clinical relevance. Interestingly, increased abundances of *Candida tropicalis* in fecal samples from CD patients have been positively correlated to ASCA concentrations (49). *Candida* is also extensively involved in bacterial interactions, demonstrating a significant influence on microbiome composition (45, 49). However, the reports show opposite effects depending on the experimental setup. Studies following the bacterial community reassembly after antibiotic treatment showed the influence of *C. albicans* on the bacterial diversity levels and possible influence on *Lachnospiraceae* colonization, a family with recognized positive effects on gut health (50, 51). In a mucosal model evaluating mouth and gut colonization, *C. albicans* triggered a dysbiosis characterized by a bloom of *Enterococcus* strains associated with increased epithelial permeability and susceptibility to invasive infections (52).

The terminal ileum is the predilection site for primary and recurrent CD and we, therefore, specifically analyzed the mucosa-associated mycobiota in the inflamed and proximal non-inflamed mucosa in patients with terminal ileitis. The alpha diversity in inflamed and proximal non-inflamed mucosa did not differ; however, a separation on beta diversity plots suggested an altered and more dysbiotic fungal composition in the inflamed ileum compared to proximal non-inflamed ileum and healthy mucosa of controls. *Lecanicillium* genera and *Candida sake* sp. were increased in the inflamed mucosa, whereas *Exophiala equina and Debaryomyces hansenii* were increased in the proximal non-inflamed mucosa. *C. sake* is frequently found in the feces of healthy humans (31), it can cause rare invasive candidemia (53) but have, however, not been associated with IBD to our knowledge. Indeed, *C. sake* is used as a biocontrol agent in the food industry to limit the decay of apples due to mold (54, 55). The literature on *E. equina* is scarce, but *E. equina* has been identified in subcutaneous abscesses with histologically granulomatous inflammation (56) and *Exophiala* has been associated with primary sclerosing cholangitis (57). *D. hansenii* is a commensal gut fungus that is found in feces of healthy adults and reported to be increased in feces of infants (31, 58), it is also frequently found in foods such as meat, fruit, cheese, beer, and wine (59). Jain et al. have recently reported *D. hansenii* to be enriched and completely dominant in inflamed compared to the non-inflamed ileum in 16 CD patients from two different cohorts (23), which is the opposite of our and Liguori et al. (16) findings. Jain et al. did not consider the relative location of inflamed and non-inflamed samples, and slight differences in DNA isolation protocols between studies could also affect results. However, *D. hansenii* was found to impair tissue healing in mice models, but intestinal damage was required for *D. hansenii* to have detrimental effect (23). The mentioned differences between studies could hypothetically be explained by the transfer of *D. hansenii* from the proximal non-inflamed mucosa to the ileum downstream and reduce wound healing, consistent with early descriptions of a beneficial effect of fecal stream diversion (2, 3).

Interestingly, we found that increased abundance of *Malassezia* genus with corresponding increases of Malasseziaceae at the family level, Malasseziomycetes class, and Malasseziales order were associated with the need for treatment escalation within 1-year follow-up. The association suggests that *Malassezia* does not only characterize CD in our and other patient cohorts (14) but it may also affect the disease course and represent a poor prognostic factor. Larger prospective studies to examine this observation would be of great interest.

Anti-TNF treatment was also associated with alterations of the fungal microbiota. CD patients on current anti-TNF treatment had lower abundances of Basidiomycota compared to CD patients not receiving medical treatment. CD patients in general have an increased Basidiomycota-to-Ascomycota ratio compared to HC, as reported in this cohort as well as by others (14, 40). A recent study investigating the bacterial and fungal communities in fecal samples before and after initiation of anti-TNF treatment found that both fungal and bacterial microbiota composition differed between anti-TNF-responders and non-responders (60), arguing that the microbiome composition is relevant for pharmacological therapy. Anti-TNF treatment could modulate the mycobiota in a potentially beneficial direction since it reversed the fungal community toward a lower Basidiomycota-to-Ascomycota ratio which is found in healthy subjects. However, the finding should be verified in larger cohorts, and the mechanisms by which this occurs need to be evaluated.

Neither endoscopic nor histologic inflammation was associated with an increased number of OTUs or altered mycobiota according to other alpha- or beta-diversity measures. On the contrary, fungal richness and diversity have previously been found to be increased in inflamed vs. non-inflamed mucosa assessed by PCR and Denaturing

Gel Gradient Electrophoresis; however, the method is less sensitive in terms of taxa identification and diversity measures compared to ITS-sequencing (61). We found that ileal sub-location seems to neither impact mycobiota diversity nor its composition. Finally, patients using anti-TNF agents did not have a mycobiome that differed from CD patients not receiving any anti-inflammatory treatment. This observation strengthens the hypothesis that altered ileal mycobiome in CD patients is related to the disease *per se*.

The role of fungi in IBD has been described and acknowledged (62), consequently the fungal microbiome is a potential therapeutic target. Factors known to affect the mycobiome include diet, antibacterial and antifungal agents, and gut bacteria (9, 45, 49, 63, 64). The risk of CD was associated with cumulative antibiotic exposure in a Swedish national cohort (65). Antibacterial therapy increases fungal abundances in fecal samples (64, 66), suggesting that fungi could mediate the increased risk of CD after exposure to antibacterial agents (46). Ingestion of meat, eggs, and cheeses seems to increase the fecal fungal load compared to vegetarian food (67), and correspondingly, fiber and fruit reduce the risk of CD in epidemiological studies (68). It has recently been found that a proportion of secretory IgA (sIgA), which have an important gut barrier function, is induced by and directed toward intestinal fungi (69). The production of sIgAs that target and coat certain fungi may be dysregulated in CD. The previously recognized risk factors and prognostic factors for IBD could be mediated by alterations of intestinal fungi. Food that either contains fungi or otherwise alters the intestinal composition of fungi could be of importance (63, 67), but considering the complex interactions between bacteria and fungi, drugs that alter microbial composition including anti-bacterial agents and proton-pump inhibitors could also be implicated (70). Before establishing treatment strategies aiming to maintain or restore a health-promoting mycobiome, prospective and interventional studies with careful monitoring of intestinal fungi are needed. Given the widespread interest in microbiota research, it would also be valuable if the numerous studies of fecal microbiota transplantation also included sequencing of not only bacteria but also fungi and viruses. Oral anti-fungal agents have the potential to reduce inflammation in IBD, and this approach should be explored further (71).

There are several challenges within the field of mycobiota research. Several methodological steps harbor a potential for variation which can impact reported mycobiota composition. This includes the method of sample collection which varies from endoscopic biopsies (22), endoscopic water-lavage samples (14), surgical samples (23), or even a combination (16). Furthermore, the storage of samples, DNA isolation protocol, choice of primer (ITS1 or ITS2), sequencing protocol, and bioinformatic pipeline vary.

Strengths of the study include analysis of the mucosa-associated microbiota, which represent only a section of the entire gut microbiota, but due to its location interacts with the host cells and the immune system, which provides original data compared to the most common studies on fecal composition. Additionally, the mucosa-associated mycobiota was assessed in the highest number of CD patients to date (14, 16, 22, 23), and the sequencing analysis of high quality and the majority of sequences were taxonomically classified. Furthermore, the clinical course was followed for 1 year after biopsy collection. Limitations include the observational study design, heterogeneity of the CD duration, and previous and current medical and surgical treatment that may affect the mycobiota. We have not correlated the bacterial and fungal microbiota, and interactions between bacteria and fungi occurring have not been assessed.

In conclusion, this relatively large study describes the mucosa-associated mycobiota in the inflamed and proximally non-inflamed ileum in CD patients and confirms several alterations found in other cohorts. We have also identified fungal taxa which are associated with the need for treatment escalation in CD. The mycobiota composition in the inflamed ileum and proximal non-inflamed ileum differ and may play a role in CD pathogenesis.

Data availability statement

The sequencing data underlying this article are available at NCBI with accession number PRJNA850908.

Ethics statement

The studies involving human participants were reviewed and approved by Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference, 2016/2164). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MO, TM, and RF were responsible for the study design. MO and RF were responsible for data acquisition. MO, MR, OR, VB, AG, and ER were responsible for analyses of biological material and data analysis. MO, MR, and RF interpreted the results and drafted the manuscript. MO, MR, VB, AG, ER, TM, AS, HS, and RF contributed to the critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by the Liaison Committee between Central Norway Regional Health Authority (RHA), the Norwegian University of Science and Technology (NTNU) (2016/29014), and by St. Olav's Hospital, Trondheim University Hospital. The GCF is funded by the Faculty of Medicine and Health Sciences at NTNU and Central Norway Regional Health Authority.

Acknowledgments

We would sincerely thank all our patients for participating in the study. We would like to thank Bjørn Munkvold for the preparation of histological sections. The 16S library prep and sequencing were performed in close collaboration with the Genomics Core Facility (GCF), Norwegian University of Science and Technology (NTNU).

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/fmed.2022.868812/full#supplementary-material

References

1. Torres J, Mehandru S, Colombel JF, Peyrin-Biroulet L. Crohn's Disease. *Lancet.* (2017) 389:1741–55. doi: 10.1016/S0140-6736(16)31711-1

2. Rutgeerts P, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R, et al. Effect of faecal stream diversion on recurrence of crohn's disease in the neoterminal ileum. *Lancet.* (1991) 338:771–4. doi: 10.1016/0140-6736(91)90663-A

3. D'Haens GR, Geboes K, Peeters M, Baert F, Penninckx F, Rutgeerts P. Early lesions of recurrent crohn's disease caused by infusion of intestinal contents in excluded ileum. *Gastroenterology.* (1998) 114:262–7. doi: 10.1016/S0016-5085(98)70476-7

4. Fornaro R, Caratto E, Caratto M, Fornaro F, Caristo G, Frascio M, et al. Post-operative recurrence in crohn's disease. Critical analysis of potential risk factors: an update. *Surgeon.* (2015) 13:330–47. doi: 10.1016/j.surge.2015.04.002

5. Rutgeerts P, Geboes K, Vantrappen G, Kerremans R, Coenegrachts JL, Coremans G. Natural history of recurrent crohn's disease at the ileocolonic anastomosis after curative surgery. *Gut.* (1984) 25:665–72. doi: 10.1136/gut.25.6.665

6. Sokol H, Brot L, Stefanescu C, Auzolle C, Barnich N, Buisson A, et al. Prominence of ileal mucosa-associated microbiota to predict postoperative endoscopic recurrence in crohn's disease. *Gut.* (2019) 69:462–72. doi: 10.1136/gutjnl-2019-318719

7. Yilmaz B, Juillerat P, Oyas O, Ramon C, Bravo FD, Franc Y, et al. Microbial network disturbances in relapsing refractory crohn's disease. *Nat Med.* (2019) 25:323–36. doi: 10.1038/s41591-018-0308-z

8. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of crohn disease patients. *Proc Natl Acad Sci U S A.* (2008) 105:16731–6. doi: 10.1073/pnas.0804812105

9. Mukherjee PK, Sendid B, Hoarau G, Colombel JF, Poulain D, Ghannoum MA. Mycobiota in gastrointestinal diseases. *Nat Rev Gastroenterol Hepatol.* (2015) 12:77–87. doi: 10.1038/nrgastro.2014.188

10. Main J, McKenzie H, Yeaman GR, Kerr MA, Robson D, Pennington CR, et al. Antibody to saccharomyces cerevisiae (bakers' yeast) in Crohn's disease. *Bmj.* (1988) 297:1105–6. doi: 10.1136/bmj.297.6656.1105

11. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature.* (2012) 491:119–24. doi: 10.1038/nature11582

12. Rivas MA, Beaudoin M, Gardet A, Stevens C, Sharma Y, Zhang CK, et al. Deep resequencing of gwas loci identifies independent rare variants associated with inflammatory bowel disease. *Nat Genet.* (2011) 43:1066–73. doi: 10.1038/ng.952

13. Glocker EO, Hennigs A, Nabavi M, Schäffer AA, Woellner C, Salzer U, et al. A homozygous card9 mutation in a family with susceptibility to fungal infections. *N Engl J Med.* (2009) 361:1727–35. doi: 10.1056/NEJMoa0810719

14. Limon JJ, Tang J, Li D, Wolf AJ, Michelsen KS, Funari V, et al. Malassezia is associated with crohn's disease and exacerbates colitis in mouse models. *Cell Host Microbe.* (2019) 25:377–88.e6. doi: 10.1016/j.chom.2019.01.007

15. Underhill D, Braun J. Current understanding of fungal microflora in inflammatory bowel disease pathogenesis. *Inflamm Bowel Dis.* (2008) 14:1147–53. doi: 10.1002/ibd.20402

16. Liguori G, Lamas B, Richard ML, Brandi G, da Costa G, Hoffmann TW, et al. Fungal dysbiosis in mucosa-associated microbiota of crohn's disease patients. *J Crohns Colitis.* (2016) 10:296–305. doi: 10.1093/ecco-jcc/jjv209

17. Ott SJ, Kuhbacher T, Musfeldt M, Rosenstiel P, Hellmig S, Rehman A, et al. Fungi and inflammatory bowel diseases: alterations of composition and diversity. *Scand J Gastroenterol.* (2008) 43:831–41. doi: 10.1080/00365520801935434

18. Zmora N, Zilberman-Schapira G, Suez J, Mor U, Dori-Bachash M, Bashiardes S, et al. Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell.* (2018) 174:1388–405.e21. doi: 10.1016/j.cell.2018.08.041

19. Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset crohn's disease. *Cell Host Microbe.* (2014) 15:382–92. doi: 10.1016/j.chom.2014.02.005

20. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, et al. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* (2012) 13:R79. doi: 10.1186/gb-2012-13-9-r79

21. Pittayanon R, Lau JT, Leontiadis GI, Tse F, Yuan Y, Surette M, et al. Differences in gut microbiota in patients with vs without

inflammatory bowel diseases: a systematic review. *Gastroenterology.* (2020) 158:930–46.e1. doi: 10.1053/j.gastro.2019.11.294

22. El Mouzan M, Wang F, Al Mofarreh M, Menon R, Al Barrag A, Korolev KS, et al. Fungal microbiota profile in newly diagnosed treatment-naive children with crohn's disease. *J Crohns Colitis.* (2017) 11:586–92. doi: 10.1093/ecco-jcc/jjw197

23. Jain U, Ver Heul AM, Xiong S, Gregory MH, Demers EG, Kern JT, et al. Debaryomyces is enriched in crohn's disease intestinal tissue and impairs healing in mice. *Science.* (2021) 371:1154–9. doi: 10.1126/science.abd0919

24. Olaisen M, Flatberg A, Granlund AVB, Røyset ES, Martinsen TC, Sandvik AK, et al. Bacterial mucosa-associated microbiome in inflamed and proximal noninflamed ileum of patients with crohn's disease. *Inflamm Bowel Dis.* (2020) 27:12–24. doi: 10.1093/ibd/izaa107

25. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a working party of the 2005 montreal world congress of gastroenterology. *Can J Gastroenterol.* (2005) 19:5a—36a. doi: 10.1155/2005/269076

26. Mearin F, Lacy BE, Chang L, Chey WD, Lembo AJ, Simren M, et al. Bowel disorders. *Gastroenterology.* (2016) 150:1393–407. doi: 10.1053/j.gastro.2016.02.031

27. Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of crohn's disease. *Gastroenterology.* (1990) 99:956–63. doi: 10.1016/0016-5085(90)90613-6

28. Mosli MH, Feagan BG, Zou G, Sandborn WJ, D'Haens G, Khanna R, et al. Development and validation of a histological index for Uc. *Gut.* (2017) 66:50–8. doi: 10.1136/gutjnl-2015-310393

29. Novak G, Parker CE, Pai RK, MacDonald JK, Feagan BG, Sandborn WJ, et al. Histologic scoring indices for evaluation of disease activity in crohn's disease. *Cochrane Database Syst Rev.* (2017) 7:Cd012351. doi: 10.1002/14651858.CD012351.pub2

30. Sandborn WJ, Feagan BG, Hanauer SB, Lochs H, Lofberg R, Modigliani R, et al. A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with crohn's disease. *Gastroenterology.* (2002) 122:512–30. doi: 10.1053/gast.2002.31072

31. Nash AK, Auchtung TA, Wong MC, Smith DP, Gesell JR, Ross MC, et al. The gut mycobiome of the human microbiome project healthy cohort. *Microbiome.* (2017) 5:153. doi: 10.1186/s40168-017-0373-4

32. Gow NAR, Latge JP, Munro CA. The fungal cell wall: structure, biosynthesis, and function. *Microbiol Spectr.* (2017) 5:5. doi: 10.1128/microbiolspec.FUNK-0035-2016

33. Tang J, Iliev ID, Brown J, Underhill DM, Funari VA. Mycobiome: approaches to analysis of intestinal fungi. *J Immunol Methods.* (2015) 421:112–21. doi: 10.1016/j.jim.2015.04.004

34. Illumina. Fungal Metagenomic Sequencing Demonstrated Protocol (2019). Available online at: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/metagenomic/fungal-metagenomic-demonstrated-protocol-1000000064940-01.pdf (accessed September 17, 2022).

35. Frogs Pipeline. Available online at: http://frogs.toulouse.inra.fr (accessed September 17, 2022).

36. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, et al. Frogs: find, rapidly, otus with galaxy solution. *Bioinformatics.* (2018) 34:1287–94. doi: 10.1093/bioinformatics/btx791

37. Nilsson RH, Larsson KH, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, et al. The unite database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* (2019) 47:D259–d64. doi: 10.1093/nar/gky1022

38. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome Biol.* (2014) 15:550. doi: 10.1186/s13059-014-0550-8

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

40. Sokol H, Leducq V, Aschard H, Pham HP, Jegou S, Landman C, et al. Fungal microbiota dysbiosis in ibd. *Gut.* (2017) 66:1039–48. doi: 10.1136/gutjnl-2015-310746

41. Ha CWY, Martin A, Sepich-Poore GD, Shi B, Wang Y, Gouin K, et al. Translocation of viable gut microbiota to mesenteric adipose drives formation of creeping fat in humans. *Cell.* (2020) 183:666–83.e17. doi: 10.1016/j.cell.2020.09.009

42. Takahashi K, Nishida A, Fujimoto T, Fujii M, Shioya M, Imaeda H, et al. Reduced abundance of butyrate-producing bacteria species in the fecal microbial community in crohn's disease. *Digestion.* (2016) 93:59–65. doi: 10.1159/000441768

43. McIlroy J, Ianiro G, Mukhopadhya I, Hansen R, Hold GL. Review article: the gut microbiome in inflammatory bowel disease-avenues for microbial management. *Aliment Pharmacol Ther.* (2018) 47:26–42. doi: 10.1111/apt.14384

44. Guslandi M, Mezzi G, Sorghi M, Testoni PA. Saccharomyces boulardii in maintenance treatment of crohn's disease. *Dig Dis Sci.* (2000) 45:1462–4. doi: 10.1023/A:1005588911207

45. Richard ML, Sokol H. The gut mycobiota: insights into analysis, environmental interactions and role in gastrointestinal diseases. *Nat Rev Gastroenterol Hepatol.* (2019) 16:331–45. doi: 10.1038/s41575-019-0121-2

46. Sokol H. Antibiotics: a trigger for inflammatory bowel disease? *Lancet Gastroenterol Hepatol.* (2020) 5:956–7. doi: 10.1016/S2468-1253(20)30208-9

47. Leonardi I, Paramsothy S, Doron I, Semon A, Kaakoush NO, Clemente JC, et al. Fungal trans-kingdom dynamics linked to responsiveness to fecal microbiota transplantation (fmt) therapy in ulcerative colitis. *Cell Host Microbe.* (2020) 27:823–9.e3. doi: 10.1016/j.chom.2020.03.006

48. Standaert-Vitse A, Sendid B, Joossens M, François N, Vandewalle-El Khoury P, Branche J, et al. Candida albicans colonization and asca in familial crohn's disease. *Am J Gastroenterol.* (2009) 104:1745–53. doi: 10.1038/ajg.2009.225

49. Hoarau G, Mukherjee PK, Gower-Rousseau C, Hager C, Chandra J, Retuerto MA, et al. Bacteriome and mycobiome interactions underscore microbial dysbiosis in familial crohn's disease. *mBio.* (2016) 7:e01250–16. doi: 10.1128/mBio.01250-16

50. Mason KL, Erb Downward JR, Mason KD, Falkowski NR, Eaton KA, Kao JY, et al. Candida albicans and bacterial microbiota interactions in the cecum during recolonization following broad-spectrum antibiotic therapy. *Infect Immun.* (2012) 80:3371–80. doi: 10.1128/IAI.00449-12

51. Erb Downward JR, Falkowski NR, Mason KL, Muraglia R, Huffnagle GB. Modulation of post-antibiotic bacterial community reassembly and host response by candida albicans. *Sci Rep.* (2013) 3:2191. doi: 10.1038/srep02191

52. Bertolini M, Ranjan A, Thompson A, Diaz PI, Sobue T, Maas K, et al. Candida albicans induces mucosal bacterial dysbiosis that promotes invasive infection. *PLoS Pathog.* (2019) 15:e1007717. doi: 10.1371/journal.ppat.1007717

53. Juneja D, Borah AK, Nasa P, Singh O, Javeri Y, Dang R. Candida sake candidaemia in non-neutropenic critically ill patients: a case series. *Crit Care Resusc.* (2011) 13:187–91.

54. Nunes C, Usall J, Teixidó N, Viñas I. Improvement of candida sake biocontrol activity against post-harvest decay by the addition of ammonium molybdate. *J Appl Microbiol.* (2002) 92:927–35. doi: 10.1046/j.1365-2672.2002.01602.x

55. Morales H, Sanchis V, Usall J, Ramos AJ, Marín S. Effect of biocontrol agents candida sake and pantoea agglomerans on penicillium expansum growth and patulin accumulation in apples. *Int J Food Microbiol.* (2008) 122:61–7. doi: 10.1016/j.ijfoodmicro.2007.11.056

56. Najafzadeh MJ, Suh MK, Lee MH, Ha GY, Kim JR, Kim TH, et al. Subcutaneous phaeohyphomycosis caused by exophiala equina, with susceptibility to eight antifungal drugs. *J Med Microbiol.* (2013) 62:797–800. doi: 10.1099/jmm.0.057406-0

57. Lemoinne S, Kemgang A, Ben Belkacem K, Straube M, Jegou S, Corpechot C, et al. Fungi participate in the dysbiosis of gut microbiota in patients with primary sclerosing cholangitis. *Gut.* (2020) 69:92–102. doi: 10.1136/gutjnl-2018-317791

58. Schei K, Avershina E, Øien T, Rudi K, Follestad T, Salamati S, et al. Early gut mycobiota and mother-offspring transfer. *Microbiome.* (2017) 5:107. doi: 10.1186/s40168-017-0319-x

59. Breuer U, Harms H. Debaryomyces hansenii–an extremophilic yeast with biotechnological potential. *Yeast.* (2006) 23:415–37. doi: 10.1002/yea.1374

60. Ventin-Holmberg R, Eberl A, Saqib S, Korpela K, Virtanen S, Sipponen T, et al. Bacterial and fungal profiles as markers of infliximab drug response in inflammatory bowel disease. *J Crohns Colitis.* (2021) 15:1019–31. doi: 10.1093/ecco-jcc/jjaa252

61. Li Q, Wang C, Tang C, He Q, Li N, Li J. Dysbiosis of gut fungal microbiota is associated with mucosal inflammation in crohn's disease. *J Clin Gastroenterol.* (2014) 48:513–23. doi: 10.1097/MCG.0000000000000035

62. Iliev ID. Mycobiota-host immune interactions in ibd: coming out of the shadows. *Nat Rev Gastroenterol Hepatol.* (2022) 19:91–2. doi: 10.1038/s41575-021-00541-2

63. Hoffmann C, Dollive S, Grunberg S, Chen J, Li H, Wu GD, et al. Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PLoS ONE.* (2013) 8:e66019. doi: 10.1371/journal.pone.0066019

64. Lewis JD, Chen EZ, Baldassano RN, Otley AR, Griffiths AM, Lee D, et al. Inflammation, antibiotics, and diet as environmental stressors of the gut

microbiome in pediatric crohn's disease. *Cell Host Microbe*. (2015) 18:489–500. doi: 10.1016/j.chom.2015.09.008

65. Nguyen LH, Örtqvist AK, Cao Y, Simon TG, Roelstraete B, Song M, et al. Antibiotic use and the development of inflammatory bowel disease: a national case-control study in sweden. *Lancet Gastroenterol Hepatol*. (2020) 5:986–95. doi: 10.1016/S2468-1253(20)30267-3

66. Samonis G, Gikas A, Anaissie EJ, Vrenzos G, Maraki S, Tselentis Y, et al. Prospective evaluation of effects of broad-spectrum antibiotics on gastrointestinal yeast colonization of humans. *Antimicrob Agents Chemother*. (1993) 37:51–3. doi: 10.1128/AAC.37.1.51

67. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. (2014) 505:559–63. doi: 10.1038/nature12820

68. Piovani D, Danese S, Peyrin-Biroulet L, Nikolopoulos GK, Lytras T, Bonovas S. Environmental risk factors for inflammatory bowel diseases: an umbrella review of meta-analyses. *Gastroenterology*. (2019) 157:647–59.e4. doi: 10.1053/j.gastro.2019.04.016

69. Doron I, Mesko M, Li XV, Kusakabe T, Leonardi I, Shaw DG, et al. Mycobiota-induced iga antibodies regulate fungal commensalism in the gut and are dysregulated in crohn's disease. *Nat Microbiol*. (2021) 6:1493–504. doi: 10.1038/s41564-021-00983-z

70. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science*. (2016) 352:565–9. doi: 10.1126/science.aad3369

71. Jena A, Dutta U, Shah J, Sharma V, Prasad KK, Shivaprakash RM, et al. Oral fluconazole therapy in patients with active ulcerative colitis who have detectable candida in the stool: a double-blind randomized placebo-controlled trial. *J Clin Gastroenterol*. (2021) 56:705–11. doi: 10.1097/MCG.0000000000001609

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership