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Gut microbiota as a weapon against infections

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Gut microbiota as a weapon against infections

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Editorial: Gut microbiota as a weapon against infections

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Editorial on the Research Topic
Gut microbiota as a weapon against infections

Gut microbiota plays a dominant role in host defense against infections. The Research Topic entitled "*Gut microbiota as a weapon against infections*" in the *Intestinal Microbiome* section is centered on new perspectives, recent advances, and current challenges in microbiome and infection. This Research Topic is focused on the significant role and potential of gut microbiota in combating infections and improving overall human health. Seven original research articles and two reviews were published on this Research Topic.

Microbiota colonization during early life is crucial for infant health and immunity. Huang et al. aimed to understand the mechanisms of antibiotic-induced fungal infection in preterm infants. They found that broad-spectrum antibiotics may promote invasive fungi disease (IFD) in preterm rats. A decreased abundance of beneficial gut microbes such as Clostridium species and *Bacteroides* accompanies this IFD. This study highlights the importance of symbiotic microbiota, which may reduce the risk of IFD. Further, Cong et al. reviewed the potential of intestinal bacteria to combat invasive fungal infections. Here, they discussed related research studies and emphasis on the ability of intestinal commensal bacteria and probiotics to restrict the invasion of pathogenic fungi.

The 16S rRNA microbial profiling approach was employed by Lee et al. to determine the microbiota composition in *Clostridioides difficile* infections (CDI) patients after high-dose vitamin D supplementation. Interestingly, they found an elevated abundance of beneficial intestinal bacteria such as *Bifidobacteriaceae* and *Christensenellaceae*. However, a large cohort study is needed to establish the potential of vitamin D as replacement therapy in patients with CDI.

In the investigation by Zhang et al. the potential role of lactic acid bacteria (LAB) in preventing yak disease-related diarrhea was demonstrated. As an essential animal species on the plateau, Yak is an important source of livelihood and economy for herders. However, yak diarrhea, especially calf diarrhea, has brought substantial economic losses to residents. In this study, authors examine the probiotic potential of four species of lactic acid bacteria to prevent/treat yak diarrhea. The findings from this article can be accommodative to the meta-analysis work in the future and may formulate good probiotic clues. Chen et al. investigated how Cyprinid herpesvirus 2 (CyHV-2) infection impacts the gut microbiota of gibel carp. Their metabolome approach found a decrease in

microbiota-regulated metabolites upon infection which correlated with the changes in the bacterial community.

Fecal microbiota transplantation (FMT) is an effective treatment for gastrointestinal disorders and highlights the importance of healthy microbiota in treating diseases. Zou et al., research on pediatric Crohn's disease (CD) patients reported that FMT in conjugation with partial enteral nutrition could be used as a first-line treatment for active CD in children. They did not consider the gut flora variation in these patients, which warrants future prospective studies.

Better knowledge of the interplay between microbiota and drugs can help develop effective disease therapies to treat diseases. The study by Wan et al. evaluated the effect of the first-class hypoglycemic drug metformin on sepsis-related liver lung in aged rats. They highlighted in their study that metformin could alleviate inflammatory response and lung injury by reversing the imbalanced gut microbiota composition. These results provide a potential treatment for sepsis-associated acute lung injury (SALI) in aged rats with sepsis.

Another critical aspect to consider is the gut-lung axis, which indicates the crosstalk between the gut and lung that can impact infections such as tuberculosis (TB). Ye et al. cross-sectional study revealed that pulmonary tuberculosis patients exhibit significantly different microbiota from healthy controls. This finding suggests that altered gut microbiota might be the possible fundamental pathophysiology of pulmonary tuberculosis. Another interesting review article by Yu et al. summarized the current findings regarding tuberculosis and the gut microbiome. They have discussed studies related to the alteration of the gut microbiome in patients with pulmonary TB (PTB) and intestinal TB (ITB). They have focused on establishing *Mycobacterium tuberculosis* in the gastrointestinal tract and potential probiotics as well as postbiotics in treating ITB in PTB patients. These studies add to the knowledge of the gut-lung axis that can be a therapeutic target to improve TB infection.

In conclusion, our Research Topic provides some appreciable insights into the crucial role of gut microbiota in controlling bacterial and fungal infections. Thus, the published articles on this Research Topic will bring the opportunities to understand the potential role of commensal microbiota and their mechanisms to modulate host immunity, which could contribute to novel microbiota based therapeutics to treat infections.

Author contributions

SN: Conceptualization, Writing – original draft, Writing – review & editing. SP: Writing – original draft, Writing – review & editing.

Conflict of interest

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

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High Dose Intramuscular Vitamin D3 Supplementation Impacts the Gut Microbiota of Patients With *Clostridioides Difficile* Infection

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Background and Aim: Current therapeutic strategies for *Clostridioides difficile* infections (CDI), including oral vancomycin, metronidazole and fecal microbial transplantation, have limited efficacy and treatment failure may occur in as many as one- third of cases. Recent studies have reported that lower concentrations of 25-hydroxyvitamin D are associated with CDI severity and recurrence. However, there have been no studies on microbiota composition after the administration of vitamin D in patients with CDI. Therefore, our study aimed to compare the microbiota composition between the two groups, including eight CDI-positive patients with vitamin D supplementation and ten CDI-positive patients without vitamin D supplementation by using 16S rRNA microbial profiling.

Methods: Twenty subjects were enrolled in this prospective randomized controlled study. One subject dropped out due to lack of contact with the guardian after discharge and one subject dropped out due to withdrawal of consent. Thus, 18 patients with CDI and vitamin D insufficiency (vitamin D level < 17 ng/mL) were divided into two groups: CDI with vitamin D supplementation (n = 8) and CDI without vitamin D supplementation (control: n = 10). Subjects with vitamin D insufficiency were randomized to receive 200,000 IU intramuscular cholecalciferol whereas patients in the control group received only oral vancomycin. Stool samples were obtained twice before vancomycin was administered and eight weeks after treatment; the V3-V4 16S rRNA metagenomic sequencing was performed using EzBioCloud.

Results: The alpha diversity of the gut microbiota in the recovery state was significantly higher than that in the CDI state. Analysis of bacterial relative abundance showed significantly lower *Proteobacteria* and higher *Lachnospiraceae*, *Ruminococcaceae*, *Akkermansiaceae*, and *Bifidobacteriaceae* in the recovery state. When comparing the control and vitamin D treatment groups after eight weeks, increase in alpha diversity and,

abundance of *Lachnospiraceae*, and *Ruminococcaceae* exhibited the same trend in both groups. A significant increase in *Bifidobacteriaceae* and *Christensenellaceae* was observed in the vitamin D group; *Proteobacteria* abundance was significantly lower in the vitamin D treatment group after eight weeks than that in the control group.

Conclusion: Our study confirmed that the increase in the abundance of beneficial bacteria such as *Bifidobacteriaceae*, and *Christensenellaceae* were prominently evident during recovery after administration of a high dose of cholecalciferol. These findings indicate that vitamin D administration may be useful in patients with CDI, and further studies with larger sample sizes are required.

Keywords: clostridioides difficile infection, vitamin D, microbiota, cholecalciferol, bifidobacteriaceae, christensenellaceae

INTRODUCTION

Clostridioides difficile is a spore-forming, and toxin-producing, gram-positive anaerobic bacterium. *C. difficile* infection (CDI) is caused by the colonization of *C. difficile* due to changes in the composition of the normal intestinal flora of hospitalized patients receiving antibiotics (Guh and Kutty, 2018). CDI is one of the most common causes of nosocomial infections, and its incidence and mortality rates are increasing worldwide (Guh et al., 2020). In addition, antibiotic use is associated with the recurrence and emergence of antibiotic-resistant bacteria. Current therapies using oral vancomycin or metronidazole are inappropriate for treating intractable severe CDI and preventing recurrent CDI (Surawicz and Alexander, 2011). In recent years, attention has been focused on treatments for the preservation and restoration of intestinal flora and the optimization of the immune response to CDI (Johnson et al., 2021).

Over the past several years, experimental studies have reported the association of vitamin and trace element deficiencies with systemic inflammation and multi-organ failure (Holick, 2007). In particular, vitamin D is involved in the maintenance of bone growth, calcium and phosphorus metabolism, and immune system functions (Chang and Lee 2019). Vitamin D-related epidemiological studies have also reported that vitamin D deficiency increases the risk of systemic infection and is associated with a poor disease course and greater disease activity in patients with chronic inflammatory diseases (Jørgensen et al., 2010; Kempker and Martin, 2013). Recent studies have provided evidence that lower concentrations of 25-hydroxyvitamin D [25(OH)D] (vitamin D level < 20 ng/mL) are associated with CDI severity and recurrence (Sahay and Ananthakrishnan, 2014; Abdelfatah et al., 2015). In another study, vitamin D protect against CDI by restoring melanocyte inducing transcription factor expression and lysosomal function in mice (Chan et al., 2022). However, to date, there have been no studies on changes in microbiota composition after the administration of vitamin D in patients with CDI.

In this prospective observational study, we aimed to compare the microbiota composition by conducting 16S rRNA microbial profiling of two groups: CDI-positive with vitamin D supplementation and CDI-positive without vitamin D supplementation.

MATERIALS AND METHODS

Study Population

This was a prospective, randomized, controlled, and interventional pilot study on 20 patients diagnosed with CDI (defined as ≥3 loose stools in 24 hours without any other cause and a positive glutamate dehydrogenase antigen test, positive Toxin A and Toxin B test or positive polymerase chain reaction) and vitamin D deficiency (serum 25(OH)D levels <17 ng/mL) at Kangwon National University Hospital between October 2019 and June 2021. Twenty subjects were randomly classified into two groups. Exclusion criteria included the use of immunosuppressants, pregnancy or plans to become pregnant in the next 3 months, disorders associated with hypercalcemia, current hypercalcemia (10.8 mg/dL albumin-corrected serum calcium. or 5.2 mg/dL ionized calcium), history of nephrolithiasis, chronic kidney disease worse than stage III, current substantial hepatic dysfunction (2.5 mg/dL total bilirubin, 1.0 mg/dL, direct bilirubin), use of probiotics (within four weeks from the date of CDI diagnosis), and inflammatory bowel disease. The study was approved by the Institutional Review Board of Kangwon National University Hospital (KNUH B-2019-04-005-011). Written informed consent was obtained from all the patients. This research was registered at the Clinical Research Information Service (identifier KCT0004335). Patients in the vitamin D treatment group were administered high-dose vitamin D3 (200,000 IU) *via* intramuscular injection once and were treated with oral vancomycin (125 mg, qid, for 14 days), whereas patients in the control group received only oral vancomycin (**Figure 1**).

Data Collection

Stool samples were collected from all patients at baseline and at eight weeks after treatment. Stool samples were immediately stored on ice and frozen at −80°C. DNA was extracted from the collected samples using a sterile container, and the composition of the microorganisms was analyzed *via* 16S rRNA sequencing using the extracted DNA.

DNA Extraction, PCR Amplification and Sequencing

Total DNA was extracted using the Maxwell® RSC PureFood GMO and Authentication Kit (Promega, USA), according to the

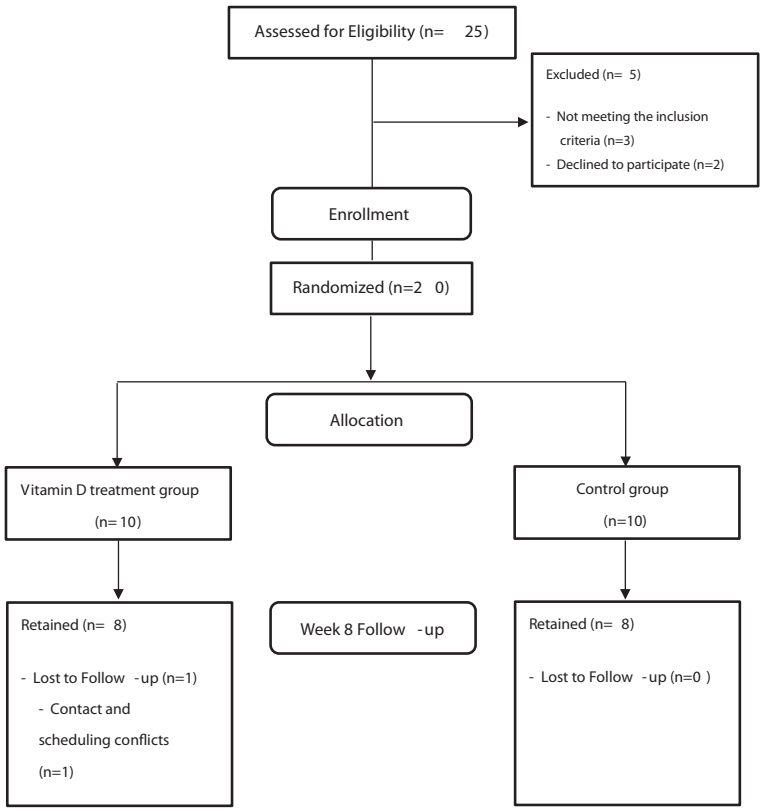

FIGURE 1 | Schematic diagram showing the recruitment process.

manufacturer's instructions. PCR amplification was performed using fusion primers targeting the V3-V4 region of the 16S rRNA gene. For bacterial amplification, the fusion primers 341F (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3'; underlined sequence indicates the target region primer) and 805R (5'- CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3') were used. The fusion primers were constructed in the following order: P5 (P7) graft-binding, i5 (i7) index, Nextera consensus, sequencing adaptor, and target region sequence. The amplification conditions were as follow: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final elongation at 72°C for 5 min. The PCR product was confirmed using 1% agarose gel electrophoresis and visualized using a Gel Doc system (Bio-Rad, Hercules, CA, USA). The amplified products were purified using CleanPCR (CleanNA). Equal concentrations of the purified products were pooled and short fragments (non-target products) were removed using CleanPCR (CleanNA). Quality and product size were assessed on the Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were pooled and sequencing was performed by Chunlab, Inc. (Seoul, Korea), using the Illumina MiSeq Sequencing system (Illumina, USA) according to the manufacturer's instructions. Sequence data were deposited in the National Center for Biotechnology Information as Bio Project ID: PRJNA824324.

Data Analysis Pipeline

The processing of raw reads started with quality check and filtering of low quality (<Q25) reads by Trimmomatic ver. 0.321. After QC pass, paired-end sequence data were merged together using the fastq_mergepairs command of VSEARCH version 2.13.42 with default parameters. Primers were then trimmed using the alignment algorithm of Myers and Miller3 at a similarity cutoff of 0.8. Non-specific amplicons that do not encode 16S rRNA were detected using nhmmer (Wheeler and Eddy, 2013) in the HMMER software package ver. 3.2.1 with hmm profiles. Unique reads were extracted and redundant reads were clustered with the unique reads by the derep_fulllength command of VSEARCH (Rognes et al., 2016). The EzBioCloud 16S rRNA database5 was used for taxonomic assignment using usearch global command of VSEARCH2 followed by more precise pairwise alignment3. Chimeric reads were filtered on reads with <97% similarity by reference based chimeric detection using the UCHIME algorithm6 and the non-chimeric 16S rRNA database from EzBioCloud. After chimeric filtering, reads that are not identified at the species level (with <97% similarity) in the

EzBioCloud database were compiled and cluster_fast command2 was used to perform *de-novo* clustering to generate additional Operational taxonomic units. Finally, OTUs with single reads (singletons) were omitted from further analyses. The secondary analysis which includes diversity calculation and biomarker discovery was conducted using in-house programs of Chunlab, Inc (Seoul, South Korea). Alpha diversity information was confirmed through the Chao1 value, and Shannon. The relationship between samples was visualized through principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity and beta diversity distances were calculated using the Bray-Curtis dissimilarity index.

Linear discriminant Effect Size (LEfSe) analysis was performed to identify bacteria that were significantly different; the degree of difference was expressed as a linear discriminant analysis (LDA) score with $\alpha = 0.05$ and LDA score threshold-2. At this time, 1% or more of the genus level was analyzed. All analyses mentioned above were performed in the EzBioCloud 16S-based MTP, which is a Chunlab bioinformatics cloud platform.

Statistical Analysis

Continuous variables were analyzed using the Mann–Whitney U test. Fisher's exact test was used to compare the categorical variables between the control and experimental group. Paired data were analyzed using the paired Wilcoxon signed-rank test. In the statistical analysis, continuous variables were analyzed using t-test when $P \geq 0.05$ and the Shapiro-Wilk and the Mann-Whitney U tests when $P < 0.05$. Statistical analyses were performed using SPSS for Windows (version 19.0; IBM Co., Armonk, NY, USA). Statistical significance was set at $P < 0.05$. GraphPad Prism 9.0 software (GraphPad Inc., San Diego, CA, USA) was used to generate the graphs.

RESULTS

Demographic and Clinical Characteristics

The demographic and clinical characteristics of the patients are summarized in **Table 1**. Twenty subjects who met the inclusion criteria were enrolled; however, one subject dropped out due to no contact with the guardian after discharge, and one subject dropped out due to withdrawal of consent. Thus, a total of 18 subjects was analyzed. No differences in age, sex, BMI, presence of hypertension, presence of diabetes, cardiovascular disease, liver disease, chronic kidney disease, or chronic respiratory disease were observed between the vitamin D treatment and control groups. In addition, no statistically significant difference between the two groups in any of the tests performed were observed, except for blood urea nitrogen in the hematological examination (**Table 1**).

Changes in the Gut Microbiota at the Time of CDI and Recovery After Eight Weeks

Alpha diversity, particularly in the Shannon index was increased (**Figure 2A**). The beta diversity using principal coordinate analysis was higher in terms of weighted UniFrac distance in CDI than in the recovery state (**Figure 2B**). When looking at the changes in individual species, the abundance of *Proteobacteria* (*Enterobacteriaceae* and *Sutterellaceae*) and *Enterococcaceae*, which are generally known to increase during CDI or antibiotic treatment, increased during CDI compared to those after recovery. In particular, a significant reduction in *Proteobacteria* (47.61 ± 37.20% in CDI vs. 13.63 ± 16.79% in recovery, $P = 0.002$) was evident during the recovery period. Conversely, the numbers of commensal bacteria and beneficial strains increased during the recovery period. A statistically

TABLE 1 | Demographic and clinical characteristics of study population (N=18).

	Control (n = 10)	Experiment (n = 8)	P-value
Age(yr)	76.9 ± 13.3	71.5 ± 21.9	0.573
Sex(M:F)	5 vs 5	4 vs 4	1
BMI(kg/m2)	20.9 ± 2.9	20.4 ± 4.6	0.46
Hypertension - no.(%)	5(50)	4(50)	1
Diabetes mellitus- no.(%)	2(20)	2(25)	1
Caridiac_disease- no.(%)	2(20)	0(0)	0.477
Liver_disaeas- no.(%)	1(10)	0(0)	1
Chronic_renal_disease - no.(%)	3(30)	0(0)	0.216
Chronic_pulmonary_disease - no.(%)	2(20)	1(12.5)	1
WBC(/ul)	17260 ± 11931	11675 ± 4062	0.36
Neutrophil_count(%)	82.3 ± 9.0	74.2 ± 12.7	0.146
Hemoglobin(g/dL)	11.5 ± 1.5	11.1 ± 1.6	0.633
Platelet_count(/ul)	211 ± 116	289 ± 104	0.237
ESR(mm/hr)	17.7 ± 32.6	45.2 ± 17.5	0.065
CRP(mg/dL)	9.9 ± 6.5	9.0 ± 6.0	0.965
BUN(mg/dL)	34.1 ± 34.4	12.8 ± 5.6	0.016
Creatine(mg/dL)	1.5 ± 1.3	0.7 ± 0.2	0.173
total_bilirubin(mg/dL)	0.7 ± 0.2	0.7 ± 0.3	0.762
Albumin(g/dL)	3.2 ± 0.8	3.2 ± 0.6	0.829
Vitamin_D(ng/mL)	10.1 ± 3.8	9.8 ± 4.4	0.897

BMI, body mass index; WBC, white blood cell count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BUN, blood urea nitrogen.

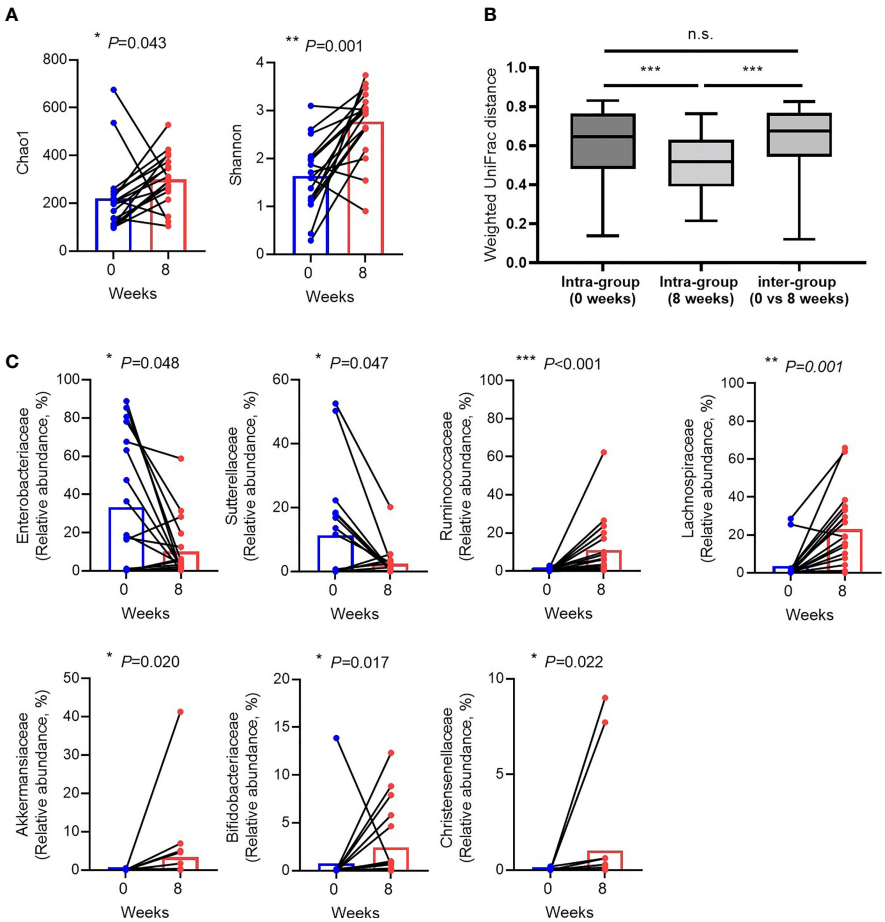

FIGURE 2 | Changes in the gut microbiota between *Clostridioides* difficile infection and recovery after eight weeks. **(A)** Alpha diversity. **(B)** Weighted UniFrac distance. **(C)** Relative abundances of individual bacteria that were significantly different between *Clostridioides* difficile infection and recovery. Blue dots indicate C. difficile infection state (0 week), red dots indicate recovery after eight weeks, and black lines represent changes in the same patient. n.s, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (Wilcoxon signed rank test and Mann-Whitney U test).

significant increase in the abundance of short-chain fatty acid (SCFA)-producing clostridia such as *Lachnospiraceae* (3.50 ± 8.17% in CDI vs. 22.92 ± 19.66% in recovery, P = 0.001 and *Ruminococcaceae* (0.49 ± 0.80% in CDI vs. 11.12 ± 15.27% in recovery, $P < 0.001$) was observed (**Figure 1C**). Beneficial strains such as *Akkermanssiaceae* (0.03 ± 0.13% in CDI vs. 3.35 ± 9.71% in recovery, P = 0.020), *Bifidobacteriaceae* (0.79 ± 3.27% in CDI vs. 2.44 ± 3.79% in recovery, P = 0.017) and *Christensenellaceae* (0.13 ± 0.04% in CDI vs. 1.02 ± 2.69% in recovery, P = 0.022) also showed increased abundance. The decrease in *Proteobacteria* abundance and the increase in *Lachnospiraceae* and *Ruminococcaceae* abundance during the recovery period showed a statistically strong association (**Figure 2C**).

Changes in the Gut Microbiota in the Control and Vitamin D Treatment Groups

No significant difference in the alpha diversity between the vitamin D treatment and control groups were observed (**Figure 3A**). Beta diversity was not significantly different between the vitamin D treatment and control groups (**Figures 3B**, **3C**). In addition, no statistically significant differences at the phylum level were observed between the two groups (**Supplementary figure 1**).

With respect to individual taxon, the abundance of *Proteobacteria*, which significantly increased during CDI, showed a downward trend in the vitamin D treatment group, but the difference was not statistically significant. At the family and genus levels, the vitamin D treatment group showed a significant decrease in *Enterobacteriaceae* (16.50 ± 18.69% in CDI vs. 2.16 ± 1.88% in recovery, P = 0.034) and *Escherichia* (4.29 ± 5.68% in CDI vs. 0.69 ± 0.85% in recovery, P = 0.027) compared to those in the control group (**Figures 3D–F** and **Supplementary Figure 2**). Furthermore, the abundance of *Christensenellaceae* and *Sutterellaceae* was higher in the vitamin D treatment group. However, the vitamin D treatment group decreased more effectively than CDI and recovery groups (**Figure 3G**, **Supplementary Figure 2** and **Supplementary Figure 3**).

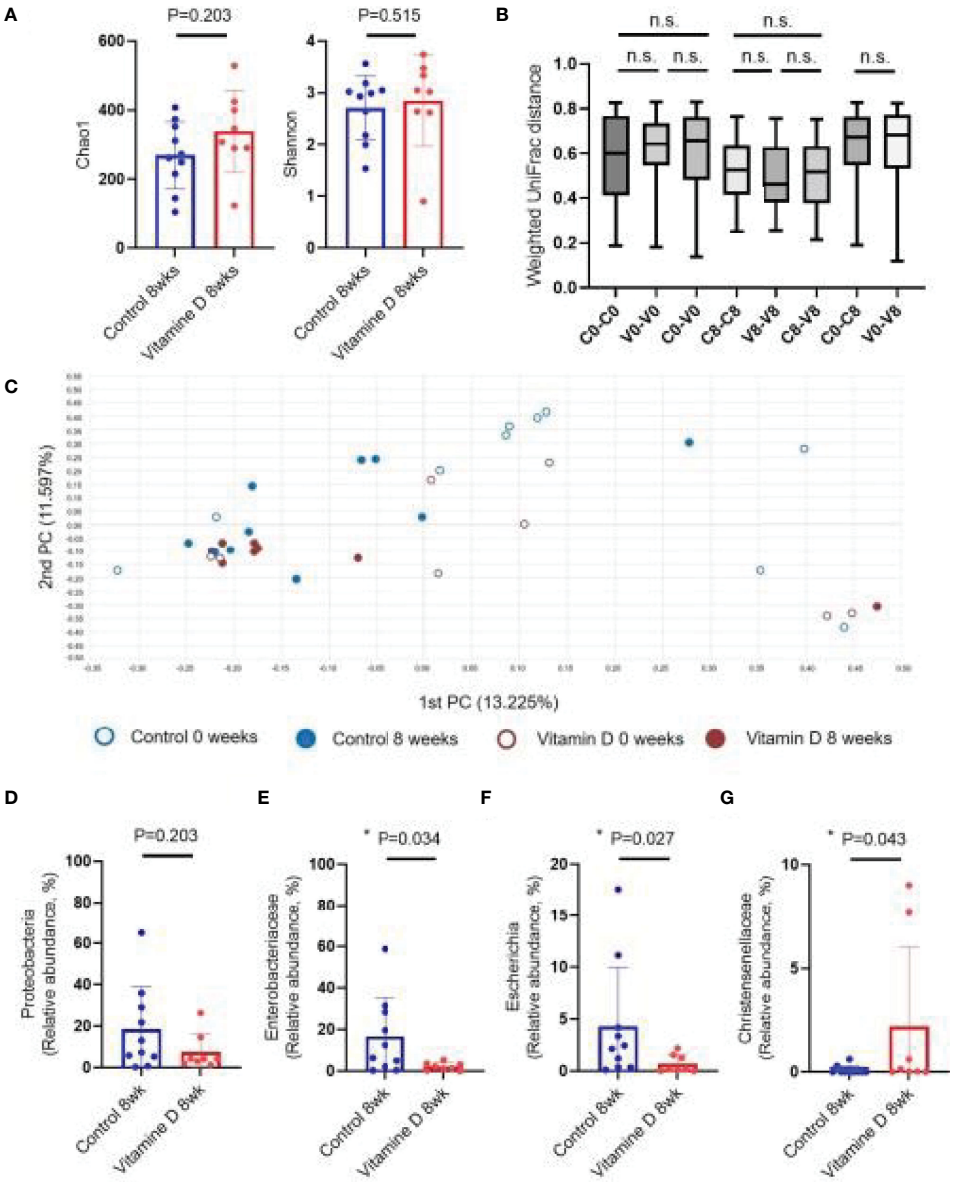

FIGURE 3 | Effect of vitamin D supplementation on the gut microbiome. **(A)** Alpha diversity. **(B)** Weighted UniFrac distance. **(C)** Principal coordinates analysis (Bray–Curtis dissimilarity). **(D)** Relative abundances of *Proteobacteria*. **(E)** Relative abundances of *Enterobacteriaceae*. **(F)** Relative abundances of Escherichia. **(G)** Relative abundances of *Christensenellaceae*. n.s, not significant; *P < 0.05 (Wilcoxon signed rank test and Mann-Whitney U test).

The increase in *Lachnospiraceae*, *Ruminococcaceae*, *Bifidobacteriaceae*, and *Christensenellaceae* were evident in the vitamin D treatment group, whereas the abundance of *Proteobacteria* decreased (**Figures 4A, B**). Changes in *Proteobacteria*, *Lachnospiraceae*, and *Ruminococcaceae* abundance were also observed in both the vitamin D treatment group and the control group (**Figures 4C–E**). However, the increase in *Bifidobacteriaceae* and *Christensenellaceae* abundance was more prominent in the vitamin D treatment group than that in the control group (**Figures 4F, G**).

DISCUSSION

This study investigated the changes in the gut microbiota between CDI and recovery in patients with and without vitamin D supplementation. Specifically, we found a significant increase in the abundance of *Proteobacteria* during CDI, and an increase in the abundance of *Lachnospiraceae*, *Ruminococcaceae*, *Akkermansiaceae*, *Bifidobacteriaceae*, and *Christensenellaceae* after recovery. The vitamin D treatment group showed a significant increase in the abundance of beneficial bacteria,

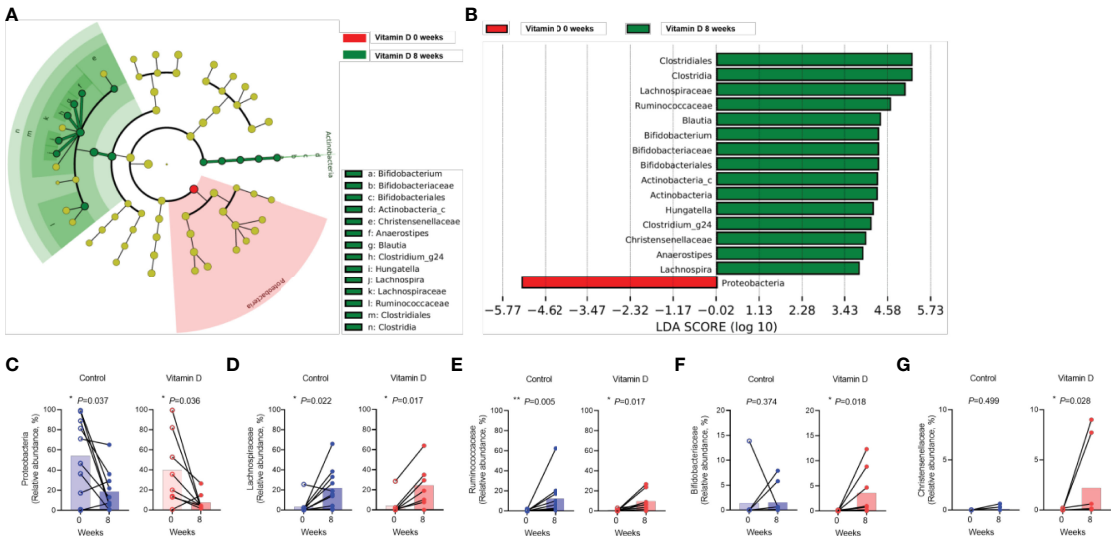

FIGURE 4 | Effect of vitamin D supplementation on the individual gut taxa. A, B. Cladogram **(A)** and linear discriminant analysis scores **(B)** using LEfSe analysis of the fecal microbiota in *Clostridioides* difficile infection versus recovery after eight weeks in the vitamin D treatment group. **(C–G)**. Differences in the abundance of individual bacteria between the control group and vitamin **(D)** supplement group. **(C)** *Proteobacteria*. **(D)** *Lachnospiraceae*. **(E)** *Ruminococcaceae*. **(F)** *Bifidobacteriaceae*. **(G)** *Christensenellaceae*. *P < 0.05, **P < 0.01 (Wilcoxon signed rank test).

such as *Bifidobacteriaceae* and *Christensenellaceae*, and the abundance of *Enterobacteriaceae* in the recovery state was significantly lower than that in the group without vitamin D treatment.

To date, the overall incidence of CDI and severe or fulminant CDI have increased, in association with an increased infection by highly virulent strains, such as NAP1/BI/027, and the increased use of antibiotics, anticancer drugs, and gastric acid inhibitors (Pépin et al., 2004; Ricciardi et al., 2007). Another problem is the increased frequency of CDI recurrence that may be caused by the reactivation of previous bacteria or re-infection with new bacteria. According to previous studies, up to 25% of patients experience recurrent CDI within 30 days of treatment (Kelly, 2012). The risk of subsequent recurrence in patients was 45% (McFarland et al., 2002). In the treatment of severe, refractory, or recurrent CDI, vancomycin has shown limitations in enhancing the effectiveness of treating or preventing recurrence. Recently, many studies have investigated fecal microbiota transplantation (FMT) as a treatment for CDI. Conventional CDI treatment with vancomycin disrupts the balance of normal colonic flora and weakens the resistance of normal flora to other proliferating bacteria. In FMT, this imbalance is restored by transplanting donor feces containing normal flora into patients with CDI (Kim, 2012). However, FMT has procedural complications and risks the transmission of infectious agents (Reumkens et al., 2016; Zellmer et al., 2021).

Vitamin D plays an important role in the maintenance of bone mineral density and has an important influence on the immune system, including the modulation of antiviral and antibacterial inflammatory immune responses (Chang and Lee 2019). Most studies emphasize maintaining vitamin D levels above 30 ng/ml to prevent osteoporosis and rickets (Dawson-Hughes et al., 2010; American Geriatrics Society Workgroup on Vitamin D Supplementation for Older Adults Workgroup on Vitamin, 2014). It also has an important influence on the immune system (Taha et al., 2021),. Vitamin D induces cathelicidin production, which can directly kill viruses and bacteria or bind to endotoxins (Liu et al., 2006). Studies on vitamin D deficiency and supplementation in inflammatory bowel disease (IBD) and inherited disorders, such as cystic fibrosis, have been published. When vitamin D was administered daily for one month to patients with IBD and vitamin D deficiency, a negative relationship between vitamin D levels and C-reactive protein was found (Jun et al., 2019). Kanhere et al. (2018) reported that in patients with cystic fibrosis and vitamin D deficiency, an imbalance in the intestinal microflora was observed, which improved when high doses of vitamin D were administered. Abdelfatah et al. (2015) suggested a significant relationship between vitamin D levels and CDI severity. In the present study, we investigated the effect of vitamin D by applying a strict standard of less than 17 ng/mL, which is lower than the vitamin D deficiency standard (Taha et al., 2021). When vitamin D3 (200,000 IU) was administered to patients with CDI at the initial stage of infection, vitamin D deficiency was quickly corrected, but the difference was not statistically significant compared to the control group in relieving microbiota dysbiosis.

Firmicutes and *Bacteroidetes* dominate the normal intestinal environment, whereas *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* are less abundant (Backhed et al., 2005). An increased prevalence of *Proteobacteria* is a marker of an imbalance in the taxonomic composition of the gut microbiota and a potential diagnostic criterion for disease (Shin et al., 2015). In particular, an increase in *Proteobacteria* is evident when the

gut microbiota changes, and metabolic homeostasis is disrupted by the use of antibiotics (Zarrinpar et al., 2018). In CDI, *Proteobacteria* and *Enterococcus* strains are the major strains that increase during dysbiosis owing to changes in the gut microbiota (Samarkos et al., 2018; Kim et al., 2020). *Proteobacteria* induces epithelial dysfunction and exacerbates intestinal inflammation, and the association between CDI and IBD is well known (Litvak et al., 2017; Caruso et al., 2020). In contrast, *Bacteroides*, *Prevotella*, and *Clostridiales* are commensal bacteria, and enterotyping is performed depending on which strain is dominant (Arumugam et al., 2011). Among them, SCFA-producing *Clostridium*, as well as *Lachnospiraceae* and *Ruminococcaceae*, play an important role in the anti-inflammatory action of intestinal immunity by increasing T reg activity (Guo et al., 2020; Park et al., 2020). In a previous study, patients with CDI showed a decrease in *Lachnospiraceae* and *Ruminococcaceae* (Antharam et al., 2013). In the present study, the increase in *Proteobacteria* abundance after CDI was substantial. Furthermore, during the recovery process, the decrease in *Proteobacteria* abundance and increase in the abundance of *Lachnospiraceae* and *Ruminococcaceae*, which are important for the secretion of metabolites such as SCFA with anti-inflammatory effects, particularly among normal flora were prominent. Therefore, methods that reduce *Proteobacteria* abundance and increase the abundance of commensal *Clostridiales*, such as *Lachnospiraceae* and *Ruminococcaceae*, could be used to treat CDI. *Akkermansia* is also a candidate probiotic that has recently received attention for its role in metabolic and systemic diseases (Plovier et al., 2017; Michalovich et al., 2019).

In addition, we observed that the abundance of *Bifidobacteriaceae* and *Christensenellaceae* significantly increased with vitamin D supplementation. *Bifidobacterium*, a well-known probiotic, is effective against inflammatory diseases by reducing inflammatory substances including cytokines, protecting the intestinal epithelial barrier, and balancing the gut microbiota (Konieczna et al., 2012). The anti-inflammatory effects of *Bifidobacterium* in animal dextran sodium sulfate models and human IBD have also been consistently reported (Singh et al., 2020; Yao et al., 2021). In a recent study involving vitamin D supplementation in healthy controls, an increase in *Bifidobacterium* abundance was observed (Meng et al., 2020). Previous studies have reported that *Bifidobacteriaceae* reduces CDI through anti-inflammatory effects (Skraban et al., 2013; Singh et al., 2020). Meanwhile, the function of *Christensenellaceae* is relatively less known, but it is a commensal bacterium in the human gut that plays an important role in human health (Waters and Ley, 2019). Further research is needed on the effects of *Bifidobacteriaceae* and *Christensenellaceae* on CDI and their relationship with vitamin D. Fifth, it was not possible to evaluate whether there were intestinal changes in increased intestinal permeability following high-dose vitamin D supplementation. In our first study plan, we tried to perform biopsy by performing sigmoidoscopy at the time of initial diagnosis and 8 weeks after treatment for CDI. Through this biopsy, it was attempted to determine whether there were any changes in the tissues depending on whether or not high-dose vitamin D was administered, but it was not possible to obtain the consent of the patients and guardians.

This study has several limitations. First, since this was a pilot study, the sample size was small, which may be statistically underpowered. To overcome this limitation, we included a control group. Second, selection bias may be possible because only patients with CDI with a vitamin D level of less than 17 ng/mL were enrolled in our study. Third, other parameters such as diet were not evaluated. Further investigations, including dietary patterns and other predisposing factors that could affect the microbiome, would help deepen our understanding of the relationship between vitamin D and the microbiome. Fourth, the short-term effects of vitamin D supplementation could not be assessed. In this study, the control group also showed a significant change after eight weeks compared to the CDI group. Therefore, to observe the effects of vitamin D, additional analysis over a short period of one–two weeks may be necessary.

In conclusion, our study is the first to identify changes in the gut microbiota upon administration of high-dose vitamin D to patients with CDI and show that the administration of a high dose of cholecalciferol may play an adjuvant role in the treatment of CDI for the first time. Furthermore, our study confirmed that the increase in *Christensenellaceae* and *Bifidobacteriaceae* abundance was enhanced in vitamin D-deficient patients with CDI recovery after the administration of a high dose of cholecalciferol. Physicians should consider the potential role of vitamin D as replacement therapy in patients with CDI. These findings require further evaluation in a larger, multicenter study.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA824324.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Kangwon National University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SL and H-KP: design of the study. SL, CK, DC and JP: data acquisition. H-KP, KL and HC: data analysis, and interpretation. S-JN, SP and GC: drafted the article and critically revised the manuscript. SL: gave final approval for the version to be

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SUPPLEMENTARY MATERIAL

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REFERENCES

Abdelfatah, M., Nayfe, R., Moftakhar, B., Nijim, A., Zoghbi, M. E., Donskey, C. J., et al. (2015). Low Vitamin D Level and Impact on Severity and Recurrence of *Clostridium Difficile* Infections. *J. Investig. Med.* 63, 17–21. doi: 10.1097/JIM.0000000000000117

American Geriatrics Society Workgroup on Vitamin D Supplementation for Older Adults Workgroup on Vitamin (2014). Recommendations Abstracted From the American Geriatrics Society Consensus Statement on Vitamin D for Prevention of Falls and Their Consequences. *J. Am. Geriatr. Soc.* 62, 147–152. doi: 10.1111/jgs.12631

Antharam, V. C., Li, E. C., Ishmael, A., Sharma, A., Mai, V., Rand, K. H., et al. (2013). Intestinal Dysbiosis and Depletion of Butyrogenic Bacteria in *Clostridium Difficile* Infection and Nosocomial Diarrhea. *J. Clin. Microbiol.* 51, 2884–2892. doi: 10.1128/JCM.00845-13

Arumugam, M., Raes, J., Pelletier, E., Paslier, D. L., Yamada, T., Mende, D. R., et al. (2011). Enterotypes of the Human Gut Microbiome. *Nature* 473, 174–180. doi: 10.1038/nature09944

Backhed, F., Sonnenburg, L. S., Peterson, D. A., and Gordon, J. I.. (2005). Host-Bacterial Mutualism in the Human Intestine. *Science* 307, 1915–1920. doi: 10.1126/science.1104816

Caruso, R., Lo, B. C., and Núñez, G. (2020). Host–microbiota Interactions in Inflammatory Bowel Disease. *Nat. Rev. Immunol.* 20, 411–426. doi: 10.1038/s41577-019-0268-7

Chan, H., Li, Q., Wang, X., Liu, W. Y., Hu, W., Zeng, J., et al. (2022). Vitamin D3 and Carbamazepine Protect Against Clostridioides Difficile Infection in Mice by Restoring Macrophage Lysosome Acidification. *Autophagy* 1–18. doi: 10.1080/15548627.2021.2016004

Chang, S.-W., and Lee, H.-C.. (2019). Vitamin D and Health - The Missing Vitamin in Humans. *Pediatr. Neonatol.* 60 (3), 237–44. doi: 10.1016/j.pedneo.2019.04.007

Dawson-Hughes, B., Mithal, A., Bonjour, J. P., Boonen, S., Burckhardt, P., Yoshimura, N., et al. (2010). IOF Position Statement: Vitamin D Recommendations for Older Adults. *Osteoporos. Int.* 21, 1154. doi: 10.1007/s00198-010-1285-3

Guh, A. Y., and Kutty, P. K. (2018). Clostridioides Difficile Infection. *Ann. Intern. Med.* 169, ITC49–ITC64. doi: 10.7326/AITC201810020

Guh, A. Y., Mu, Y., Winston, L. G., Johnston, H., Olson, D., Farley, M. M., et al. (2020). Trends in U.S. Burden of Clostridioides Difficile Infection and Outcomes. *N. Engl. J. Med.* 382, 1320–1330. doi: 10.1056/NEJMoa1910215

Guo, P., Zhang, K., Ma, X., and He, P. (2020). Clostridium Species as Probiotics: Potentials and Challenges. *J. Anim. Sci. Biotechnol.* 11, 24. doi: 10.1186/s40104-019-0402-1

Holick, M. F. (2007). Vitamin D Deficiency. *N. Engl. J. Med.* 357, 266–281. doi: 10.1056/NEJMra070553

Jørgensen, S. P., Agnholt, J., Glerup, H., Lyhne, S., Villadsen, G. E., Hvas, C. L., et al. (2010). Clinical Trial: Vitamin D3 Treatment in Crohn's Disease: A Randomized Double-Blind Placebo-Controlled Study. *Aliment Pharmacol. Ther.* 32, 377–383. doi: 10.1111/j.1365-2036.2010.04355.x

Johnson, S., Lavergne, V., Skinner, A. M., Gonzales-Luna, A. J., Garey, K. W., Kelly, C. P., et al. (2021). Clinical Practice Guideline by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA): 2021 Focused Update Guidelines on Management of Clostridioides Difficile Infection in Adults. *Clin. Infect. Dis.* 73, e1029–e1044.

Jun, J. C., Yoon, H., Choi, Y. J., Shin, C. M., Park, Y. S., Kim, N., et al. (2019). When Daily Vitamin D Was Administered for One Month in IBD Patients With Vitamin D Deficiency, a Negative Relationship Between Vitamin D Levels and C-Reactive Protein Was Found. *Intest Res.* 17, 210–217. doi: 10.5217/ir.2018.00081

Kanhere, M., He, J., Chassaing, B., Ziegler, T. R., Lvie, E. A., Hao, L., et al. (2018). Bolus Weekly VitaminD3 Supplementation Impacts Gut and Airway Microbiota in Adults With Cystic Fibrosis: A Double-Blind, Randomized, Placebo-Controlled Clinical Trial. *J. Clin. Endocrinol. Metab.* 103, 564–574. doi: 10.1210/jc.2017-01983

Kelly, C. P. (2012). Can We Identify Patients at High Risk of Recurrent *Clostridium Difficile* Infection? *Clin. Microbiol. Infect.* 18 Suppl 6, 21.

Kempker, J. A., and Martin, G. S. (2013). Vitamin D and Sepsis: From Associations to Causal Connections. *Inflamm. Allergy Drug Targets* 12, 246–252. doi: 10.2174/18715281131299900048

Kim, S. W. (2012). Treatment of Refractory or Recurrent *Clostridium Difficile* Infection. *Korean J. Gastroenterol.* 60, 71–78. doi: 10.4166/kjg.2012.60.2.71

Kim, J., Cho, Y., Seo, M.-R., Bae, M. H., Rho, M., Pai, H., et al. (2020). Quantitative Characterization of *Clostridioides Difficile* Population in the Gut Microbiome of Patients With C. Difficile Infection and Their Association With Clinical Factors. *Sci. Rep.* 10, 17608.

Konieczna, P., Akdis, C. A., Quigley, E. M., Shanahan, F., and O'Mahony, L. (2012). Portrait of an Immunoregulatory Bifidobacterium. *Gut Microbes* 3, 261–266. doi: 10.4161/gmic.20358

Litvak, Y., Byndloss, M. X., Tsolis, R. M., and Bäumler, A. J. (2017). Dysbiotic Proteobacteria Expansion: A Microbial Signature of Epithelial Dysfunction. *Curr. Opin. Microbiol.* 39, 1–6. doi: 10.1016/j.mib.2017.07.003

Liu, P. T., Stenger, S., Li, H., Li, H., Wenzel, L., Tan, B. H., Krutzik, S. R., et al. (2006). Toll-Like Receptor Triggering of a Vitamin D-Mediated Human Antimicrobial Response. *Science* 311, 1770–1773. doi: 10.1126/science.1123933

McFarland, L. V., Elmer, G. W., and Surawicz, C. M. (2002). Breaking the Cycle: Treatment Strategies for 163 Cases of Recurrent *Clostridium Difficile* Disease. *Am. J. Gastroenterol.* 97, 1769–1775. doi: 10.1111/j.1572-0241.2002.05839.x

Meng, D., Sommella, E., Salviati, E., Campiglia, P., Ganguli, K., Djebali, K., et al. (2020). Indole-3-Lactic Acid, a Metabolite of Tryptophan, Secreted by *Bifidobacterium Longum* Subspecies Infantis Is Anti-Inflammatory in the Immature Intestine. *Pediatr. Res.* 88, 209–217. doi: 10.1038/s41390-019-0740-x

Michalovich, D., Rodriguez-Perez, N., Smolinska, S., Pirozynski, M., Mayhew, D., Uddin, S., et al. (2019). Obesity and Disease Severity Magnify Disturbed Microbiome-Immune Interactions in Asthma Patients. *Nat. Commun.* 10, 5711. doi: 10.1038/s41467-019-13751-9

Park, H. K., Choi, Y., Lee, D. H., Kim, S., Lee, J.-M., Choi, S. W., et al. (2020). Altered Gut Microbiota by Azithromycin Attenuates Airway Inflammation in Allergic Asthma. *J. Allergy Clin. Immunol.* 145, 1466–1469.e8. doi: 10.1016/j.jaci.2020.01.044

Pépin, J., Valiquette, L., Alary, M. E., Villemure, P., Pelletier, A., Forget, K., et al. (2004). *Clostridium Difficile*-Associated Diarrhea in a Region of Quebec From 1991 to 2003: A Changing Pattern of Disease Severity. *CMAJ* 171, 466–472. doi: 10.1503/cmaj.1041104

Plovier, H., Everard, A., Druart, C., Depommier, C., Hul, M. V., Geurts, L., et al. (2017). A Purified Membrane Protein From *Akkermansia Muciniphila* or the Pasteurized Bacterium Improves Metabolism in Obese and Diabetic Mice. *Nat. Med.* 23, 107–113. doi: 10.1038/nm.4236

Reumkens, A., Rondagh, E. J., Bakker, C. M., Winkens, B., Masclee, A. M., and Sanduleanu, S.. (2016). Post-Colonoscopy Complications: A Systematic

Review, Time Trends, and Meta-Analysis of Population-Based Studies. *Am. J. Gastroenterol.* 111, 1092–1101. doi: 10.1038/ajg.2016.234

Ricciardi, R., Rothenberger, D. A., Madoff, R. D., and Baxter, N. N. (2007). Increasing Prevalence and Severity of Clostridium Difficile Colitis in Hospitalized Patients in the United States. *Arch. Surg.* 142, 624–631. doi: 10.1001/archsurg.142.7.624

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: A Versatile Open Source Tool for Metagenomics. *PeerJ* 4, e2584. doi: 10.7717/peerj.2584

Sahay, T., and Ananthakrishnan, A. N. (2014). Vitamin D Deficiency is Associated With Community-Acquired Clostridium Difficile Infection: A Case–Control Study. *BMC Infect. Dis.* 14, 661. doi: 10.1186/s12879-014-0661-6

Samarkos, M., Mastrogianni, E., and Kampouropoulou, O. (2018). The Role of Gut Microbiota in Clostridium Difficile Infection. *Eur. J. Intern. Med.* 50, 28–32. doi: 10.1016/j.ejim.2018.02.006

Shin, N. R., Whon, T. W., and Bae, J. W. (2015). Proteobacteria: Microbial Signature of Dysbiosis in Gut Microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Singh, S., Bhatia, R., Khare, P., Sharma, S., Rajarammohan, S., Bishnoi, M., et al. (2020). Anti-Inflammatory Bifidobacterium Strains Prevent Dextran Sodium Sulfate Induced Colitis and Associated Gut Microbial Dysbiosis in Mice. *Sci. Rep.* 10, 18597. doi: 10.1038/s41598-020-75702-5

Singh, P., Rawat, A., Alwakeel, M., Sharif, E., and Al Khodor, S. (2020). The Potential Role of Vitamin D Supplementation as a Gut Microbiota Modifier in Healthy Individuals. *Sci. Rep.* 10, 21641. doi: 10.1038/s41598-020-77806-4

Skraban, J., Dzeroski, S., Zenko, B., Mongus, D., Gangl, S., Rupnik, M., et al. (2013). Gut Microbiota Patterns Associated With Colonization of Different *Clostridium Difficile* Ribotypes. *PLos One* 8, e58005. doi: 10.1371/journal.pone.0058005

Surawicz, C. M., and Alexander, J. (2011). Treatment of Refractory and Recurrent Clostridium Difficile Infection. *Nat. Rev. Gastroenterol. Hepatol.* 8, 330–339. doi: 10.1038/nrgastro.2011.59

Taha, R., Abureesh, S., Alghamdi, S., Hassan, R. Y., Cheikh, M. M., Bagabir, R. A., et al. (2021). The Relationship Between Vitamin D and Infections Including COVID-19: Any Hopes? *Int. J. Gen. Med.* 14, 3849–3870. doi: 10.2147/IJGM.S317421

Waters, J. L., and Ley, R. E. (2019). The Human Gut Bacteria Christensenellaceae Are Widespread, Heritable, and Associated With Health. *BMC Biol.* 17, 83. doi: 10.1186/s12915-019-0699-4

Wheeler, T. J., and Eddy, S. R. (2013). Nhmmer: DNA Homology Search With Profile HMMs. *Bioinformatics* 29 (19), 2487–2489. doi: 10.1093/bioinformatics/btt403

Yao, S., Zhao, Z., Wang, W., and Liu, X. (2021). Bifidobacterium Longum: Protection Against Inflammatory Bowel Disease. *J. Immunol. Res.* 2021, 8030297. doi: 10.1155/2021/8030297

Zarrinpar, A., Chaix, A., Xu, Z. Z., Chang, M. W., Marotz, C. A., Saghatelian, A., et al. (2018). Antibiotic-Induced Microbiome Depletion Alters Metabolic Homeostasis by Affecting Gut Signaling and Colonic Metabolism. *Nat. Commun.* 9, 2872. doi: 10.1038/s41467-018-05336-9

Zellmer, C., Sater, M. R.A., Huntley, M. H., Osman, M., Olesen, S. W., Ramakrishna, B., et al. (2021). Shiga Toxin-Producing *Escherichia Coli* Transmission *via* Fecal Microbiota Transplant. *Clin. Infect. Dis.* 72, e880. doi: 10.1093/cid/ciaa1486

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Antibiotic-induced depletion of *Clostridium* species increases the risk of secondary fungal infections in preterm infants

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Preterm infants or those with low birth weight are highly susceptible to invasive fungal disease (IFD) and other microbial or viral infection due to immaturity of their immune system. Antibiotics are routinely administered in these vulnerable infants in treatment of sepsis and other infectious diseases, which might cause perturbation of gut microbiome and hence development of IFD. In this study, we compared clinical characteristics of fungal infection after antibiotic treatment in preterm infants. As determined by 16S rRNA sequencing, compared with non-IFD patients with or without antibiotics treatment, *Clostridium* species in the intestinal tracts of patients with IFD were almost completely eliminated, and *Enterococcus* were increased. We established a rat model of IFD by intraperitoneal inoculation of *C. albicans* in rats pretreated with meropenem and vancomycin. After pretreatment with antibiotics, the intestinal microbiomes of rats infected with *C. albicans* were disordered, as characterized by an increase of proinflammatory conditional pathogens and a sharp decrease of *Clostridium* species and *Bacteroides*. Immunofluorescence analysis showed that *C. albicans*-infected rats pretreated with antibiotics were deficient in IgA and IL10, while the number of Pro-inflammatory $CD11c^+$ macrophages was increased. In conclusion, excessive use of antibiotics promoted the imbalance of intestinal microbiome, especially sharp decreases of short-chain fatty acids (SCFA)-producing *Clostridium* species, which exacerbated the symptoms of IFD, potentially through decreased mucosal immunomodulatory molecules. Our results suggest that inappropriate use of broad-spectrum antibiotics may promote the colonization of invasive fungi. The results of this study provide new insights into the prevention of IFD in preterm infants.

KEYWORDS

neonatal fungal infection, antibiotics, intestinal microbiome, *Clostridium* species, SCFA, IL10, IgA

Introduction

Invasive fungal disease (IFD) is one of the late-onset severe infections in premature infants (Kilpatrick et al., 2021). Because premature infants and those with low birth weights are at increased risk, neonatal intensive care units (NICU) tend to be locales of relatively high incidences of candidiasis. IFD affects 7 to 10% of preterm infants with birth weights of less than 1500 g (Benjamin et al., 2010; Weimer et al., 2021). Infants with birth weights of less than 750 g are at disproportionate risk: the incidence of IFD in these extremely low birth-weight infants due to infection with *Candida* species is two times higher than in infants with birth weights of 750 to 1000 g (Aliaga et al., 2014; Autmizguine et al., 2018). The signs of the disease are usually subtle, and despite the existence of effective antifungal treatments, the consequences for preterm infants are severe (Aliaga et al., 2014; Autmizguine et al., 2018). After *Candida* infection, almost 70% of infants born with birth weights under 1000 g lost their lives or developed severe neurodevelopmental disorders (Benjamin, 2006). Therefore, reducing the risk of invasive fungal infection in preterm infants is crucial.

The heightened vulnerability of hospitalized preterm infants to infection means that they often undergo long-term exposure to antibiotics. Increasing evidence shows that some specific antibiotics are associated with significantly increased risks of neonatal IFD (Benjamin, 2006; Lee et al., 2013; Fu et al., 2018; Warris et al., 2020; Kilpatrick et al., 2021). For example, a study of 3702 infants demonstrated that in extremely low birth-weight infants, long-term use of broad-spectrum antibiotics for at least 3 days after birth was associated with invasive candidiasis. This effect was especially strong for the use of third-generation cephalosporins, with a correlation coefficient of 0.67 (P = 0.017) (Cotten et al., 2006). In another study, 691 neonates who had been exposed to third-generation cephalosporins were found to be infected with *Candida*, which indicated a strong correlation between cephalosporins use and the incidence of invasive candidiasis (Benjamin, 2006). Thus, these studies suggest that either genetic or pharmacological interference with the host microbiota or the microbial environment may destroy the delicate balance and lead to the invasive growth of *Candida albicans* (*C. albicans*), causing candidiasis (Gutierrez et al., 2020; Costantini et al., 2022; Qin et al., 2022). However, while antibiotics are generally thought to increase the risk of fungal infection, the specific mechanisms leading to this increased risk are not entirely clear.

The microbiota during early life is important for infant health (Gasparrini et al., 2019; Rao et al., 2021). Importantly, the presence and activity of *C. albicans* have been found to be related to imbalances of the intestinal microbiome that can lead to health disturbances (Fox et al., 2014; Fan et al., 2015; Bergeron et al., 2017; Nogueira et al., 2019). Most studies of bacterial-fungal interactions have focused on interactions between pathogenic *C. albicans* and various bacteria (Nogueira et al., 2019), such as *Pseudomonas aeruginosa* (Bergeron et al., 2017), *Bacteroides fragilis* (Valentine et al., 2019), *Clostridium* species (Fan et al., 2015) and *Enterococcus faecalis* (Brown et al., 2019). IFD and the resulting microbial disruptions caused by various *Candida* species have been found to be associated with several factors. For instance, IFD is considered to be more likely to occur in patients with low immune function (Baptista et al., 2016; Brown et al., 2019; Ferreras-Antolin et al., 2019). In addition, either gestational age, mode of antibiotic treatment, host epithelial barrier function, immune ontogeny or diet may affect the composition of the microbiota (La Rosa et al., 2014; Bäckhed et al., 2015; Bokulich et al., 2016; DiBartolomeo and Claud, 2016; Gibson et al., 2016; Gregory et al., 2016; Shao et al., 2019; Ronan et al., 2021). However, due to these complexities, the impact of any single factor on microbiota development remains unclear, and understanding how antibiotics affect the microbiota of preterm infants and whether they promote fungal infection is a major challenge.

In order to clarify mechanisms by which antibiotics affect the microbiota and risk of IFD, we compared the clinical characteristics of fungus-infected and non-fungus-infected newborns after antibiotic treatment. The feces of these volunteers were studied through gene sequencing and analyses of 16S rRNA, and the compositions of their intestinal microbiomes were compared. Then, we constructed a rat fungal infection model and compared the responses of the microbiomes of rats with and without *C. albicans* infection to treatment with antibiotics. Finally, by using intestinal tissue immunofluorescence assays, the distribution and expression of IgA and IL10, the key molecules of immune regulation in the cecum, were detected. In summary, through this study, we aimed at elucidating the possible mechanisms of antibiotic-induced fungal infection in preterm infants.

Materials and methods

Participants

This retrospective study enrolled infants who were patients of the Guangzhou Women and Children Medical Care Center from January 2019 to December 2019. Three infants with clinically diagnosed fungal infection who had been admitted to the NICU constituted the fungal group, five infants who were treated with antibiotics but were not diagnosed with fungal infection constituted the non-fungal group, and four infants who were not treated with antibiotics and were not diagnosed with fungal infection constituted the control group. The inclusion criterion for patients with fungal infection was positive symptoms of fungal infection and an increased serum (1,3)-β-d-glucan level as confirmed with multiple blood tests. The inclusion criteria for the non-fungal group were as follows: admission to the NICU during the same time period, antibiotic

use after birth due to other diseases, and without symptoms of fungal infection. The criteria for the control group were as follows: admission to the NICU during the same time period, no antibiotic use during hospitalization, and no evidence of fungal infection. All the studies followed the guidelines for the ethical treatment of human specimens and were approved by the Ethics Committee of Guangzhou Women and Children's Medical Center. According to the Declaration of Helsinki, written informed consent was obtained from the parents of all enrolled neonates.

We recorded the general clinical data of all infants in the study, including sex, gestational age, birth weight, mode of delivery, premature rupture of membranes, 5 min Apgar score, and other diseases. The Apgar score comprises 5 components: (1) color, (2) heart rate, (3) reflexes, (4) muscle tone, and (5) respiration (Watterberg et al., 2015). The Apgar score was used to evaluate neonatal asphyxia. We also recorded additional clinical data of fungal group, including the types and days of antibiotic treatments, serum 1,3-β-D-glucan levels during hospitalization, the types and days of antifungal drugs, and the amount and duration of daily feeding.

Collection of feces

Samples were taken from the fresh stool by using a sampling spoon, and the stool samples were quickly placed in a sterilized microcentrifuge tube. Then, the labeled tubes were transferred to a -80°C freezer for storage. After the diagnosis of IFD, the fecal samples of neonates in the fungal group were collected before antifungal treatment. The fecal samples of neonates in the non-fungal group were collected at the end of antibiotic treatment. The fecal samples of the control group were collected under normal feeding.

Establishment of animal models

Animals in this study were approved by Guangzhou Medical University's Institutional Animal Care and Use Committee and conducted in accordance with institutional guidelines. SPF-grade 0 to 2 day-old Sprague-Dawley (SD) rats, male or female, weighing 6 to 10 g, were provided by the Experimental Animal Center of Southern Medical University.

Increased risk of IFD in neonates has been associated with broad-spectrum antibiotic exposure, in particular with exposure to carbapenems (Fu et al., 2016; Esaiassen et al., 2017; Jiang et al., 2020). Therefore, we used meropenem in the establishment of a rat model of IFD. As in our ward, the infants who underwent co-administration of meropenem and vancomycin were more susceptible to IFD, we also included vancomycin in this treatment. In the fungal infection group (n = 9), the body weight (g) of rats were measured daily, and meropenem (2.4 mg/g) and vancomycin (0.9 mg/g) were injected intramuscularly once per day for 14 days. An intraperitoneal injection of 0.3 mL of *C. albicans* inoculum (10^8 CFU/mL) was given on the 15th day. Saline solution was utilized in the vehicle group (n = 7) for 14 days, and then *C. albicans* inoculum was administered on the 15th day. Rats in the control group (n = 4) received regular feedings.

Animal sampling

Fresh fecal pellets were taken from each rat on the 10th day after injection of *C. albicans*. Fresh fecal samples were obtained by placing a rat in an empty cage for a period of time during which its feces were excreted. The collected fecal pellets were put into a sterilized microcentrifuge tube and quickly stored at -80°C. All rats were sacrificed by cervical dislocation on the 10th day, and the severity of fungal infection was observed and intestinal samples were collected.

Immunofluorescence staining

Sections of frozen ileum tissue (7 mm) were prepared and fixed with 4% PFA. Goat serum was added for 1 hour at room temperature, then the primary antibodies were applied overnight in a wet chamber at 4°C. Following the washing with PBS, the sections were incubated for 1 hour at room temperature with secondary antibodies and mounted them with VECTASHIELD Antifade Mounting Medium with DAPI to stain the nucleus. Using a Leica TCS SP8 Inverted Fluorescence Microscope (Leica Microsystems), immunofluorescent images were acquired. ImageJ software (National Institutes of Health, Bethesda, MD) was used to calculate the average fluorescence intensity of IgA and IL-10, and the number of CD11c clusters per square millimeter.

The primary antibodies and their concentrations were as follows: anti-IL-10 (bs-0698r, Bioss, China) (1:100), anti-IgA (MARA-1, Origene, USA) (1:100), and anti-CD11c (orb621157, Biorbyt, Cambridge, UK) (1:50). The secondary antibodies and their concentrations were as follows: goat anti-mouse IgG H&L-Cy3 (GB21301, Servicebio, China) (1:500), goat anti-rabbit IgG H&L-Cy3 (GB21303, Servicebio, China) (1:500), and goat anti-mouse IgG H&L-FITC (GB22301, Servicebio, China) (1:500).

16S rRNA analyses

According to the manufacturer's instructions, DNA was extracted from microbial community using MagPure Stool DNA KF kit B (Magen, China). Degenerate PCR primers were used for amplification of variable region V4 of the bacterial 16S rRNA gene, primers 515F (5'-GTGCCAGCMGCCGCGGTAA-

3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 45 s, with a final extension of 10 minutes at 72°C. We purified the PCR products using Agencourt AMPure XP beads, followed by elution in elution buffer. The Agilent Technologies 2100 bioanalyzer was used to qualify libraries. In the sequencing process, the validated libraries were analyzed using an Illumina HiSeq 2500 platform (BGI, Shenzhen, China) following the standard Illumina pipelines, and two 250 bp paired-end reads were obtained.

Then, according to the 97% similarity criterion, we use *de novo* OTU screening with USEARCH (v7.0.1090) software platform to group reads into discrete operational taxonomic unit (OTU) clusters (Caporaso et al., 2010). These clusters were classified taxonomically using the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/) for bacteria. Based on annotations of OTUs, we generated phylogenetic relative abundance profiles at multiple taxa levels (phylum, class, order, family and genus). The Shannon diversity index, Simpson diversity index, and Chao1 diversity were used to estimate Alpha diversity. A weighted UniFrac distance was calculated using the QIIME (v1.80) pipeline to determine beta diversity (Edgar, 2010). R software (v3.1.1) was used to draw rank-abundance (Alroy, 2015) and Venn diagram analysis (Chen and Boutros, 2011). R software (v3.1.1, ade4 package) was used to draw PCA analysis. R software (v3.1.1, vegan package) was used to draw Nonmetric Multidimensional Scaling (NMDS) analysis. R software (v3.4.1) was used to draw a Spearman correlation heat map between dominant microbiome. PICRUSt2 software package (v2.2.0-b) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/) were used to predict the functional content of microbial communities as KEGG ortholog profiles (Kanehisa et al., 2012). After obtaining the different gene functional pathways (Kruskal-Wallis tests), GraphPad Prism 8 (GraphPad Software Inc., USA) was used to represent data graphically. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to find significant microbiome of different groups (Segata et al., 2011). Only taxa with LDA >3 at a P value <0.05 were considered significantly enriched.

Statistical analysis

SPSS 25.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., USA) were used for statistical analyses. All data were first tested for normality and homogeneity of variance, and were statistically analyzed by analysis of variance. Composition and diversity of the gut microbiota were reported as mean ± SEM. The nonparametric Mann-Whitney U and Kruskal-Wallis tests were used for comparison of these factors. A permutational multivariate ANOVA PERMANOVA was used to test PCoA comparisons. NMDS comparisons were performed using ANOSIM. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of premature infants with invasive fungal infections

This study involved a retrospective analysis of patients at our hospital. We collected the clinical information from three groups of infants. One group of infants (fungal group, n = 3) was diagnosed with fungal infections after being treated with broad-spectrum antibiotics for bacterial sepsis. A second group of infants (non-fungal group, n = 5) did not have any symptoms of fungal infection after broad-spectrum antibiotics therapy for bacterial sepsis. We identified a third group of infants (control group, n = 4) who did not have symptoms of fungal or bacterial infections, and were not treated with antibiotics. Fungal infections were diagnosed by combining clinical characteristics and blood levels of 1,3-β-D-glucan according to standard guidelines (Calley and Warris, 2017; King et al., 2017; Clancy and Nguyen, 2018; Warris et al., 2019; Weimer et al., 2021). We found that the average gestational age of infants with fungal infection was less than 30 weeks (29.5 ± 0.7 weeks) and the birth weight was less than 1500 g (1120 ± 226 g) (Table 1). There was no significant difference in the premature rupture of membranes, mode of delivery or the age of achieving full enteral feeding ($P > 0.05$) (Table 1).

We found that patients diagnosed with a fungal infection tended to have received injections of broad-spectrum antibiotics for more than 2 weeks prior to the fungal infection. In two cases, the treatment lasted for more than 1 month (Table 2). The average exposure time to broad-spectrum antibiotics in the fungal group was longer than that in the non-fungal group. All of the cases in fungal group received carbapenem therapy. The group of infants with fungal infection had more severe complications than those in the non-fungal group and the control group (Table 2).

Previous studies demonstrated that low birth weight, use of broad-spectrum antibiotics (e.g. third-generation cephalosporins or carbapenems) for greater than 7 days, mechanical ventilation, central vascular catheter, and delayed full feeding were risk factors of invasive fungal infections (Feja et al., 2005; Hsieh et al., 2012; Lee et al., 2013; King et al., 2017). These findings are consistent with our results regarding the characteristics of the fungal group. However, we noticed that two infants in the non-fungal group who had been exposed to third-generation cephalosporins or carbapenems for more than one month had no invasive fungal infections. We further found that the time required for infants in the fungal group to achieve total enteral feeding was longer than that in the non-fungal group (50.67 ± 18.93 vs 22.0 ± 16.6 days, Table 1; Figure 1). Thus, there

TABLE 1 Comparison of clinical data among healthy volunteers, infants with fungal infection and infants without fungal infection (n=12).

Clinical information	Control group n=4	Fungal group n=3	Non-fungal group n=5	P value
Gestational age (weeks)	38.80 ± 1.3	29.5 ± 0.8#	35.6 ± 4.7	0.013
Birth weight (g)	3105 ± 346.1	1130.0 ± 226.1#	2072.0 ± 1016.8	0.017
Male infants (n)	2	3	1	0.134
Caesarean section (n)	0	1	1	0.697
Premature rupture of membrane (n)	1	1	0	0.470
Apgar score ≤7 at 5 minutes (n)	0	1	0	0.250
Age of complete feeding& (days)	*	50.67 ± 18.93	22.0 ± 16.6	0.064+

*The patients had achieved the full enteral feeding when they were admitted to the NICU. & A newborn feeding amount of 150ml/kg per day means complete feeding. The p value was analyzed by One-way ANOVA or fisher exact test. #P<0.05 vs Control. +P value was analyzed by two-tailed Student t test. The data are presented as the mean ± SD.

were significant differences in specific tested clinical parameters between fungal and non-fungal group.

Characteristics of intestinal microbiomes in neonates with *C. albicans* infection after antibiotic use

It has been shown that the type of birth, gestational age, the type of feeding, and antibiotic treatment affect the microbial colonization of preterm infants (Parra-Llorca et al., 2018; Zwittink et al., 2018; Healy et al., 2022). Therefore, we collected the feces of these three groups of infants and analyzed the intestinal microbiomes in order to explore the relationship between the intestinal microbiome and invasive fungal infection. After performing quality control of the sequences, a total of 704,264 high-quality sequences were obtained from all patient samples.

By applying the USEARCH software platform, OTUs clusters were identified based on nonrepetitive sequences. 53, 77 and 79 OTUs were found from the control group, non-fungal group and fungal group, respectively. The analysis also showed that there were 24 identical OTUs in the three groups, and 28 OTUs were identified as being specific to the fungal group (Figure 2A).

We evaluated the composition of the microbiome communities in the intestines of these infants by calculating

TABLE 2 Clinical and laboratory information among the three groups of individuals (n=12).

Groups	Fungal group (F1-3)			Non-Fungal group (N1-5)					Control group (C1-4)			
Infants	F1	F2	F3	N1	N2	N3	N4	N5	C1	C2	C3	C4
Types and duration of antibiotics before fungal infection (d)	Carbapenems (19)	Carbapenems (12) Penicillins (1) Glycopeptides (10) Cephalosporins (9)	Carbapenems (44) Penicillins (37)	Penicillins (10)	Carbapenems (33) Penicillins (6) Glycopeptides (14) Cephalosporins (46)	Penicillins (17) Cephalosporins (30)	Cephalosporins (6)	Carbapenems (17)	–	–	–	–
Age of fungal infection onset (d)	19	27	131	–	–	–	–	–	–	–	–	–
Level of 1,3-β-d-glucan before using antifungal drugs (pg/ml)	152.53	189.37	>600	–	–	–	–	–	–	–	–	–
Categories and duration of antifungal drugs (d)	Azoles (47)	Azoles (77)	Azoles (3)	–	–	–	–	–	–	–	–	–
Complication	Encephalopathy, Pneumonia, Atelectasis, ROP*, NEC*	Pneumonia, Laryngomalacia, Gastroesophageal reflux, Encephalopathy, BPD*	RDS*, Sepsis, Pneumonia, BPD*, PDA*, IVH*, Hyperbilirubinemia	Intraventricular hemorrhage, Ischemic stroke	BPD*, RDS*, Intraventricular hemorrhage, HIE*, Sepsis	Congenital retinitis pigmentosa, Albinism	Pneumonia, Cleft palate, Pierre Robin sequence	Intraventricular hemorrhage, Subdural hematoma, Skull fractures	–	–	–	–

*NEC, Neonatal necrotizing enterocolitis; RDS, Respiratory distress syndrome; ROP, Retinopathy of Prematurity; HIE, Hypoxic-ischemic encephalopathy; BPD, Bronchopulmonary Dysplasia; PDA, Patent ductus arteriosus; IVH, Intraventricular hemorrhage. F1-3 stand for patients in fungal group, N1-5 stand for patients in non-fungal group, C1-4 stand for patients in control group.

alpha diversity and beta diversity. Regarding alpha diversity, the increase of the Chao1 index represents the richness of the intestinal bacterial community, and a lower Simpson index or higher Shannon index suggests a higher diversity of intestinal bacterial communities. The fungal group showed more estimated richness according to the Chao1 index than did the control group, but the differences between these groups did not rise to the level of statistical significance (sFigure1A). The Shannon index of the fungal group was decreased (sFigure1B), while the Simpson index was increased (sFigure1C), suggesting that the diversity of the microbiome changed upon infection, but there was no significant difference between the groups.

Next, we estimated beta diversity of gut microbiota based on their relative abundances and shared OTUs. A Principal Component Analysis (PCA) illustrated the similarities and differences of the three groups of bacteria based on the total number of OTUs. As shown in sFigure1D, there were some notable separations among the microbial populations of the fungal group, the control group and the non-fungal group. The percentages attributed to variations in Principle Component (PC)1 and PC2 were 38.15% and 22.08%, respectively. NMDS (Figure 2B) provided another method to identify the similarities and differences among the three groups; in this analysis, the stress coefficient was found to have explanatory significance (stress < 0.2, $P = 0.0588$, ANOSIM).

According to the absolute abundances of OTUs and the species annotation information, the microbiota compositions of each group at the family and genus levels were analyzed statistically. At the family level (Figures 2C, D), the composition of the intestinal microbiome in neonates with fungal infections was clearly altered. Interestingly, the relative abundance of inflammation-associated microbiota, including *Enterococcaceae* (72.00 vs 3.30%, $P = 0.034$) and *Pseudomonadaceae* (9.04 vs 0%, $P = 0.028$) in infants with a fungal infection was significantly higher than that of control group. This difference was especially clear with the *Enterococcaceae* family. The abundance of *Enterobacteriaceae* was decreased in the fungal group and non-fungal group relative to the control group, and it was especially decreased in the fungal group. *Clostridiaceae* was almost absent from the fungal group, but it was abundant in the non-fungal group. *Veillonellaceae* and *Pasteurellaceae* were detected at low levels in the fungal and non-fungal group.

These major changes observed at the family level were similarly observed in downstream taxa. At the genus level (sFigures 1E, F), we confirmed that *Enterococcus* (72.01 vs 3.30%, $P = 0.034$) and *Pseudomonas* (9.04 vs 0%, $P = 0.028$) were enriched in fungal group compared to control group, while the number of species of the genus *Escherichia* (*Enterobacteriaceae*) (0 vs 36.77%, $P = 0.028$) was lower. *Clostridium_sensu_stricto*, which are linked to the production of butyric acid, almost disappeared in the intestines of the three patients in the fungal group. Although the patient sample size was small, clear trends indicating a decrease of *Clostridiaceae* and increase of *Enterococcaceae* in the fungal infection group were noted.

A GraPhlAn plot was created to show the overall composition of the microbiota at the phylum-to-genus level of all samples. We observed that the abundance of *Enterococcus* was higher in the fungal group and the abundance of *Enterobacteriaceae* and *Clostridiaceae* were enriched in the

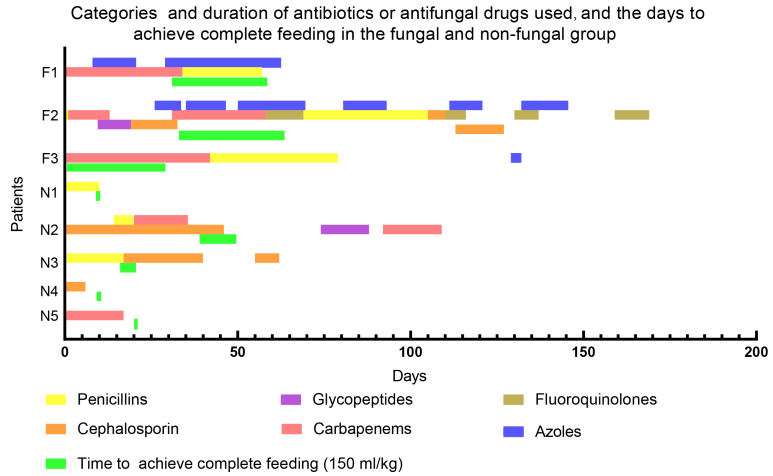

FIGURE 1
Categories and duration of antibiotics or antifungal drugs used, and the time to achieve complete feeding in the fungal and non-fungal group. F1-3 stand for patients in fungal group, N1-5 stand for patients in non-fungal group. A newborn feeding amount of 150ml/kg per day means complete feeding.

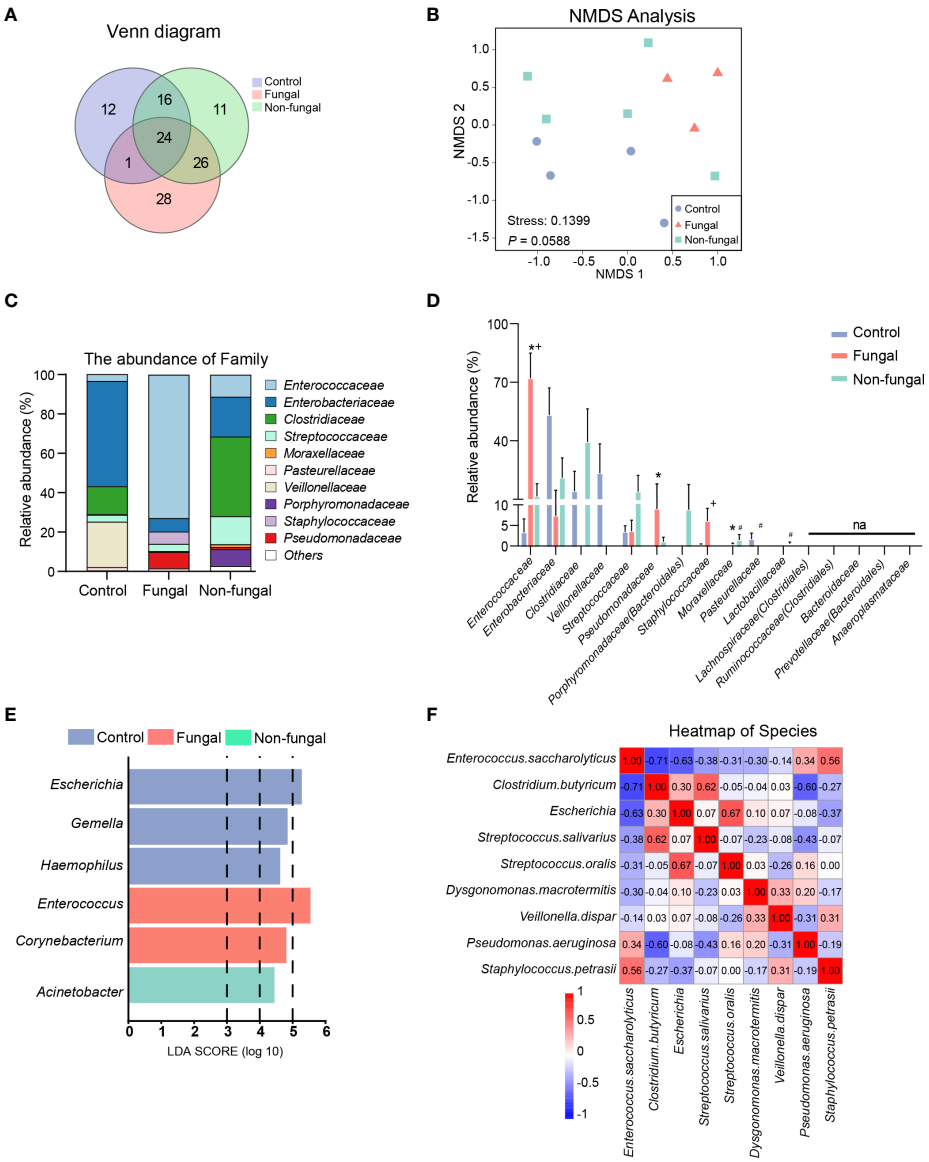

FIGURE 2
Evaluation of microbiome composition in newborns without antibiotics and without fungal infection (control group), newborns treated with antibiotics (non-fungal group) and newborns infected with fungi (fungal group). **(A)** A Venn diagram shows the common and unique OTUs of the three groups, where Core represents the common OTUs of the three groups. **(B)** Nonmetric Multidimensional Scaling (NMDS): groups were compared using ANOSIM. Relative proportions of sequences read at the family **(C)** levels assigned to different bacteria. The microbiome abundance was less than 0.5% in all samples, and the unannotated microbiomes were all merged into Others. Comparison of relative abundance of bacteria at family **(D)** levels. The results are mean ± SEM, and the P values of the differences between groups were tested by the Mann-Whitney U test. *P < 0.05 vs Control, #P < 0.05 vs Control, +P < 0.05 vs Fungal. **(E)** Differential enrichment of intestinal microbiota of rats in each group at the genus level according to linear discriminant analysis (LDA). Only taxa with LDA >3 at a P value <0.05 were considered significantly enriched. **(F)** Heat map: the number in the box is the Spearman coefficient, whose color deepens with the increase of the absolute value. Spearman values range from -1 (blue) to 1 (red).

control group and non-fungal group (sFigure 1G). The distribution of enriched bacteria at the genus level was identified using LEfSe analysis (Figure 2E and Supplement 1). Although the patient sample size was small, an enrichment of *Enterococcus* (LDA = 5.55, P = 0.024) in the fungal group attracted our attention. A heat map based on the Spearman coefficient showed the correlation between the microbiome at the species level; we found that there was a strong negative correlation between *Enterococcus* and *Clostridium butyricum* (*C. butyricum*), with a Spearman coefficient of -0.71 (Figure 2F).

Then, we used KEGG analysis to predict metabolic pathways of the infant intestinal microbiome (Figure 3). At the third hierarchical level of KEGG pathways (KEGG level 3), the metabolism of vancomycin (1.75 and 2.09 vs 1.13) and streptomycin (1.42 and 1.60 vs 0.96) were predicted to increase significantly in the fungal group and the non-fungal group compared to control group; these changes were likely related to the use of antibiotics in these neonates. In addition, bacterial infection (0.54 and 0.20 vs 0.05) and bacterial phosphotransferase system (3.0 and 1.72 vs 1.11) pathways were significantly enriched in the fungal and non-fungal groups. Glucose metabolism, amino acid metabolism and vitamin metabolism in the fungal and non-fungal group were disordered. Interestingly, butanoate metabolism (0.71 vs 1.04) decreased significantly in the fungal group compared to control group, while the demand for fatty acid (2.13 vs 1.49) and ketone synthesis (1.20 vs 0.41) increased.

Association of *C. albicans* infection with gut microbial changes in a rat model

Based on the strong negative correlation between *Enterococcus* and *C. butyricum* in intestinal microbiomes of infants infected with fungi, we hypothesized that these microbes play important roles in the process of neonatal fungal infection. Therefore, we constructed a rat fungal infection model to test our hypothesis. In a previous work, we showed that compared with untreated neonatal rats, the immunity of neonatal rats to *C. albicans* infection was significantly impaired after antibiotic treatment. This damage was manifested by increased levels of fungal glucan in peripheral blood, intestinal congestion, ischemia, multiple caseous fungal infections in the abdominal cavity and reduction of intestinal villi (Wang et al., 2020). These symptoms were similar to those of IFD.

Therefore, we further analyzed and evaluated the microbiome in the rat model of fungal infection. This analysis led to the identification of 537 OTUs in control rats (control group), 124 OTUs in rats infected with fungi after antibiotic treatment (fungal group) and 554 OTUs in rats infected with fungi without antibiotic treatment (vehicle group). We also identified 17 OTUs that were specific to the fungal group, indicating that the abundance and composition of microbial communities in the fungal group changed dramatically with antibiotic treatment (Figure 4A).

A detailed examination of the microbiota by investigation of alpha diversity and beta diversity showed important differences between groups. Regarding alpha diversity, the Chao1 index decreased sharply in the fungal group (Figure 4B), suggesting that the microbiome abundance decreased in this group. A decreased Shannon index (Figure 4C) and an increased Simpson index (Figure 4D) indicated that *C. albicans* infection significantly reduced the diversity of the intestinal microbiome in rats after antibiotic pretreatment. Upon rank-abundance curve analysis, which can reflect the richness and uniformity

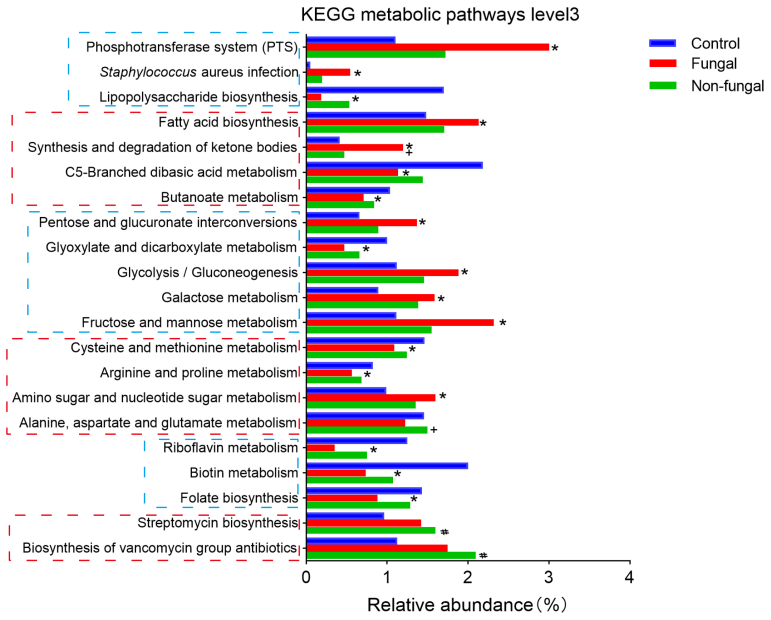

FIGURE 3
Differences of neonates in intestinal KEGG metabolic pathways level 3. *P < 0.05 vs Control, #P < 0.05 vs Control, +P < 0.05 vs Fungal. The P value was calculated with the Kruskal–Wallis test.

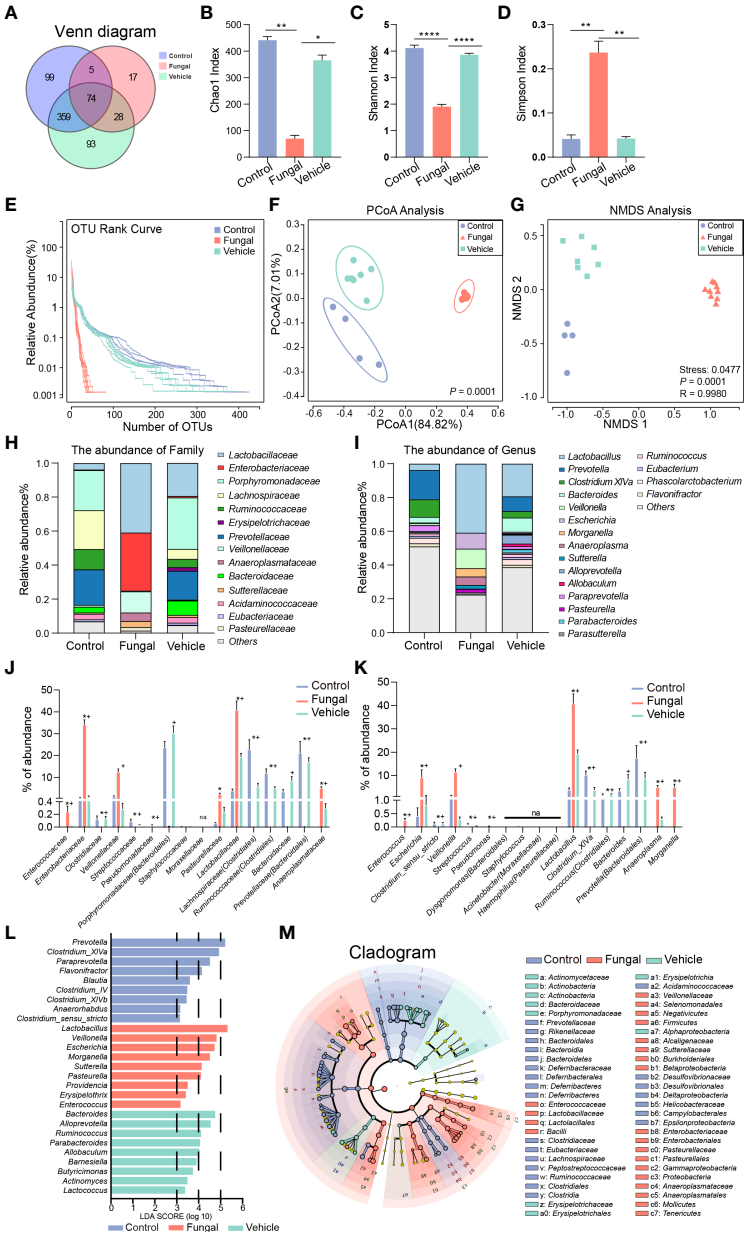

FIGURE 4
Comparison of intestinal microbial abundance and community composition among rats without any treatment (Control group), rats infected with fungi after antibiotic treatment (Fungal group) and rats inoculated with fungi only (Vehicle group). **(A)** A Venn diagram analysis shows the common and unique OTUs among the three groups. Three indexes of alpha diversity are displayed: **(B)** the Chao1 index, **(C)** the Shannon index, and **(D)** the Simpson index. The data are presented as the mean ± SEM. The P value was calculated with a Kruskal–Wallis test. *P < 0.05, **P < 0.01, ****P < 0.0001. **(E)** The RNK curve: the abscissa is sorted by sample OTUs, and the ordinate is OTU abundance. **(F)** Principal co-ordinate analysis (PCoA). PERMANOVA was used for comparison between groups. **(G)** Nonmetric Multidimensional Scaling (NMDS); groups were compared using Anosim. The Stress represents the difference between the distance of a point in two-dimensional space and that in multi-dimensional space. Stress < 0.05 is a perfect representation. Relative proportions of sequence read at the family **(H)** and genus **(I)** levels assigned to different bacteria. The microbiome abundance was less than 0.5% in all samples, and the unannotated microbiomes were all merged into Others. Comparison of relative abundance of bacteria at the family **(J)** and genus **(K)** levels. The results are presented as mean ± SEM, and the P value was calculated with a Kruskal–Wallis test. *P < 0.05 vs Control, #P < 0.05 vs Control, +P < 0.05 vs Fungal. **(L)** Differential enrichment of intestinal microbiota of rats in each group at the genus level according to linear discriminant analysis (LDA). Only taxa with LDA >3 at a P value <0.05 were considered significantly enriched. **(M)** Cladogram shows the community composition of the gut microbiota in rats based on LEfSe analysis.

of sample microbiome, there was no significant difference found in the length and steepness of the curves between the control group and the vehicle group, while the curve of the fungal group was steep and the length was shortened. These rank-abundance curves reflected the fact that a few dominant phylotypes comprise the major proportion of microbial communities in the rats of the fungal group, whereas more communities were seen in rats of the control and vehicle groups (Figure 4E). A principle component analysis (PCoA) of weighted UniFrac distance identified a clear separation of the microbiomes of the three groups, and each group was highly aggregated (Figure 4F). The percentages attributed to variations in PCoA1 and PCoA2 were 84.82% and 27.01%, respectively. NMDS (Figure 4G) analysis also confirmed the noted differences among the three groups (stress = 0.0477, $P < 0.001$, ANOSIM).

Next, we analyzed differences in the rat gut microbial communities at the levels of the family and genus that were caused by *C. albicans* infection after antibiotic pretreatment. When comparing the abundances, we ranked the strains on the abscissa according to their abundances within the human intestinal microbiome (Figure 2D). Although the abundances and compositions of rat microbiome were not completely consistent with the human microbiome, this comparison can still emphasize similar trends of several bacteria.

At the family level (Figures 4H, J), *Clostridiaceae* (0.00 vs 0.12%, P =0.019), *Ruminococcaceae* (*Clostridiales*) (0.001 vs 11.78%, $P < 0.001$) and *Lachnospiraceae* (*Clostridiales*) (0.002 vs 22.74%, $P < 0.001$) nearly disappeared in the fungal group compared to the control group. This finding was consistent with the change of *Clostridiaceae* in neonates infected with fungi (Figure 2D). Interestingly, lower levels of *Bacteroidaceae* (0.003 vs 3.42%, $P = 0.084$), *Prevotellaceae* (*Bacteroidales*) (0.003 vs 21.03%, $P = 0.006$) and *Porphyromonadaceae* (*Bacteroidales*) (0.009 vs 23.61%, $P = 0.088$) and higher levels of *Enterococcaceae* (0.24 vs 0%, $P = 0.005$), *Enterobacteriaceae* (34.14 vs 0.40%, $P = 0.028$), *Veillonellaceae* (12.43 vs 1.13%, $P = 0.088$), *Lactobacillaceae* (40.80 vs 3.83%, $P < 0.001$), *Pasteurellaceae* (2.18 vs 0.06%, $P = 0.014$) and *Anaeroplasmataceae* (5.06 vs 0%, $P < 0.001$) were observed in the fungal group as compared to the control group.

We further investigated changes of the microbiomes at the genus level. At the genus level (Figures 4I, K), *Clostridium_XlVa* (0 vs 10.28%, $P < 0.001$), *Clostridium_sensu_stricto* (0.001 vs 0.12%, $P = 0.019$), *Ruminococcus (Clostridiales)* (0 vs 1.42%, $P = 0.027$) and *Prevotella* (*Bacteroidales*) (0.002 vs 17.52%, $P = 0.003$) nearly disappeared in the fungal group compared to the control group. We noted that *Lactobacillus* was significantly enriched in the fungal group and the vehicle group. *Lactobacillus* was the dominant resident bacteria in the control group, and the increase of its relative abundance seemed to be caused by decreases of other bacteria. In addition, higher levels of *Enterococcus* (0.24 vs 0%, $P = 0.008$), *Escherichia* (9.24 vs 0.40%, $P = 0.044$), *Veillonella* (11.53 vs 1.13%, $P = 0.088$), *Anaeroplasma* (5.06 vs 0%, $P < 0.001$) and *Morganella* (5.06 vs 0.001%, $P = 0.028$) were observed in the fungal group as compared to the control group.

The distribution of enriched bacteria was identified at the genus level using LEfSe analysis. The LDA scores of 9 differentially abundant taxa in the fungal group were higher than 3 (Figures 4L, M; Supplement 1). These taxa were *Lactobacillus*, *Veillonella*, *Escherichia*, *Morganella* (*Enterobacteriaceae*), *Sutterella*, *Pasteurella*, *Providencia* (*Enterobacteriaceae*), *Erysipelothrix* and *Enterococcus*. Compared with rats subjected to fungal infection, *Prevotella*, *Clostridium_XlVa*, *Paraprevotella*, *Flavonifractor* (*Ruminococcaceae*), *Blautia* (*Clostridiales*), *Clostridium_IV*, *Clostridium_XlVb*, *Anaerorhabdus* (*Bacteroidaceae*) and *Clostridium_sensu_stricto* had higher LDA scores in the control group. Most of these microbes are well known to produce short-chain fatty acids (SCFAs), such as acetic acid and butyric acid. *Enterococcus* (LDA = 3.20, $P < 0.001$) in the fungal group, *Clostridium_XlVa* (LDA = 4.93, $P < 0.001$) in the control group and *Bacteroides* (LDA = 4.78, $P < 0.001$) and *Ruminococcus* (LDA = 4.14, $P < 0.001$) in the vehicle group were other findings that attracted our attention. Taken together, we have found that the intestinal microbiomes of infants with clinical fungal infections and rats in a model of fungal infection were enriched in proinflammatory *Enterococcus* and decreased in SCFA-producing *Clostridium* species.

Finally, we used PICRUST2 to predict the KEGG metabolic pathway level 3 of the intestinal microbiomes in the three groups of rats (Figure 5). The functions of amino acid metabolism and glucose metabolism in the fungal group were disordered. Compared with control group, *C. albicans* infection significantly increased the bacterial phosphotransferase system (1.23 vs 0.24), weakened bacterial chemotaxis (0.41 vs 1.94), and increased bacterial secretion (1.04 vs 0.78), invasion (0.06 vs 0.002) and infection (0.19 vs 0.02). Interestingly, pathways involving fatty acid synthesis (1.91 vs 1.59) and degradation (0.51 vs 0.32) increased in the fungal group compared to control group; these pathways included the metabolism of butyrate (0.91 vs 0.71), propionate (0.85 vs 0.64) and ketone bodies (0.61 vs 0.48). In addition, the normal activation of the NOD-like receptor signaling pathway (0.004 vs 0.04) in the intestinal microbiome of rats infected with *C. albicans* seemed to be inhibited.

Association of *Clostridium* species with loss of intestinal immunosuppression

As mentioned above, the intestinal microbiomes of rats infected with fungi showed significant changes after antibiotic treatment. Increases of *Enterococccus* and other opportunistic pathogens can induce inflammatory intestinal injury (Fiore et al., 2019). Of more concern is the disappearance of the

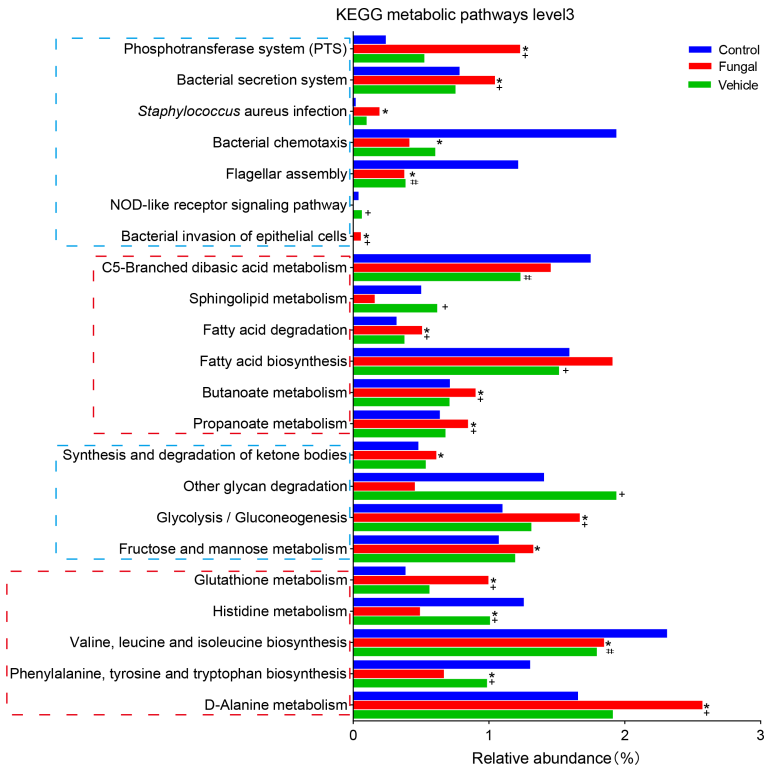

FIGURE 5
Differences in intestinal KEGG metabolic pathways level 3 among control rats, rats infected with fungi after antibiotic treatment and rats inoculated with fungi only. *P < 0.05 vs Control, #P < 0.05 vs Control, +P < 0.05 vs Fungal. The P value was calculated with a Kruskal–Wallis 4test.

genera *Clostridium* and the decrease of *Ruminococcus*, *Lachnospiraceae* and *Bacteroidacea*. Bacteria from these genera are dominant commensal bacteria that are known to be producers of SCFAs, which tend to control inflammation (Fan et al., 2015; Stoeva et al., 2021). In particular, the butyrate molecule can be shown to be effective in maintaining T regulatory (Treg) cell differentiation (Furusawa et al., 2013).

SCFAs promote intestinal IgA immune function and induce intestinal IL-10 expression to play an anti-inflammatory role (Kanai et al., 2015; Wu et al., 2017; Ariyoshi et al., 2020). Pro-inflammatory $CD11c^+$ macrophages produce cytokines that promote intestinal inflammation and mucosal injury, while $CD11c^-$ macrophage-like cells produce IL-10 (Arnold et al., 2016; Girard-Madoux et al., 2016; Bernardo et al., 2018). Compared with the control group and vehicle group, the expression of IgA and IL10 in the intestinal tissue of rats infected with *C. albicans* pretreated with antibiotics was significantly decreased (Figures 6A–D). In addition, CD11c expression in proinflammatory macrophages increased significantly (Figures 6B, E). The microbiota metabolite butyrate promotes intestinal IgA immune function (Isobe et al., 2020). Butyrate-producing *C. butyricum* induces the increase of IL10 expression in the intestine to play an anti-inflammatory role (Kanai et al., 2015; Ariyoshi et al., 2020). Therefore, the susceptibility to fungal infections after antibiotic use may be due to dysregulation of mucosal immune regulatory molecules such as IL10 and IgA. SCFA-producing *Clostridium* species may also play a key role in this process.

Discussion

Gut microbes play a crucial role in host health and disease of the host all through life, but its influence on diseases at the early stage of life is unclear. Neonatal IFD due to *Candida* is an important cause of morbidity and mortality in critical neonates, especially in premature infants (Benjamin, 2006; Kilpatrick et al., 2021). Using broad-spectrum antibiotics is a risk factor for neonatal *Candida* infection, because it can eliminate healthy bacterial microbiome and lead to fungal overgrowth (Cotten et al., 2006; Tripathi et al., 2012; Kilpatrick et al., 2021). Previous studies revealed that long-term exposure to antibiotics leads to a disordering of the intestinal microbiome that is mainly manifested as decreased probiotics and increased antibiotic-

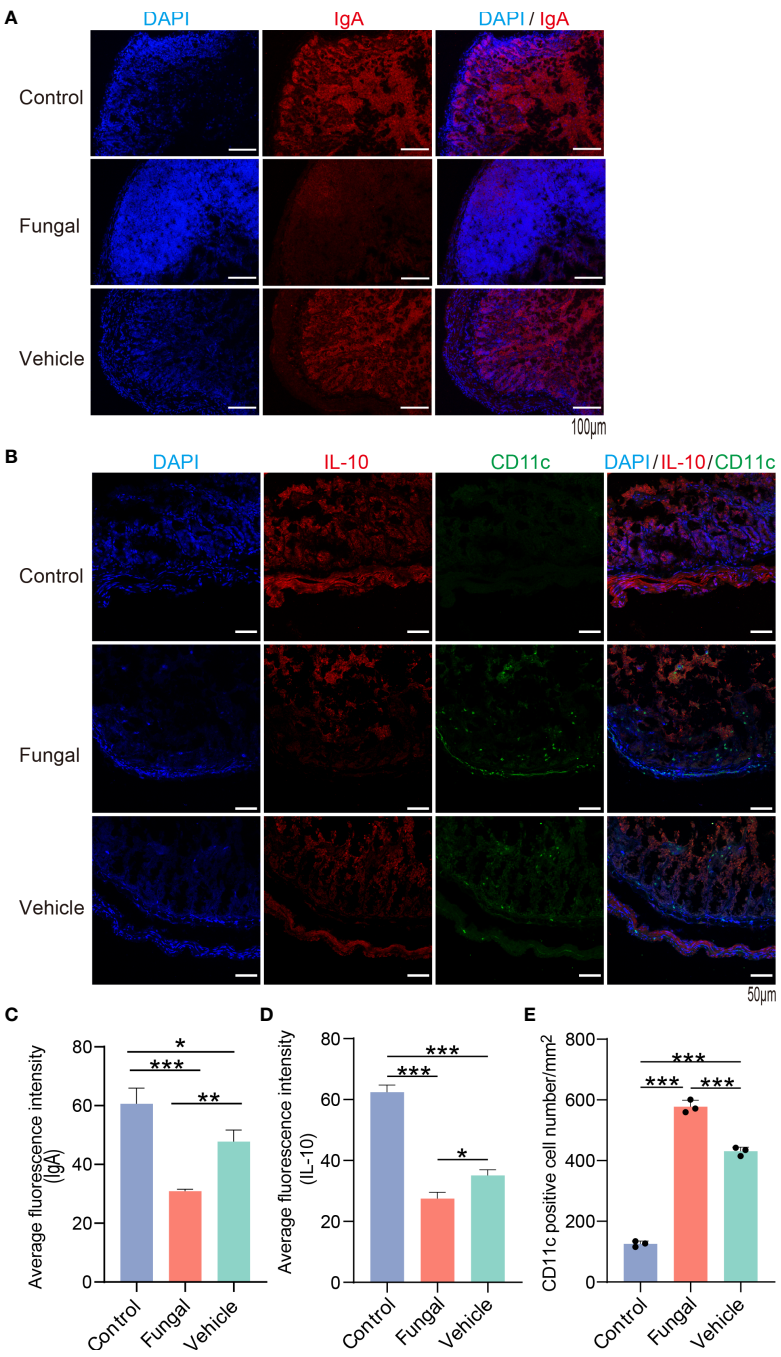

FIGURE 6
The expression of IL10 and IgA in intestinal tissues of rats with fungal infection decreased after antibiotic treatment. **(A)** IgA (red) and DAPI for nuclei (blue) and **(B)** IL10 (red), CD11c (green) and DAPI for nuclei (blue) in intestinal tissues of rats of the three group. **(C, D)**: The average fluorescence intensity of IgA and IL10. **(E)** Number of $CD11c^{+}$ cells/mm^2. *P < 0.05, **P < 0.01, ***P < 0.001, by one-way ANOVA with Tukey's multiple comparisons test. Data are presented as the mean ± SD of at least 3 independent experiments.

resistant pathogens (Tripathi et al., 2012; Gasparrini et al., 2019; Ramirez and Cantey, 2019), but the change in intestinal microbiome of neonates with IFD is unknown. In our study, clear trends indicating a decrease of *Clostridium* species in neonates with IFD was noted. We also confirmed that SCFA-producing *Clostridium* species almost disappeared from the intestinal microbiome of a rat model of IFD, while *Enterococcus* increased significantly. The expression of IgA and

IL10 in the intestinal tissue of fungus-infected rats after antibiotic treatment decreased significantly, suggesting that antibiotics may affect intestinal immune homeostasis by changing neonatal intestinal microecology. The improper use of broad-spectrum antibiotics can lead to the increase of opportunistic pathogens and the depletion of SCFA-producing *Clostridium* species and *Bacteroides*, which can affect immune homeostasis and induce IFD (Figure 7).

We found that the levels of *Clostridium* species and *Bacteroidales*, which produce SCFA (including acetic acid and butyric acid), decreased sharply in both IFD infants and rats. *Clostridium _ XlVa* and *Bacteroides* have been confirmed to activate HIF-1α and IL-37 to inhibit the colonization of *C. albicans* (Fan et al., 2015). Moreover, animal studies revealed that *Clostridium* species clusters IV, XIVa and XVIII are involved in SCFA production and have been demonstrated to promote the induction of colonic Tregs (Atarashi et al., 2011; 2013). The gut microbiome regulates the effects of local and systemic immunity through SCFAs, especially butyrate (Markowiak-Kopec and Slizewska, 2020). The gut epithelial cells use butyric acid as 70% of their energy to regulate immune cells and anti-inflammatory factors (such as IL-10, IgA and TGF-β) (Siddiqui and Cresci, 2021). In this study, we found a significant depletion of *C. butyricum* in the gut microbiota of IFD rats. According to the KEGG metabolic pathway, abnormalities in pathways related to the metabolism of SCFAs (including butyric acid, propionic acid and ketone bodies) were related to alterations in the abundance of these probiotics that produce SCFAs. We believe that the depletion of *Clostridium* species weakened the resistance of rats to fungal colonization.

Enterococcus naturally exists in the environment and is an important component of human and animal intestinal microbiomes (Wada et al., 2019). After the use of antibiotics, however, the number of *Enterococci* will likely continue to increase, which may be the key event to induce fungal infection after the use of antibiotics (Elvers et al., 2020). At the same time, drug-resistant *Enterococcus* is one of the severe challenges faced in the NICU, as this organism causes immune-mediated inflammatory injury to the intestinal mucosal (Arias and Murray, 2012; Fiore et al., 2019). For the first time, we found an increase in the abundance of *Enterococcus* in the intestinal tracts of infants infected with fungi after the use of antibiotics. Similarly, a significant increase in opportunistic pathogens, including *Enterococcus*, was clearly demonstrated in rats infected with fungi after antibiotic use. We also found a strong negative correlation between *Enterococcus* and *C. butyricum* in IFD rats. We consider that the increased *Enterococcus* may take part in the susceptibility to fungi after the use of antibiotics.

The gut and other mucosal tissues are rich in IgA (Pietrzak et al., 2020). As part of mucosal immunity, secretory IgA is involved in pathogen elimination and the regulation of the intestinal microbiome (Tsuji et al., 2008; Pabst and Slack, 2020; Rollenske et al., 2021). For example, *Bacteroides* can induce the production of IgA in the large intestine through the formation of germinal centers and an increase in the number of B cells that produce IgA (Yanagibashi et al., 2013). In this study,

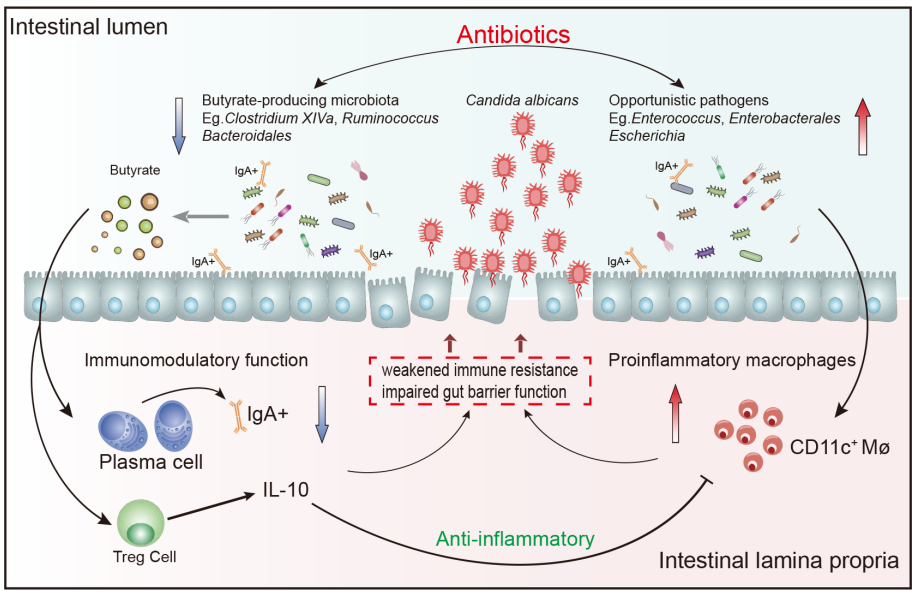

FIGURE 7
Schematic drawing showing relationship between the intestinal microbiome and immune imbalance.

it was confirmed that *Clostridium* species and *Bacteroides* are decreased and *Enterococcus* are increased after antibiotic use, and these are closely related to the key proteins IgA and IL10 in the maintenance of intestinal homeostasis. We considered that the decrease in intestinal immune resistance mediated by SCFA promoted the colonization of invasive fungi. In the future, we plan to quantify changes to the butyric acid content and to further test its role in resisting fungal colonization.

The data in this study show that one of the most important preventive measures is to avoid the irregular use of broad-spectrum antibiotics in order to protect the appropriate intestinal microbiome. In addition, *Clostridium* probiotics and their metabolites have been shown to enhance the gastrointestinal mucosal barrier and help regulate Treg cell immune responses (Atarashi et al., 2013; Cao et al., 2019; Liu et al., 2019; Guo et al., 2020; Hagihara et al., 2020). Therefore, we propose that reintroducing symbiotic microbiota may reduce the risk of IFD. However, a limitation of this study is that the sample size was small, and further exploration of underlying mechanisms was not conducted. In the future, genetically engineered rats and aseptic rats will be used to further explore the molecular mechanisms to clarify the relationships between specific bacteria and intestinal immunity.

Conclusion

In summary, the gut microbiota is highly affected by the use of antibiotics and is closely related to the pathogenesis of IFD. The inappropriate use of broad-spectrum antibiotics can lead to the increase of opportunistic pathogens and the depletion of *Clostridium* species and *Bacteroides* in preterm infants. The depletion of these SCFA-producing bacteria ultimately reduces the IgA- and IL-10-mediated immune resistance in the host intestinal tissue, while harmful pathogenic bacteria cause inflammatory intestinal injury. This eventually leads to the colonization and outbreak of invasive fungi in preterm infants.

Data availability statement

The datasets presented in this study can be found in online repositories. The datasets have been submitted to NCBI (https://www.ncbi.nlm.nih.gov) with accession PRJNA821364 and PRJNA821375.

Ethics statement

All the studies followed the guidelines for the ethical treatment of human specimens and were approved by the Ethics Committee of Guangzhou Women and Children's Medical Center. According to the Declaration of Helsinki, written informed consent was obtained from the parents of all enrolled neonates. Animals in this study were approved by Guangzhou Medical University's Institutional Animal Care and Use Committee and conducted in accordance with institutional guidelines.

Author contributions

DH, HL and PW contributed to the study design. JL and CL joined in experiments. BH and PW performed and/or contributed critically to all experiments and analyzed the data. PW and YL recruited the study cohort and collected samples. YL and YK collated and counted the clinical data of volunteers. BH and DH wrote the manuscript. BH, YK and WZ put forward valuable amendments to the article. All the authors have read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.981823/full#supplementary-material

SUPPLEMENTARY FIGURE 1

Evaluation of microbiome composition in newborns. Three indexes of alpha diversity are displayed: **(A)** the Chao1 index, **(B)** the Shannon index, and **(C)** the Simpson index. The results are shown as mean ± SEM. There were no statistical differences in the three indexes. **(D)** Principal component analysis (PCA): color matching of each sample in groups. The fraction of diversity captured by the coordinate is given as a percentage. Relative proportions of sequences read at the genus **(E)** levels assigned to different bacteria. The microbiome abundance was less than 0.5% in all samples, and the unannotated microbiomes were all merged into Others. Comparison of relative abundance of bacteria at genus **(F)** levels. The results are mean ± SEM, and the P values of the differences between groups were tested by the Mann-Whitney U test. *P < 0.05 vs Control, #P < 0.05 vs Control, +P < 0.05 vs Fungal. **(G)** A GraPhlAn was used to examine the overall microbiota composition at the phylum-to-genus level of all samples.

References

Aliaga, S., Clark, R. H., Laughon, M., Walsh, T. J., Hope, W. W., Benjamin, D. K., et al. (2014). Changes in the incidence of candidiasis in neonatal intensive care units. *Pediatrics* 133 (2), 236–242. doi: 10.1542/peds.2013-0671

Alroy, J. (2015). The shape of terrestrial abundance distributions. *Sci. Adv.* 1 (8), e1500082. doi: 10.1126/sciadv.1500082

Arias, C. A., and Murray, B. E. (2012). The rise of the enterococcus: Beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10 (4), 266–278. doi: 10.1038/nrmicro2761

Ariyoshi, T., Hagihara, M., Eguchi, S., Fukuda, A., Iwasaki, K., Oka, K., et al. (2020). Clostridium butyricum MIYAIRI 588-induced protectin D1 has an anti-inflammatory effect on antibiotic-induced intestinal disorder. *Front. Microbiol.* 11, 587725. doi: 10.3389/fmicb.2020.587725

Arnold, I. C., Mathisen, S., Schulthess, J., Danne, C., Hegazy, A. N., and Powrie, F. (2016). CD11c(+) monocyte/macrophages promote chronic helicobacter hepaticus-induced intestinal inflammation through the production of IL-23. *Mucosal Immunol.* 9 (2), 352–363. doi: 10.1038/mi.2015.65

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of clostridia strains from the human microbiota. *Nature* 500 (7461), 232–236. doi: 10.1038/nature12331

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., et al. (2011). Induction of colonic regulatory T cells by indigenous clostridium species. *Science* 331 (6015), 337–341. doi: 10.1126/science.1198469

Autmizguine, J., Smith, P. B., Prather, K., Bendel, C., Natarajan, G., Bidegain, M., et al. (2018). Effect of fluconazole prophylaxis on candida fluconazole susceptibility in premature infants. *J. Antimicrob. Chemother.* 73 (12), 3482–3487. doi: 10.1093/jac/dky353

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al. (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* 17 (5), 690–703. doi: 10.1016/j.chom.2015.04.004

Baptista, M. I., Nona, J., Ferreira, M., Sampaio, I., Abrantes, M., Tome, M. T., et al. (2016). Invasive fungal infection in neonatal intensive care units: A multicenter survey. *J. Chemother.* 28 (1), 37–43. doi: 10.1179/1973947814Y.0000000222

Benjamin, D. K. (2006). Neonatal candidiasis among extremely low birth weight infants: Risk factors, mortality rates, and neurodevelopmental outcomes at 18 to 22 months. *Pediatrics* 117 (1), 84–92. doi: 10.1542/peds.2004-2292

Benjamin, D. K., Stoll, B. J., Gantz, M. G., Walsh, M. C., Sanchez, P. J., Das, A., et al. (2010). Neonatal candidiasis: Epidemiology, risk factors, and clinical judgment. *Pediatrics* 126 (4), e865–e873. doi: 10.1542/peds.2009-3412

Bergeron, A. C., Seman, B. G., Hammond, J. H., Archambault, L. S., Hogan, D. A., and Wheeler, R. T. (2017). Candida albicans and pseudomonas aeruginosa interact to enhance virulence of mucosal infection in transparent zebrafish. *Infect. Immun.* 85 (11), e00475-17. doi: 10.1128/IAI.00475-17

Bernardo, D., Marin, A. C., Fernandez-Tome, S., Montalban-Arques, A., Carrasco, A., Tristan, E., et al. (2018). Human intestinal pro-inflammatory CD11c(high) CCR2(+) CX3CR1(+) macrophages, but not their tolerogenic CD11c(-) CCR2(-) CX3CR1(-) counterparts, are expanded in inflammatory bowel disease. *Mucosal Immunol.* 11 (4), 1114–1126. doi: 10.1038/s41385-018-0030-7

Bokulich, N. A., Chung, J., Battaglia, T., Henderson, N., Jay, M., Li, H., et al. (2016). Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci. Transl. Med.* 8 (343), 343r–382r. doi: 10.1126/scitranslmed.aad7121

Brown, A. O., Graham, C. E., Cruz, M. R., Singh, K. V., Murray, B. E., Lorenz, M. C., et al. (2019). Antifungal activity of the enterococcus faecalis peptide EntV requires protease cleavage and disulfide bond formation. *mBio* 10 (4), e01334-19. doi: 10.1128/mBio.01334-19

Calley, J. L., and Warris, A. (2017). Recognition and diagnosis of invasive fungal infections in neonates. *J. Infect.* 74 Suppl 1, S108–S113. doi: 10.1016/S0163-4453(17)30200-1

Cao, G., Tao, F., Hu, Y., Li, Z., Zhang, Y., Deng, B., et al. (2019). Positive effects of a clostridium butyricum-based compound probiotic on growth performance, immune responses, intestinal morphology, hypothalamic neurotransmitters, and colonic microbiota in weaned piglets. *Food Funct.* 10 (5), 2926–2934. doi: 10.1039/c8fo02370k

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7 (5), 335–336. doi: 10.1038/nmeth.f.303

Chen, H., and Boutros, P. C. (2011). VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinf.* 12, 35. doi: 10.1186/1471-2105-12-35

Clancy, C. J., and Nguyen, M. H. (2018). Diagnosing invasive candidiasis. *J. Clin. Microbiol.* 56 (5), e01909-17. doi: 10.1128/JCM.01909-17

Costantini, C., Nunzi, E., Spolzino, A., Merli, F., Facchini, L., Spadea, A., et al. (2022). A high-risk profile for invasive fungal infections is associated with altered nasal microbiota and niche determinants. *Infect. Immun.* 90 (4), e4822. doi: 10.1128/iai.00048-22

Cotten, C. M., McDonald, S., Stoll, B., Goldberg, R. N., Poole, K., and Benjamin, D. K. (2006). The association of third-generation cephalosporin use and invasive candidiasis in extremely low birth-weight infants. *Pediatrics* 118 (2), 717–722. doi: 10.1542/peds.2005-2677

DiBartolomeo, M. E., and Claud, E. C. (2016). The developing microbiome of the preterm infant. *Clin. Ther.* 38 (4), 733–739. doi: 10.1016/j.clinthera.2016.02.003

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26 (19), 2460–2461. doi: 10.1093/bioinformatics/btq461

Elvers, K. T., Wilson, V. J., Hammond, A., Duncan, L., Huntley, A. L., Hay, A. D., et al. (2020). Antibiotic-induced changes in the human gut microbiota for the most commonly prescribed antibiotics in primary care in the UK: A systematic review. *BMJ Open* 10 (9), e35677. doi: 10.1136/bmjopen-2019-035677

Esaiassen, E., Fjalstad, J. W., Juvet, L. K., van den Anker, J. N., and Klingenberg, C. (2017). Antibiotic exposure in neonates and early adverse outcomes: A systematic review and meta-analysis. *J. Antimicrob. Chemother.* 72 (7), 1858–1870. doi: 10.1093/jac/dkx088

Fan, D., Coughlin, L. A., Neubauer, M. M., Kim, J., Kim, M. S., Zhan, X., et al. (2015). Activation of HIF-1alpha and LL-37 by commensal bacteria inhibits candida albicans colonization. *Nat. Med.* 21 (7), 808–814. doi: 10.1038/nm.3871

Feja, K. N., Wu, F., Roberts, K., Loughrey, M., Nesin, M., Larson, E., et al. (2005). Risk factors for candidemia in critically ill infants: A matched case-control study. *J. Pediatr.* 147 (2), 156–161. doi: 10.1016/j.jpeds.2005.02.021

Ferreras-Antolin, L., Sharland, M., and Warris, A. (2019). Management of invasive fungal disease in neonates and children. *Pediatr. Infect. Dis. J.* 38 (6S Suppl 1), S2–S6. doi: 10.1097/INF.0000000000002317

Fiore, E., Van Tyne, D., and Gilmore, M. S. (2019). Pathogenicity of enterococci. *Microbiol. Spectr.* 7 (4), 10.1128/microbiolspec.GPP3-0053-2018. doi: 10.1128/microbiolspec.GPP3-0053-2018

Fox, E. P., Cowley, E. S., Nobile, C. J., Hartooni, N., Newman, D. K., and Johnson, A. D. (2014). Anaerobic bacteria grow within candida albicans biofilms and induce biofilm formation in suspension cultures. *Curr. Biol.* 24 (20), 2411–2416. doi: 10.1016/j.cub.2014.08.057

Fu, J., Ding, Y., Jiang, Y., Mo, S., Xu, S., and Qin, P. (2018). Persistent candidemia in very low birth weight neonates: Risk factors and clinical significance. *BMC Infect. Dis.* 18 (1), 558. doi: 10.1186/s12879-018-3487-9

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., et al. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* 504 (7480), 446–450. doi: 10.1038/nature12721

Fu, J., Wang, X., Wei, B., Jiang, Y., and Chen, J. (2016). Risk factors and clinical analysis of candidemia in very-low-birth-weight neonates. *Am. J. Infect. Control* 44 (11), 1321–1325. doi: 10.1016/j.ajic.2016.03.026

Gasparrini, A. J., Wang, B., Sun, X., Kennedy, E. A., Hernandez-Leyva, A., Ndao, I. M., et al. (2019). Persistent metagenomic signatures of early-life hospitalization and antibiotic treatment in the infant gut microbiota and resistome. *Nat. Microbiol.* 4 (12), 2285–2297. doi: 10.1038/s41564-019-0550-2

Gibson, M. K., Wang, B., Ahmadi, S., Burnham, C. D., Tarr, P. I., Warner, B. B., et al. (2016). Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nat. Microbiol.* 1 (4), 16024. doi: 10.1038/nmicrobiol.2016.24

Girard-Madoux, M. J., Ober-Blobaum, J. L., Costes, L. M., Kel, J. M., Lindenbergh-Kortleve, D. J., Brouwers-Haspels, I., et al. (2016). IL-10 control of CD11c+ myeloid cells is essential to maintain immune homeostasis in the small and large intestine. *Oncotarget* 7 (22), 32015–32030. doi: 10.18632/oncotarget.8337

Gregory, K. E., Samuel, B. S., Houghteling, P., Shan, G., Ausubel, F. M., Sadreyev, R. I., et al. (2016). Influence of maternal breast milk ingestion on acquisition of the intestinal microbiome in preterm infants. *Microbiome* 4 (1), 68. doi: 10.1186/s40168-016-0214-x

Guo, P., Zhang, K., Ma, X., and He, P. (2020). Clostridium species as probiotics: Potentials and challenges. *J. Anim. Sci. Biotechnol.* 11, 24. doi: 10.1186/s40104-019-0402-1

Gutierrez, D., Weinstock, A., Antharam, V. C., Gu, H., Jasbi, P., Shi, X., et al. (2020). Antibiotic-induced gut metabolome and microbiome alterations increase the susceptibility to candida albicans colonization in the gastrointestinal tract. *FEMS Microbiol. Ecol.* 96 (1), fiz187. doi: 10.1093/femsec/fiz187

Hagihara, M., Kuroki, Y., Ariyoshi, T., Higashi, S., Fukuda, K., Yamashita, R., et al. (2020). Clostridium butyricum modulates the microbiome to protect intestinal barrier function in mice with antibiotic-induced dysbiosis. *iScience* 23 (1), 100772. doi: 10.1016/j.isci.2019.100772

Healy, D. B., Ryan, C. A., Ross, R. P., Stanton, C., and Dempsey, E. M. (2022). Clinical implications of preterm infant gut microbiome development. *Nat. Microbiol.* 7 (1), 22–33. doi: 10.1038/s41564-021-01025-4

Hsieh, E., Smith, P. B., Jacqz-Aigrain, E., Kaguelidou, F., Cohen-Wolkowiez, M., Manzoni, P., et al. (2012). Neonatal fungal infections: When to treat? *Early Hum. Dev.* 88 Suppl 2, S6–S10. doi: 10.1016/S0378-3782(12)70004-X

Isobe, J., Maeda, S., Obata, Y., Iizuka, K., Nakamura, Y., Fujimura, Y., et al. (2020). Commensal-bacteria-derived butyrate promotes the T-cell-independent IgA response in the colon. *Int. Immunol.* 32 (4), 243–258. doi: 10.1093/intimm/dxz078

Jiang, S., Yang, C., Yang, C., Yan, W., Shah, V., Shah, P. S., et al. (2020). Epidemiology and microbiology of late-onset sepsis among preterm infants in China 2015-2018: A cohort study. *Int. J. Infect. Dis.* 96, 1–9. doi: 10.1016/j.ijid.2020.03.034

Kanai, T., Mikami, Y., and Hayashi, A. (2015). A breakthrough in probiotics: Clostridium butyricum regulates gut homeostasis and anti-inflammatory response in inflammatory bowel disease. *J. Gastroenterol.* 50 (9), 928–939. doi: 10.1007/s00535-015-1084-x

Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., and Tanabe, M. (2012). KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* 40 (Database issue), D109–D114. doi: 10.1093/nar/gkr988

Kilpatrick, R., Scarrow, E., Hornik, C., and Greenberg, R. G. (2021). Neonatal invasive candidiasis: Updates on clinical management and prevention. *Lancet Child Adolesc. Health.* 6 (1), 60–70. doi: 10.1016/S2352-4642(21)00272-8

King, J., Pana, Z., Lehrnbecher, T., Steinbach, W. J., and Warris, A. (2017). Recognition and clinical presentation of invasive fungal disease in neonates and children. *J. Pediatr. Infect. Dis. Soc.* 6 (suppl_1), S12–S21. doi: 10.1093/jpids/pix053

La Rosa, P. S., Warner, B. B., Zhou, Y., Weinstock, G. M., Sodergren, E., Hall-Moore, C. M., et al. (2014). Patterned progression of bacterial populations in the premature infant gut. *Proc. Natl. Acad. Sci.* 111 (34), 12522–12527. doi: 10.1073/pnas.1409497111

Lee, J. H., Hornik, C. P., Benjamin, D. K., Herring, A. H., Clark, R. H., Cohen-Wolkowiez, M., et al. (2013). Risk factors for invasive candidiasis in infants >1500 g birth weight. *Pediatr. Infect. Dis. J.* 32 (3), 222–226. doi: 10.1097/INF.0b013e3182769603

Liu, L., Zeng, D., Yang, M., Wen, B., Lai, J., Zhou, Y., et al. (2019). Probiotic clostridium butyricum improves the growth performance, immune function, and gut microbiota of weaning rex rabbits. *Probiotics Antimicrob. Proteins* 11 (4), 1278–1292. doi: 10.1007/s12602-018-9476-x

Markowiak-Kopec, P., and Slizewska, K. (2020). The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients* 12 (4), 1107. doi: 10.3390/nu12041107

Nogueira, F., Sharghi, S., Kuchler, K., and Lion, T. (2019). Pathogenetic impact of bacterial-fungal interactions. *Microorganisms* 7 (10), 459. doi: 10.3390/microorganisms7100459

Pabst, O., and Slack, E. (2020). IgA and the intestinal microbiota: The importance of being specific. *Mucosal Immunol.* 13 (1), 12–21. doi: 10.1038/s41385-019-0227-4

Parra-Llorca, A., Gormaz, M., Alcantara, C., Cernada, M., Nunez-Ramiro, A., Vento, M., et al. (2018). Preterm gut microbiome depending on feeding type: Significance of donor human milk. *Front. Microbiol.* 9, 1376. doi: 10.3389/fmicb.2018.01376

Pietrzak, B., Tomela, K., Olejnik-Schmidt, A., Mackiewicz, A., and Schmidt, M. (2020). Secretory IgA in intestinal mucosal secretions as an adaptive barrier against microbial cells. *Int. J. Mol. Sci.* 21 (23), 9254. doi: 10.3390/ijms21239254

Qin, Y., Havulinna, A. S., Liu, Y., Jousilahti, P., Ritchie, S. C., Tokolyi, A., et al. (2022). Combined effects of host genetics and diet on human gut microbiota and incident disease in a single population cohort. *Nat. Genet.* 54 (2), 134–142. doi: 10.1038/s41588-021-00991-z

Ramirez, C. B., and Cantey, J. B. (2019). Antibiotic resistance in the neonatal intensive care unit. *Neoreviews* 20 (3), e135–e144. doi: 10.1542/neo.20-3-e135

Rao, C., Coyte, K. Z., Bainter, W., Geha, R. S., Martin, C. R., and Rakoff-Nahoum, S. (2021). Multi-kingdom ecological drivers of microbiota assembly in preterm infants. *Nature* 591 (7851), 633–638. doi: 10.1038/s41586-021-03241-8

Rollenske, T., Burkhalter, S., Muerner, L., von Gunten, S., Lukasiewicz, J., Wardemann, H., et al. (2021). Parallelism of intestinal secretory IgA shapes functional microbial fitness. *Nature* 598 (7882), 657–661. doi: 10.1038/s41586-021-03973-7

Ronan, V., Yeasin, R., and Claud, E. C. (2021). Childhood development and the microbiome-the intestinal microbiota in maintenance of health and development of disease during childhood development. *Gastroenterology* 160 (2), 495–506. doi: 10.1053/j.gastro.2020.08.065

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

Shao, Y., Forster, S. C., Tsaliki, E., Vervier, K., Strang, A., Simpson, N., et al. (2019). Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* 574 (7776), 117–121. doi: 10.1038/s41586-019-1560-1

Siddiqui, M. T., and Cresci, G. (2021). The immunomodulatory functions of butyrate. *J. Inflamm. Res.* 14, 6025–6041. doi: 10.2147/JIR.S300989

Stoeva, M. K., Garcia-So, J., Justice, N., Myers, J., Tyagi, S., Nemchek, M., et al. (2021). Butyrate-producing human gut symbiont, clostridium butyricum, and its role in health and disease. *Gut Microbes* 13 (1), 1–28. doi: 10.1080/19490976.2021.1907272

Tripathi, N., Cotten, C. M., and Smith, P. B. (2012). Antibiotic use and misuse in the neonatal intensive care unit. *Clin. Perinatol.* 39 (1), 61–68. doi: 10.1016/j.clp.2011.12.003

Tsuji, M., Suzuki, K., Kitamura, H., Maruya, M., Kinoshita, K., Ivanov, I. I., et al. (2008). Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin a generation in the gut. *Immunity* 29 (2), 261–271. doi: 10.1016/j.immuni.2008.05.014

Valentine, M., Benade, E., Mouton, M., Khan, W., and Botha, A. (2019). Binary interactions between the yeast candida albicans and two gut-associated bacteroides species. *Microb. Pathog.* 135, 103619. doi: 10.1016/j.micpath.2019.103619

Wada, Y., Harun, A. B., Yean, C. Y., and Zaidah, A. R. (2019). Vancomycin-resistant enterococcus: Issues in human health, animal health, resistant mechanisms and the malaysian paradox. *Adv. Anim. Vet. Sci.* 11 (7), 1021–1034. doi: 10.17582/journal.aavs/2019/7.11.1021.1034

Wang, P., Yao, J., Deng, L., Yang, X., Luo, W., and Zhou, W. (2020). Pretreatment with antibiotics impairs Th17-mediated antifungal immunity in newborn rats. *Inflammation* 43 (6), 2202–2208. doi: 10.1007/s10753-020-01287-w

Warris, A., Lehrnbecher, T., Roilides, E., Castagnola, E., Bruggemann, R., and Groll, A. H. (2019). ESCMID-ECMM guideline: Diagnosis and management of invasive aspergillosis in neonates and children. *Clin. Microbiol. Infect.* 25 (9), 1096–1113. doi: 10.1016/j.cmi.2019.05.019

Warris, A., Pana, Z. D., Oletto, A., Lundin, R., Castagnola, E., Lehrnbecher, T., et al. (2020). Etiology and outcome of candidemia in neonates and children in europe: An 11-year multinational retrospective study. *Pediatr. Infect. Dis. J.* 39 (2), 114–120. doi: 10.1097/INF.0000000000002530

Watterberg, K., Aucott., S., Benitz, W. E., Cummings, J. J., Eichenwald, E. C., Goldsmith, J., et al. (2015). The apgar score. *Pediatrics* 136 (4), 819–822. doi: 10.1542/peds.2015-2651

Weimer, K., Smith, P. B., Puia-Dumitrescu, M., and Aleem, S. (2021). Invasive fungal infections in neonates: A review. *Pediatr. Res.* 91 (2), 404–412. doi: 10.1038/s41390-021-01842-7

Wu, W., Sun, M., Chen, F., Cao, A. T., Liu, H., Zhao, Y., et al. (2017). Microbiota metabolite short-chain fatty acid acetate promotes intestinal IgA response to microbiota which is mediated by GPR43. *Mucosal Immunol.* 10 (4), 946–956. doi: 10.1038/mi.2016.114

Yanagibashi, T., Hosono, A., Oyama, A., Tsuda, M., Suzuki, A., Hachimura, S., et al. (2013). IgA production in the large intestine is modulated by a different mechanism than in the small intestine: Bacteroides acidifaciens promotes IgA production in the large intestine by inducing germinal center formation and increasing the number of IgA+ b cells. *Immunobiology* 218 (4), 645–651. doi: 10.1016/j.imbio.2012.07.033

Zwittink, R. D., Renes, I. B., van Lingen, R. A., van Zoeren-Grobben, D., Konstanti, P., Norbruis, O. F., et al. (2018). Association between duration of intravenous antibiotic administration and early-life microbiota development in late-preterm infants. *Eur. J. Clin. Microbiol. Infect. Dis.* 37 (3), 475–483. doi: 10.1007/s10096-018-3193-y

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In vitro investigation on lactic acid bacteria isolated from Yak faeces for potential probiotics

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In order to evaluate the potential and safety of lactic acid bacteria (LAB) isolated from faeces samples of Ganan yak as probiotic for prevention and/or treatment of yak diarrhea, four strains of LAB including *Latilactobacillus curvatus* (FY1), Weissella cibaria (FY2), *Limosilactobacillus mucosae* (FY3), and *Lactiplantibacillus pentosus* (FY4) were isolated and identified in this study. Cell surface characteristics (hydrophobicity and cell aggregation), acid resistance and bile tolerance, compatibility, antibacterial activity and *in vitro* cell adhesion tests were also carried out to evaluate the probiotic potential of LAB. The results showed that the four isolates had certain acid tolerance, bile salt tolerance, hydrophobicity and cell aggregation, all of which contribute to the survival and colonization of LAB in the gastrointestinal tract. There is no compatibility between the four strains, so they can be combined into a mixed probiotic formula. Antimicrobial tests showed that the four strains were antagonistic to *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. Moreover, the *in vitro* safety of the four isolates were determined through hemolytic analysis, gelatinase activity, and antibacterial susceptibility experiments. The results suggest that all the four strains were considered as safe because they had no hemolytic activity, no gelatinase activity and were sensitive to most antibacterial agents. Moreover, the acute oral toxicity test of LAB had no adverse effect on body weight gain, food utilization and organ indices in Kunming mice. In conclusion, the four LAB isolated from yak feces have considerable potential to prevent and/or treat yak bacterial disease-related diarrhea.

Abbreviations: LAB, Lactic acid bacteria; MRS, De Man, Rogosa, and Sharpe; MH, Mueller Hinton; LB, Luria Bertani; CFU, Colony-forming unit; PCR, polymerase chain reaction; PBS, Phosphate buffer saline; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; SPF, specified pathogen-free; CFC, Cell-free culture supernatants; *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*; *Salm. Typhimurium*, *Salmonella Typhimurium*; *L. curvatus*, *Latilactobacillus curvatus*; *W. cibaria*, *Weissella cibaria*; *L. pentosus*, *Lactiplantibacillus pentosus*; *L. mucosae*, *Limosilactobacillus mucosae*.

KEYWORDS
Yak, diarrhea, lactic acid bacteria, potential probiotics, antimicrobial activity, Caco-2

1 Introduction

As is a common disease caused by bacterial invasion (*E. coli*, *salmonella species*, *S. aureus*, *Shigella species*, *Campylobacter species*), parasitic infections, dietary changes and viral infections, diarrhea is often associated with disturbance of the intestinal flora and damage to the intestinal mucosal barrier (Kim et al., 2021; Liu et al., 2022). Moreover, diarrhea is prevalent in calves, which may seriously affect the growth and health and even cause death of calves, resulting in high treatment and breeding costs in the breeding industry and hence considerable economic losses (Dioso et al., 2020; Liu et al., 2022). In modern livestock production, antibacterials are widely used for growth promotion and disease prevention or treatment (Kim et al., 2021). However, accumulating evidence indicates that the overuse and misuse of antimicrobial agents cause extensive food safety problems and environmental pollution, and resistance of antibacterials may selectively spread from animals to humans, posing a public health risk (Hu and Cheng, 2016). Due to the increasing antimicrobial resistance of pathogens, since 2006, the European Union (EU) ratified usage prohibition of antibacterials as animal growth supplements and disease prevention. At present, many countries are following suit (More, 2020). Therefore, there is an urgent need for non antibacterial substitutes to promote animal growth and prevent diseases.

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Food and Agriculture Oraganization/ World Health Organization [FAO/WHO], 2006). Probiotics can produce organic acids (mainly acetic acid and lactic acid), hydrogen peroxide, bacteriocins, bacteriocin-like inhibitory substances, short-chain fatty acids (SCFAs), conjugated linoleic acid (CLA), gamma-aminobutyric acid (GABA), vitamins (especially vitamin B and vitamin K) and other substances (Upadrasta and Madempudi, 2016; Zhang et al., 2022). Probiotics are a potential living biological therapy for maintaining gastrointestinal microecology by stimulating immunity, competing for nutrients, synthesizing antimicrobial peptides and metabolites for inhibiting epithelial and mucosal adhesion of pathogens, balancing unfavorable intestinal pH (Rolfe, 2000). Probiotics are considered as natural substitutes for antimicrobia. They are considered as capable of stabilizing the intestinal flora and normalizing peristaltic disorders. Besides, they could also inhibit the development of pathogenic microorganisms, prevent or reduce the course of bacterial, viral and antibiotic diarrhea, eliminate or reduce the symptoms of lactose intolerance and so on (Jarocki et al., 2020). Probiotics are widely used in treatment of human and animal gastrointestinal disorders because of their biological properties of promoting intestinal peristalsis and maintaining intestinal homeostasis (Wang et al., 2018; Jarocki et al., 2020; Zhang et al., 2020). The European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN group) recommended the usage of probiotics as an adjunctive/ preventive treatment for different types of diarrhea in pediatrics (Dioso et al., 2020). So far, probiotics are considered a safe and viable natural alternative to antibacterials in improving livestock performance due to their multiple beneficial effects on the host and have attracted a lot of attention from researchers (Li et al., 2019; Alayande et al., 2020).

Many projects have been undertaken to better understand the impact of probiotics on the intestinal ecosystem and its impact on health and disease. Probiotic feeding based on milk substitutes has the potential to control diseases, including neonatal calf diarrhea (Kayasaki et al., 2021). Liu et al. (2022) found that newborn calves in the complex probiotic group had tightly clustered intestinal bacterial communities and lower rates of diarrhea.

Diarrhea has been reported occurring in yak calves and is a major cause of calf death (Wang et al., 2018; Dong et al., 2020; Liu et al., 2022). Yak, which has a strong ability to adapt to the harsh natural environment, e.g. low temperature, food scarcity, especially low oxygen, is an ancient and primitive livestock species unique to the Qinghai Tibet Plateau. Not only used for farming and transportation, yaks can also local herdsmen with production and daily necessities such as milk, meat, wool, labor and fuel, which make them an important source of life and economy for herdsmen. The Gannan yak is one of the indigenous yak of China (Goshu et al., 2018).

However, the information we have about probiotic LAB from yak intestines is limited. Although some probiotics are generally recognized as safe (GRAS) there are a few reports of local or systemic infections, such as endocarditis and sepsis, that may be associated with the ingestion of certain lactobacilli (Bourdichon et al., 2012; Aroutcheva et al., 2016). Therefore, caution is still needed when choosing probiotics. To ensure the efficiency and safety of LAB for application, they must be systematically identified and characterized. Therefore, this study aims to providing a theoretical basis for the prevention and/or treatment of diarrhea associated with bacterial diseases in yaks *via* isolating and

identifying LAB strains from Gannan yak, and evaluating their probiotic potential and safety.

2 Materials and methods

2.1 Sample collection, bacterial strains, cells and culture conditions

Samples were randomly collected from free-ranging yaks in Hezuo Forest Park in Gannan Tibetan Autonomous Prefecture. Twenty fecal samples were initially stored on-site by location in special sterile faecal sampling tubes filled with Phosphate buffer saline (PBS) (Figure S1). Then, samples were kept on ice and transported to Gansu Agricultural University in Lanzhou and stored at -80°C for further experiments.

E. coli (ATCC 25922), *S. aureus* (ATCC 25923), and *Salm. Typhimurium* (CMCC 50115) were purchased from Beijing Biobw Biotechnology Co., Ltd. They were incubated in Mueller Hinton (MH) agar plates or broth (Solarbio, China) and Luria Bertani (LB) broth (Solarbio, China) at 37°C aerobically for 24 h to study cell aggregation and antibacterial activity. The human colon cancer cell lines Caco-2 (BNCC350772) were purchased from the BeNa Culture Collection (BNCC; Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM with high glucose, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Pansera, Aidenbach, Germany) in 5% CO_2 at 37°C.

2.2 Ethical statement and animal care

Experimental procedures adopted in this study were approved by the Animal Ethics Committee of College of Veterinary Medicine, Gansu Agricultural University (License no. GSAU-AEW-2019-0010).

Fifty (25 males and 25 females) specified pathogen-free (SPF) Kunming mice (Swiss albino mice origin, 4 weeks old, 18-22g) were obtained from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. They were housed in standard plastic cages (5 per cage, segregated by sex) under controlled atmosphere (temperature 22 ± 3°C, humidity 55 ± 5%) with a light/dark cycle of 12/12 h. During the whole study, mice were freely consuming the same basic diet and plain water, and were monitored regularly for health status.

2.3 Isolation of LAB

The feces (ca. 1 g) were diluted by 10 times gradient in turn, and the bacterial suspensions of 10^{-1}, 10^{-2} and 10^{-3} gradients were uniformly coated onto De Man, Rogosa, and Sharpe (MRS) agar (Solarbio, China) added with 2% (w/v) $CaCO_3$ (Solarbio, China) incubated at 37°C anaerobically for 48 h. After the reaction of lactic acid with $CaCO_3$, a clear area was formed around the colony. So, the milky white colony with clear zone was selected as tentative LAB for purification (Chang et al., 2007). All purified strains were mixed with equal volume of 50% (w/v) sterile glycerol and stored at -80°C for subsequent experiments.

2.4 Molecular identification

Molecular identification was performed according to Screening Criteria of Lactic Acid Bacteria for Feeding Aquatic Animals (TCSWSL016-2019). Four isolates were incubated overnight at 37 °C in MRS broth, and then genomic DNA was extracted using TIANamp bacteria DNA extraction kit (TIANGEN, Beijing, China). Molecular identification was performed by amplifying the 16S rRNA gene using universal primers (27F 5′-AGAGTTTGATCCTGGCTCAG -3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′) and previously described polymerase chain reaction (PCR) reaction conditions (Vaz-Moreira et al., 2009). PCR products were separated by 1.2% agarose gel electrophoresis and confirmed by sequencing (Genewiz, Suzhou, China). The obtained 16S rRNA sequences of the strains were compared with the EzBioCloud databases to identify the species (https://www.ezbiocloud.net/). Phylogenetic tree was constructed based on 16S rRNA sequences by MEGA software (version 7.0) with a Kimura two-parameter model for distance options and a Neighbor Joining (NJ) method for clustering with 1000 bootstrap replicates (Kumar et al., 2016).

2.5 Screening of probiotic strains

2.5.1 Hydrophobicity

The degree of cell surface hydrophobicity of the LAB isolates was assessed *via* measuring microbial adhesion to hydrocarbons (MATH) in xylene (Macklin, Shanghai, China) according to the method in Zhang et al. (2022). Briefly, overnight cultures of four isolates were centrifuged at 5,000 × g for 3 min at 4 °C. The pellets were washed twice with PBS and resuspended in PBS, then the OD600 of the isolates was adjusted by spectrophotometry (Biochrom genequant, UK) in the range of 1.0 ± 0.1. Each bacterial suspension (3 mL) was mixed with 1 mL of xylene (Macklin, Shanghai, China), swirled for 2 min, then incubated at 37 °C for 1 h. The water layer was carefully aspirated and measured at OD600 (Biochrom genequant, UK). Cell surface hydrophobicity (%) was calculated the equation as follows:

$$\text{Hydrophobicity } \% = [(1 - A_t)/A_0] \times 100$$

Where A_0 denotes the optical density at 0 h and A_t stands for the optical density at 1 h (Zeng et al., 2021).

The affinity of LAB to solvent was divided into hydrophilicity (< 10%), medium hydrophilicity (10-34%), medium hydrophobicity (35-70%) or high hydrophobicity (71-100%) (Qureshi et al., 2020)

2.5.2 Cell aggregation

Aggregation properties of the selected LAB were performed according to the procedure described by El-Deeb et al. (2020) and Li et al. (2020). Briefly, four strains were incubated in MRS medium for 18 h at 37°C, then harvested by 5000 × g, centrifugation for 10 min. The pellets washed 3 times with PBS (pH 7.0), then resuspended in PBS and incubated (aerobic and static) at room temperature for 4 h. Then, the bacterial supernatant was pipetted out carefully for OD600 measurement. Three replicates were made for each strain. Autoaggregation (%) was calculated using the following equation:

$$\text{Autoaggregation } \% = [1 - A_0/A_t] \times 100$$

Where A_0 represents the absorbance at 0 h and A_t indicates the absorbance at 4 h.

Bacterial suspension for coaggregation was prepared as described above. Four isolates were assessed for their ability to co-aggregate with *E. coli*, *S. aureus* and *Salm. typhimurium*. The suspension of each LAB strain (2 mL) was mixed with the same volume of each indicator strain and three replicates were made. The OD600 of the suspensions were measured by spectrophotometry (Biochrom genequant, UK) at 0 h and 4 h. The coaggregation rates were calculated as follows.

$$\text{Coaggregation } \% = [(A_p + A_i) - 2A_{mix}(A_p + A_i)] \times 100$$

Where A_p and A_i represent OD600nm of three standard bacterial strains and four isolates before mixing, A_{mix} is the pool absorbance at final time.

While A_p denotes the OD600 nm of the three indicator strains at 0 h, A_i is the OD600 nm of the four isolates before mixing, and A_{mix} is the absorbance after 4 h of the mixture.

2.5.3 Acid resistance and bile tolerance

The viability of the isolates in acidic environments was determined according to the method described in Li et al. (2020) and Dowarah et al. (2018). Acid resistance was assessed with MRS broth (pH 3.0), and bile tolerance was tested with MRS broth supplemented with 0.3% (w/v) bile salt (Solarbio, China). Then, 2700 µl of each solution was mixed with 300 µl of each overnight culture in a 5 mL tube and incubated at 37°C for 4 h. The mixture was retrieved with a pipette and measured at OD600. Survival rates were calculated as follows:

$$\text{Survival rate} = A_t/A_0 \times 100$$

Where A_0 stands for the optical density at 0 h and A_t denotes the optical density at 4 h.

2.5.4 Compatibility

The compatibility study was performed with minor modifications to previously described procedures (Aristimuno Ficoseco et al., 2018). Firstly, 10 mL of 1.5% sterilized agar was spread in each Petri dish, and after agar solidification, the Oxford cups were placed evenly on top. Then, 15 mL MRS solid medium was taken when it was cooled to 45°C and mixed vigorously with 200 µL of each LAB overnight culture and poured into Petri dishes. After cooling, the Oxford cups were gently removed and wells of 8 mm in diameter would appear on the agar. Then 50 µL of Cell-free culture supernatants (CFC) from each LAB strain was placed into each well, then the dishes were incubated aerobically at 37°C for 24 h. Finally, the diameter of the inhibition zone was observed and measured with a vernier caliper.

2.5.5 Antibacterial activities

The agar well diffusion method was used to evaluate the antimicrobial activities of CFC from four isolates against *E. coli*, *S. aureus* and *Salm. typhimurium*. Four isolates were activated and cultured in MRS broth and indicator strains were incubated in LB broth at 37°C for 18 h. CFC of LAB were prepared by centrifugation (10000 × g, 10 min, 4 °C) and filtration (Millipore 0.22 µm). The bacterial suspension of indicator bacteria was adjusted to 10^7 CFU/mL with LB and 100 µL of bacterial fluid was spread on the surface of MH agar plate. After the plate is dried, the Oxford cups (8 mm) were placed evenly on the plates and 100 µL CFC was loaded into the Oxford cup. MRS medium with pH 6.5 was added as control. Plates were incubated at 37°C for 24 h before measuring the inhibition zone. The inhibition zone diameter was scored as follows: negative (-), ≤9 mm; weak (+), 9-12mm; strong (++), 12-16mm; very strong (+++), ≥16mm (Qureshi et al., 2020).

2.5.6 *In vitro* cell adhesion assay

The adhesion capacity was determined based on the previous method with minor adjustments (Li et al., 2020; Chen et al., 2022). Briefly, Caco-2 cells were seeded in 24-well plate (5×10^4/well) and cultured in DMEM with 10% FBS to achieve a confluent monolayer cell. Four isolates were cultured in MRS broth for 24 h at 37°C and harvested using centrifugation (5000 × g, 3 min, 4°C). The pellets were washed twice in sterile PBS. Then, the bacterial solution was fluorescently labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA SE, Beyotime, China, 10 µg/mL) in PBS at 37°C for 10 min in the dark, and finally the cell density was adjusted to 10^8 CFU/mL with DMEM medium. Cell monolayer were washed thrice with PBS, then 1 mL of DMEM serum-free medium and 50 µL of bacterial suspension were added into each well in three replicates, and 24-well plates were incubated for 1 h at 37°C in 5% CO_2.

First, culture medium was aspirated and each well was carefully washed twice with sterile PBS to remove non-adherent bacteria. Then, 100 μL of trypsin was added into each well, the plates were incubated at 37°C for 10 min to completely digest the cells. Finally, cell culture medium was added into each well to stop the reaction. The fluorescence intensity was performed at 488 nm of excitation, and at 518 nm of emission wavelength by using the fluorescence microplate reader (SpectraMax i3x, Molecular Devices, USA). The percentage of adhesion was calculated as follows:

$$\text{Adhesion rate}\ (\%) = A_a/A_b \times 100$$

where A_b indicates the fluorescence intensity before adhesion and A_a represents the fluorescence intensity after adhesion.

2.6 Safety assessment

2.6.1 Hemolytic activity

The four isolates and *S. aureus* were incubated in MRS medium and LB broth, respectively, for 24 h at 37°C. Each bacterial solution was streaked on blood agar plates containing 5% defibrinated sheep blood (Solarbio, China) and incubated at 37°C for 48 h. The clear zone around the *S. aureus* colony (β-hemolysis) was detected and used as a positive control (Moreno et al., 2018).

2.6.2 Gelatinase activity

The gelatinase activity of LAB was conducted by using a previously reported method with minor adjustments (Perin et al., 2014; Rastogi et al., 2020). Briefly, 1 μL of 24 h incubated LAB was spotted on MRS agar with 5% (w/v) gelatin (Solarbio, China), and the plates were incubated anaerobically at 37°C for 72 h, then cooled at 4°C for 4 h. The opaque halo around the colony is considered to be a positive result of gelatinase production.

2.6.3 Antimicrobial susceptibility

The disk diffusion method was used to determine the antimicrobial susceptibility patterns of all strains. Twelve antimicrobial paper disks (μg/disc) including ceftriaxone (30), ciprofloxacin (5), ampicillin (10), rifampicin (5), kanamycin (30), streptomycin (10), tetracycline (30), gentamicin (10), chloramphenicoll (30), erythromycin (15), clindamycin (2) and cephalothiophene (30) were purchased from Hangzhou Binhe Microorganism Reagent Co,. First, 100 μL of each bacterial solution (1×10^8 CFU/mL) was evenly coated on MRS agar plates, and then antibacterial paper was placed on the plate at equal intervals. The plates were incubated at 37°C for 24 h. Finally, the diameter of the inhibition zone around the antibacterial paper was measured with vernier caliper and evaluated based on Clinical and Laboratory Standards Institute (CLSI, 2018). The sensitivity of LAB was classified as sensitive (S), intermediate (I), or resistant (R) to 12 antibiotics.

2.6.4 Acute oral toxicity in mice

The acute oral toxicity test in mice was carried out following guidelines provided by Organisation of Economic Cooperation and Development (OECD) guidelines 423 (OECD, 2001). After 5 days of acclimation, 50 mice were divided into 5 groups (5 males and 5 females). Four groups were gavaged with FY1, FY2, FY3 and FY4 at a concentration of 5×10^{12} CFU/mL, and the control group were fed with 200μL of saline. All mice were monitored for special attention during the first 4 hours and daily for 14 days thereafter for clinical signs or behavioral changes, body weight, food intake, and mortality. After mice were sacrificed, organs were taken to observe lesions, weighed and organ indices (heart, liver, spleen, lung, kidney, uterus, ovary or testis) were calculated for LAB safety assessment. Organ index were determined as follows: organ weight/mouse body weight.

2.7 Statistical analysis

Phylogenic tree were conducted using the MEGA 7 software and graphs were plotted by Originpro 2019b and GraphPad Prism 8.0. Date were expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using SPSS 26.0, and statistical significance was defined as $P<0.05$ by one-way ANOVA following least significant difference (LSD) and Waller-Duncan's post-treatment multiple comparisons.

3 Results

3.1 Isolation of LAB

After purification, four presumptive LAB (FY1, FY2, FY3, FY4) were selected according to the size of calcium dissolving ring and colony morphology. Gram-staining and microscopic examinations revealed that 4 strains were Gram-positive bacilli (Figure S2). Sequence analysis of 16S rRNA revealed that FY1, FY2, FY3, FY4 had 99-100% similarity with, *Latilactobacillus curvatus* (*L.* curvatus), *Weissella cibaria* (*W. cibaria*), *Lactiplantibacillus pentosus* (*L. pentosus*), and *Limosilactobacillus mucosae* (*L. mucosae*), respectively (Figure S3, Data Sheet 1). Phylogenetic trees (Figure 1) were constructed based on Neighbor-Joining algorithm and Kimura two-parameter model using the MEGA 7.0.

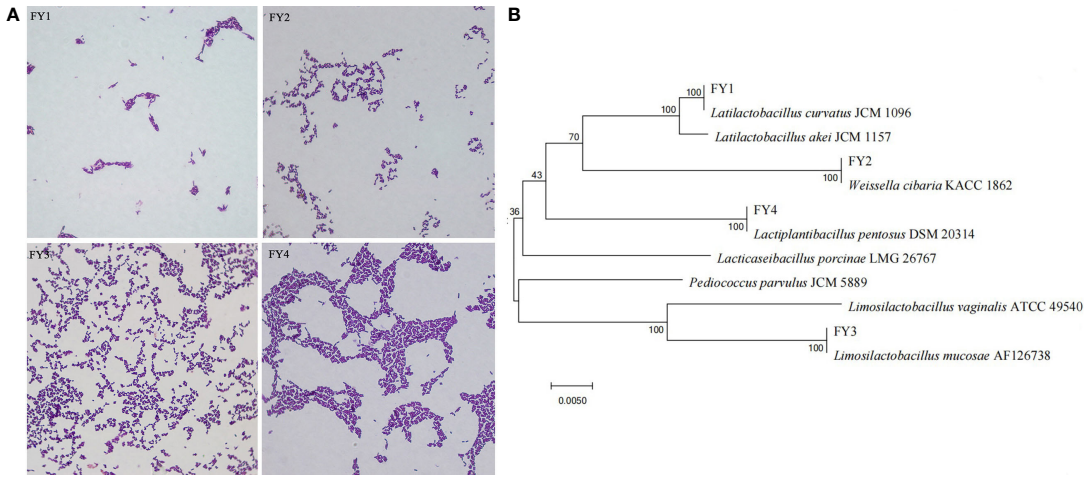

FIGURE 1
Phylogenetic tree of four isolates based on Neighbor-Joining distance analysis of 16S rRNA gene sequences. **(A)** The diagram shows the results of Gram staining for four isolates: FY1 (*L. curvatus*), FY2 (*W. cibaria*), FY3 (*L. mucosae*), and FY4 (*L. pentosus*). **(B)** Phylogenetic tree constructed by using a neighbor-joining method on the basis for 16S rRNA gene sequences.

3.2 Accession numbers

The 16S rRNA nucleotide sequences of four strains were deposited in the GenBank database under the following accession numbers: FY1: *Latilactobacillus curvatus* (ON758920), FY2: *Weissella cibaria* (ON758921), FY3: *Lactiplantibacillus pentosus* (ON758922), and FY4: *Limosilactobacillus mucosae* (ON758923).

3.3 Hydrophobicity

Figure 2A shows that four isolates have varied cell surface hydrophobicity xylene. FY4 exhibited highly hydrophobic (84.33%), FY3 showed moderately hydrophobic (37.79%), while FY2 and FY1 had moderately hydrophilic (17.32%, 13.65%).

3.4 Acid resistance and bile tolerance

Acid resistance and bile tolerance of the four strains were shown in Figure 2B. Acid resistance of the four strains are very approximate, FY1 (69.96%), FY2 (68.29%), FY3 (68.86), and FY4 (68.59%). After 3 h exposure to bile salt condition, FY1 showed the highest bile tolerance (45.24%), followed by FY3 (30.10%), FY2 (29.33%), and FY4 (29.09%).

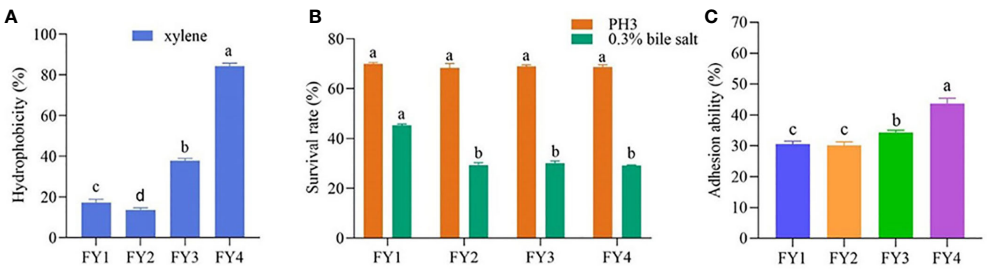

FIGURE 2
The hydrophobicity, acid and bile salt resistance and adhesion ability of LAB isolates. **(A)** Hydrophobicity percentages of LAB isolates to xylene **(B)** The acid and bile salt tolerance of LAB isolates. Values expressed as mean ± SD. Different letters represent significant difference, P< 0.05. **(C)** Percent adhesion values of LAB to Caco-2. Values expressed as mean ± SD. Different letters represent significant difference, P < 0.05.

TABLE 1 The percentage of autoaggregation and coaggregation with *E. coli*, *S. aureus*, and *Salm. typhimurium* by four isolates.

Isolates	Autoaggregation	Coaggregation		
		E. coli	*S. aureus*	*Salm. typhimurium*
FY1	15.67 ± 0.29 d	24.68 ± 2.65 c	23.00 ± 0.54 c	22.59 ± 0.38 b
FY2	23.88 ± 0.47 b	31.79 ± 0.38 b	24.92 ± 0.87 b	21.93 ± 0.72 b
FY3	19.24 ± 0.48 c	26.28 ± 0.99 c	26.13 ± 0.85 b	18.76 ± 0.34 c
FY4	49.19 ± 0.10 a	38.43 + 0.20 a	38.44 ± 0.41 a	34.81 ± 0.64 a

Values expressed as mean ± SD. Different letters represent significant difference, $P < 0.05$.

3.5 Cell aggregation

The percentage of autoaggregation and coaggregation of four isolates against three pathogenic bacteria were shown in Table 1. All the four strains have large ranges of autoaggregation ability (15.67-49.19%) and coaggregation ability (18.76-38.44%) to three pathogens. Among them, FY4 exhibited high autoaggregation ability (49.19%) and coaggregation ability with *E. coli* (38.43%), *S. aureus*(38.44%), and *Salm. Typhimurium* (34.81%).

3.6 Compatibility

No inhibition halo was observed from the compatibility results among the four strains, indicating that four isolates can be combined as a mixed probiotic formula (Figure S4).

3.7 Antibacterial activities

The antibacterial activity results of four strains are shown in Table 2. FY3 exhibited the strongest antimicrobial activity towards *E. coli* (15.83 mm) and *S. aureus* (15.33 mm); FY4 showed the most potent antimicrobial activity to *Salm. typhimurium* (12.10 mm).

3.8 *In vitro* cell adhesion assay

The adhesion ability of four isolates to Caco-2 cells was shown in Figure 2C. Four isolates showed varying adhesion properties, ranging from 30.06% to 43.69%. FY4 has the maximum adhesion property (43.69%), followed by FY3 (34.33%), FY1 (30.61%) and FY2 (30.06%).

3.9 Safety assessment

3.9.1 Antimicrobial susceptibility

The susceptibility of four isolates to 12 different antimicrobials are provided in Table 3 and Figure 3. Four strains were susceptible to ceftriaxone, tetracycline, chloramphenicol and cephalothiophene, but showed resistant to kanamycin. FY1 showed intermediate susceptibility to erythromycin and clindamycin, resistant to ciprofloxacin, kanamycin, streptomycin and gentamicin. FY2 showed intermediate susceptibility to clindamycin, but resistant to kanamycin, streptomycin and gentamicin. FY3 showed intermediate susceptibility to ampicillin, but resistant to rifampicin, kanamycin, and streptomycin. FY4 was sensitive to all antimicrobials except kanamycin.

TABLE 2 The inhibition zone diameters (mm) of four strains against *E. coli*, *S. aureus* and *Salm. typhimurium.*

Isolates	Indicator pathogens					
	E. coli		*S. aureus*		*Salm. typhimurium*	
FY1	9.60 ± 0.16	+	10.43 ± 0.33	+	9.77 ± 0.21	+
FY2	10.77 ± 0.25	+	10.83 ± 0.35	+	9.87 ± 0.31	+
FY3	15.83 ± 0.29	++	15.33 ± 0.29	++	11.83 ± 0.35	+
FY4	12.83 ± 0.35	++	14.67 ± 0.42	++	12.10 ± 0.36	++

Values expressed as mean ± SD. Less than or equal to 9 mm (negative, -), 9-12mm (weak, +), 12-16mm (strong, ++), and more than 16mm (very strong, +++).

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TABLE 3 Antibiotic susceptibility of selected LAB strains.

Strains	Antibiotic susceptibility zone of inhibition in mm											
	CRO	CIP	AM	R	K	S	TE	GM	C	E	CC	CE
FY1	S	R	S	S	R	R	S	R	S	I	I	S
FY2	S	S	S	S	R	R	S	R	S	S	I	S
FY3	S	S	I	R	R	R	S	S	S	S	S	S
FY4	S	S	S	S	R	S	S	S	S	S	S	S

Ceftriaxone (CRO), ciprofloxacin (CIP), ampicillin (AM), rifampicin (R), kanamycin (K), streptomycin (S), tetracycline (TE), gentamicin (GM), chloramphenicol (C), erythromycin (E), clindamycin (CC), cephalothiophene (CE). Erythromycin results based on R ≤ 13 mm; I: 13–23 mm; S ≥ 23 mm. Gentamycin results based on R ≤ 6 mm; I, 7–9 mm; S ≥ 10 mm. Streptomycin results based on R ≤ 11 mm; I, 12–14 mm; S ≥ 15 mm. S, susceptible (zone diameter, ≥21). I: intermediate (zone diameter, 15–20 mm); R, resistant (zone diameter, ≤15 mm).

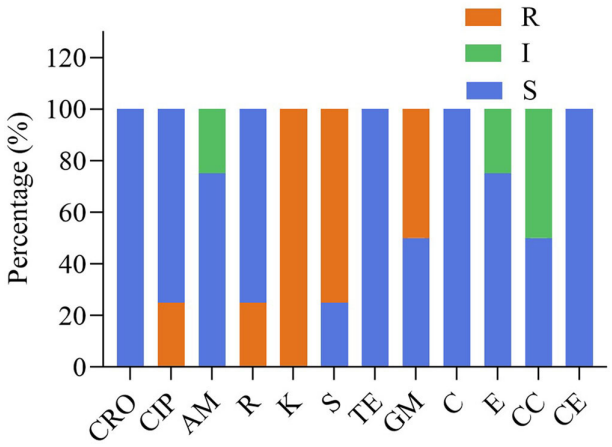

FIGURE 3
The antibiotic resistance of the LAB isolates against 12 tested antibiotics. Ceftriaxone (CRO), ciprofloxacin (CIP), ampicillin (AM), rifampicin (R), kanamycin (K), streptomycin (S), tetracycline (TE), gentamicin (GM), chloramphenicol (C), erythromycin (E), clindamycin(CC), cephalothiophene (CE). The total number of LAB strains was taken as 100%. S, sensitive; I, intermediately resistant; R, resistant.

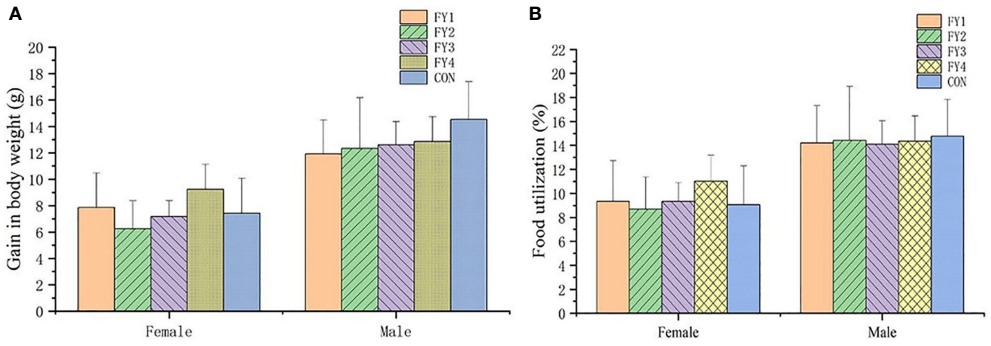

FIGURE 4
Effect of LAB on body weight gain and food utilization rate of experimental mice. **(A)** Body weight gain of female and male mice in each group. **(B)** Food utilization rate of female and male mice in each group.

3.10 Hemolytic activity

There was obvious hemolytic zone (β-hemolysis) around *S. aureus* colony on blood agar. While, colonies of four isolates had no zone effect (γ-hemolysis), indicating that they had no hemolytic activity (Figure S5).

3.11 Gelatinase activity

Four isolates did not have the property of breaking down gelatin, thus, they were considered as safe (Figure S6).

3.12 Acute toxicity in mice

No adverse effects or mortality were observed in the acute toxicity assay in mice. Compared with the control group, there was no significant difference in weight gain and food utilization rate of mice in the experimental group (Figure 4). In addition, no significant differences were found in the organ index of the same organ in each group (Figure 5).

4 Discussion

Our experimental designing were mainly based on previously published studies, Guidelines for the Evaluation of Probiotics in Food (Food and Agriculure Organization/ World Health Organization [FAO/WHO], 2006), Criteria TCSWSL016-2019 and OECD guidelines 423. The research can be divided into four sections: (1) identification of bacteria, (2) *In vitro* potential testing of probiotics, (3) proving *in vitro*ly safe probiotics, (4) proving *in vitro*ly safe probiotics. In our study, four LAB (*L. curvatus*, *W. cibaria*, *L. pentosus* and *L. mucosae*) were isolated, identified and evaluated for probiotic properties.

Hydrophobicity is an important property of probiotics that requires consideration during potential probiotic candidates selection (Reuben et al., 2019). Many studies have shown that hydrophobicity plays a crucial role in bacterial aggregation, colony formation, and initial cell adhesion to host cells (Myint et al., 2010; Pachla et al., 2021). In this study, only FY4 exhibited high hydrophobicity, which is consistent with previous studies that only a few LAB isolated from cattle showed high hydrophobicity (Otero et al., 2006; Maldonado et al., 2012). Hydrophobicity correlates with the physical and chemical properties of the bacteria, with high hydrophobicity indicating the presence of hydrophobic components on or embedded in the bacterial surface. When hydrophobicity exceeds 40%, probiotics are considered to have greater host adhesion and better pathogen competition inhibition (Lin et al., 2020). Studies have shown that hydrophobicity is a strain-specific characteristic and that hydrophobicity values differ significantly even between strains of the same LAB (Tokatli et al., 2015; Boranbayeva et al., 2020).

Probiotic strains need to tolerate acidic and bile salt environments in order to remain viable during gastrointestinal transport and maintain functional in the intestine (Yao et al., 2021). The pH of gastric acid fluctuates from 1.5 to 4.5, with a medium value of 3.0, and the range of bile salt concentration in the upper intestine is 0.03-0.3% (wt/vol) (Ding et al., 2017). Like many studies, we assayed the tolerance of isolates in pH 3 and in 0.3% bile salt. In this study, four isolates all had close viability under acidic condition, but different viability in bile salt, which may be attributed to the expression of resistance-associated proteins in strains and species.

Autoaggregation, coaggregation and hydrophobicity of bacteria are considered to be important properties affecting their colonization in the intestine (Diale et al., 2021). Autoaggregation is the ability of the same cell types to self-adhere, and coaggregation is the combination of organisms of different species (Del Re et al., 2000; Kolenbrander, 2000). A high self-aggregation capacity ensures

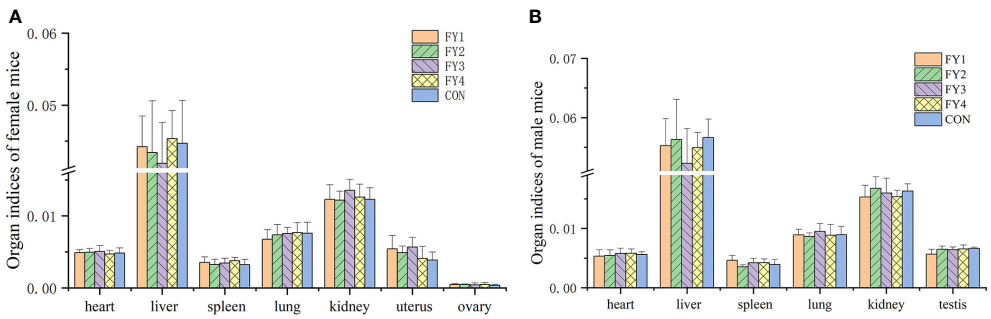

FIGURE 5
Organ indices of mice in each experimental group. **(A)** Organ indices of female mice (heart, liver, spleen, lung, kidney, uterus and ovary) **(B)** Organ indices of male mice (heart, liver, spleen, lung, kidney and testis).

that microorganisms achieve higher population density and stability in the host's gut, reducing exposure to unfavorable conditions (Slizewska et al., 2021). A high coaggregation capacity can prevents the host's gastrointestinal tract from being colonized by pathogens (Dlamini et al., 2019). Some studies reported significant correlations between self-aggregation and hydrophobicity in some Lactobacillus strains, while others pointed to positive correlations between cell hydrophobicity and the presence of protein surface coatings, leading to aggregation and adhesion capacity (Aleksandrzak-Piekarczyk et al., 2015; Cozzolino et al., 2020). In this study, FY4 exhibited adhesion ability, high hydrophobicity, strong autoaggregation and coaggregation ability. These results are consistent with previous studies that strong adhesion is correlated with high hydrophobicity and high aggregation ability (Wang et al., 2018; Sharma et al., 2021).

Antibacterial activity against pathogens is another functional requirement for probiotics. The antibacterial effect of probiotics is complex and multifactorial, mainly by fighting against binding sites and nutrients, stimulating the host immune system to competitively reject pathogens and producing inhibitory metabolites against unfavorable microorganisms (Slizewska et al., 2021). Its metabolisms with antibacterial effect are mainly organic acids (mainly acetic acid and lactic acid), hydrogen peroxide, ethanol, bacteriocin and bacterioids (Azhar et al., 2017). Like many previous studies, the CFC (metabolites) of LAB for *in vitro* bacterial inhibition experiments were adopted in this study, and the results showed that four isolates have antibacterial activity against enteric pathogenic bacteria.

The adhesion of LAB in the intestine is not only beneficial for intestinal colonization and pathogens elimination, but also for immunomodulation and synthesis of healthy molecules (Donaldson et al., 2016; Slizewska et al., 2021). The adhesion of probiotics to the host is related to their cell surface properties, such as charges on bacterial cell and host cell, teichoic acids, extracellular polysaccharides, pili, and cell wall-anchored proteins (Donaldson et al., 2016; Slizewska et al., 2021).

In vitro adhesion of LAB with Caco-2 cells, which has been widely applied in the identification of potential probiotics (Yeo et al., 2016), was adopted in this study. The adhesion rate of four isolates was 30.06% to 43.69%. Consistent with the previous studies that cell adhesion is proportional to hydrophobicity, FY4 has the highest cell adhesion and hydrophobicity in this study Albeit LAB are GRAS microorganisms, and safety properties should be evaluated prior to administration. In this study, four isolates were tested on their capabilities for hemolytic analysis, gelatinase activity, and antibacterials resistance. Some bacteria are known to produce enzymes that break down phospholipids and cause rupture of the cell membrane of red blood cells (RBCs). Hemolytic activity is considered to be an important virulence factor in pathogenesis, facilitating the acquisition of iron or other "growth factors" to pathogens, which leads to host anemia or edema, etc. (Vesterlund et al., 2007). Therefore, bacterial hemolytic activity is the first *in vitro* safety parameter need to be evaluated. There are three types of bacterial hemolytic activities, α-hemolysis (green-hued zone around colonies), β-hemolysis (transparent zone around colonies), and γ-hemolysis (no change around colonies). Alpha hemolysis is not true hemolysis, since it actually is the oxidation of hemoglobin to green methemoglobin by the hydrogen peroxide produced by the bacteria, which gives the bacterial colonies a green color. Beta-hemolysis is the complete lysis of RBCs around the colony and in the medium below, giving the area a pale (yellow) and transparent appearance. Gamma-hemolysis is the absence of hemolytic activity and causes no change around the colony (Pradhan et al., 2020).

Gelatinase is a kind of Zn metalloproteinase secreted by pathogenic bacteria. It can effectively attack the host by digesting the protein components of tissue, so as to facilitate the spread of bacteria (Salamone et al., 2019). Therefore, probiotics must be unable to cause hemolysis and gelatin liquefaction in the host. In our study, four strains appear to be safe, as they did not cause erythrocyte lysis in sheep blood and had no gelatinase activity.

Antibacterial susceptibility assays were performed on 12 antimicrobials for phenotypic resistance according to international standards and guidelines. The results confirmed that most strains were susceptible to most antimicrobials and showed strain-dependent characteristics. The results showed consistency with previous studies on a wide range of antimicrobials, therefore no data on resistance genes or cellular localization of resistance genes were available. All strains were resistant to kanamycin, three-quarters were resistant to streptomycin, half were resistant to gentamicin and one-quarter were resistant to ciprofloxacin and rifampicin.

This result is consistent with previous reports that LAB is usually highly resistant to aminoglycosides such as kanamycin, gentamicin and streptomycin. It is considered to be intrinsic in Lactobacillus due to the lack of cytochrome-mediated electron transfer that mediates drug and food uptake (Pradhan et al., 2020).According to previous reports, the intrinsic resistance is chromosomally encoded and poses no risk of horizontal metastasis in non-pathogenic bacteria. In contrast, acquired resistance, caused by the transfer of resistance genes from the environment or from other bacteria, and might be horizontally spread among bacteria (Jeong et al., 2021).

Previous studies showed that 34% of LAB isolated from microbial food and drug additives were resistant to ciprofloxacin (Liu et al., 2009) and 26% of *Lactobacillus* spp. isolated from dairy products were resistant to ciprofloxacin (Erginkaya Z and Tatlı 2017). *L. acidophilus* and *L. brevis* have been reported to be resistant to ciprofloxacin (Shazali et al., 2014). As Reported by Liu et al. (2009) *L. lactis* strains were all resistant to rifampicin, but he mechanism is not yet clear. Fguiri et al. (2016) showed that *L. plantarum*, *L. pentosus*, *L. brevis*, *L. Lactis* and *Pediococcus pentosaceus* isolated from raw camel milk were resistant to rifampicin. The resistance mechanism of some LAB to ciprofloxacin and rifampicin is still unclear, and we assumed it is a unique characteristic of the strain, which may be related to its origin and evolution.

Finally, comparative analysis of the experimental results showed that FY4 performed best in terms of aggregation, hydrophobicity, antibacterial activities and adherence to Caco-2 cells. FY3 exhibited a high degree of self-aggregation and the strongest antimicrobial activity against *E. coli* and *Salm. Typhimurium*; and FY4 possessed the most potent antimicrobial activity toward three pathogenic bacteria. Previous experimental studies have shown that a mixture of LAB to prevent and treat diarrhea has yielded encouraging results. We speculate that a mixture of FY3 and FY4 may be effective in the prevention and/or treatment of bovine diarrhea. This hypothesis needs to be validated by additional experiments, such as *in vivo* safety and efficacy evaluation to support its practical application.

Toxicity is a complex phenomenon and *in vitro* safety assays may involve false negatives, as virulence properties may be inactive under the specific conditions of the assay. Genome sequencing is currently an approach to assess the safety risk of non-expression (Papadimitriou et al., 2015). In recent years, the safety evaluation of LAB based on the whole genome sequence has been reported. Due to experimental design and economic budget, multi-genomic studies have to be carried out later. Since some toxicities require active interaction with the host to be triggered, oral toxicity tests were conducted in mice as a basis for assessing the safety of probiotics.

Acute toxicity tests in mice showed that LAB did not cause death. Weight change is an indicator of the health of the mice, too rapid increase or decrease may be a sign of immune dysregulation, organ damage and organism disorder. Compared with controls, there were no significant organ damage and no significant differences in body weight, food intake and organ indices in mice. Therefore, these four strains were considered as non-toxic, safe and effective probiotics.

In vitro screening of probiotics was applied in our study, it is a simpler and less costly method. Although some tests seem to be outdated, *In vitro* screening are still used in recent reports and its most important advantage is the ability to screen multiple strains at the same time (Papadimitriou et al., 2015). In recent decades, with the rapid development of bioinformatics and high-throughput technologies, LAB studies have expanded from a few genes of interest to quantify whole-genomes, transcriptomes, proteomes and metabolomes changes. The genetic function, metabolic networks, population inheritance and species divergence of LAB can be analyzed by omics techniques, which can accelerate the selection and transform superior strains and provide a basis for the efficient use of LAB, and hence improve the industrial control of fermentation.

5 Conclusions

Four LAB were isolated from yak feces in this study, which were *Latilactobacillus curvatus*, *Weissella cibaria*, *Limosilactobacillus mucosae*, and *Lactiplantibacillus pentosus*. The probiotic properties of LAB were evaluated by hydrophobicity, acid and bile salt resistance, cell aggregation, compatibility, antimicrobial activity and cell adhesion tests. The safety attributes toward hemolytic activity, gelatinase activity and antimicrobial susceptibility were assessed. The results showed that the four strains had probiotic and safety profiles, which were sensitive to spectral antimicrobials, without hemolytic or gelatinase activity. Among them, FY3 and FY4 exhibited outstanding performances in hydrophobicity, aggregation, antibacterial activity and adhesion to Caco-2 cells. The acute oral toxicity test of LAB had no adverse effect on the weight gain, food utilization rate or organ index of mice. All results indicate that these four LAB strains, especially FY3 and FY4 could be potential probiotics for the prevention and/or treatment of bacterial disease-related diarrhea in yak.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by College of Veterinary Medicine, Gansu Agricultural University (License no. GSAU-AEW-2019-0010, Figure S7).

Author contributions

QZh, MW, YP, and QZe conceived and designed the experiments. QZh, MW, XM, ZL, and CJ contributed reagents, materials, and analysis tools. QZh wrote the manuscript. YP, MW, and QZe revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.984537/full#supplementary-material

References

Alayande, K. A., Aiyegoro, O. A., and Ateba, C. N. (2020). Probiotics in animal husbandry: Applicability and associated risk factors. *Sustainability* 12(3):1087. doi: 10.3390/su12031087

Aleksandrzak-Piekarczyk, T., Koryszewska-Baginska, A., Grynberg, M., Nowak, A., Cukrowska, B., Kozakova, H., et al. (2015). Genomic and functional characterization of the unusual plock 0919 plasmid harboring the spacba pili cluster in lactobacillus casei lock 0919. *Genome Biol. Evol.* 8, 202–217. doi: 10.1093/gbe/evv247

Aristimuno Ficoseco, C., Mansilla, F. I., Maldonado, N. C., Miranda, H., Fatima Nader-Macias, M. E., and Vignolo, G. M. (2018). Safety and growth optimization of lactic acid bacteria isolated from feedlot cattle for probiotic formula design. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02220

Aroutcheva, A., Auclair, J., Frappier, M., Millette, M., Lolans, K., de Montigny, D., et al. (2016). Importance of molecular methods to determine whether a probiotic is the source of lactobacillus bacteremia. *Probiotics Antimicrob. Proteins* 8, 31–40. doi: 10.1007/s12602-016-9209-y

Azhar, N. S., Md Zin, N. H., and Hamid, T. (2017). Lactococcus lactis strain a5 producing nisin-like bacteriocin active against gram positive and negative bacteria. *Trop. Life Sci. Res.* 28, 107–118. doi: 10.21315/tlsr2017.28.2.8

Beijing Biological Feed Industry Technology Innovation Strategic Alliance (2019) *TCSWSL016-2019 screening criteria of lactic acid bacteria for feeding aquatic animals*. Available at: http://down.foodmate.net/standard/yulan.php?itemid=63858.

Boranbayeva, T., Karahan, A. G., Tulemissova, Z., Myktybayeva, R., and Ozkaya, S. (2020). Properties of a new probiotic candidate and lactobacterin-tk(2) against diarrhea in calves. *Probiotics Antimicrob. Proteins* 12, 918–928. doi: 10.1007/s12602-020-09649-4

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., et al. (2012). Food fermentations: Microorganisms with technological beneficial use. *Int. J. Food Microbiol.* 154, 87–97. doi: 10.1016/j.ijfoodmicro.2011.12.030

Chang, J. Y., Lee, H. J., and Chang, H. C. (2007). Identification of the agent from lactobacillus plantarum kfri464 that enhances bacteriocin production by leuconostoc citreum gj7. *J. Appl. Microbiol.* 103, 2504–2515. doi: 10.1111/j.1365-2672.2007.03543.x

Chen, Y. M., Limaye, A., Chang, H. W., and Liu, J. R. (2022). Screening of Lactic Acid Bacterial Strains with Antiviral Activity Against Porcine Epidemic Diarrhea. *Probiotics and Antimicrobial Proteins* 143:546–559. doi: 10.1007/s12602-021-09829-w

CLSI (2018). *Performance standards for antimicrobial susceptibility testing, M100, 28st ed* (PWayne, PA: Clinical and Laboratory Standards Institute).

Cozzolino, A., Vergalito, F., Tremonte, P., Iorizzo, M., Lombardi, S. J., Sorrentino, E., et al. (2020). Preliminary evaluation of the safety and probiotic potential of akkermansia muciniphila dsm 22959 in comparison with lactobacillus rhamnosus gg. *Microorganisms* 8(2): 189. doi: 10.3390/microorganisms8020189

Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of bifidobacterium longum. *Lett. Appl. Microbiol.* 31, 438–442. doi: 10.1046/j.1365-2672.2000.00845.x

Diale, M. O., Kayitesi, E., and Serepa-Dlamini, M. H. (2021). Genome in silico and *in vitro* analysis of the probiotic properties of a bacterial endophyte, bacillus paranthracis strain mhsd3. *Front. Genet.* 12. doi: 10.3389/fgene.2021.672149

Ding, W., Shi, C., Chen, M., Zhou, J., Long, R., and Guo, X. (2017). Screening for lactic acid bacteria in traditional fermented tibetan yak milk and evaluating their probiotic and cholesterol-lowering potentials in rats fed a high-cholesterol diet. *J. Funct. Foods* 32, 324–332. doi: 10.1016/j.jff.2017.03.021

Dioso, C. M., Vital, P., Arellano, K., Park, H., Todorov, S. D., Ji, Y., et al. (2020). Do your kids get what you paid for? evaluation of commercially available probiotic products intended for children in the republic of the philippines and the republic of korea. *Foods* 9(9):1229. doi: 10.3390/foods9091229

Dlamini, Z. C., Langa, R. L. S., Aiyegoro, O. A., and Okoh, A. I. (2019). Safety evaluation and colonisation abilities of four lactic acid bacteria as future probiotics. *Probiotics Antimicrob. Proteins* 11, 397–402. doi: 10.1007/s12602-018-9430-y

Donaldson, G. P., Lee, S. M., and Mazmanian, S. K. (2016). Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.* 14, 20–32. doi: 10.1038/nrmicro3552

Dong, H., Liu, B., Li, A., Iqbal, M., Mehmood, K., Jamil, T., et al. (2020). Microbiome analysis reveals the attenuation effect of lactobacillus from yaks on diarrhea *via* modulation of gut microbiota. *Front. Cell Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.610781

Dowarah, R., Verma, A. K., Agarwal, N., Singh, P., and Singh, B. R. (2018). Selection and characterization of probiotic lactic acid bacteria and its impact on growth, nutrient digestibility, health and antioxidant status in weaned piglets. *PloS One* 13, e0192978. doi: 10.1371/journal.pone.0192978

El-Deeb, W. M., Fayez, M., Elsohaby, I., Ghoneim, I., Al-Marri, T., Kandeel, M., et al. (2020). Isolation and characterization of vaginal Lactobacillus spp. in dromedary camels (Camelus dromedarius): in vitro evaluation of probiotic potential of selected isolates. *PeerJ*. 8:e8500. doi: 10.7717/peerj.8500

Erginkaya Z, T. E., and Tatlı, D. (2017). Determination of antibiotic resistance of lactic acid bacteria isolated from traditional turkish fermented dairy products. *Iranian J. Vet. Res.* 19(1):53. doi: 10.22099/IJVR.2018.4769

Fguiri, I., Ziadi, M., Atigui, M., Ayeb, N., Arroum, S., Assadi, M., et al. (2016). Isolation and characterisation of lactic acid bacteria strains from raw camel milk for potential use in the production of fermented Tunisian dairy products. *International Journal of Dairy Technology*. 69(1):103–113. doi: 10.1111/1471-0307.12226

Food and Agriculture Organization/ World Health Organization [FAO/WHO] (2006). "Guidelines for the evaluation of probiotics in food," in *Report ofa joint FAO/WHO working group.Safety evaluation of certain contaminants in food* (Vol. 82). Food and Agriculture Org

Goshu, H., Wu, X., Chu, M., Bao, P., Ding, X., and Yan, P. (2018). Copy number variations of klf6 modulate gene transcription and growth traits in chinese datong yak (bos grunniens). *Animals* 8(9):145. doi: 10.3390/ani8090145

Hu, Y., and Cheng, H. (2016). Health risk from veterinary antimicrobial use in china's food animal production and its reduction. *Environ. pollut.* 219, 993–997. doi: 10.1016/j.envpol.2016.04.099

Jarocki, P., Komon-Janczara, E., Glibowska, A., Dworniczak, M., Pytka, M., Korzeniowska-Kowal, A., et al. (2020). Molecular routes to specific identification of the lactobacillus casei group at the species, subspecies and strain level. *Int. J. Mol. Sci.* 21(8):2694. doi: 10.3390/ijms21082694

Jeong, C. H., Sohn, H., Hwang, H., Lee, H. J., Kim, T. W., Kim, D. S., et al. (2021). Comparison of the probiotic potential between lactiplantibacillus plantarum isolated from kimchi and standard probiotic strains isolated from different sources. *Foods* 10(9):2125. doi: 10.3390/foods10092125

Kayasaki, F., Okagawa, T., Konnai, S., Kohara, J., Sajiki, Y., Watari, K., et al. (2021). Direct evidence of the preventive effect of milk replacer-based probiotic feeding in calves against severe diarrhea. *Vet. Microbiol.* 254, 108976. doi: 10.1016/j.vetmic.2020.108976

Kim, H. S., Whon, T. W., Sung, H., Jeong, Y. S., Jung, E. S., Shin, N. R., et al. (2021). Longitudinal evaluation of fecal microbiota transplantation for ameliorating calf diarrhea and improving growth performance. *Nat. Commun.* 12, 161. doi: 10.1038/s41467-020-20389-5

Kolenbrander, P. E. (2000). Oral microbial communities: Biofilms, interactions, and genetic systems. *Annu. Rev. Microbiol.* 54, 413–437. doi: 10.1146/annurev.micro.54.1.413

Kumar, S., Stecher, G., and Tamura, K. (2016). Mega7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lin, W. C., Ptak, C. P., Chang, C. Y., Ian, M. K., Chia, M. Y., Chen, T. H., et al. (2020). Autochthonous lactic acid bacteria isolated from dairy cow feces exhibiting promising probiotic properties and *in vitro* antibacterial activity against foodborne pathogens in cattle. *Front. Vet. Sci.* 7. doi: 10.3389/fvets.2020.00239

Liu, B., Wang, C., Huasai, S., Han, A., Zhang, J., He, L., et al. (2022). Compound probiotics improve the diarrhea rate and intestinal microbiota of newborn calves. *Anim. (Basel)* 12(3):322. doi: 10.3390/ani12030322

Liu, C., Zhang, Z.-Y., Dong, K., Yuan, J.-P., and Guo, X.-K. (2009). Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. *Biomed. Environ. Sci.* 22, 401–412. doi: 10.1016/s0895-3988(10)60018-9

Li, M., Wang, Y., Cui, H., Li, Y., Sun, Y., and Qiu, H. J. (2020). Characterization of lactic acid bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics. *Front. Vet. Sci.* 7. doi: 10.3389/fvets.2020.00049

Li, A., Wang, Y., Li, Z., Qamar, H., Mehmood, K., Zhang, L., et al. (2019). Probiotics isolated from yaks improves the growth performance, antioxidant activity, and cytokines related to immunity and inflammation in mice. *Microb. Cell fact.* 18, 112. doi: 10.1186/s12934-019-1161-6

Maldonado, N. C., de Ruiz, C. S., Otero, M. C., Sesma, F., and Nader-Macias, M. E. (2012). Lactic acid bacteria isolated from young calves–characterization and potential as probiotics. *Res. Vet. Sci.* 92, 342–349. doi: 10.1016/j.rvsc.2011.03.017

More, S. J. (2020). European Perspectives on efforts to reduce antimicrobial usage in food animal production. *Ir Vet. J.* 73, 2. doi: 10.1186/s13620-019-0154-4

Moreno, I., Marasca, E. T. G., de Sa, P., de Souza Moitinho, J., Marquezini, M. G., Alves, M. R. C., et al. (2018). Evaluation of probiotic potential of bacteriocinogenic lactic acid bacteria strains isolated from meat products. *Probiotics Antimicrob. Proteins* 10, 762–774. doi: 10.1007/s12602-018-9388-9

Myint, A. A., Lee, W., Mun, S., Ahn, C. H., Lee, S., and Yoon, J. (2010). Influence of membrane surface properties on the behavior of initial bacterial adhesion and biofilm development onto nanofiltration membranes. *Biofouling* 26, 313–321. doi: 10.1080/08927010903576389

Organisation for Economic Cooperation and Development (OECD) (2001). *Guideline for the Testing of Chemicals 423 on Acute Oral Toxicity––Acute Toxic Class Method*. Paris, France. doi: 10.1787/9789264071001-en

Otero, M. C., Morelli, L., and Nader-Macias, M. E. (2006). Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. *Lett. Appl. Microbiol.* 43, 91–97. doi: 10.1111/j.1472-765X.2006.01914.x

Pachla, A., Ptaszynska, A. A., Wicha, M., Kunat, M., Wydrych, J., Olenska, E., et al. (2021). Insight into probiotic properties of lactic acid bacterial endosymbionts of apis mellifera l. derived from the polish apiary. *Saudi J. Biol. Sci.* 28, 1890–1899. doi: 10.1016/j.sjbs.2020.12.040

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

Perin, L. M., Miranda, R. O., Todorov, S. D., Franco, B. D., and Nero, L. A. (2014). Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. *Int. J. Food Microbiol.* 185, 121–126. doi: 10.1016/j.ijfoodmicro.2014.06.001

Pradhan, D., Mallappa, R. H., and Grover, S. (2020). Comprehensive approaches for assessing the safety of probiotic bacteria. *Food Control* 108:106872. doi: 10.1016/j.foodcont.2019.106872

Qureshi, N., Gu, Q., and Li, P. (2020). Whole genome sequence analysis and *in vitro* probiotic characteristics of a lactobacillus strain lactobacillus paracasei zfm54. *J. Appl. Microbiol.* 129, 422–433. doi: 10.1111/jam.14627

Rastogi, S., Mittal, V., and Singh, A. (2020). *In vitro* evaluation of probiotic potential and safety assessment of lactobacillus mucosae strains isolated from donkey's lactation. *Probiotics Antimicrob. Proteins* 12, 1045–1056. doi: 10.1007/s12602-019-09610-0

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

Rolfe, R. D. (2000). The role of probiotic cultures in the control of gastrointestinal health. *J. Nutr.* 130, 396S–402S. doi: 10.1093/jn/130.2.396S

Salamone, M., Nicosia, A., Ghersi, G., and Tagliavia, M. (2019). Vibrio proteases for biomedical applications: Modulating the proteolytic secretome of v Alginolyticus and v. parahaemolyticus for improved enzymes production. *Microorganisms* 7(10):387. doi: 10.3390/microorganisms7100387

Sharma, A., Lavania, M., Singh, R., and Lal, B. (2021). Identification and probiotic potential of lactic acid bacteria from camel milk. *Saudi Journal of Biological Sciences* 28(3):1622–1632. doi: 10.1016/j.sjbs.2020.11.062

Shazali, N., Foo, H. L., Loh, T. C., Choe, D. W., and Abdul Rahim, R. (2014). Prevalence of antibiotic resistance in lactic acid bacteria isolated from the faeces of broiler chicken in malaysia. *Gut Pathog.* 6, 1. doi: 10.1186/1757-4749-6-1

Slizewska, K., Chlebicz-Wojcik, A., and Nowak, A. (2021). Probiotic properties of new lactobacillus strains intended to be used as feed additives for monogastric animals. *Probiotics Antimicrob. Proteins* 13, 146–162. doi: 10.1007/s12602-020-09674-3

Tokatli, M., Gulgor, G., Bagder Elmaci, S., Arslankoz Isleyen, N., and Ozcelik, F. (2015). *In vitro* properties of potential probiotic indigenous lactic acid bacteria originating from traditional pickles. *BioMed. Res. Int.* 2015, 315819. doi: 10.1155/2015/315819

Upadrasta, A., and Madempudi, R. S. (2016). Probiotics and blood pressure: current insights. *Integr. Blood Press Control* 9, 33–42. doi: 10.2147/IBPC.S73246

Vaz-Moreira, I., Faria, C., Lopes, A. R., Svensson, L., Falsen, E., Moore, E. R., et al. (2009). Sphingobium vermicomposti sp. nov., isolated from vermicompost. *Int. J. Syst. Evol. Microbiol.* 59, 3145–3149. doi: 10.1099/ijs.0.006163-0

Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., and Ouwehand, A. C. (2007). Safety assessment of lactobacillus strains: Presence of putative risk factors in faecal, blood and probiotic isolates. *Int. J. Food Microbiol.* 116, 325–331. doi: 10.1016/j.ijfoodmicro.2007.02.002

Wang, Y., Li, A., Jiang, X., Zhang, H., Mehmood, K., Zhang, L., et al. (2018). Probiotic potential of leuconostoc pseudomesenteroides and lactobacillus strains isolated from yaks. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02987

World Health Organization (2006). "Food and agricultural organization of the united nations," in *Probiotics in food: Health and nutritional properties and guidelines for evaluation* (Rome: Food and Nutrition. World Health Organization).

Yao, M., Lu, Y., Zhang, T., Xie, J., Han, S., Zhang, S., et al. (2021). Improved functionality of ligilactobacillus salivarius li01 in alleviating colonic inflammation by layer-by-layer microencapsulation. *NPJ Biofilms Microbiomes*. 7, 58. doi: 10.1038/s41522-021-00228-1

Yeo, S., Lee, S., Park, H., Shin, H., Holzapfel, W., and Huh, C. S. (2016). Development of putative probiotics as feed additives: Validation in a porcine-specific gastrointestinal tract model. *Appl. Microbiol. Biotechnol.* 100, 10043–10054. doi: 10.1007/s00253-016-7812-1

Zeng, Z., He, X., Li, F., Zhang, Y., Huang, Z., Wang, Y., et al. (2021). Probiotic properties of bacillus proteolyticus isolated from tibetan yaks, china. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.649207

Zhang, X., Ali Esmail, G., Fahad Alzeer, A., Valan Arasu, M., Vijayaraghavan, P., Choon Choi, K., et al. (2020). Probiotic characteristics of lactobacillus strains isolated from cheese and their antibacterial properties against gastrointestinal tract pathogens. *Saudi J. Biol. Sci.* 27, 3505–3513. doi: 10.1016/j.sjbs.2020.10.022

Zhang, Q., Pan, Y., Wang, M., Sun, L., Xi, Y., Li, M., et al. (2022). *In vitro* evaluation of probiotic properties of lactic acid bacteria isolated from the vagina of yak (bos grunniens). *PeerJ* 10:e13177. doi: 10.7717/peerj.13177

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The correlation between dysfunctional intestinal flora and pathology feature of patients with pulmonary tuberculosis

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Introduction: Recent studies have provided insights into the important contribution of gut microbiota in the development of Pulmonary Tuberculosis (PTB). As a chronic consumptive infectious disease, PTB involves many pathological characteristics. At present, research on intestinal flora and clinical pathological Index of PTB is still rare.

Methods: We performed a cross-sectional study in 63 healthy controls (HCs) and 69 patients with untreated active PTB to assess the differences in their microbiota in feces via 16S rRNA gene sequencing.

Results: Significant alteration of microbial taxonomic and functional capacity was observed in PTB as compared to the HCs. The results showed that the alpha diversity indexes of the PTB patients were lower than the HCs ($P<0.05$). Beta diversity showed differences between the two groups ($P<0.05$). At the genus level, the relative abundance of Bacteroides, Parabacteroides and Veillonella increased, while Faecalibacterium, Bifidobacterium, Agathobacter and CAG-352 decreased significantly in the PTB group, when compared with the HCs. The six combined genera, including Lactobacillus, Faecalibacterium, Roseburia, Dorea, Monnoglobus and [Eubacterium]_ventriosum_group might be a set of diagnostic biomarkers for PTB (AUC=0.90). Besides, the predicted bacterial functional pathway had a significant difference between the two groups ($P<0.05$), which was mainly related to the nutrient metabolism pathway. Significant alterations in the biochemical index were associated with changes in the relative abundance of specific bacteria, the short chain fatty acid (SCFA)-producing bacteria enriched in HCs had a positively correlated with most of the biochemical indexes.

Discussion: Our study indicated that the gut microbiota in PTB patients was significantly different from HCs as characterized by the composition and metabolic pathway, which related to the change of biochemical indexes in the PTB group. It was hypothesized that the abovementioned changes in the

gut microbiota could exert an impact on the clinical characteristics of PTB through the regulation of the nutrient utilization pathway of the host by way of the gut-lung axis.

KEYWORDS

pulmonary tuberculosis, 16S rRNA gene sequencing, gut microbiota, biochemical index, SCFA

Introduction

A large number of microorganisms exist in the human body, especially the intestinal microbiome, which plays a crucial role in human physiology and pathology (de Vos et al., 2022). The gut microbiota will not only directly modulate the intestinal tract but also impact the distal organs, such as the brain, liver and lung (Zhu et al., 2021). Recently, an increasing amount of evidence has indicated that the gut microbiota is closely related to respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD), lung cancer and respiratory infection (Dumas et al., 2018; Barcik et al., 2020; Li et al., 2021; Zhao et al., 2021). Pulmonary tuberculosis (PTB) is one of the most common infectious diseases and remains a major infectious cause of death worldwide (Pezzella, 2019). Emerging scientific research hints at a possible role of the gut microbiota in the pathophysiology of tuberculosis (TB) (Comberiati et al., 2021).

A study performed in South Africa depicted the distinct stool microbiome in the cases of tuberculosis with features of enriched anaerobes, which related to the upregulation of pro-inflammatory immunological pathways (Naidoo et al., 2021). Another study reported in India showed different results, researchers observed that butyrate and propionate-producing bacteria were significantly enriched in TB patients (Maji et al., 2018). As a country with a high burden of tuberculosis, researches on tuberculosis and flora have been widely implemented in China. In the east of China, compared with the healthy controls, the gut microbiota in the TB group was reflected by a striking decrease in short-chain fatty acids (SCFAs)-producing bacteria as well as metabolically associated pathways (Hu et al., 2019). Importantly, a study performed in the west of China showed a positive correlation between the gut microbiota and peripheral $CD4^+$ T cell counts in the TB patients (Luo et al., 2017), which highlighted the associations between gut microbiota with tuberculosis and its clinical outcomes. Although many studies described that the change of bacterial function is related to PTB (Wang et al., 2022; Yang et al., 2022), the specific mechanism between flora and clinical characteristics remains unclear.

Herein, we have performed a comparative analysis of 63 healthy controls (HCs) and 69 PTB patients based on 16S rRNA sequencing. We found that the intestinal flora of the PTB group was characterized by the increase of proinflammatory bacteria and the decrease of SCFAs-producing bacteria, which was related to the metabolic pathway of nutrients. Notably, we set a series of specific genera as novel biomarkers for identification between PTB patients and HCs. More importantly, we found that some probiotics enriched in HCs were positively correlated with most of the biochemical indexes, indicating that the enrichment of probiotics in the intestine was closely related to the healthy physiological state of the host. In general, we aim to identify the association with the microbiome, biochemical index and pulmonary tuberculosis.

Material and methods

Ethics

This study was approved by the Ethics Review Committee of Affiliated Dongyang Hospital of Wenzhou Medical University (2022-YX-268), and written informed consent was obtained from all participants.

Participant recruitment

A total of 69 pulmonary tuberculosis patients and 63 healthy controls were initially enrolled from the Affiliated Dongyang Hospital of Wenzhou Medical University, Zhejiang, China, from May 2021 to June 2022. All patients were recruited on the basis of TB symptomatic features (WS288-2008). Healthy controls (HCs) were recruited in hospital health examination center, who did not have close contact with TB patients at least one year. All participants had not taken any broad-spectrum antibiotics in the previous six months and without other basic diseases or major illness.

Biochemical analysis

Blood samples were collected for biochemical analysis using the automated equipment and standard methods. The level of

Albumin (Alb), Serum fasting blood glucose (Glu), Total Cholesterol (TC), Triglycerides (TG), High-Density Lipoprotein cholesterol (HDL), Low-Density Lipoprotein cholesterol (LDL), and Creatinine (Cr) were examined using a Hitachi 7600 automatic biochemical analyzer (HITACHI, Japan). The Hemoglobin (Hb) and White Blood Cell (WBC) were examined by Sysmex pocH-100i automatic blood Analyzer (SYSMEX, Japan).

Fecal sample collection and microbe DNA extraction

Fresh stool samples (morning samples, 200 mg/aliquot) were collected using GUHE Flora Storage buffer (GUHE Laboratories, Hangzhou, China), and stored at room temperature until DNA extraction. DNA was extracted with nucleic acid extraction or purification reagent (GUHE Laboratories, GHFDE100, Hangzhou, China), and the quantity and quality of extracted DNAs were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

16S rRNA gene sequencing

The V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from each sample using the 515F(5'-GTGCCAGCMGCCGCGGTAA-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') primers as previously described prior to sequencing. Phusion High-Fidelity PCR Master Mix with HF Buffer (PHUSION high fidelity DNA polymerase) was used for PCR. The following primary PCR cycling conditions were used: (1) Pre-denaturatio at 98℃ for 30 s; (2) 25 cycles of PCR at 98℃ for 15 s, 58℃ for 15 s, and 72℃ for 15 s; (3) a final extension at 72℃ for 1 min. PCR products were purified with AMPure XP Beads (Beckman Coulter, Indianapolis, IN), and the library was quantified with Qubit. After quantification, the Illumina NovaSeq6000 platform at GUHE Info Technology (GUHE, Hangzhou, China) was used for sequencing. The sequence data of this study have been uploaded in the GenBank Sequence Read Archive (SRA) of NCBI under the accession code BioProject PRJNA901399.

Sequence analysis

The original data of each sample were split according to the Barcode sequence and the primer sequence. The low-quality sequences were filtered through following criteria (Gill et al., 2006; Chen and Jiang, 2014): sequences that had a length of <150 bp, sequences that had average Phred scores of <20, sequences that contained ambiguous bases, and sequences that contained mononucleotide repeats of >8 bp. Paired-end reads were assembled using Vsearch V2.4.4 (–fastq_mergepairs –fastq_minovlen 5), than Operational taxonomic unit (OTU) picking using Vsearch v2.15.0, included dereplication(–derep_fulllength), cluster(-cluster_unoise), detectection of chimeras(-uchime3_denovo) (Rognes et al., 2016). The final effective tags were obtained by removing the chimeric sequences.

OTU/ASV clustering and species annotation

The filtered clean reads were clustered to get ASV statistical results. The representative sequences of OTUs were selected using default parameters. The representative sequences were annotated through QIIME2 (v2020.6) based on the SILVA138 database to further generate the OTU list. At each classification level, phylum, family, and genus were used to count the community composition of each sample.

Bioinformatics and statistical analysis

Sequence reads for 16S rRNA gene amplicons were analyzed using QIIME2 software and R package (v3.2.0). QIIME2 was used to calculate alpha diversity index in OTU level, including Chao1, ACE, Shannon, Simpson index. Beta diversity analysis was calculated through Qimme software based on UniFrac distance metric (Lozupone and Knight, 2005; Lozupone et al., 2007). "Vegan" from R package was used to evaluate the markers of microbial community structure differentiation between groups through PERMANOVA (Permanent multivariate analysis of variance) (McArdle and Anderson, 2001). The classification and abundance of bacteria were visualized by MEGAN software and GraPhlAn. And the R package "VennDiagram" was used to generate a Venn diagram (Zaura et al., 2009). Taxa abundances at the phylum, class, order, family, genus and species levels were statistically compared among samples or groups by Kruskal test from R stats package (metagenomeSeq packages). LEfSe (Linear discriminant analysis effect size) was performed to detect differentially abundant taxa across groups using the default parameters (Segata et al., 2011). Microbial functions were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states, https://github.com/picrust/picrust2/), based on high-quality sequences. For each sample, functions were profiling by using the Omixer-RPM version 1.0 (https://github.com/raeslab/omixer-rpm) with KO redundants predicted from PICRUSt2. Spearman's rank correlation between the intestinal flora and biochemical indexes was calculated with our own python scripts (Ramsey, 1989). Random forest analysis was applied to discriminating the samples from different groups using the R package "random

Forest" with 1,000 trees and all default settings. Values were expressed as the mean ± SEM. All other comparisons were calculated by two-tailed Student's t-test or one-way ANOVA followed by Tukey's test using GraphPad Prism 6 software.

Results

Participant cohort

Table 1 showed the detailed clinical characteristics of the study participants. 69 PTB patients and 63 age- and sex-matched healthy controls (HCs) were enrolled in this study. Among the clinical biochemical indexes tested in the study, a significant increase in serum fasting blood glucose (Glu) was observed in PTB patients ($P<0.05$). The levels of Albumin (Alb), Creatinine (Cr), Hemoglobin (Hb), Triglycerides (TG), Total cholesterol (TC), High-density lipoprotein cholesterol (HDL) and Low-density lipoprotein cholesterol (LDL), were all found to be significantly lower in PTB patients than in HCs ($P<0.001$). There was no statistical difference in White blood cells (WBC) between the two groups.

Dysbiosis of gut microbiota in PTB patients

In total, we generated 16,259,048 high-quality reads, and the reads were clustered into 7296 operational taxonomic units (OTUs) based on a similarity of 97%. A Venn diagram showed that 1141 OTUs and 732 OTUs were unique in the healthy controls and PTB patients, respectively (Figure 1A). The rarefaction curves showed that the curve entered in plateau, indicating that the sequencing data was large enough to reflect the bacterial diversity of the intestinal flora in HCs was higher than that in the PTB patients (Figure 1B). The alpha diversity indexes, including Shannon, Simpson, Chao1 and ACE reflected that the diversity of fecal microbiota in PTB patients was significantly lower than in HCs ($P<0.01$, $P<0.05$, $P<0.001$, $P<0.001$, respectively) (Figure 1C). The principal coordinate analysis (PCoA), based on Bray-Curtis, unweighted UniFrac and weighted UniFrac distances were performed to identify the difference in fecal microbiota composition between PTB patients and HCs. As shown in Figure 1D, there was a tendency of separation and had a partial overlap between two groups, which demonstrated that the total community of gut microbiota in the two groups was different.

The compositions of the fecal microbiota in the PTB patients and the HCs were assessed at different taxonomic levels, and the top 20 relative abundance of bacteria were shown in Figure 2A. At the phylum level, we observed an enrichment of *Bacteroidota* (33.10% vs. 27.08%), *Proteobacteria*(12.99% vs. 9.83%) and *Fusobacteriota*(0.58% vs. 0.56%, $P<0.05$), and a remarkable decrease of *Firmicutes*(48.07% vs. 55.89%, $P<0.05$) and *Actinobacteriota*(2.44% vs. 5.83%, $P<0.01$) in PTB patients compared with HCs (Figure 2B). At the family level, the relative abundance of *Bacteroidaceae* (24.59% vs. 16.14%, $P<0.05$), *Tannerellaceae* (2.88% vs. 0.90%, $P<0.05$) and *Oscillospiraceae* (2.19% vs. 1.18%, $P<0.05$) increased significantly in PTB patients, while *Bifidobacteriaceae* (1.7% vs. 5.03%, $P<0.01$), *Butyricicoccaceae* (0.45% vs. 0.81%, $P<0.05$) and *Ruminococcaceae* (10.74% vs. 22.20%, $P<0.001$) decreased drastically (Figure 2B). At the genus level, compared with the HCs, the PTB patients had a significantly higher relative abundance of *Bacteroides* (25.59% vs. 16.14%, $P<0.05$), *Parabacteroides* (2.88% vs. 0.90%, $P<0.05$) and *Veillonella* (4.24% vs. 0.84%, $P<0.05$), and lower relative abundance of *Faecalibacterium* (5.31% vs. 14.80%, $P<0.05$), *Bifidobacterium* (1.67% vs. 5.02%, $P<0.05$), *Agathobacter* (0.86% vs. 3.32%, $P<0.05$) and *CAG-352* (0.60% vs. 2.21%, $P<0.05$) (Figure 2B). Our data indicated the altered gut microbiota composition in PTB patients.

TABLE 1 Clinical characteristics of the participants.

Variable	HC (n=63) mean (range)	PTB (n=69) mean (range)	Student's t test (*P* values)
Gender, Man, n (%)	40 (63.49%)	44 (63.77%)	n.s.
Age (year)	49 (34-67)	51 (30-69)	n.s.
Albumin (g/L)	46.86 (41.60-51.80)	32.62 (14.90-44.60)	< 0.001
Creatinine (mmol/L)	75 (48-105)	63(35-110)	< 0.001
Hemoglobin (g/L)	150 (110-170)	121 (70-175)	< 0.001
Triglycerides (mmol/L)	1.64 (0.54-5.04)	1.03(0.31-2.71)	< 0.001
Total cholesterol (mmol/L)	4.34 (2.33-6.35)	3.34(1.36-5.99)	< 0.001
High-density lipoprotein (mmol/L)	1.26(0.79-1.98)	0.96(0.12-1.88)	< 0.001
Low-density lipoprotein (mmol/L)	3.03 (0.95-5.31)	2.13(0.46-3.89)	< 0.001
Serum fasting blood glucose (mmol/L)	5.08 (4.22-7.04)	6.19(4.27-21.29)	< 0.05
White blood cell ($\times10^9$/L)	6.13(3.46-9.10)	6.36(3.07-13.55)	n.s.

HC, healthy control; PTB, pulmonary tuberculosis; n.s., non-significance difference.

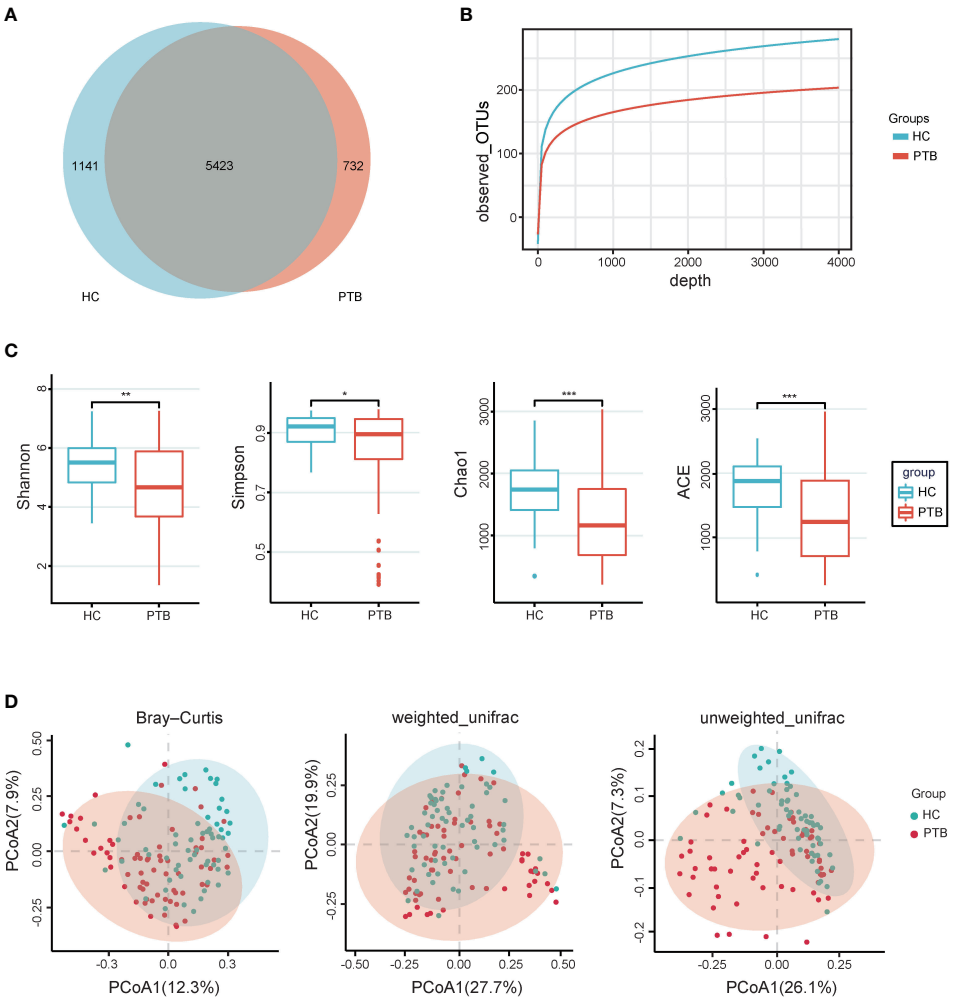

FIGURE 1
Intestinal microbial diversity. **(A)** Venn diagram showing the shared and unique OTUs in the gut microbiota of the two groups. **(B)** The rarefaction curves of two groups tended to be flat, indicating that the amount of sequencing data is large enough to reflect the majority of microbial species information in the sample. **(C)** The alpha diversity was assessed using the ACE, Chao1, Shannon and Simpson indexes, which showed significant differences between the PTB and HCs groups. **P*<0.05, ***P*<0.01, ****P*<0.001. Wilcoxon rank-sum test followed by Benjamini–Hochberg false discovery rate (FDR) correction. **(D)** PCoA based on Bray-Curtis, weighted_unifrac and Unweighted_Unifrac distance showed that gut microbiota in PTB separated from HCs (*P*<0.05). The significance was assessed by permutational multivariate analysis of variance (PERMANOVA) using the R package "vegan". PTB, pulmonary tuberculosis; HCs, healthy controls.

LEfSe analysis demonstrated that many key taxa were clearly different between PTB patients and HCs. According to the LDA score, the significantly enriched taxa in the gut microbiome of the PTB patients were *Lactobacillus*, *Blautia*, *Erysipelatoclostridium* and *Fusobacterium*. While the abundance of *Faecalibacterium*, *Bifidobacterium*, *Butyricicoccus*, *Roseburia* and *Lachnospiraceae_NK4A136_group* were higher in HCs (Figure 2C). The structure of the intestinal flora was determined by dynamic interactions between these community members. Based on the relative abundance of OTUs, PTB patients showed a more complex network of interactions than the HCs, with more positive and negative correlations among the microbiota (Figure S1).

The biochemical index was correlated with gut microbiota

A heat map was used to examine the associations between intestinal bacteria and the biochemical index (Figure 3). TC, HDL, LDL and Hb were positively correlated with 5 genera that were enriched in HCs, namely *Lachnospiraceae_ND3007_group*, *Haemophilus*, *Faecalibacterium*, *Roseburia*, *Lachnospira* (*P*<0.05). The levels of TG, Hb, LDL and TC were negatively associated with *Lactobacillus*, *Erysipelatoclostridium* and *Megasphaera* (*P*<0.05). Particularly, Alb was positively correlated with SCFA producers which were enriched in HCs,

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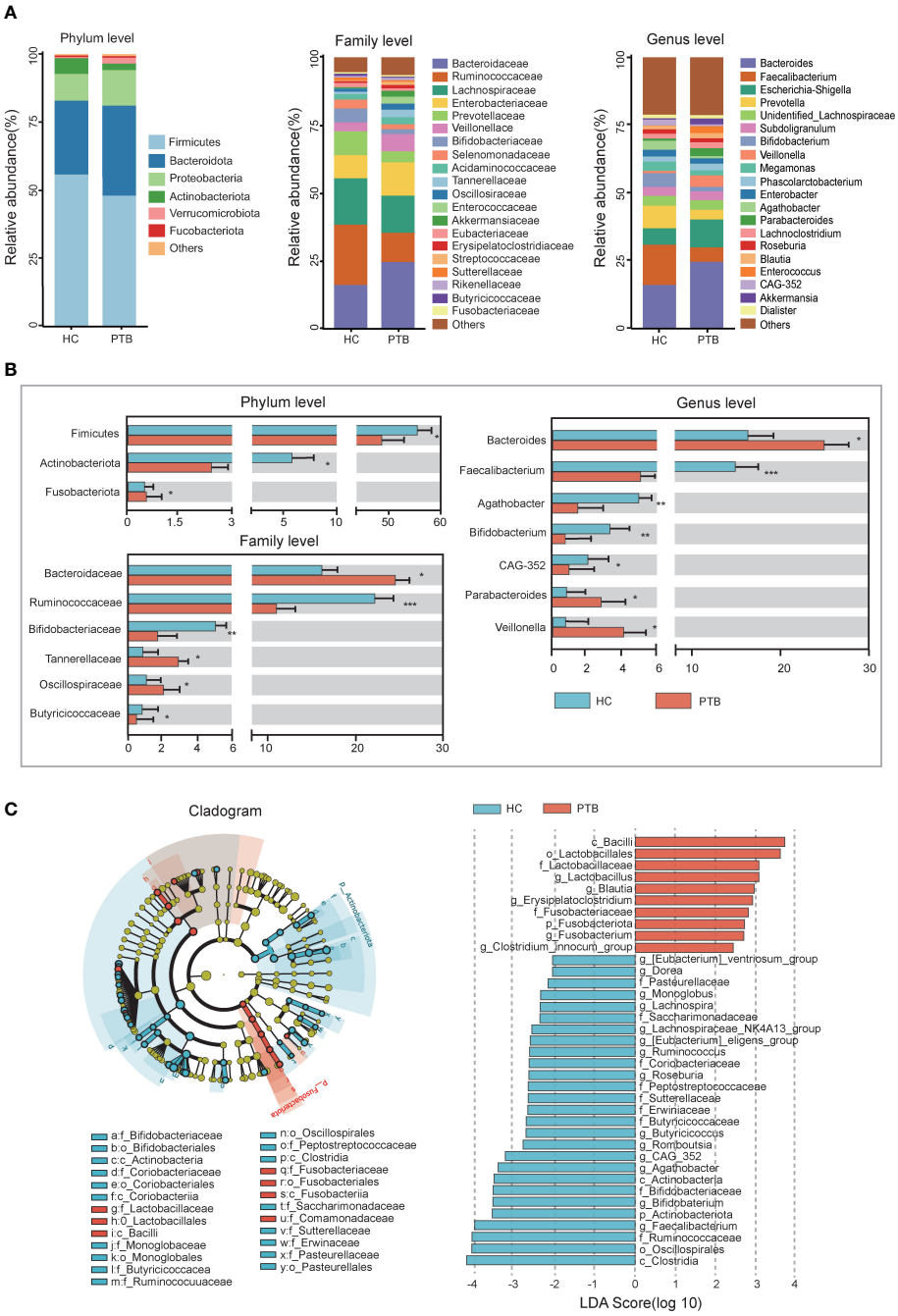

FIGURE 2
Relative abundance and LEfSe analysis of intestinal microbiota. **(A)** The relative abundance of intestinal flora between the PTB patients (red) and HCs (blue) at the phylum level, the family level and the genus level. **(B)** The statistical difference of intestinal flora between two groups. **P*<0.05, ***P*<0.01, ****P*<0.001. The significance was assessed by Student's t test. **(C)** The cladogram identified the differentially abundant taxa between the PTB patients and HCs. The HC-enriched taxa were indicated with a negative LDA score (blue), and the taxa enriched in the patients with PTB were characterized by a positive score (red).

such as *Lachnospiraceae_NK4A136_group*, *Faecalibacterium*, *Dorea*, *Butyricicoccus* and *Bifidobacterium* (*P*<0.001), and negatively associated with *Lactobacillus*, *Erysipelatoclostridium*, *Megasphaera* and *Sellimonas*, most of which were enriched in PTB patients. Moreover, Cr was positively correlated with some bacteria enriched in HCs, and negatively correlated with bacteria enriched in PTB group (*P*<0.05). Besides, WBC had no direct relation with the genera in our study. It is also unusual in that

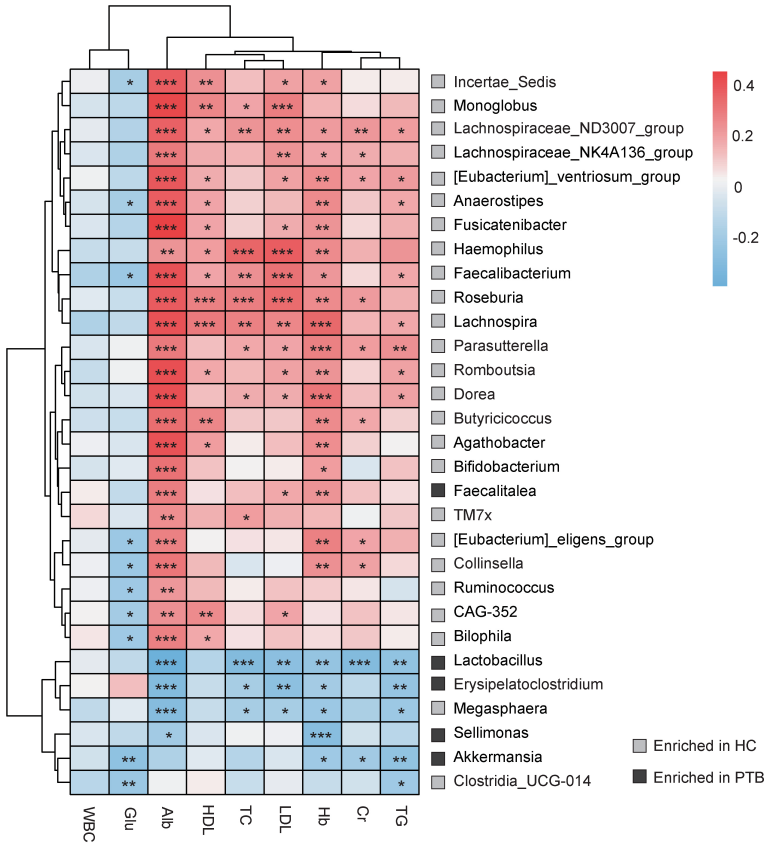

FIGURE 3
Correlation analysis between intestinal flora and biochemical indexes. The depth of the color in the heat maps signifies the strength of the correlation: red represents a positive correlation, whereas blue indicates a negative correlation. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. The correlation were assessed by the Spearman test. Grey represents features enriched in HCs, while black represents features enriched in PTB patients.

Glu only had a negative correlation with *Incertae_Sedis*, *Faecalibacterium*, *[Eubactrium]_eligens_group*, *Collinsella*, *Ruminococcus*, *CAG-352*, *Bilophila*, *Akkermanisia* and *Clostridia_UCG-014* ($P<0.05$). Taken together, there was an intimate relationship between the deferential genera of PTB patients and the biochemical index, indicating that the altered gut microbiota may be the key pathophysiology of PTB.

Microbial functional dysbiosis in PTB patients

PICRUSt2 was used to identify the metabolic and functional changes in the fecal microbiota between the PTB patients and the HCs. The results of PICRUSt2 based on the KEGG classification showed that the predominant predicted bacterial functions were related to cellular processes, genetic system, metabolism and organismal system (Figure 4A). Top 20 pathways were shown in Figure 4B, among which 18 were overrepresented in the PTB patients, while only 2 were enriched in healthy controls. Compared with HCs, 7 biosynthesis pathways related to ubiquinone, polyketide, siderophore, folate, lipopolysaccharide, N-glycan and steroid hormone were highly represented in PTB patients. Interestingly, 5 metabolism pathways related to lipoic acid, ascorbate, taurine, fructose and biotin were strikingly increased in PTB patients. Moreover, the degradation capacities for different types of substrates, such as amino acid and glycan were increased in patients, while the dioxin degradation pathway was increased in HCs. Notably, PTB patients had more pathways involved in toxin degradation and energy circulation, specifically reflected in the rise of peroxisome and TCA cycle pathways. However, HCs obviously had more proteasome, indicating a superior protein utilization capacity (Figure 4B). Thus, these results further confirmed that *Mycobacterium tuberculosis* infection would disturb the functional of the fecal microbiota and might participate in the pathogenesis and development of PTB.

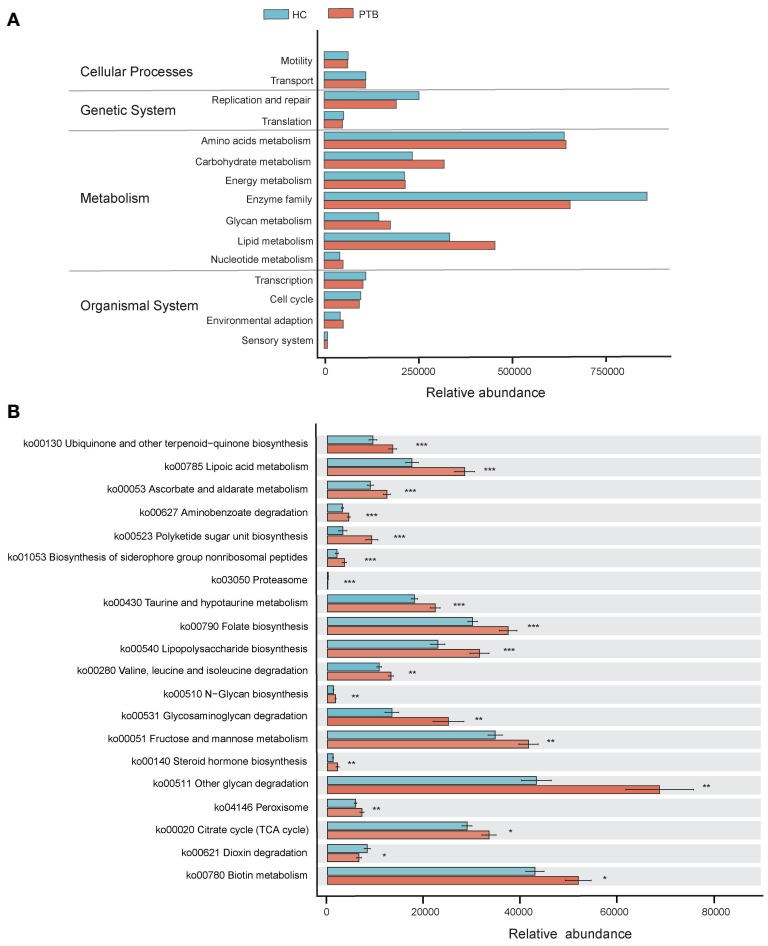

FIGURE 4
PICRUSt2-based examination of the fecal microbiome of the PTB patients and the healthy controls. **(A)** The different bacterial functions were evaluated between two groups. **(B)** A comparison of enriched KEGG pathways in PTB and HCs. * P<0.05, ** P<0.01, *** P<0.001. Functions and pathways were enriched in PTB group (red); Functions and pathways were enriched in the HC group (blue).

Significant biomarkers based on gut microbiota for PTB diagnosis

To further explore the diagnostic value of the gut microbiota for PTB, we constructed a random forest model to accurately distinguish PTB patients from HCs. 30 genus markers were selected from the model. The training of the random forest classifier involved the calculation of the feature importance of these 30 genera and their relative abundances (Table S1). The vital genera identified based on the model were described based on a mean decrease in accuracy (Figure 5A). ROC analysis was used to verify the diagnostic ability of these biomarkers. The area under the curve (AUC) obtained using the training datasets was 0.91, showing that patients with PTB could be successfully distinguished from the HCs. The AUC values for six genera, *Lactobacillus* (AUC=0.758), *Faecalibacterium* (AUC=0.803), *Roseburia* (AUC=0.829), *Dorea* (AUC=0.851), *Monnoglobus* (AUC=0.867) and *[Eubacterium]_ventriosum_group* (AUC=0.88), had an adequate diagnostic efficacy, respectively. Importantly, the model including all six genera had a relatively better diagnostic ability (AUC=0.90) (Figure 5B), indicating the combination of six genera may be a set of new diagnostic biomarkers for PTB.

Discussion

Homeostatic interactions with the microbiome are central to healthy human physiology and nutrition is the main driving force shaping the microbiome (Dominguez-Bello et al., 2019; Gomaa, 2020). Malnutrition is one of the major factors in *Mycobacterium tuberculosis* (*Mtb*) infection, which has been associated with the gut microbiota (Martin and Sabina, 2019; Tellez-Navarrete et al., 2021). Once TB sets in, it leads to an

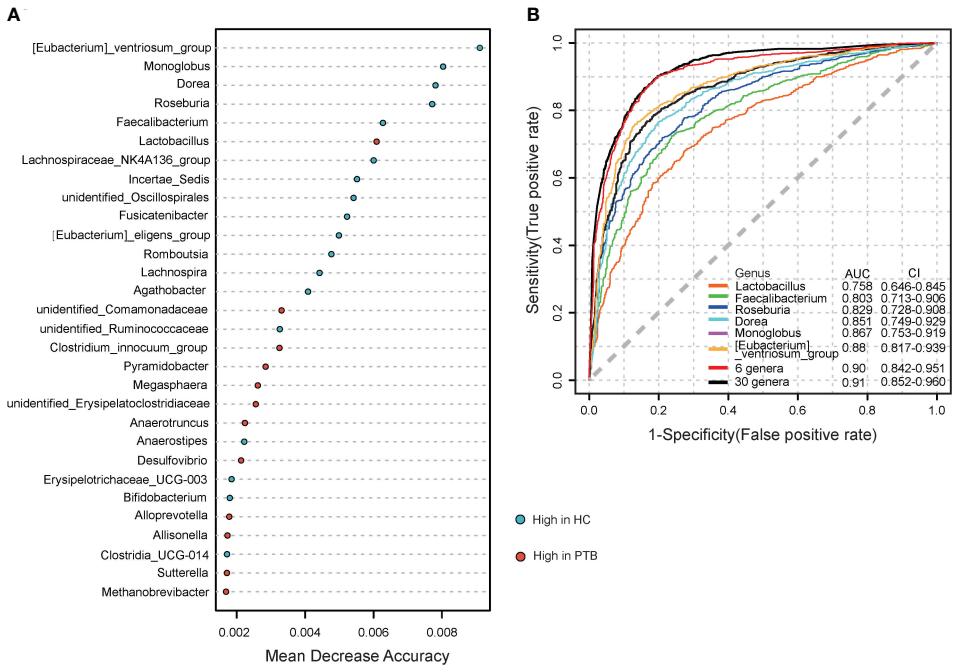

FIGURE 5
Establishing random forest models to predict the biomarkers of gut microbiota in PTBs. **(A)** The top 30 genera were selected to establish a random forest classifier for the diagnosis of PTB. The mean value enriched in HCs(blue); The mean value enriched in PTB patients (red). **(B)** Receiver operating characteristic (ROC) analysis of *Lactobacillu*, *Faecalibacterium, Roseburia, Dorea, Monnoglobus, [Eubacterium]_ventriosum*_group, 6 genera mentioned above and the 30 genera.

increase in metabolism and a decrease in appetite that compounds the already present malnutrition (Hernández-Garduño and Pérez-Guzmán, 2007; Kant et al., 2015; Mashabela et al., 2019). Emerging studies have indicated cross-talk occurs between the gut microbiota and the pathogenesis of pulmonary tuberculosis (PTB) through a gut-lung axis (Dang and Marsland, 2019; Saint-Criq et al., 2021). Our study group consists of participants from eastern China, which is relatively less explored and has no consistent results in the existing research. We updated the data on the structure and function of the intestinal flora of Chinese tuberculosis patients and attempted to contact the interplay and mechanism between host nutritional status, pathology, and the gut microbiome.

We observed microbial dysbiosis in PTB patients, which was reflected in the reduction of diversity and the change of taxonomic composition (Figures 1, 2), parallel to the recent study (Khaliq et al., 2021). In this study, at the phylum level, we found an enrichment of *Bacteroidota*, *Proteobacteria* and *Fusobacteriota* in PTB patients, many of which produced lipopolysaccharide (LPS), suggesting an increase of gut-derived LPS in PTB patients. Chronic infection and persistent inflammatory reaction of tuberculosis may affect the integrity of the intestinal mucosal barrier, facilitating the translocation of the bacteria, leading to the increase of circulating LPS, which further aggravated the inflammation of the host (Gallucci et al., 2021). Especially, as compared with the HCs, at the genus level, the abundance of *Bacteroides*, *Parabacteroides* and *Veillonella* increased markedly in PTB patients. The stability of gut microbiota is essential for the host's health, while the enrichment of anaerobic bacteria, for example, *Bacteroides* and *Veillonella*, were associated with disease status, leading to inflammation (Wei et al., 2020; Zafar and Saier, 2021). Moreover, we observed a significant decrease in the abundance of SCFAs-producing genera in PTB patients, such as *Faecalibacterium*, *Bifidobacterium*, *Agathobacter* and *CAG-352* (Figure 2A). Especially, the depletion of *Faecalibacterium* and *Bifidobacterium* was related to the TB cases (Khaliq et al., 2021; He et al., 2021). It is known that SCFAs, especially, acetate, propionate and butyrate are key mediators of the beneficial effects elicited by the gut microbiome (Martin-Gallausiaux et al., 2021). In addition to maintain intestinal function, evidence is accumulating that SCFAs directly modulate host metabolic health related to appetite regulation, energy expenditure, glucose homeostasis and immunomodulation (Canfora et al., 2015; Blaak et al., 2020). More importantly, we found a set of novel non-invasive diagnostic biomarkers for PTB, the combination of 6 genera, containing *Lactobacillus*, *Faecalibacterium*, *Roseburia*, *Dorea*, *Monnoglobus* and

[Eubacterium]_ventriosum_group specifically (Figure 5), had a relatively better diagnostic ability (AUC=0.90) than previous studies (Hu et al., 2019; Wang et al., 2022), which improved the accuracy and reduced the complexity.

Pulmonary tuberculosis (PTB) is a chronic consumptive disease, the malnutrition of PTB may cause anemia, hyperglycemia and other complications (Moon, 2014; Kant et al., 2015; Chhabra et al., 2021). In this study, we found that significantly lower lipid (TC, TG, HDL and LDL), Alb, Cr and Hb levels were other interesting characteristics of PTB patients (Table 1), which may result from a decrease in the utilization of protein and fat due to malnutrition (Kant et al., 2015). Moreover, we found that the levels of lipid, Alb and Hb were positively correlated with the abundances of SCFA-producing probiotics which enriched in HCs, such as *Lachnospiraceae_ND3007_group*, *Faecalibacterium*, *Roseburia*, *Dorea* and *Butyricicoccus* (Figure 3), indicating the enrichment of probiotics were related to the healthy physiological state of the host. *Faecalibacterium* and *Roseburia* are major producers of butyrate, which decreased *Mtb*-induced inflammation as well as insulin resistance (Jo et al., 2021; Faden, 2022). Meanwhile, the mentioned indexes were negatively correlated with the abundances of *Lactobacillus*, *Erysipelatoclostridium* and *Sellimonas*, which enriched in PTB patients (Figure 3). Previous study found that *Lactobacillus* was significantly increased in newly treated patients with PTB (Xie et al., 2021), and lactate, the product of *Lactobacillus*, was found to provide additional carbon matrix for *Mtb (*Billig et al., 2017). Besides, *Erysipelatoclostridium* was one of the major genera associated with active-TB, also associated with inflammation as well as lipid metabolism (Jo et al., 2021; Khaliq et al., 2021; Chang et al., 2022). In this study, we observed the level of Glu increased significantly in the PTB group (Table 1), and there was only a negative correlation between Glu level and the abundance of SCFAs producers, such as *[Eubacterium]_eligens_group*, *Ruminococcus* and *Akkermansia* (Figure 3) (Wang et al., 2022), different from the previous result (Hayashi et al., 2014). Although *Akkermansia* was reported to regulate blood glucose (Yoon et al., 2021), its ability might be reversed by other bacteria in the PTB group due to relative abundance or flora interaction. Thus, this study indicated that the imbalance of intestinal flora, especially the down-regulation of SCFA-producing bacteria, is related to the pathological indexes of PTB patients. In light of these observations, we considered this hypothesis that an upsurge of SCFAs-producing bacteria might have positive consequences on the host.

In addition to the SCFAs, we speculated another way that gut microbiota affected the pathological status of PTB patients was by regulating the bioavailability of nutrients such as amino acids, vitamins and glycan (Kant et al., 2015). The microbial function analysis revealed that PTB patients had a significant increase in the metabolism/degradation pathway of amino acids and glycan (Figure 4B), indicating an increased basal metabolic rate, which was consistent with the low fever pathology of PTB (El Amrani et al., 2016). The upregulation of these pathways probably resulted from the complicated microbial interaction network (Figure S1), which demanded much more nutrients to maintain in PTB patients than HCs. Notably, the lipopolysaccharide (LPS) biosynthesis pathway was significantly up-regulated in the PTB group, which was consistent with the up-regulated relative abundance of LPS-producing bacteria in PTB patients. Previous studies have found that LPS may be a sign of malnutrition and bacterial infection (Ubenauf et al., 2007; Patterson et al., 2021). Besides, LPS can induce insulin resistance, which is related to hyperglycemia (Salazar et al., 2020). The up-regulated LPS biosynthesis pathway may provide some explanations for the high blood glucose level and low blood lipid level of PTB patients in this study, which reaffirmed the involvement of gut microbiota in the process of PTB disease. Moreover, the biosynthesis of folic acid in this study increased significantly in PTB patients, it might be due to the enrichment of *Lactobacillus* in the PTB group, which was known as folic acid-producing bacteria (Levit et al., 2018; Hajian et al., 2019). Based on these results, the enrichment of proinflammatory bacteria in the PTB group seems to explain the pathologically elevated basal metabolic rate and the clinical characteristics of the host. Known as SCFAs can inhibit inflammation and regulate basic metabolism (Morrison and Preston, 2016; He et al., 2020), studies on the treatment of probiotics in diseases have been implemented (Xiong et al., 2021; Jiang et al., 2022). It seems feasible to treat PTB by replenishing the probiotics, for their excellent ability in improving the flora structure and increasing the production of SCFAs.

The results of this study provide a new framework for understanding the changes of intestinal microorganisms in the east China cohort exposed to *Mtb* and provide a new diagnostic marker for non-invasive diagnosis of PTB. We speculate that the enrichment of pro-inflammatory bacteria and the reduction of SCFA-producing probiotics in the PTB group caused the imbalance of intestinal flora structure and function, which led to the pathological metabolism in the PTB group, explaining the abnormality of clinical indexes in PTB patients. Therefore, it is meaningful to take probiotics to regulate the structure and function of the flora. The gut microbiota will be affected by environment, diet and living habits, diseases, antibiotics, etc. To increase the universality and strictness of this study, we need more large-scale follow-up research, including expanding cohort, upgrading sequencing methods, conducting animal experiments, etc., to obtain new insights from current findings. These studies will help us better understand the gut-lung axis and have potential significance for the non-invasive diagnosis and treatment of PTB.

Data availability statement

The data presented in the study are deposited in the BioProject repository, accession number is PRJNA901399. The link for data can be found below: https://www.ncbi.nlm.nih.gov/bioproject/901399.

Ethics statement

The studies involving human participants were reviewed and approved by Affiliated Dongyang Hospital of Wenzhou Medical University, Zhejiang, China. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study concept and design: XL, YL; Specimen collection: LW, SY, QD; Analysis and interpretation of data and statistical analysis: SL, SY, XW, XJ; Drafting the manuscript: XL, SY, YW. The authors thank the GUHE Info technology, Co., Ltd., (Hangzhou, China) for expert technical advice. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1090889/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The interaction network of intestinal flora in HCs and PTB patients. The interaction network of intestinal flora in HCs showed a simple relationship between the community members, and the interaction network of intestinal flora in PTBs had more positive and negative correlations among the bacteria. The correlation coefficients were calculated with the Sparse Correlations for Compositional (SparCC) data algorithm. Cytoscape version 3.4.0 was used for network construction.

References

Barcik, W., Boutin, R. C. T., Sokolowska, M., and Finlay, B. B. (2020). The role of lung and gut microbiota in the pathology of asthma. *Immunity* 52 (2), 241–255. doi: 10.1016/j.immuni.2020.01.007

Billig, S., Schneefeld, M., Huber, C., Grassl, G. A., Eisenreich, W., and Bange, F. C., et al. (2017). Lactate oxidation facilitates growth of mycobacterium tuberculosis in human macrophages. *Sci. Rep.* 7 (1), 6484. doi: 10.1038/s41598-017-05916-7

Blaak, E. E., Canfora, E. E., Theis, S., Frost, G., Groen, A. K., Mithieux, G., et al. (2020). Short chain fatty acids in human gut and metabolic health. *Benef Microbes* 11 (5), 411–455. doi: 10.3920/BM2020.0057

Canfora, E. E., Jocken, J. W., and Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. *Nat. Rev. Endocrinol.* 11 (10), 577–591. doi: 10.1038/nrendo.2015.128

Chang, Z.-Y., Liu, H.-M., Leu, Y.-L., Hsu, C.-H., and Lee, T.-Y. (2022). Modulation of gut microbiota combined with upregulation of intestinal tight junction explains anti-inflammatory effect of corylin on colitis-associated cancer in mice. *Int. J. Mol. Sci.* 23 (5), 2667. doi: 10.3390/ijms23052667

Chen, H., and Jiang, W. (2014). Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. *Front. Microbiol.* 5, 508. doi: 10.3389/fmicb.2014.00508

Chhabra, S., Kashyap, A., Bhagat, M., Mahajan, R., and Sethi, S. (2021). Anemia and nutritional status in tuberculosis patients. *Int. J. Appl. Basic Med. Res.* 11 (4), 226–230. doi: 10.4103/ijabmr.ijabmr_76_21

Comberiati, P., Di Cicco, M., Paravati, F., Pelosi, U., Di Gangi, A., Arasi, S., et al. (2021). The role of gut and lung microbiota in susceptibility to tuberculosis. *Int. J. Environ. Res. Public Health* 18, 12220. doi: 10.3390/ijerph182212220

Dang, A. T., and Marsland, B. J. (2019). Microbes, metabolites, and the gut-lung axis. *Mucosal Immunol.* 12 (4), 843–850. doi: 10.1038/s41385-019-0160-6

de Vos, W., Tilg, H., Van Hul, M., and Cani, P. D. (2022). Gut microbiome and health: Mechanistic insights. *Gut* 71 (5), 1020–1032. doi: 10.1136/gutjnl-2021-326789

Dominguez-Bello, M. G., Godoy-Vitorino, F., Knight, R., and Blaser, M. J. (2019). Role of the microbiome in human development. *Gut* 68 (6), 1108–1114. doi: 10.1136/gutjnl-2018-317503

Dumas, A., Bernard, L., Poquet, Y., Lugo-Villarino, G., and Neyrolles, O. (2018). The role of the lung microbiota and the gut-lung axis in respiratory infectious diseases. *Cell Microbiol.* 20 (12), e12966. doi: 10.1111/cmi.12966

El Amrani, M., Asserraji, M., Bahadi, A., El Kabbaj, D., and Benyahia, M. (2016). Tuberculosis in hemodialysis. *Med. Sante Trop.* 26 (3), 262–266. doi: 10.1684/mst.2016.0569

Faden, H. (2022). The role of faecalibacterium, roseburia and butyrate in inflammatory bowel disease. *Dig Dis* 40 (6), 793–795. doi: 10.1159/000522247

Gallucci, G., Santucci, N., Díaz, A., Bongiovanni, B., Bértola, D., Gardeñez, W., et al. (2021). Increased levels of circulating LPS during tuberculosis prevails in patients with advanced pulmonary involvement. *PloS One* 16 (9), e0257214. doi: 10.1371/journal.pone.0257214

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., et al. (2006). Metagenomic analysis of the human distal gut microbiome. *Science* 312 (5778), 1355–1359. doi: 10.1126/science.1124234

Gomaa, E. Z. (2020). Human gut microbiota/microbiome in health and diseases: A review. *Antonie Van Leeuwenhoek* 113 (12), 2019–2040. doi: 10.1007/s10482-020-01474-7

Hajian, B., Scocchera, E., Shoen, C., Krucinska, J., Viswanathan, K., G-Dayanandan, N., et al. (2019). Drugging the folate pathway in mycobacterium tuberculosis: The role of multi-targeting agents. *Cell Chem. Biol.* 26 (6), 781–791.e6. doi: 10.1016/j.chembiol.2019.02.013

Hayashi, S., Takeuchi, M., Hatsuda, K., Ogata, K., Kurata, M., Nakayama, T., et al. (2014). The impact of nutrition and glucose intolerance on the development of tuberculosis in Japan. *Int. J. Tuberc Lung Dis.* 18 (1), 84–88. doi: 10.5588/ijtld.13.0495

He, J., Zhang, P., Shen, L., Niu, L., Tan, Y., Chen, L., et al. (2020). Short-chain fatty acids and their association with signalling pathways in inflammation, glucose and lipid metabolism. *Int. J. Mol. Sci.* 21 (17), E6356. doi: 10.3390/ijms21176356

He, C., Wang, H., Yu, C., Peng, C., Shu, X., Liao, W., Zhu, Z, et al. (2021). Alterations of gut microbiota in patients with intestinal tuberculosis that different from crohn's disease. *Front. Bioeng Biotechnol.* 9, 673691. doi: 10.3389/fbioe.2021.673691

Hernández-Garduño, E., and Pérez-Guzmán, C. (2007). Appetite and tuberculosis: Is the lack of appetite an unidentified risk factor for tuberculosis? *Med. Hypotheses* 69 (4), 869–872. doi: 10.1016/j.mehy.2007.02.006

Hu, Y., Feng, Y., Wu, J., Liu, F., Zhang, Z., Hao, Y., et al. (2019). The gut microbiome signatures discriminate healthy from pulmonary tuberculosis patients. *Front. Cell Infect. Microbiol.* 9, 90. doi: 10.3389/fcimb.2019.00090

Jiang, L., Wang, J., Xu, L., Cai, J., Zhao, S., and Ma, A., et al. (2022). Lactobacillus casei modulates inflammatory cytokines and metabolites during tuberculosis treatment: A *post hoc* randomized controlled trial. *Asia Pac J. Clin. Nutr.* 31 (1), 66–77. doi: 10.6133/apjcn.202203_31(1).0008

Jo, J.-K., Seo, S. H., Park, S. E., Kim, H. W., Kim, E. J., Kim, J. S., et al. (2021). Gut microbiome and metabolome profiles associated with high-fat diet in mice. *Metabolites* 11 (8), 482. doi: 10.3390/metabo11080482

Kant, S., Gupta, H., and Ahluwalia, S. (2015). Significance of nutrition in pulmonary tuberculosis. *Crit. Rev. Food Sci. Nutr.* 55 (7), 955–963. doi: 10.1080/10408398.2012.679500

Khaliq, A., Ravindran, R., Afzal, S., Jena, P. K., Akhtar, M. W., Ambreen, A., et al. (2021). Gut microbiome dysbiosis and correlation with blood biomarkers in active-tuberculosis in endemic setting. *PloS One* 16 (1), e0245534. doi: 10.1371/journal.pone.0245534

Levit, R., Savoy de Giori, G., de Moreno de LeBlanc, A., LeBlanc, J. G., and LeBlanc, J. G (2018). Folate-producing lactic acid bacteria reduce inflammation in mice with induced intestinal mucositis. *J. Appl. Microbiol.* 125 (5), 1494–1501. doi: 10.1111/jam.14038

Li, N., Dai, Z., Wang, Z., Deng, Z., Zhang, J., Pu, J., et al. (2021). Gut microbiota dysbiosis contributes to the development of chronic obstructive pulmonary disease. *Respir. Res.* 22, 274. doi: 10.1186/s12931-021-01872-z

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73 (5), 1576–1585. doi: 10.1128/AEM.01996-06

Lozupone, C., and Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71 (12), 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Luo, M., Liu, Y., Wu, P., Luo, D. X., Sun, Q., Zheng, H., et al. (2017). Alternation of gut microbiota in patients with pulmonary tuberculosis. *Front. Physiol.* 8, 822. doi: 10.3389/fphys.2017.00822

Maji, A., Misra, R., Dhakan, D. B., Gupta, V., Mahato, N. K., Saxena, R., et al. (2018). Gut microbiome contributes to impairment of immunity in pulmonary tuberculosis patients by alteration of butyrate and propionate producers: Gut microbiome of TB patients. *Environ. Microbiol.* 20 (1), 402–419. doi: 10.1111/1462-2920.14015

Martin-Gallausiaux, C., Marinelli, L., Blottière, H. M., Larraufie, P., and Lapaque, N. (2021). SCFA: Mechanisms and functional importance in the gut. *Proc. Nutr. Soc.* 80 (1), 37–49. doi: 10.1017/S0029665120006916

Martin, S. J., and Sabina, E. P. (2019). Malnutrition and associated disorders in tuberculosis and its therapy. *J. Diet Suppl.* 16 (5), 602–610. doi: 10.1080/19390211.2018.1472165

Mashabela, G. T., de Wet, T. J., and Warner, D. F. (2019). Mycobacterium tuberculosis metabolism. *Microbiol. Spectr.* 7 (4). doi: 10.1128/microbiolspec.GPP3-0067-2019

McArdle, B. H., and Anderson, M. J. (2001). Fitting multivariate models to community data: A comment on distance-based redundancy analysis. *Ecology* 82, 290–297. doi: 10.1890/0012-9658(2001)082[0290:FMMTCD]2.0.CO;2

Moon, M.-S. (2014). Tuberculosis of spine: Current views in diagnosis and management. *Asian Spine J.* 8 (1), 97–111. doi: 10.4184/asj.2014.8.1.97

Morrison, D. J., and Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7 (3), 189–200. doi: 10.1080/19490976.2015.1134082

Naidoo, C. C., Nyawo, G. R., Sulaiman, I., Wu, B. G., Turner, C. T., Bu, K., et al. (2021). Anaerobe-enriched gut microbiota predicts pro-inflammatory responses in pulmonary tuberculosis. *EBioMedicine* 67, 103374. doi: 10.1016/j.ebiom.2021.103374

Patterson, G. T., Manthi, D., Osuna, F., Muia, A., Olack, B., Mbuchi, M., et al. (2021). Environmental, metabolic, and inflammatory factors converge in the pathogenesis of moderate acute malnutrition in children: An observational cohort study. *Am. J. Trop. Med. Hyg* 104 (5), 1877–1888. doi: 10.4269/ajtmh.20-0963

Pezzella, A. T. (2019). History of pulmonary tuberculosis. *Thorac. Surg. Clin.* 29 (1), 1–17. doi: 10.1016/j.thorsurg.2018.09.002

Ramsey, P. H. (1989). Critical values for spearman's rank order correlation. *J. Educ. Stat* 14 (3), 245–253. doi: 10.3102/10769986014003245

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ* 4, e2584. doi: 10.7717/peerj.2584

Saint-Criq, V., Lugo-Villarino, G., and Thomas, M. (2021). Dysbiosis, malnutrition and enhanced gut-lung axis contribute to age-related respiratory diseases. *Ageing Res. Rev.* 66, 101235. doi: 10.1016/j.arr.2020.101235

Salazar, J., Angarita, L., Morillo, V., Navarro, C., Martínez, M. S., Chacín, M., et al. (2020). Microbiota and diabetes mellitus: Role of lipid mediators. *Nutrients* 12 (10), E3039. doi: 10.3390/nu12103039

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

Tellez-Navarrete, N. A., Ramon-Luing, L. A., Muñoz-Torrico, M., Osuna-Padilla, I. A., and Chavez-Galan, L. (2021). Malnutrition and tuberculosis: The gap between basic research and clinical trials. *J. Infect. Dev. Ctries* 15 (3), 310–319. doi: 10.3855/jidc.12821

Ubenauf, K. M., Krueger, M., Henneke, P., and Berner, R. (2007). Lipopolysaccharide binding protein is a potential marker for invasive bacterial infections in children. *Pediatr. Infect. Dis. J.* 26 (2), 159–162. doi: 10.1097/01.inf.0000253064.88722.6d

Wang, S., Yang, L., Hu, H., Lv, L., Ji, Z., Zhao, Y., et al. (2022). Characteristic gut microbiota and metabolic changes in patients with pulmonary tuberculosis. *Microb. Biotechnol.* 15, 262–275. doi: 10.1111/1751-7915.13761

Wei, Y., Li, Y., Yan, L., Sun, C., Miao, Q., Wang, Q., et al. (2020). Alterations of gut microbiome in autoimmune hepatitis. *Gut* 69 (3), 569–577. doi: 10.1136/gutjnl-2018-317836

Xie, J. H., Yu, R., Shi, G. M., Ma, X. H., Xiao, S. F., Yi, Y. H., et al. (2021). [Correlation study between changes in intestinal microflora structure and immune indexes in newly treated patients with pulmonary tuberculosis]. *Zhonghua Yu Fang Yi Xue Za Zhi* 55 (12), 1486–1490. doi: 10.3760/cma.j.cn112150-20210728-00721

Xiong, K., Cai, J., Liu, P., Wang, J., Zhao, S., Xu, L., et al. (2021). Lactobacillus casei alleviated the abnormal increase of cholestasis-related liver indices during tuberculosis treatment: A *Post hoc* analysis of randomized controlled trial. *Mol. Nutr. Food Res.* 65 (16), e2100108. doi: 10.1002/mnfr.202100108

Yang, F., Yang, Y., Chen, L., Zhang, Z., Liu, L., Zhang, C., et al. (2022). The gut microbiota mediates protective immunity against tuberculosis *via* modulation of lncRNA. *Gut Microbes* 14 (1), 2029997. doi: 10.1080/19490976.2022.2029997

Yoon, H. S., Cho, C. H., Yun, M. S., Jang, S. J., You, H. J., Kim, J. H., et al. (2021). Akkermansia muciniphila secretes a glucagon-like peptide-1-inducing protein that improves glucose homeostasis and ameliorates metabolic disease in mice. *Nat. Microbiol.* 6 (5), 563–573. doi: 10.1038/s41564-021-00880-5

Zafar, H., and Saier, M. H. (2021). Gut bacteroides species in health and disease. *Gut Microbes* 13 (1), 1–20. doi: 10.1080/19490976.2020.1848158

Zaura, E., Keijser, B. J. F., Huse, S. M., and Crielaard, W. (2009). Defining the healthy 'core microbiome' of oral microbial communities. *BMC Microbiol.* 9, 259. doi: 10.1186/1471-2180-9-259

Zhao, Y., Liu, Y., Li, S., Peng, Z., Liu, X., Chen, J., et al. (2021). Role of lung and gut microbiota on lung cancer pathogenesis. *J. Cancer Res. Clin. Oncol.* 147 (8), 2177–2186. doi: 10.1007/s00432-021-03644-0

Zhu, W., Wu, Y., Liu, H., Jiang, C., and Huo, L. (2021). Gut–lung axis: Microbial crosstalk in pediatric respiratory tract infections. *Front. Immunol.* 12, 741233. doi: 10.3389/fimmu.2021.741233

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Cyprinid herpesvirus 2 infection changes microbiota and metabolites in the gibel carp (*Carassius auratus gibelio*) midgut

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Cyprinid herpesvirus 2 (CyHV-2) infects gibel carp (*Carassius auratus gibelio*) and causes severe losses. Microbiota in animal guts involves nutrition intake, development, immunity, and disease resistance. However, the relationship between gibel carp gut microbiota and CyHV-2 infection is not well known. Herein, we analyzed the gut microbiota composition and metabolite profiles in CyHV-2-infected and -uninfected fish using high-throughput sequencing and gas chromatography/mass spectrometry. Results showed that CyHV-2 infection significantly changed gut microbiota and metabolite profiles ($p < 0.05$). High-throughput sequencing demonstrated that the relative abundance of *Aeromonas* in the midgut increased dramatically while *Cetobacterium* decreased. Time-course analysis showed that the number of *Aeromonas* in the midgut of infected fish increased more than 1,000 times within 5 days post infection. Metabolome analysis illustrated that CyHV-2 infection significantly altered 24 metabolites in the midgut of gibel carp, annotating to the anomaly of digestion and metabolisms of amino acids, carbohydrates, and lipids, such as tryptophan (Trp) metabolism. The Mantel test demonstrated that gut microbiota and metabolite profiles were well related ($r = 0.89$). Furthermore, Trp metabolism responded to CyHV-2 infection closely was taken as one example to prove the correlation among CyHV-2 infection, metabolites and microbiota in the midgut, and host immunity. Results showed that modulating Trp metabolism could affect the relative abundance of *Aeromonas* in the midgut of fish, transcription of antiviral cytokines, and CyHV-2 infection. Therefore, we can conclude that CyHV-2 infection significantly perturbed the gut microbiome, disrupted its' metabolic functions, and caused the proliferation of the opportunistic pathogen *Aeromonas*.

This study also suggests that modulation of the gut microbiome will open a therapeutic opportunity to control CyHV-2 infection in gibel carp.

KEYWORDS

CyHV-2, gut microbiota-mediated metabolites, *Aeromonas*, *Cetobacterium*, tryptophan metabolism

1 Introduction

Gibel carp (*Carassius auratus gibelio*), an omnivorous freshwater fish, is one of the leading aquaculture species worldwide. More than 2,700,000 tons of gibel carps were produced in 2020 with an economic value of over 5 billion dollars (Ministry of Agriculture and Rural Affairs of China, 2021), and most of its production farms are located in Yancheng, Province Jiangsu, China (Liu et al., 2018). However, the disease caused by Cyprinid herpesvirus 2 (CyHV-2), also known as the goldfish hematopoietic necrosis virus (GFHNV), limits the sustainable aquaculture of gibel carp (Lu et al., 2022; Zhu et al., 2022). CyHV-2 infection in gibel carp was first reported in 1999 in China (Chang et al., 1999), and the massive mortality reached over 90% in many breeding ponds (Wang et al., 2012). The CyHV-2-infected fish displays lethargic, anorexic, and widespread bleeding in the body, gill, and swim bladder. When the gill cover opens and closes or the fish's body is stressed or jumping, blood will flow out from the gills (some infected fish may not have gill-bleeding symptoms). Some infected fish have erythema on the gill cover, which is called a beauty spot. Internal organs are congested or bleeding, the fish's swim bladder has obvious bleeding points compared to the hemorrhages caused by *Aeromonas* spp., and the tail fin is whitish (Wu et al., 2013). CyHV-2 can be naturally detected in the water and sediments in the culture ponds, and gibel carp is sensitive to this virus (Yao et al., 2016). Gills are the critical organ for the initial invasion of CyHV-2 into fish, while the kidney and spleen are the primary organs for viral replication (Ding et al., 2014; Zhu et al., 2015; Yao et al., 2016). However, there is still no effective strategy to control this infection, which threatens this species' sustainable culture industry.

Recent studies reveal that modulating the gut microbiota is an effective strategy to control disease development in human beings (Liu et al., 2013), but few studies were reported on the fish (Ran et al., 2021). Gut microbiota is considered as a good indicator of animal health, and plays a vital role in regulating host immune homeostasis and metabolic function (Guarner and Malagelada, 2003; Hu, 2017). The following four roles related to gut microflora have been reported: 1) metabolic activities in the fermentation of non-digestible dietary residue and endogenous mucus: the salvage of energy as short-chain fatty acids, the production of vitamin K, and the absorption of ions; 2) critical trophic effects on intestinal epithelial cell growth and differentiation, and interactions between intestinal bacteria and host immunity at the mucosal interface; 3) protection or mucous integrity-dependent barrier against pathogen invasion by alien microbes; and 4) relation to certain pathological disorders, including multisystem organ failure and inflammatory diseases (Guarner and Malagelada, 2003; Shapira, 2016; Cong et al., 2022). Thus, determining causal links of gut microbiota to diseases can contribute to developing new therapeutic interventions (Guarner and Malagelada, 2003; Li et al., 2017; Agus et al., 2018).

Although the gut microbiota of fish is affected by many factors, including host factors (e.g., genetics, gender, weight, and immunity), environmental factors (e.g., water, salinity, diet, and medicine), and microbial factors (Wang et al., 2018), the composition of the gut core microbiota is relatively stable in fish (Wang et al. 2018). Among them, water, diet, and feeding habits have been studied the most. *Aeromonas*, *Pseudomonas*, and *Bacteroides* type A dominate the gut microbiota of freshwater fish species. *Plesiomonas*, *Enterobacteriaceae*, *Micrococcus*, *Acinetobacter*, *Clostridium*, *Bacteroides* type B, and *Fusariumas* are less abundant. Additionally, many studies have used the combined metabolomic and metagenomic methods to investigate the correlation between metabolic alterations and gut microbiota changes, providing new insights into the disease pathology and therapeutic intervention (Guarner and Malagelada, 2003; Wang et al., 2018). For gibel carp, the gut microbiota of fish post-CyHV-2 infection was changed, and *Plesiomonas* was highly abundant in the infected fish Rong et al., 2017. CyHV-2 and CyHV-3 infections ruin the gut integrity of carps. These findings raised the possibility of the direct interaction between CyHV-2 and commensal bacteria (Rong et al., 2017; Ran et al., 2021). However, few studies have been conducted to investigate the effect of CyHV-2 infection on the gut microbial ecology by using combined metabolomic and metagenomic analysis. It will hinder the control and prevention development of CyHV-2 infection in gibel carp culture.

Thus, in this study, the changes of microbiota and metabolites in the midgut of CyHV-2-infected gibel carp and the correlation among them were firstly investigated. Then, the time-dependent

number changes of target bacteria associated with CyHV-2 infection were analyzed through the culture method. Furthermore, tryptophan (Trp) metabolism was taken as one of the examples to prove the correlation between CyHV-2 infection and the metabolites since 1) the changes of microbiota and metabolites in the fish midgut post-CyHV-2 infection in this study had been annotated to Trp metabolism; 2) Trp metabolism has been reported to play a crucial role in gut microbiota–host cross-talk in health and disease *via* the aryl hydrocarbon receptor (AhR) (Hao and Whitelaw, 2013; Agus et al., 2018; Natividad et al., 2018; Sun et al., 2019; Wyant and Moslehi, 2022); 3) AhR, a nuclear protein upon association with certain endogenous and exogenous ligands, can translocate signals into the nucleus, bind DNA, and regulate gene expression. Trp metabolites are one of the most important endogenous AhR ligands (Ghiboub et al., 2020; Willem et al., 2022); and 4) Trp metabolism is known to regulate the intestinal barrier function and host immunity, and Trp metabolites/AhR signaling modulation are a therapeutic perspective in human diseases (Lamas et al., 2016; Lamas et al. 2018; Trikha and Lee, 2019; Platten et al., 2019; Ghiboub et al., 2020; Rosser et al., 2020). Taken together, these results about microbiome-modulated metabolites from this study will provide valuable information to control CyHV-2 infection from a disrupted equilibrium to clinical opportunities.

2 Materials and methods

2.1 Ethics statement

In the present study, all the animal experiments were handled in accordance with the guidelines for the Care and Use of Experimental Animals of China. The Committee for the Welfare and Ethics of Laboratory Animals was approved by the Animal Care and Use Committee of the Center for Applied Aquatic Genomics at the Chinese Academy of Fishery Sciences.

2.2 Fish acclimation

Gibel carp (43.17 ± 2.9 g) was obtained from a fish farm at Yancheng City, Jiangsu province China. After bringing the fish to the lab, we first performed PCR to make sure that the fish did not carry the CyHV-2 and then did further research (Figure S1). The primers for PCR are listed in Table S1. PCR in the reaction volume of 20 μl was performed following the protocols as one cycle of 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and final elongation at 72°C for 5 min (Zhu et al., 2015). The PCR products were checked by 1% agarose gel electrophoresis.

Then, the fish were acclimated in a tank with static water at a water temperature of 23°C–25°C, pH of 7.8–8.2, and dissolved oxygen (DO) higher than 5 mg L^{-1} and provided with 24-h continuous aeration circulation for 2 weeks prior to the experiments. Gibel carp was fed three times each day with commercial diets (Tongwei, Yancheng, China) to satiation. The diets contained 32.25% crude protein, 5.90% crude lipid, 1.18% calcium, and 1.23% total phosphorus.

2.3 CyHV-2 solution preparation and viral quantification

The CyHV-2 solution was prepared as the previous description by Qiao et al. (2020). In brief, the kidney from naturally CyHV-2-infected gibel carp with typical signs of hemorrhage in the gill and swim bladder was sampled, homogenized by mixing with 10 times volume of phosphate-buffered saline (PBS, 0.01 M, pH7.2), centrifuged at 12,000 × g for 30 min and filtered by a 0.45/0.22-μm biofilter to remove bacteria.

DNA from the kidney was extracted using the viral DNA extraction kit (TaKaRa BIO Co., Ltd., Dalian, China), and the extracted DNA was assessed and adjusted to 50 ng μl^{-1} by Biophotometer Plus (Eppendorf). The primers (CyHV-2-RT-F and CyHV-2-RT-R) for quantitative real-time PCR (qPCR) are listed in Table S1. The qPCR reactions were performed using the SYBR® Premix ExTaq™ kit (Takara Bio Co., Ltd., Dalian, China) in a total volume of 25 μl, containing 12.5 μl SYBR Premix Ex Taq II (TaKaRa, Japan), 2 μl template DNA, 1 μl each primer and 8.5 μl ddH_2O. Distilled water replacing the extracted DNA was set as a negative control for each new run. The thermal cycling condition was as follows: one cycle of 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and 55°C for 30 s and then by dissociation curve analysis (65°C–95°C: increment 0.5°C for 5 s) (Qiao et al., 2020). After amplification, melting curve analysis was performed, and Ct values were obtained. The standard curve of the viral DNA copy number is shown in Figure S2, which was generated as described by Xu et al. (2014). Plasmid DNA containing the sequence of the CyHV-2 helicase gene was selected to serve as the standard for virus quantification. The amplified DNA fragment was gel-purified using a Fermentas Gel Extraction Kit (Thermo). The resulting DNA fragment was inserted into the pMD19-T plasmid to produce pMD-CyHV-2. A 10-fold dilution series of pMD-CyHV-2 was used as the standard template of CyHV-2 in the qPCR. Finally, a viral stock solution at a concentration of 9.7×10^7 copies μl^{-1} was prepared.

2.4 Fish infection with CyHV-2 through gills (simulated to natural infection)

The gut microbiota diversity of gibel carp is related to the environment, diets, and feeding habits (Chen et al., 2021), but the composition of core microbiota in the gut is relatively stable under the same cultural circumstance (Wang et al. 2018; Chen

et al., 2021). Therefore, allogynogenetic gibel carp, after acclimation for 14 days in the lab, was house-infected by CyHV-2 to avoid the effect of gender, water, and diet on gut microbiota. After acclimation in part 2.2, fish were divided randomly into two groups, namely, a control group with PBS and the CyHV-2-infection group. Each group included six tanks, three of them (volume of 100 L) with 8 individuals were used to calculate cumulative mortality (CM) and another three (volume of 400 L) with 30 individuals was set to sample for gut microbiota and metabolite analysis.

The 'per-gill infection method' mocked natural infection, which directly exposes virus to only gills, had been used to infect gibel carp like KHV and CHNV (Miyazaki et al., 2008; Somamoto et al., 2015) since gills are one of the first organs of aquatic animals exposed to pathogens (Murray et al., 2017; Andrews et al., 2010) and key invasion portal for CyHV-2 in gibel carp (Ding et al., 2014). Briefly, fish were anesthetized with tricaine methane sulfonate (MS-222) at 100 mg L^{-1} and inoculated with a CyHV-2 working solution (9.7×10^6 copies μl^{-1}) at 10 μl $fish^{-1}$ into their gills (both sides) in air. An equal volume of PBS was inoculated into gills as the control group. After gill exposure to the viral solution or PBS, fish were wrapped with wet papers and kept in air for 5–7 min at 25°C to allow the virus to adsorb into the gill tissue. Then, the fish were returned to the tank and maintained at 25°C with 24-h continuous aeration circulation. During the challenge test, no diets were fed (Qiao et al., 2020).

2.5 CyHV-2 infection confirmation

2.5.1 Clinical symptom observation and cumulative mortality

Clinical symptoms were observed daily, focusing on lethargy, anorexia, and hemorrhage in the body, eye, gill, and swim bladder. CM within 14 days post infection (dpi) was counted.

2.5.2 CyHV-2 load measurement

The fish with typical hemorrhagic symptoms at the 5th dpi were sampled. The viral load at 5 dpi in different tissues, including the gill, foregut, midgut, hindgut, liver, kidney, spleen, brain, and muscle, was detected by qPCR as described in part 2.3. Viral load was converted into copies per ng DNA.

2.6 Microbiome analysis of fish gut post-CyHV-2 infection

2.6.1 Midgut microbiota analysis through high-throughput sequencing

The midgut of three individuals with typical hemorrhagic symptoms at 5 dpi from each tank was collected and pooled as one sample. Resultantly, a total of nine fish for each group and three biological replicates were conducted for gut microbiome analysis. Microbial DNA was extracted from the midgut using the HiPure Soil DNA kits (Magen, Guangzhou, China) according to the manufacturer's protocols. DNA samples were amplified to target the V4 region of bacterial 16S rRNA (Qiao et al., 2020). PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Suzhou, China). The primers 341F and 806R were used, and sequences are listed in Table S1 (Guo et al., 2017). Purified amplicons were pooled in the equimolar and paired-end sequences on an Illumina platform (MiSeq, Illumina Inc., Guangzhou, China) (2 × 250) following the standard protocols. The effective tags were clustered into operational taxonomic units (OTUs) of ≥97% similarity using the UPARSE (version 9.2.64) pipeline (Edgar, 2013). The tag sequence with the highest abundance was selected as a representative sequence within each cluster. A Venn diagram using the R package (version 1.6.16) to analyze and draw plots was performed in R project UpSetR package (version 1.3.3) to identify unique and common OTUs (Conway et al., 2017). The species comparison between groups was calculated by Welch's *t*-test. Principal component analysis (PCA) was performed to distinguish the gut microbiota profiles between control and CyHV-2-infection groups. All α-diversity indexes were calculated in QIIME (version 1.9.1), and the alpha index comparison between groups was calculated by Welch's *t*-test (Caporaso et al., 2010). Sequence alignment was performed using muscle (version 3.8.31), and phylogenetic tree was constructed using FastTree (version 2.1) (Edgar, 2004; Price et al., 2010); then, the weighted UniFrac distance matrix was generated by R project GuniFrac package (version 1.0) (Lozupone and Knight, 2006). The Kyoto Encyclopedia of Genes and Genomes KEGG pathway analysis of the OTUs was inferred using PICRUSt (version 2.1.4), and the analysis of functional difference between groups was calculated by Welch's *t*-test (Langille et al., 2013). Canonical correspondence analysis (CCA) was executed in the R Project Vegan package (version 2.5.3) to clarify the influence of treatment on community composition (Oksanen et al., 2011). The biomarker features in each group were screened by the CCA effect size (LEfSe) software (version 1.0) (Edgar, 2013). Student's *t*-test was used to evaluate the statistical differences of biological parameters between control and CyHV-2-infection groups. The significant difference was set at $p < 0.05$.

2.6.2 Verification of time-dependent *Aeromonas* abundance within 5 dpi by the culture method

Based on the results from part 2.6.1 and *Aeromonas* frequent infection in gibel carp culture practice, the time-course abundance of *Aeromonas* in the midgut of fish after infected by CyHV-2 was detected. The midgut of fish at 0, 1, 4, and 5 dpi was collected and homogenized in precold PBS. Then, the

homogenate was diluted and spread on the RS selective medium, which was cultured at 28°C for 24–48 h. Bacterial colonies were counted and converted into the CFU g^{-1} midgut. Meanwhile, the number of *Aeromonas* in culture water during the whole experiment was counted.

2.7 Metabolomic analysis of fish gut post-CyHV-2 infection

2.7.1 Metabolomic analysis of fish midgut by Jiangsu Liquid Chromatography-Tandem Mass Spectrometry LC-MS/MS

The midgut from each fish with typical hemorrhagic symptoms at 5 dpi was sampled, cut off, and then flushed with PBS to collect metabolites. Six biological replicates were set for each group. LC-MS/MS analysis was performed using a Ultra High Performance Liquid Chromatography UHPLC system (1290, Agilent Technologies). Mass Spectrometer MS raw data (.raw) files were converted to the mzML format using ProteoWizard and processed by R package XCMS (version 3.2), including retention time alignment, peak detection, and peak matching. The OSI-SMMS (version 1.0, Dalian Chem Data Solution Information Technology Co. Ltd.) was used for peak annotation after data processing with an in-house MS/MS database. Partial least squares discriminant analysis (PLS-DA) is a supervised dimensionality reduction method and was applied in comparison groups using R package models (http://www.r-project.org/) (Worley and Powers, 2013). The metabolite resonances were identified according to the Human Metabolome Database (Deng et al., 2014). Significantly changed metabolites were identified based on the following criteria: fold change >2.00 or <0.56, VIP > 1, and $p < 0.05$.

2.7.2 Detection of time-dependent concentration of aryl hydrocarbon receptor (AhR) within 5 days post-CyHV-2 infection

The results from parts 2.6 and 2.7.1 showed that 1) CyHV-2 infection increased the relative abundance of *Aeromonas* spp. and decreased *Cetobacterium* in the midgut significantly; 2) the concentration of indoleacetaldehyde decreased significantly; and 3) a strong positive correlation between them was also found. Additionally, indole derivatives such as indoleacetaldehyde are the products of Trp metabolism and activate the AhR signaling pathway and its downstream signals (Agus et al., 2018). AhR activation has been reported to play important roles in host health and diseases through modulating T-cell differentiation, the expression of cytokines, transcription factors, autoimmunity, and inflammation (Quintana et al., 2008; Kerkvliet et al., 2009; Apetoh et al., 2010; Jeremy et al., 2016). Thus, the time-dependent concentration of AhR in the midgut of infected fish at 0, 6, 24, 72, and 120 hpi was detected using the AhR assay kit (Jiangsu Jianglai Biotechnology Co. Ltd., Suzhou, China) according to the manufacturers' protocol.

2.7.3 Effect verification of *AhR* expression on CyHV-2 infection

6-Formylindolo[3,2-b]carbazole (FICZ), a Trp photoproduct postulated as a candidate physiological ligand of AhR (Rannug et al., 1987; Oberg et al., 2005; Jeong et al., 2012), was used to evaluate the effect of *AhR* expression on the bacterial diversity in the midgut, the expression of antiviral cytokines, and viral infection progress.

2.7.3.1 Optimal FICZ working concentration assessment in gibel carp

FICZ (MedChemExpress, State of New Jersey USA) at the concentration of 1 μg $fish^{-1}$ was injected by I.P., and an equal volume of PBS (100 μl $fish^{-1}$) was injected as control. At 0, 12, 24, and 48 hpi, the fish midgut from two groups was sampled, immersed in RNA later (Sigma, Shanghai, China), and immediately stored at -80°C. Total RNA extraction, complementary DNA cDNA synthesis, and RT-qPCR were conducted as described in our previous study (Qiao et al., 2020). Briefly, RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA, USA), and genomic DNA was erased through DNase I (Qiagen). A Nanodrop ND-1000 spectrophotometer was used to detect the quantity of RNA and concentration. Then, cDNA was synthesized using the $oligo^{dT}$ primer and Prime-script™ first-strand cDNA synthesis kit (Takara Bio, Dalian, China). Genes *AhR1* and *AhR2* and downstream gene *Cyp1A1* (Cytochrome P450, family 1, member A1) were chosen to investigate the effect of FICZ on the expression of key genes involved in the AhR signaling pathway (Jeong et al., 2012). The gene-specific primers are listed in Table S1. The RT-qPCR was performed through SYBR® Premix Ex Taq™ (Takara, Dalian, China) in a CFX96 Real-Time PCR Detection System (Bio-Rad, Shanghai, China) (Qiao et al., 2020). The relative fold changes of a specific gene in the FICZ injection group were compared to that in the control PBS-injected group using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7.3.2 Effect of FICZ on the expression of cytokines

FICZ was injected at 1 μg $fish^{-1}$ by I.P., and an equal volume of PBS (100 μl $fish^{-1}$) injected as control. At 12 hpi based on the results from part 2.7.3.1, the head kidney was sampled, immersed in RNA later, and immediately stored at -80°C. Total RNA extraction, cDNA synthesis, RT-qPCR, and the relative expression calculation of cytokines, including *IFN-γ* (interferon), *MX1* (myxovirus resistance 1), *ISG15* (interferon-stimulated gene 15), *JAK* (Janus kinase), *TNF-α* (tumor necrosis factor-α), *MAPK3* (mitogen-activated protein kinase 3), *IL-4* (interleukin-4), *Gata3* (Gata binding protein 3), and *T-Bet1* (T-box expressed in T cells 1), were performed as described in part

2.7.3.1. The genes and gene-specific primers are listed in Table S1.

2.7.3.3 Effect of FICZ on the cumulative mortality of fish post-CyHV-2 infection

There were four groups, namely, the PBS single-injection group (10 μl $fish^{-1}$), FICZ single-injection group, 'FICZ+CyHV-2'-infection group, and 'PBS+CyHV-2'- infection group. FICZ (1 μg $fish^{-1}$) was injected into fish by I.P., PBS, and CyHV-2 immersed fish through the 'per-gill' method as described in part 2.4. Additionally, two viral concentrations were set, which were 9.7×10^6 and 2.4×10^7 copies μl^{-1}. Each group included triplicates. Death within 14 days was recorded, and CM was calculated.

2.7.3.4 Effect of FICZ on the viral replication *in vivo* post-CyHV-2 infection

The fish from both the FICZ (1 μg $fish^{-1}$, I.P.) group and control group (PBS, 100 μl $fish^{-1}$) were infected by CyHV-2 through the 'per-gill' method as described in part 2.4. At 6, 12, 24, 48, 72, and 120 hpi, the kidney from 'FICZ +CyHV-2-infected' fish and 'PBS +CyHV-2-infected' fish was sampled, and viral load was quantified by qPCR as described in part 2.3.

2.8 Correlation between the gut microbiome and metabolites

Pearson correlation coefficient between gut microbial composition and experimental treatment was calculated with the R-project psych package (version 1.8.4) (Hornik, 2012). A network of correlation coefficients was generated using Omicsmart, a dynamic real-time interactive online platform for data analysis (http://www.omicsmart.com). In order to further prove the change of metabolites on the relative abundance change of main bacteria species, the effect of *AhR* expression on the relative abundance of *Aeromonas* in the midgut of fish post-CyHV-2 infection was investigated. Briefly, based on the results from part 2.7.3.1, the optimal working model of FICZ *in vivo* was injected by I.P. at 1 μg $fish^{-1}$ for 12 h. Four groups were set, including 'PBS+ PBS' single injection group (100 μl $fish^{-1}$), 'FICZ+ PBS' injection group, 'FICZ +CyHV-2' injection group, and 'PBS+CyHV-2' injection group. FICZ and PBS were injected into fish by I.P. and CyHV-2-infected fish through the 'per-gill' method as described in part 2.4. They were conducted at 12-h intervals. At 0, 1, 4, 5, and 7 dpi, the midgut of fish in four groups was sampled and homogenized in precold PBS. Then, the homogenate was diluted and spread on TSA and RS-selective medium, which was cultured at 28°C for 24–48 h. The TSA medium was used to count the total culturable bacteria, and the RS medium was used for *Aeromonas* spp. Bacterial colonies were counted and converted into the CFU g^{-1} midgut.

2.9 Statistical analysis

Results are expressed as mean ± standard deviation and subjected to one-way analysis of variance using the statistical software program Statistical Product and Service Solutions SPSS version 17.0 (SPSS Inc., IL, USA). The significant difference was determined using Tukey's multiple comparison test for more than three experimental groups and Student's *t*-test for two experimental groups. Statistical significance was considered at P-values less than 0.05, and the results were expressed as mean ± SE (standard error).

3 Results

3.1 Viral load *in vivo* post-CyHV-2 infection through 'per-gill' method

No CyHV-2 was detected by PCR in the fish before the experiment (Figure S1A). Gibel carp could be infected by CyHV-2 through the 'per-gill' method in the lab. Like natural infection, hemorrhage in the fish skin and bulging eyes was observed at 5 dpi (Figure 1A). Severe hemorrhage was observed in the gills and swim bladder wall (Figures 1B, C). In the control group, no CyHV-2 was detected and no clinical symptoms were observed (Figures S1A, B). CM in the CyHV-2-infection (9.7×10^7 copies per fish) group was 50%, and no mortality was observed in the PBS control group (Table S2). The standard curve of viral load using the qPCR method is shown in Figure S2. CyHV-2 loads among various tissues were significantly different (Figure 2). The viral loads in the kidney, spleen, midgut, hindgut, and gill were 4.4×10^5, 3.5×10^5, 3.0×10^5, 1.6×10^5, and 1.3×10^5 copies ng^{-1} DNA, respectively. Less than 1.0×10^2 copies ng^{-1} DNA were detected in the liver, foregut, muscle, and brain. Thus, different organs had different viral loads.

3.2 CyHV-2 infection changed gut microbiome

3.2.1 OTU statistics and α-diversity analysis

We examined the microbiome in the midgut of infected and uninfected fish. The raw data about the microbiome and metabolites have been deposited in the NCBI (https://www.ncbi.nlm.nih.gov/sra/PRJNA655390), and the accession number is PRJNA655390. The abundance statistics of OTUs are shown in Table S3. Chao1 richness estimators in control and CyHV-2-infection groups were 926.60 and 985.54, respectively. The Simpson index in the CyHV-2-infection group was higher

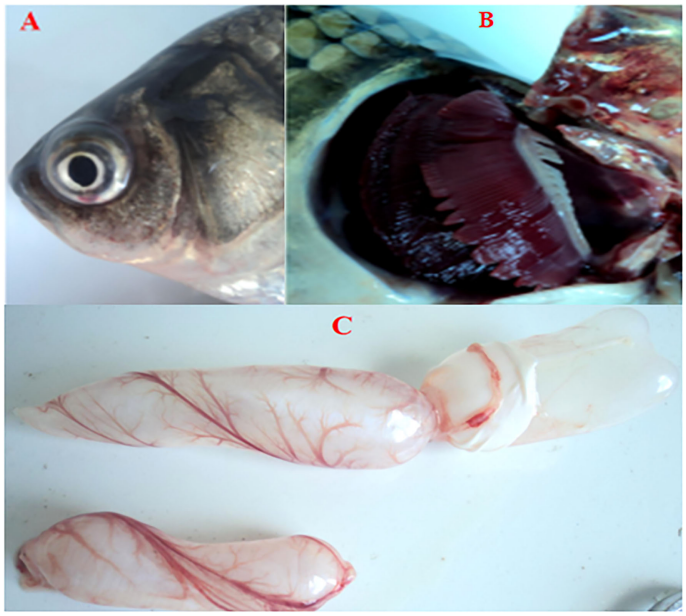

FIGURE 1
Cross-pathological signs of artificially infected gibel carp (*Carassius auratus gibelio*) by Cyprinid herpesvirus 2 (CyHV-2) through the per-gill method. **(A)** Severe hemorrhages on the body surface and bulging eye; **(B)** severe hemorrhages on gills; and **(C)** a petechial hemorrhage presented on the bladder wall.

than that in the control group, and the Shannon index was lower. The less Simpson index, the higher the bacterial community diversity, while the Simpson index is inverse. The present results suggested that there was no significant difference in bacterial diversity between CyHV-2-infection and control groups (Table S4). Following the microbiota diversity analysis, we classified bacteria in fish midguts. A total of 383 OTUs were shared by the control and CyHV-2-infection groups. A total of 264 OTUs in control and 273 OTUs in the CyHV-2-infection group were unique, respectively (Figure S3).

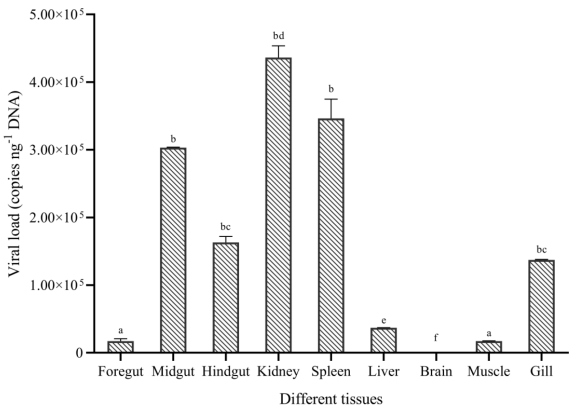

FIGURE 2
Viral replication in different tissues of gibel carp (*C. auratus gibelio*) challenged by CyHV-2 using the quantitative real-time PCR method. One-way ANOVA was conducted with SPSS 17.0 software. Different letters mean significant difference among tissues ($p < 0.05$).

3.2.2 Microbiota composition

At the phylum level, the microbiota in both control and CyHV-2-infection groups were dominated by Proteobacteria, Fusobacteria, Firmicutes, and Bacteroidetes (Figure 3A). Notably, compared to the control group, the relative abundance of Proteobacteria and Firmicutes in the CyHV-2-infection group was significantly increased, while the abundance of Fusobacteria decreased ($p < 0.05$). At the genus level, the gut microbiota in both control and CyHV-2-infection groups were dominated by *Aeromonas*, *Cetobacterium*, ZOR0006, *Flavobacterium*, *Shewanella*, *Vibrio*, *Gemmobacter*, *Bacteroides*, *Acinetobacter*, and *Pseudomonas*. Compared to the control group, the relative abundance of *Aeromonas* in the CyHV-2-infection group increased from 41.81% to 67.00%, while *Cetobacterium* decreased from 38.36% to 4.41% (Figure 3B). Multivariate statistical analysis–Principal Co-ordinates Analysis PCoA was used to reveal the difference in midgut microbiome patterns that responded to CyHV-2 infection. The gut microbiota in the CyHV-2-infection group was clearly separated from the control group, with 69.12% and 22.65% variation explained by PC1 and PC2 at the genus level, respectively (Figure S4A). The result from hierarchical clustering analysis with unweighted pair-group method with arithmetic means UPGMA was consistent with the PCoA plot, i.e., all OTUs in both control and CyHV-2-infection groups were

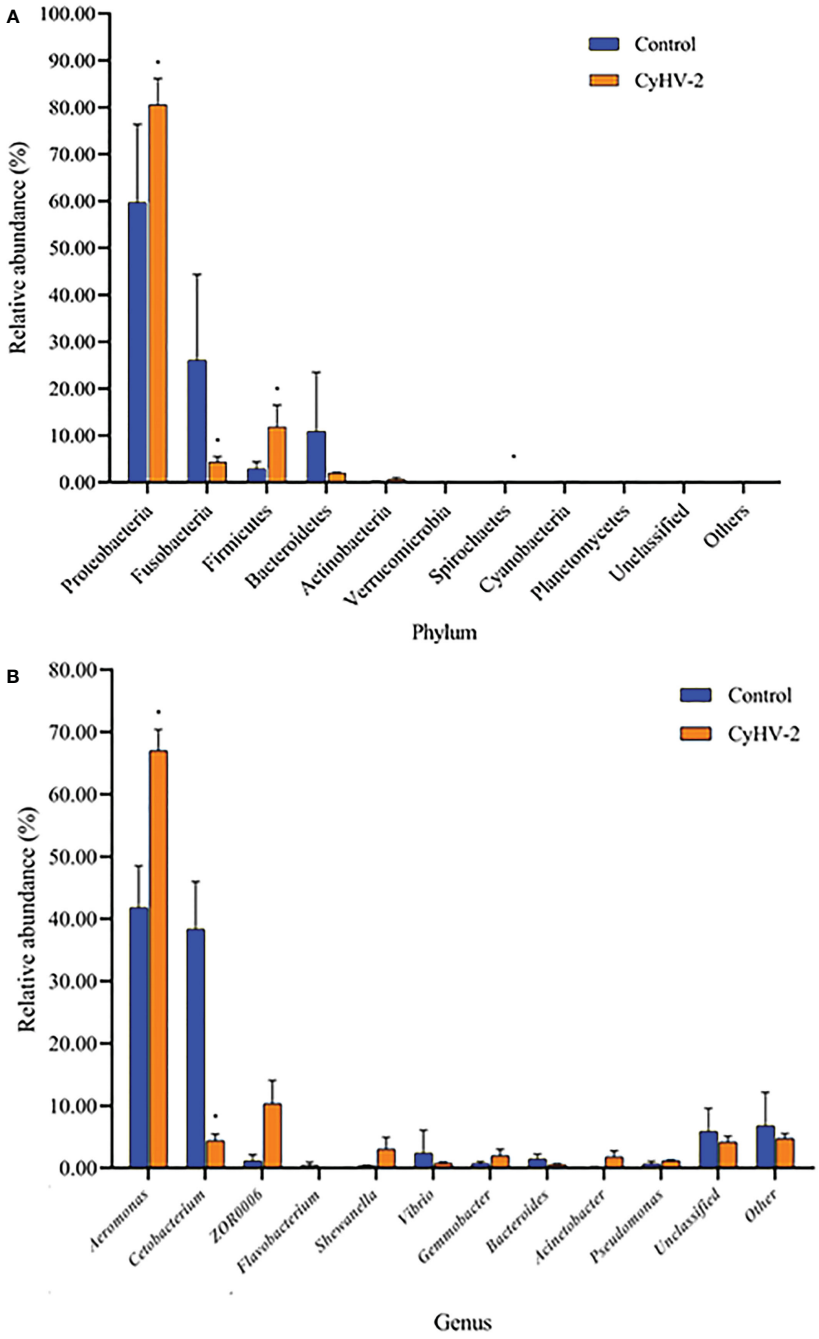

FIGURE 3
The gut microbiome composition individual profiles at the phylum **(A)** and genus **(B)** levels in the control and CyHV-2-infection groups. * means a significant difference between the two groups ($p<0.05$).

clustered in their own cluster except sample 2 by CyHV-2 infection (Figure S4B). Additionally, the analysis of similarities ANOSIM analysis showed that the R-value between the control and CyHV-2-infection groups was 0.63, illustrating that the middle difference between the two groups was found (Figure S4C). These differences of midgut microbiome patterns in different groups can be directly attributed to the relative abundance changes of dominant species. Therefore, CyHV-2 infection changed the abundance of different classes of bacteria in the midgut of fish.

LEfSe and CCA analysis showed that the specific main species in each group could be found. The most relevant species in the midgut-related CyHV-2 infection was *Aeromonas*. In the control group, *Fusobacteria* and

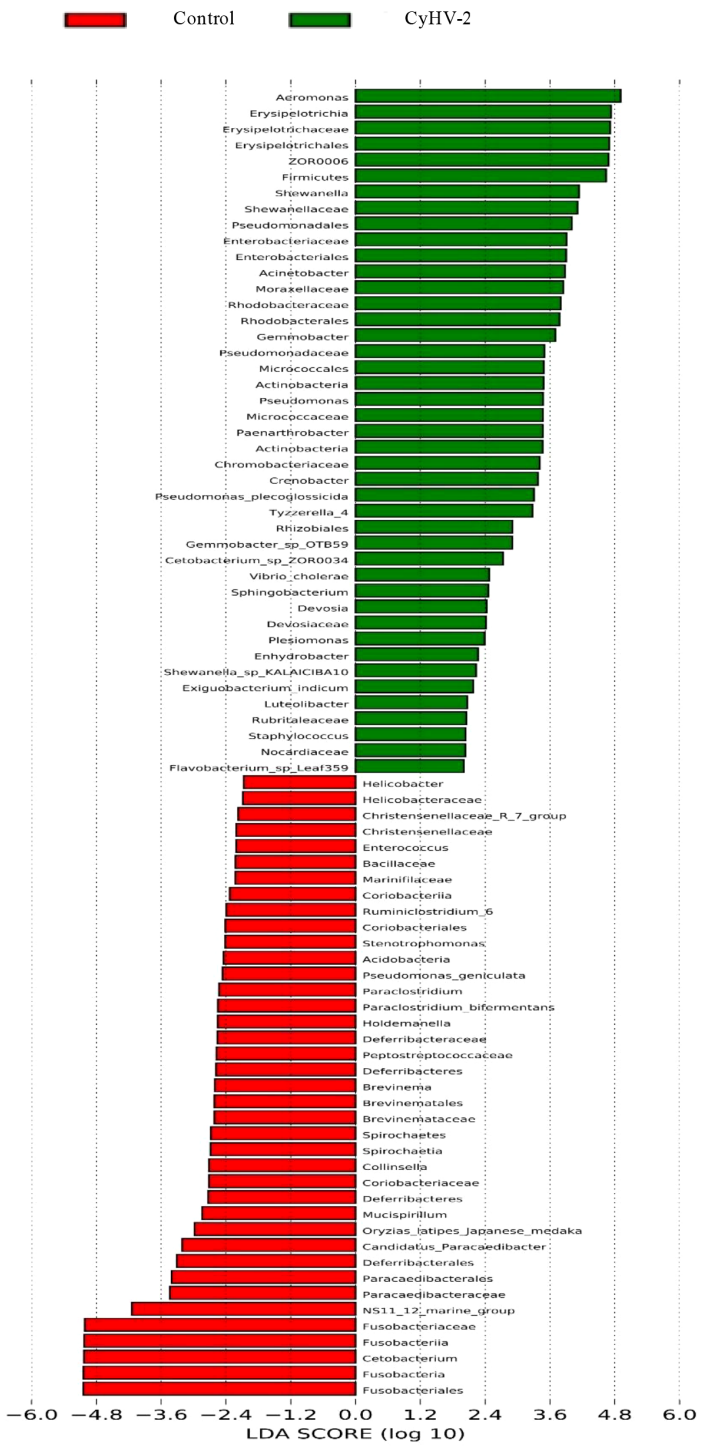

FIGURE 4
Linear discriminant analysis coupled with effect size measurements to identify the most deferentially abundant taxon between control and CyHV-2 infection groups.

Cetobacterium among different individuals were donated (Figure 4). *Aeromonas* presented a closely positive association with the viral infection (Figure 5). *Aeromonas* accounted for 41.81% in the control group and 67.00% in the CyHV-2-infection group (Figure 3B). A time-dependent abundance of *Aeromonas* in the midgut of gibel carp post-CyHV-2 infection by the culture method increased from 3.8×10^4 CFU g^{-1} midgut at 0 dpi to 6.4×10^5 CFU g^{-1} midgut at 1 dpi, 2.8×10^6 CFU g^{-1}

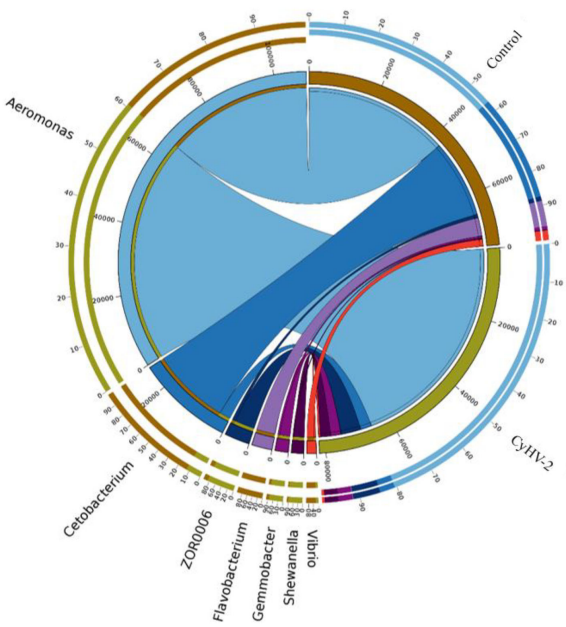

FIGURE 5
The relationship between the gut microbial composition and experimental treatments at the genus level by Circos based on canonical correspondence analysis (CCA).

midgut at 4 dpi, and 7.6 × 10^7 CFU g^{-1} midgut at 5 dpi, respectively. The abundance of *Aeromonas* at 5 dpi increased almost 1,000 times compared to 0 hpi, which was in accordance with the microbiome analysis by high-throughput sequencing. There was no significant difference in *Aeromonas* (less than 8 CFU ml^{-1} water) in culture water between the two groups during the whole challenge test. These results illustrated that CyHV-2 infection increased the relative abundance of *Aeromonas* and decreased *Cetobacterium*.

3.2.3 Functional prediction of gut microbiota through PICRUSt

A total of 35 KEGG functions of all the midgut microbiota from two groups were annotated through PICRUSt. The major functions were donated into three categories, namely, metabolism, genetic information processing, and environmental information processing. The functions regulated by the top 10 gut microbiota were membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair, energy metabolism, translation, metabolism of cofactors and vitamins, nucleotide metabolism, cell motility, and lipid metabolism (Figure S5A). The different microbiota between the control and CyHV-2-infection groups were annotated into 16 functions (Figure S5B).

3.3 CyHV-2 infection altered metabolite profiles in fish gut

3.3.1 Basic analysis of midgut metabolites from two groups

The PLS-DA model was conducted on the NMR data sets of all individuals, and the first two primary components PC1 and PC2 were selected to identify the discrimination between the control and CyHV-2-infection groups. Results showed that the metabolite profiles between the two groups were separated with 24% and 46% variation explained by PC1 and PC2, respectively (Figure 6).

3.3.2 Different metabolites between two groups

Compared to the control, the concentrations of the main 24 metabolites were significantly changed in the CyHV-2-infection group. Among them, the concentrations of 8 metabolites (norizalpinin, D-maltose, (dibromomethylene)-17beta-hydroxy-androst-4-en-3-one propiote, deoxy-D-altro-heptulose 7-phosphate, ellagic acid, alanyl-valine, N-a-acetyl-L-arginine, and phosphophosphite) increased and 16 metabolites decreased significantly (Table 1). These metabolites are related to various metabolism pathways, such as the digestive system (p-cresol, D-glucose, and D-maltose), amino acid metabolism (2-oxo-4-methylthiobutanoic acid, phenylpyruvic acid, hydroxyphenyllactic acid, ketoleucine, O-phospho-L-serine, picolinic acid, 2-oxoarginine, and 2-oxoadipate), metabolism of other amino acids

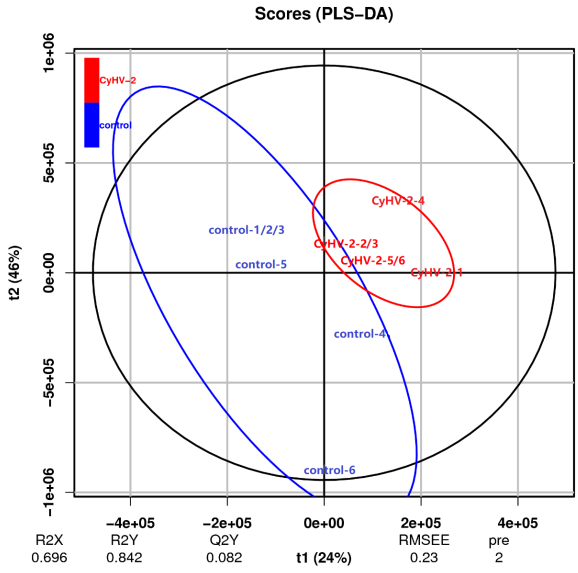

FIGURE 6
Scatter plot of partial least squares discriminant analysis for metabolite profiles from control and CyHV-2-infection groups. Each group was conducted in six replicates and named control-1/2/3/4/5/6 and CyHV-2-1/2/3/4/5/6. The distance of each point represents their correlation.

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TABLE 1 Differences in the metabolite profiles between the control and Cyprinid herpesvirus 2 (CyHV-2)-infection groups .

Metabolites	Class	Fold change	*P*-value	VIP
o-Cresol	Phenols	<0.01	<0.01	3.71
Phenylpyruvic acid	Benzene and substituted derivatives	0.05	<0.01	11.11
Indoleacetaldehyde	Indoles and derivatives	0.51	0.04	2.32
2-Oxo-4-methylthiobutanoic acid	Fatty acyls	0.02	<0.01	13.84
D-Lactic acid	Hydroxy acids and derivatives	0.53	<0.01	20.20
Succinic anhydride	Oxolanes	0.26	<0.01	2.00
8,11,14-Eicosatrienoic acid	Fatty acyls	0.56	0.02	6.95
Ketoleucine	Keto acids and derivatives	0.18	0.02	19.90
Iloprost	Fatty acyls	0.48	<0.01	3.26
Norizalpinin	Flavonoids	3.37	0.02	2.85
L-Selenocysteine	Carboxylic acids and derivatives	0.20	0.02	2.12
Convincing	–	0.51	0.01	3.37
6-(Dibromomethylene)-17beta-hydroxy-androst-4-en-3-one propiote	–	2.19	0.02	7.72
1-Deoxy-D-altro-heptulose 7-phosphate	–	36.00	<0.01	3.31
Ellagic acid	Tannins	6.43	<0.01	2.90
Picolinic acid	Pyridines and derivatives	0.43	0.02	4.64
D-Maltose	Organooxygen compounds	4.29	0.04	2.33
2,3,4,5-Tetrahydropiperidine-2-carboxylate	–	0.40	<0.01	4.85
Alanyl-Valine	–	3.37	0.04	3.58
4-Coumaroyl-2-hydroxyputrescine	Cinnamic acids and derivatives	0.07	<0.01	3.19
N-a-Acetyl-L-arginine	Carboxylic acids and derivatives	4.59	<0.01	8.86
2-Oxoarginine	Keto acids and derivatives	0.25	<0.01	7.28
Phosphophosphite	–	2.76	0.02	2.29
Nitrosylsulfuric acid	Non-metal oxoanionic compounds	0.31	<0.01	2.84

(L-selenocysteine), carbohydrate metabolism (D-maltose), lipid metabolism (phytosphingosine), xenobiotics biodegradation, and metabolism (cyclophosphamide).

3.3.3 Function annotation of metabolites

All of the metabolites from both control and CyHV-2-infection groups were enriched, and the top 20 KEGG pathways are shown in Figure 7A. The first seven pathways were metabolic pathways, 2-oxocarboxilic acid metabolism, carbohydrate digestion and absorption, Trp metabolism, starch and sucrose metabolism, taste transduction, and biosynthesis of amino acids (Figure 7A). Different metabolites between the two groups were enriched in 11 signaling pathways ($p < 0.05$) (Figure 7B). Among them, the most obviously different signaling pathways were Trp metabolism, starch and sucrose metabolism, carbohydrate digestion and absorption, metabolic pathways, and taste transduction (Figure 7). Accordingly, different metabolites were mainly annotated to the digestive system, amino acid metabolism, carbohydrate metabolism, and lipid metabolism (Table 2). Taken together with metabolites and their related signaling pathways, we found that five metabolites were involved in these significantly different signaling pathways, including picolinic acid (PLA), 2-oxoadipate, and indoleacetaldehyde participated in Trp metabolism, phytosphingosine entangled in non-alcoholic fatty liver disease, D-maltose and D-glucose annotated in carbohydrate digestion and absorption, starch and sucrose metabolism, taste transduction, and ATP-binding cassette ABC transporters. Therefore, CyHV-2 infection changed midgut metabolites significantly, further affecting metabolisms, such as Trp metabolism.

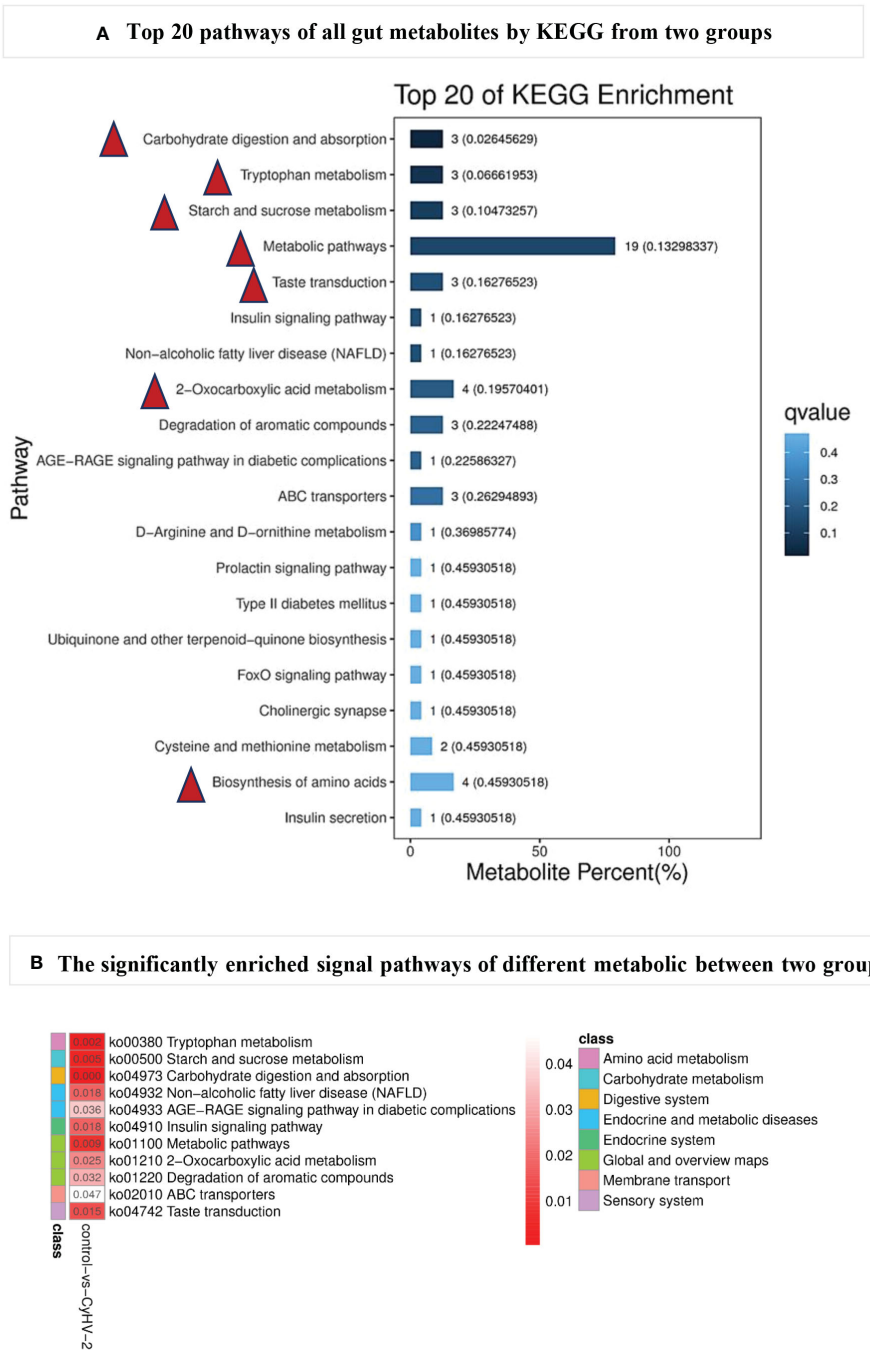

FIGURE 7
The top 20 pathways **(A)** of all gut metabolites from control and CyHV-2-infection groups through KEGG annotation and enriched signal pathways **(B)** with significant changes between two groups. The most top pathways of all gut metabolites are marked with a red triangle in **(A)** and the most significantly different pathways with a red arrow in **(B)** The colors and numbers in panel B represent the significance of metabolic pathways. The higher significance, the smaller the number and the darker the color.

TABLE 2 The correlation between some differentially specific microbes and metabolites.

Metabolites	Bacteria	Cor value	*P*-value	Class	KEGG annotation
PC	*Aeromonas*	-0.98	0.0006	Glycerophospholipids	Cancers
Glycyrrhetinic acid		0.97	0.001	Prenol lipids	Unknown
Sedoheptulose 1-phosphate		0.98	0.0008	Organooxygen compounds	Unknown
1-kestose		-0.98	0.0008	Organooxygen compounds	Unknown
Donepezil		0.98	0.0004	Piperidines	Unknown
8-iso-15-keto-PGE2		-0.98	0.0002	Fatty acyls	Unknown
2-oxoarginine		0.97	0.05	Keto acids and derivatives	Amino acid metabolism
Indoleacetaldehyde		-0.97	0.0007	Indoles and derivatives	Amino acid metabolism
Adenosine monophosphate	*Cetobacterium*	0.99	0.0002	Organooxygen compounds	Aging
LysoPC(24:0)		0.99	0.0001	Glycerophospholipids	Cancers
4-ketocyclophosphamide		0.98	0.0008	Organonitrogen compounds	Xenobiotics
D-glucono		0.98	0.0008	Organooxygen compounds	Carbohydrate metabolism
cis-aconitate		0.97	0.0009	Carboxylic acids and derivatives	Carbohydrate metabolism
cis-4-Carboxymethylenebut-2-en-4-olide		0.98	0.0007	Dihydrofurans	Global and overview maps
4-Pyridoxate		1.00	0.0000	Pyridines and derivatives	Metabolism of cofactors and vitamins
4-Hydroxybenzaldehyde		0.99	0.0002	Organooxygen compounds	Global and overview maps
Pyrrolidonecarboxylic acid		0.97	0.0013	Carboxylic acids and derivatives	Metabolism of other amino acids
Indoleacetaldehyde		0.99	0.0003	Indoles and derivatives	Amino acid metabolism
13-oxoODE		0.97	0.0011	Fatty acyls	Lipid metabolism
L-Cysteine		0.99	0.0003	Carboxylic acids and derivatives	Amino acid metabolism
Ketoleucine		0.98	0.0008	Keto acids and derivatives	Amino acid metabolism
4-hydroxyphenylpyruvic acid		0.99	0.0001	Benzene and substituted derivatives	Amino acid metabolism
Deoxyribose 5-phosphate		0.99	0.0001	Organooxygen compounds	Carbohydrate metabolism
stearoyl sphingomyelin		0.98	0.0007	Sphingolipids	Unknown
Phytosphingosine		-0.97	0.05	Organonitrogen compounds	Lipid metabolism
2-oxoarginine		0.97	0.003	Keto acids and derivatives	Amino acid metabolism
PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)	*Flavobacterium*	0.97	0.0011	Glycerophospholipids	Cancers
3',5'-cyclic GMP		-0.98	0.0007	Purine nucleotides	Cellular community—eukaryotes

(Continued)

TABLE 2 Continued

Metabolites	Bacteria	Cor value	*P*-value	Class	KEGG annotation
Squalene		-0.98	0.0006	Prenol lipids	Lipid metabolism
Sedoheptulose 7-phosphate	*Shewanella*	0.98	0.0006	Organic oxygen compounds	Carbohydrate metabolism
Palmitic acid		-0.97	0.0010	Fatty acyls	Lipid metabolism
2-amino-4-hydroxy-6-(D-erythro-1,2,3-trihydroxypropyl)-7,8-dihydropteridine		-0.97	0.0012	Pteridines and derivatives	Metabolism of cofactors and vitamins
Deoxyadenosine	*Vibrio*	0.99	0.0002	Purine nucleosides	Nucleotide metabolism
cis-aconitate		0.97	0.0010	Carboxylic acids and derivatives	Carbohydrate metabolism
6-methylthiopurine5'-monophosphate ribonucleotide	*Gemmobacter*	0.97	0.0011	Purine nucleotides	Xenobiotic biodegradation and metabolism
sn-glycerol 3-phosphate	*Bacteroides*	0.98	0.0009	Glycerophospholipids	Cancers
2,5-furandicarboxylate		-0.97	0.0011	Furans	Global and overview maps
GMP		-0.98	0.0005	Purine nucleotides	Nucleotide metabolism
Triethanolamine	*Acinetobacter*	-0.97	0.0013	Amines	Lipid metabolism
6-methylthiopurine5'-monophosphate ribonucleotide		0.99	0.0002	Purine nucleotides	Xenobiotic biodegradation and metabolism

3.4 Verification of the correlation between AhR and CyHV-2 infection

3.4.1 CyHV-2 infection decreased the concentration of AhR in the midgut

In the control group, there was no significant difference on the concentration of AhR among 0, 6, 24, 72, and 120 hpi. However, in the CyHV-2-infection group, the concentration decreased from 5.38 pM (0 hpi) to 3.77 pM (120 hpi) (Figure 8A).

3.4.2 *AhR* expression affected the transcription of cytokines

The optimal FICZ injection (I.P.) was 1 μg fish^{-1} for 12 hpi (Figure 8B). At 12 hpi, the highest expression of *AhR1* and *Cyp1A1* was observed, and *AhR2* upregulated significantly (Figure 8B). The transcriptional levels of cytokines *JAK*, *IFN-γ*, *ISG15*, *MX1*, *IL-4*, *Gata3*, and *T-Bet1* were upregulated significantly by 9.75-fold, 9.92-fold, 7.05-fold, 5.56-fold, 2.03-fold, 4.14-fold, and 2.75-fold, respectively. However, the mRNA expression of genes *TNF-α* and *MAPK3* was not regulated significantly (Figure 8C).

3.4.3 Higher *AhR* expression reduced the cumulative mortality of fish and inhibited CyHV-2 replication *in vivo*

Compared to the 'PBS+CyHV-2' infection group, the CM of fish post-CyHV-2 infection in the 'FICZ+CyHV-2' group was much lower. CM in the 'FICZ+CyHV-2' group at the viral concentrations of 2.4 × 10^7 and 9.7×10^6 copies μl^{-1} was 41.7% and 33.3%, respectively. They were 83.8% and 58.3% in the 'PBS +CyHV-2' group. The fish in the 'FICZ+CyHV-2' group died later than that in the 'PBS+CyHV-2' group. No death was observed in PBS single and FICZ single injection groups (Table 3).

Compared to the 'PBS+CyHV-2'-infection group, the viral replication in the 'FICZ+CyHV-2' group was slower, suggesting that higher *AhR* expression inhibited CyHV-2 replication *in vivo*. The viral load in the 'FICZ+CyHV-2' group at 6 hpi (6.9 × 10^3 copies ng^{-1} DNA) and 120 hpi (2.3 × 10^4 copies ng^{-1} DNA) was lower than that in the 'PBS+CyHV-2' group, which were 1.4 × 10^4 copies ng^{-1} DNA at 6 hpi and 5.5 × 10^4 copies ng^{-1} DNA at 120 hpi, respectively (Figure 8D). These findings and in-depth verification suggested that Trp metabolism is one of the cross-talk ways between a viral infection and a host.

3.5 Correlation between the altered microbiota and metabolites

In the CyHV-2-infection group, the relative abundance of *Aeromonas* significantly increased, while *Cetobacterium* decreased. We generated a scatter plot using Pearson correlation coefficient analysis to investigate the potential association between metabolites and bacterial composition. The heatmap of the correlation between gut metabolites and

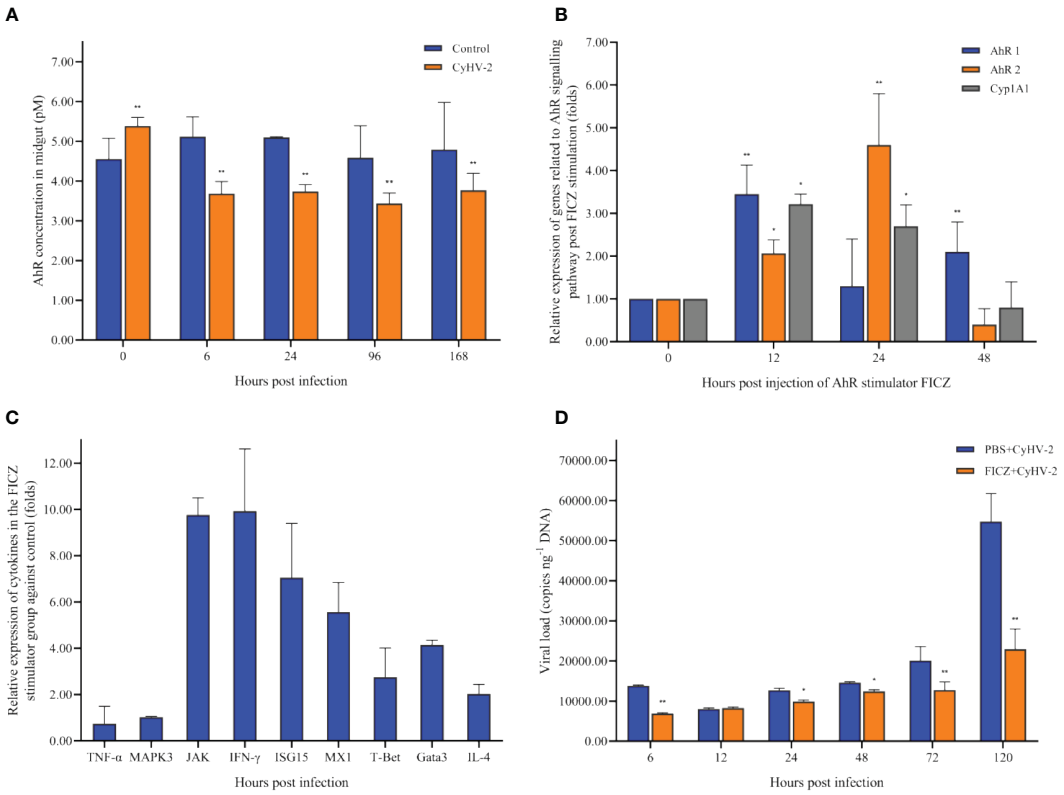

FIGURE 8
Time-dependent changes of the aryl hydrocarbon receptor (AhR) concentration in the midgut of fish post-CyHV-2 infection **(A)**, the effect of 6-Formylindolo[3,2-b]carbazole (FICZ) on the AhR signaling pathway **(B)**, the effect of FICZ on the transcription of cytokines **(C)**, and effect of FICZ on the viral replication *in vivo* **(D)**. All measurements are done in the midgut. * means a significant difference between two groups at a *p*-value from 0.01 to 0.05. ** means a *p*-value less than 0.01.

microbiota is shown in Figure 9. The apparent correlations were identified between the perturbed bacteria genus and altered metabolites (Mantel test, $r = 0.89$) (Figure 10 and Table 2). For example, the OTUs assigned to *Aeromonas* were positively correlated with glycyrrhetinic acid, sedoheptulose 1-phosphate, donepezil, and 2-oxoarginine and negatively correlated with indoleacetaldehyde, 1-kestose, and 8-iso-15-keto-PGE2. OTUs belonging to *Cetobacterium* were positively correlated with indoleacetaldehyde, adenosine monophosphate, 4-ketocyclophosphamide, LysoPC (24:0), D-glucono, cis-aconitatecis-4-carboxymethylenebut-2-en-4-olide, 4-pyridoxate, 4-hydroxybenzaldehyde, pyrrolidonecarboxylic acid, 13-oxoODE, L-cysteine, ketoleucine, stearoyl sphingomyelin, deoxyribose 5-phosphate, phytosphingosine, 2-oxoarginine and 4-hydroxyphenylpyruvic acid (Figure 10 and Table 2). In this study, the most noteworthy functional genes were involved in ABC transporters and glycerophospholipid metabolism related to membrane synthesis and gut integrity, followed by primary immunodeficiency (Figure S5B). Our data demonstrated that CyHV-2 infection changed both the gut microbiome and metabolome, and these two changes were correlated.

3.6 Alteration of AhR expression affected microbiota in the midgut of fish post CyHV-2 infection

Compared to the PBS injection group, CyHV-2-infection group, and 'FICZ+CyHV-2' group, FICZ stimulation at 4 and 5 dpi increased the number of total culturable bacteria by approximately two folds significantly. There was no significant difference among the PBS injection group, CyHV-2-infection group, and 'FICZ+CyHV-2'-infection group (Figure 11A). However, compared to PBS injection group and FICZ injection group, the number of *Aeromonas* spp. in the CyHV-2-infection group and 'FICZ+CyHV-2'- infection group from 1 to 7 dpi increased significantly. There was still a significant difference between the CyHV-2-infection group and the 'FICZ +CyHV-2' infection group from 1 to 7 dpi. The number of *Aeromonas* spp. in the CyHV-2-infection group was significantly higher than that in the 'FICZ+CyHV-2' infection group from 1 to 7 dpi. FICZ injection could inhibit the replication of *Aeromonas* spp. in the midgut of fish post-CyHV-2 infection (Figure 11B).

TABLE 3 Effect of aryl hydrocarbon receptor (AhR) expression on the cumulative mortality of fish post-CyHV-2 infection.

Groups	Days post infection														Cumulative mortality (%) (mean ± SD)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
PBS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBS+CyHV-2 (2.4×10^7 copies μl^{-1})	0	0	1	0	0	0	0	2	2	0	1	1	0	0	
PBS+CyHV-2 (2.4×10^7 copies μl^{-1})	0	0	0	0	1	0	0	2	0	0	1	1	1	0	83.8 ± 7.2
PBS+CyHV-2 (2.4×10^7 copies μl^{-1})	0	1	1	0	0	0	0	2	1	0	1	1	0	0	
PBS+CyHV-2 (9.7×10^6 copies μl^{-1})	0	0	0	0	0	0	0	0	1	1	1	0	0	1	
PBS+CyHV-2 (9.7×10^6 copies μl^{-1})	0	0	2	0	0	0	0	0	0	0	0	2	1	0	58.3 ± 7.2
PBS+CyHV-2 (9.7×10^6 copies μl^{-1})	0	0	1	1	0	0	0	1	0	1	0	1	0	0	
FICZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FICZ+CyHV-2 (2.4×10^7 copies μl^{-1})	0	0	1	2	0	0	0	0	0	0	0	1	0	0	
FICZ+CyHV-2 (2.4×10^7 copies μl^{-1})	0	0	0	0	0	0	0	2	0	0	0	1	0	0	41.7 ± 7.2
FICZ+CyHV-2 (2.4×10^7 copies μl^{-1})	0	0	0	0	1	0	0	1	0	1	0	0	0	0	
FICZ+CyHV-2 (9.7×10^6 copies μl^{-1})	0	0	0	0	0	0	0	1	0	0	0	0	0	1	
FICZ+CyHV-2 (9.7×10^6 copies μl^{-1})	0	0	1	0	0	0	0	0	1	0	1	0	0	0	33.3 ± 7.2
FICZ+CyHV-2 (9.7×10^6 copies μl^{-1})	0	0	0	0	0	1	0	1	0	0	0	0	0	1	

FICZ, 6-formylindolo [3,2-b] carbazole, a tryptophan photoproduct postulated as a candidate physiological ligand of AhR.

4 Discussion

Gibel carp can be artificially infected by CyHV-2 through the 'per-gill' method. Like natural infection, the typical clinical syndromes of hemorrhagic gills and the swim bladder were observed post-CyHV-2 infection through the 'per-gill' method, and CyHV-2 could be quantified in different inner organs. The hemorrhage of gills is mainly observed during the transition from spring to summer and in autumn, similar to the epidemiological characteristics of the hematopoietic necrosis (HVHN) of goldfish infected with CyHV-2 (Jung and Miyazaki, 1995; Zhu et al., 2015). Until now, there is no effective method to control this disease, although some studies on a vaccine are undergoing in the lab (Takafumi and Yukio, 2015; Yan et al., 2020). Early prevention before disease outbreaks becomes more important to control this epidemic disease. Growing pieces of evidence have demonstrated that the gut microbiota plays an important role in health and diseases. The gut microbiota in fish can regulate the expression of 212 genes, of which some are related to the promotion of nutrient metabolism, stimulation of epithelial proliferation, and immunity. The absence of gut microbiota in fish may lead to epithelial cell dysfunction and weaker immune responses (Wang et al., 2018). Colonization by commensal in newly hatched zebrafish (*Denio rerio*) primes neutrophils and induces several genes encoding proinflammatory and antiviral mediators (Galindo-Villegas et al., 2012).

To our knowledge, this study was the first to address the relationship among CyHV-2 infection, gut microbiota, and metabolite profiles in the gibel carp. The present results showed (1) gut microbiota dysbiosis, especially the relative abundance increment of *Aeromonas* and decrement of *Cetobacterium*; (2) metabolome alternation regarding amino acid metabolism, carbohydrate metabolism, and lipid metabolism, especially Trp metabolism; (3) the strong correlation between gut microbiota and metabolite profiles; and (4) the interference of viral infection through modulating gut ecology. The predominant genera in the gibel carp were *Aeromonas*, *Cetobacterium*, *Flavobacterium*, *Shewanella*, *Vibrio*, *Gemmobacteria*, *Bacteroides*, *Acinetobacter*, and *Pseudomonas*. Accordingly, *Aeromonas*, *Cetobacterium*, *Pseudomonas*, *Acinetobacter*, and *Bacteroides* were reported as the predominant genera in freshwater fish (Li et al., 2017; Wang et al., 2018). However, there are some differences in genus composition; *Flavobacterium*, *Shewanella*, and *Vibiro* were found in our experimental fish, while these genera are mainly reported in marine fish (Onarheim et al., 1994; Blanch et al., 1997). It is supposed that this difference is mainly attributed to the culture environment, especially salinity, since our experimental fish was obtained from Yancheng and the culture sediment in the pond was closer to the mudflat (Chen et al., 2021). The culture farm in Yancheng is mainly located along the coastline with the longest and biggest mudflat in China. Consistent to the previous report (Zhang et al., 2016), the

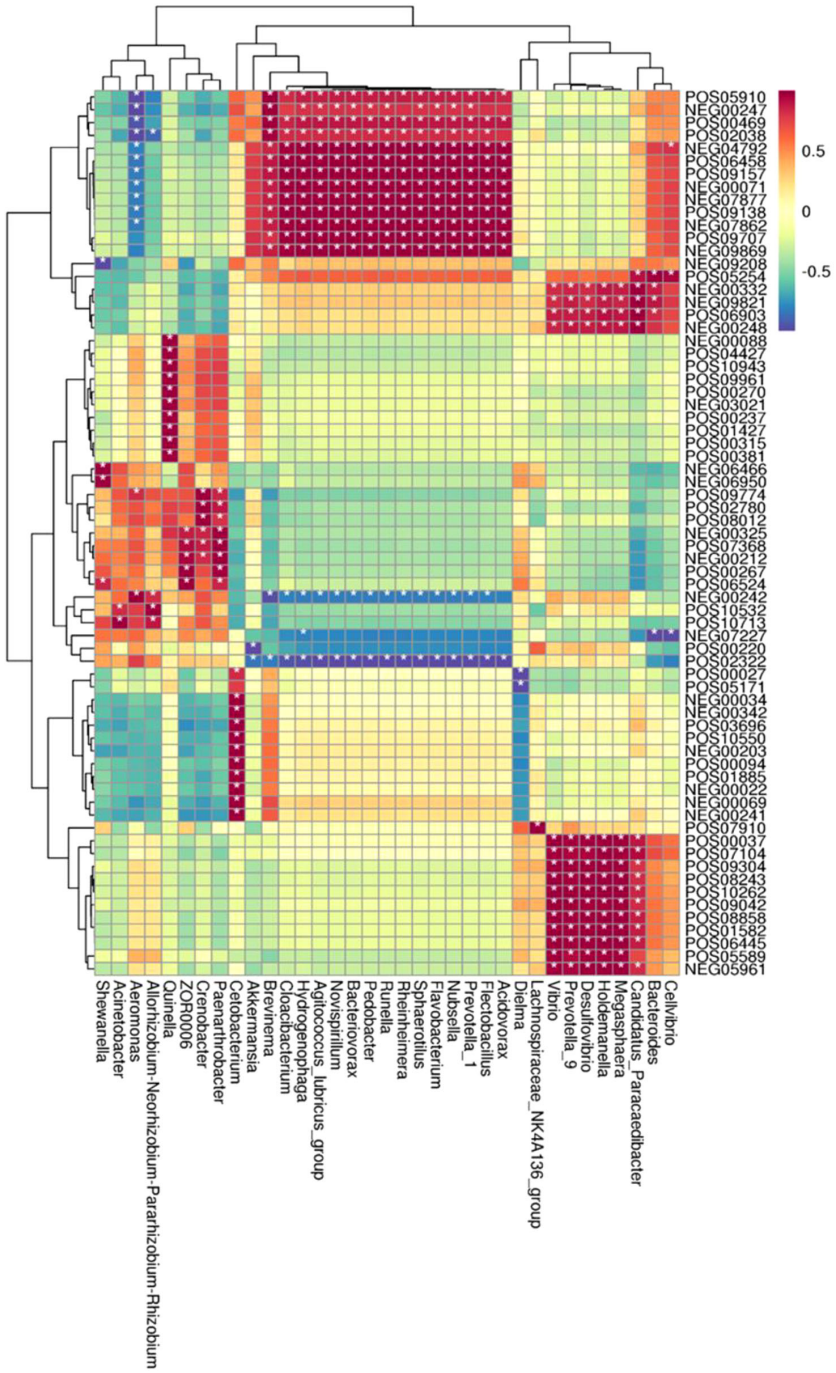

FIGURE 9
Heatmap of the correlation between gut metabolites and the microbiota. The horizontal axis represents bacterial species, and the vertical axis represents the metabolites. Grid means the correlation index. The color from white to red means a positive correlation from weak to strong, and white to blue means a negative correlation. *, significant correlation with a *p*-value less than 0.05; **, highly significant correlation with a *p*-value less than 0.01.

abundance of dominant species such as *Cetobacterium* increases along with higher salinity.

The microbiota depends on a functional equilibrium related to the host, environment, and dietary factors. The gut microbiome is responsible for maintaining gut metabolic homeostasis and coordinating immune responses (Kau et al., 2011; Shapira, 2016). A disturbance in the functional equilibrium can lead to changes in the microbial diversity and

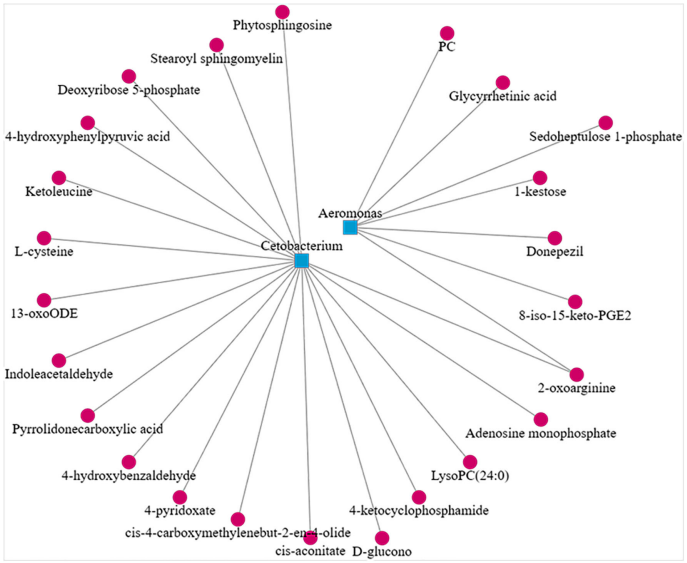

FIGURE 10
Scatter plot showing the correlation between *Aeromonas*, *Cetobacterium*, and significant different metabolites from the control and CyHV-2-infection groups.

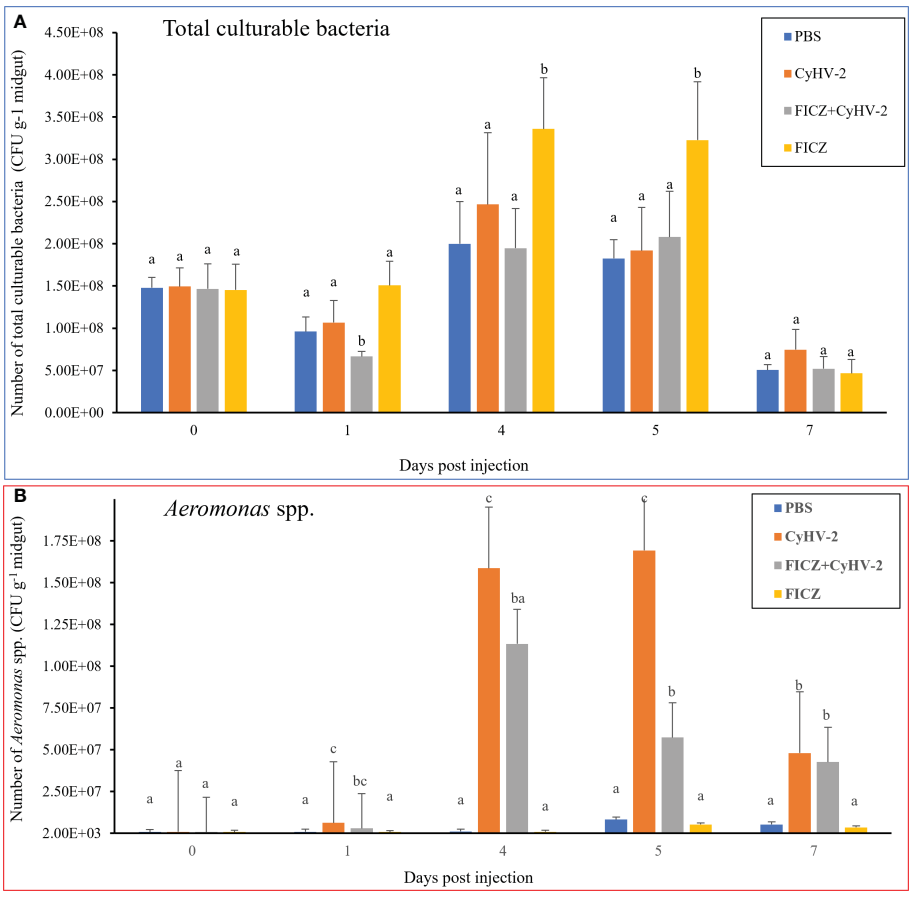

FIGURE 11
Effect of AhR expression on the number of total culturable bacteria and *Aeromonas* spp. in the midgut of fish post-CyHV-2 infection The data showing as 'mean±SE' is based on four biological replicates. The significant differences among different treatments are indicated by lowercase letters above each column (one-way ANOVA followed by the LSD test, $P < 0.05$) in **(A, B)**.

abundance of certain bacteria, which are beneficial or harmful to fish (Liu et al., 2013; Ling et al., 2014; Lukens et al., 2014). Thus, we used a laboratory infection model instead of naturally infected fish to study the effect of CyHV-2 infection on the gut microbiota and microbiome. After acclimation, the gut microbiota is more stable (Chen et al., 2021). In this study, the midgut of fish was chosen as the target organ to study the microbiome and metabolite changes post-CyHV-2 infection since the viral load in the midgut was higher than that in the foregut and hindgut (Figure 2). Accordingly, the microbial abundance and gene *gata*5 expression in the midgut of zebrafish are higher than that in the foregut and hindgut (Hu, 2017; Rong et al., 2017). The *gata*5 gene regulates gut mucus secretion and gut epithelial differentiation (Hu, 2017).

CyHV-2 infection changed the gut microbiota of fish. The abundance of genera *Aeromonas*, *Pseudomonas*, and *Flavobacterium* were significantly increased in the CyHV-2-infected fish. Particularly, the relative abundance of *Aeromonas* in the CyHV-2-infected fish increased significantly from 48.74% to 67.00%, consistent with reports in crucian carp *(C. carassius)* (Caruso et al., 2017). Accordingly, a time-dependent abundance of *Aeromonas* in the midgut of gibel carp increased along with the CyHV-2 infection progress. However, it is different from the previous report (Rong et al., 2017), which showed that *Plesiomonas* was highly abundant in CyHV-2-infected gibel carp and could be used as a microbial biomarker for CyHV-2 infection. In this study, no dominant *Plesiomonas* was detected in both uninfected and infected gibel carp, as in the previous report by Chen et al. (2021). This difference might be related to the environment salinity, water, and developmental stage, which have been widely reported to affect the fish gut microbiota (Wang et al., 2018). This study suggests that the abundance of *Aeromonas* could be attractively monitored during the disease development progress. *Aeromonas* are ubiquitous inhabitants of freshwater and estuary environments as potential etiological agents in diseases of fish, terrestrial animals, and humans. It can cause motile aeromonad septicemia with high mortality and severe economic losses (Ran et al., 2018).

The gut metabolic disorders were observed in the CyHV-2-infected fish. For instance, three digestive pathways related to metabolites of p-cresol, D-glucose, and D-maltose and eight amino acid metabolism–related pathways to the metabolites of 2-oxo-4-methylthiobutanoic acid, phenylpyruvic acid, hydroxyphenyllactic acid, ketoleucine, O-phospho-L-serine, PLA, 2-oxoarginine, and 2-oxoadipate were changed in the gut of CyHV-2-infected fish. PLA has broad and effective antimicrobial activity against both bacteria and fungi (Dieuleveux et al. 1998; Lavermicocca et al., 2003). PLA is a major metabolic biomarker for oxidative damage to the cerebral cortex, phenylketonuria, and alcohol-induced liver disease (Manna et al., 2011). More recently, PLA has been well documented to play a positive role in the progression of different cancers (Lavermicocca et al., 2003; Fong et al., 2011) as shown in this study. Glucose as a good carbon source for cofactor regeneration in Trp metabolism is consistent with the phenomenon that the glucose content increased, while the corresponding maltose content decreased significantly in this study. D-maltose and D-glucose are involved in the digestive system. Sugars are the primary carbon source for bacterial cells, and they play important roles in bacterial pathogenesis (Pacheco et al., 2012; Ng et al., 2013). Both pathogenic and commensal bacteria compete for carbon sources such as glucuronate, mannose, fucose, and ribose to colonize and proliferate in the gut (Lustri et al., 2017). Sugar availability influences the microbiota composition and expansion of bacterial pathogens (Lustri et al., 2017). The change of glucose concentration in the environment regulates the expression of the locus of enterocyte effacement and catabolic, and osmotic stress (Njoroge et al., 2012; Njoroge et al. 2013).

In this study, an apparent correlation between specific microbes and metabolites was observed. For instance, *Cetobacterium* OTUs are negatively correlated with phytosphingosine, which is involved in membrane synthesis. The concentration increment of phytosphingosine raises the permeability of endothelial cells and leads to the barrier function decline (Luther et al., 2013). Another interesting metabolite is 1-kestose, the smallest component of Fructooligosaccharide FOS in the probiotics from the human gut. These findings suggest that 1-kestose is a potential new prebiotic targeting drug (Tochio et al., 2018). It has been shown that the hepatocyte membrane has abundant glycyrrhetinic acid (GA) receptors (Shan et al., 2017). Some documents have authenticated that GA is combined with the GA receptors of hepatocyte membrane to enhance the targeted therapy of liver (Shan et al., 2017; Wang et al., 2018). A more interesting finding is that *Cetobacterium* OTUs are correlated with indoleacetaldehyde positively (Natividad et al., 2018). Both indoleacetaldehyde and PLA from this study were annotated to Trp metabolism. Actually, most indole derivatives are formed from Trp metabolism (Lamas et al., 2018). Indole substances are the endogenous ligands of AhR and thus participate in many important biological processes of the body, such as cell differentiation, apoptosis, and inflammation (Agus et al., 2018). Indole and Trp metabolites are a major source of endogenous AhR ligand precursors (Bjeldanes et al., 1991; Perdew and Babbs, 1991). It has been reported that the gut microbiota affects health and disease through the regulation of Trp metabolism and the activation of AhR (Lamas et al., 2016; Lamas et al. 2018; Agus et al., 2018; Natividad et al., 2018; Trikha and Lee, 2019). CARD9 was reported to impact colitis by altering the gut microbiota metabolism of Trp into AhR ligands (Lamas et al., 2016).

AhR is a member of the basic helix–loop–helix–(bHLH) superfamily of transcription factors, which are associated with cellular responses to environmental stimuli. Early AhR studies

focused on understanding the role of AhR in mediating the toxicity and carcinogenesis properties of the prototypic ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Recently, AhR has been highly receptive to a wide array of endogenous and exogenous ligands. Its activation leads to a myriad of crucial host physiological functions, such as intestinal barrier function and immune cells, as well as intestinal homeostasis (Lamas et al., 2018). The byproducts of Trp photooxidation possess a high AhR-binding capacity, and are able to induce the expression of *Cyp*1A1 and other AhR target genes (Paine, 1976; Paine and Francis, 1980; Goerz et al., 1996). In fish, two kinds of AhR (AhR1 and AhR2) have been reported mostly (Prasch et al., 2003; Aluru et al., 2011). Thus, the AhR pathway related to Trp metabolism in this study was chosen to prove the effect of gut micro-ecology changes on the viral infection process through FICZ stimulation, which is a prime example of a Trp photoproduct and can agonistically stimulate AhR activity in as low as picomolar ranges (Jeong et al., 2012). The present results showed that 1) CyHV-2 infection reduced AhR concentration in the midgut (Figure 8A) and serum (data not shown), 2) higher *AhR* expression upregulated the mRNA expression of antiviral genes in the head kidney; 3) higher *AhR* expression reduced the relative abundance of *Aeromonas* spp. in the midgut of fish post-CyHV-2 infection; 4) higher *AhR* expression reduced the 14-day CM of fish post-CyHV-2 infection and inhibited viral replication *in vivo*. In mammals, AhR is known to express by different intestinal immune cells, such as intestinal epithelial cells (IECs), Th17 cells, innate lymphoid cells (ILCs), macrophages, and neutrophils. The AhR signaling pathway is pivotal to the differentiation and proliferation of T cells and the regulation of mucosal intestinal immune responses (Qiu et al., 2012; Goudot et al., 2017). Although the correlation between metabolite changes and CyHV-2 infection focusing on Trp metabolism was proven, further in-depth studies on the correlation among gut microbes, gut barrier disturbance, *Aeromonas* translocation, Trp metabolism, immunity, and antiviral infection need to be conducted. Especially, regulating Trp metabolism in the gut by diets including probiotics will attract more attention as reported in mammals (Whitfield-Cargile et al., 2016; Cervantes-Barragan et al., 2017). These actionable studies will probably provide an excellent immunotherapeutic intervention to control carp diseases and thus advance the sustainable culture.

5 Conclusion

This study found a significant difference in microbiota-regulated metabolites in the fish midgut post-CyHV-2 infection. Notably, the relative abundance of *Aeromonas* increased while *Cetobacterium* decreased. The different metabolites were annotated to the metabolism of the digestive system, amino acid, carbohydrate, lipid, and xenobiotic biodegradation, especially Trp metabolism. An in-depth analysis showed that some of these altered metabolites were highly correlated with the genera *Aeromonas* and *Cetobacterium*. Our results supported that CyHV-2 infection–induced changes in the midgut, bacterial community structure disturbed the metabolic functions of the gut microbiome, and the viral infection process could be interrupted through the modulation of gut microecology. These findings will provide a new therapeutic strategy to prevent disease and thus advance the sustainable culture.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Center for Biotechnology Information (NCBI) BioProject, https://www.ncbi.nlm.nih.gov/bioproject/, PRJNA655390.

Ethics statement

This study was conducted in accordance with the regulations for the administration of laboratory animals in Jiangsu province, China.

Author contributions

PC and MZ performed project administration, original draft, editing, and funding acquisition. JL and XW help with data interpreting and writing. YZ and YC performed formal analysis, software, and visualization. TL performed conceptualization and data curation. LC performed conceptualization and investigation. ZZ provided fish. ZL and ZM performed methodology and software. GQ, ZGZ, and ZW performed conceptualization, funding acquisition, project administration, resources, supervision, validation and visualization, review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1017165/full#supplementary-material

References

Agus, A., Planchais, J., and Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell Host Microbe* 23, 716–724. doi: 10.1016/j.chom.2018.05.003

Aluru, N., Karchner, S. I., and Hahn, M. E. (2011). Role of DNA methylation of AHR1 and AHR2 promoters in differential sensitivity to PCBs in Atlantic killifish (*Fundulus heteroclitus*). *Aquat. Toxicology.* 101, 288–294. doi: 10.1016/j.aquatox.2010.10.010

Andrews, M., Battaglene, S., Cobcroft, J., Adams, M., Noga, E., and Nowak, B. (2010). Host response to the chondracanthid copepod (*Chondracanthus goldsmidi*), a gill parasite of the striped trumpeter *Latris lineata* (Forster), in Tasmania. *J. Fish Diseases.* 33, 211–220. doi: 10.1111/j.1365-2761.2009.01107.x

Apetoh, L., Quintana, F. J., Pot, C., Joller, N., Xiao, S., Kumar, D., et al. (2010). The aryl hydrocarbon receptor interacts with c-maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat. Immunol.* 11, 854–861. doi: 10.1038/ni.1912

Bjeldanes, L. F., Kim, J. Y., Grose, K. R., Bartholomew, J. C., and Bradfield, C. A. (1991). Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *PNAS Proc. Natl. Acad. Sci. U.S.A.* 88, 9543–9547. doi: 10.1073/pnas.88.21.9543

Blanch, A. R., Alsina, M., Simon, M., and Jofre, J. (1997). Determination of bacteria associated with reared turbot (*Scophthalmus maximus*) larvae. *J. Appl. Microbiol.* 82, 729–734. doi: 10.1046/j.1365-2672.1997.00190.x

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caruso, C., Pastorino, P., Burioli, E. A. V., Peletto, S., Righetti, M., Bona, C., et al. (2017). *CyHV-2 outbreak associated with aeromonas spp. in crucian carp (Carassius carassius) in piedmont (Italy)* Bamberg, Banagiotis Berillis. 307–314.

Cervantes-Barragan, L., Chai, J. N., Tianero, M. D., DiLuccia, B., Ahern, P. P., Merriman, J., et al. (2017). *Lactobacillus reuteri* induces gut intraepithelial $CD4^+$ $CD8\alpha\alpha^+$ T cells. *Science* 357, 806–810. doi: 10.1126/science.aah5825

Chang, P. H., Shu-Hwae, L., Chiang, H. C., and Jong, M. H. (1999). Epizootic of herpes-like virus infection in goldfish (*Carassius auratus*) in Taiwan. *Fish Pathology.* 34, 209–210.

Chen, P., Sun, Q. R., Zhang, H. H., Wang, S., Wang, J. W., Xu, Y., et al. (2021). Intestinal microbiota analysis in gibel carp (*Carassius auratus gibelio*) based on 16S rRNA gene sequence. *Fisheries Science.* 8, 1–10. doi: 10.16378/j.cnki.1003-1111.20208

Cong, J., Zhou, P., and Zhang, R. Y. (2022). Intestinal microbiota-derived short chain fatty acids in host health and disease. *Nutrients* 14:1977. doi: 10.3390/nu14091977

Conway, J. R., Lex, A., and Gehlenborg, N. (2017). UpSetR: an r package for the visualization of intersecting sets and their properties. *Bioinformatics* 18, 2938–2940. doi: 10.1093/ bioinformatics/btx364

Deng, Y. F., Zhang, Y., Zhang, R., Wu, B., Ding, L., Xu, K., et al. (2014). Mice *in vivo* toxicity studies for monohaloacetamides emerging disinfection byproducts based on metabolomic methods. *Environ. Sci. Technology.* 48, 8212. doi: 10.1021/es502010v

Dieuleveux, V., Lemarinier, S., and Guéguen, M. (1998). Antimicrobial spectrum and target site of d-3-phenyllactic acid. *Int. J. Food Microbiol.* 40, 177–183. doi: 10.1016/S0168-1605(98)00031-2

Ding, Z. F., Xia, S. Y., Zhao, Z. M., Xia, A. J., Shen, M. F., Tang, J. Q., et al. (2014). Histopathological characterization and fluorescence *in situ* hybridization of cyprinid herpesvirus 2 in cultured Prussian carp (*Carassius auratus gibelio*) in China. *J. Virological Methods* 206, 76–83. doi: 10.1016/j.jviromet.2014.05.011

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.2460/ajvr.69.1.82

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

Fong, M. Y., McDunn, J., and Kakar, S. S. (2011). Identification of metabolites in the normal ovary and their transformation in primary and metastatic ovarian cancer. *PloS One* 6, e19963. doi: 10.1371/journal.pone.0019963

Galindo-Villegas, J., Garcia-Moreno, D., Oliveira, S. D., Meseguer, J., and Mulero, V. (2012). Regulation of immunity and disease resistance by commensal microbes and chromatin modifications during zebrafish development. *PNAS Proc. Natl. Acad. Sci. U.S.A.* 109 (39), E2605–E2614.

Ghiboub, M., Verburgt, C. M., Sovran, B., Benninga, M. A., and Limbergen, J. (2020). Nutritional therapy to modulate tryptophan metabolism and aryl hydrocarbon-receptor signaling activation in human diseases. *Nutrients* 12 (9), 2846. doi: 10.3390/nu12092846

Goerz, G., Barnstorf, W., Winnekendonk, G., Bolsen, K., Fritsch, C., Kalka, K., et al. (1996). Influence of UVA and UVB irradiation on hepatic and cutaneous P450 isoenzymes. *Arch. Dermatol. Res.* 289, 46–51. doi: 10.1007/s004030050151

Goudot, C., Coillard, A., Villani, A. C., Gueguen, P., Cros, A., Sarkizova, S., et al. (2017). Aryl hydrocarbon receptor controls monocyte differentiation into dendritic cells versus macrophages. *Immunity* 47, 582–596. doi: 10.1016/j.immuni. 2017.08.016

Guarner, F., and Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet* 361, 512–519. doi: 10.1016/S0140-6736(03)12489-0

Guo, M. J., Wu, F. H., Hao, G. E., Qi, Q., Li, R., Li, N., et al. (2017). *Bacillus subtilis* improves immunity and disease resistance in rabbits. *Front. Immunol.* 8. doi: 10.3389/fimmu.2017.00354

Hao, N., and Whitelaw, M. L. (2013). The emerging roles of AhR in physiology and immunity. *Biochem. Pharmacol.* 86, 561–570. doi: 10.1016/j.bcp.2013.07.004

Hornik, K. (2012). *The comprehensive r archive network* Vol. 4 (Wiley Interdisciplinary Reviews-Computational Statistics), 394–398. doi: 10.1002/wics.1212

Hu, J. (2017). *The homeostasis of gut adhesive microbiota as a marker for evaluation of fish health. [dissertation's thesis]* (WuHan: Huazhong Agricultural University).

Jeong, K.-T., Hwang, S.-J., Oh, G.-S., and Park, J.-H. (2012). FICZ, a tryptophan photoproduct, suppresses pulmonary eosinophilia and Th2-type cytokine production in a mouse model of ovalbumin-induced allergic asthma. *Int. Immunopharmacology.* 13, 377–385. doi: 10.1016/j.intimp.2012.04.014

Jeremy, A. G., Roopali, G., Jessica, E. K., Ada, Y., Gopal, M., Sharmila, S., et al. (2016). AHR activation is protective against colitis driven by t cells in humanized mice. *Cell Rep.* 17, 1318–1329. doi: 10.1016/j.celrep.2016.09.082

Jung, S. J., and Miyazaki, T. (1995). Herpesviral haematopoietic necrosis of goldfish (*Carassius auratus* l.). *J. Fish Diseases.* 18, 211–220. doi: 10.1111/j.1365-2761.1995.tb00296.x

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., and Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature* 474, 327–336. doi: 10.1038/nature10213

Kerkvliet, N. I., Steppan, L. B., Vorachek, W., Oda, S., Farrer, D., Wong, C. P., et al. (2009). Activation of aryl hydrocarbon receptor by TCDD prevents diabetes in NOD mice and increases Foxp3+ T cells in pancreatic lymph nodes. *Immunotherapy* 1, 539–547. doi: 10.2217/IMT.09.24

Lamas, B., Natividad, J. M., and Sokol, H. (2018). Aryl hydrocarbon receptor and intestinal immunity. *Mucosal Immunol.* 11, 1024–1038. doi: 10.1038/s41385-018-0019-2

Lamas, B., Richard, M. L., Leducq, V., Pham, H.-P., Michel, M.-L., Costa, G. D., et al. (2016). CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. *Nat. Med.* 22, 598–605. doi: 10.1038/nm.4102

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., Mcdonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lavermicocca, P., Valerio, F., and Visconti, A. (2003). Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Appl. Environ. Microbiol.* 69, 634–640. doi: 10.1128/AEM.69.1.634-640.2003

Li, T. T., Long, M., Li, H., Gatesoupe, F. J., Zhang, X. J., Zhang, Q. Q., et al. (2017). Multi-omics analysis reveals a correlation between the host phylogeny, gut microbiota and metabolite profiles in cyprinid fishes. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00454

Ling, Z. X., Liu, X., Jia, X. Y., Cheng, Y. W., Luo, Y. Q., Yuan, L., et al. (20147485). Impacts of infection with different toxigenic *Clostridium difficile* strains on faecal microbiota in children. *Sci. Rep.* 4, 7485. doi: 10.1038/srep07485

Liu, Z. J., Cao, A. T., and Cong, Y. Z. (2013). Microbiota regulation of inflammatory bowel disease and colorectal cancer. *Semin. Cancer Biol.* 23, 543–552. doi: 10.1016/j.semcancer.2013.09.002

Liu, W., Wu, J. P., Li, Z., Duan, Z. Y., and Wen, H. (2018). Effects of dietary coated protease on growth performance, feed utilization, nutrient apparent digestibility, intestinal and hepatopancreas structure in juvenile gibel carp (*Carassius auratus gibelio*). *Aquaculture Nutr.* 24, 47–55. doi: 10.1111/anu.12531

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ method. *Methods* 25, 402–408.

Lozupone, C., and Knight, R. (2006). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lukens, J. R., Gurung, P., Vogel, P., Johnson, G. R., Carter, R. A., McGoldrick, D. J., et al. (2014). Dietary modulation of the microbiome affects autoinflammatory disease. *Nature* 516, 246–249. doi: 10.1038/nature13788

Lustri, B. C., Sperandio, V., and Moreira, C. G. (2017). Bacterial chat: Intestinal metabolites and signals in host-microbiota-pathogen interactions. *Infection Immunity.* 85, e00476–e00417. doi: 10.1128/IAI.00476-17

Lu, C. Y., Tang, R. Z., Su, M. Z., Zou, J. X., and Lv, L. Q. (2022). Induction of reactive oxygen species is necessary for efficient onset of cyprinid herpesvirus 2 replication: implications for novel antiviral strategy with antioxidants. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.792655

Luther, J. A., Enes, J., and Birren, S. J. (2013). Neurotrophins regulate cholinergic synaptic transmission in cultured rat sympathetic neurons through a p75-dependent mechanism. *J. Neurophysiology.* 109, 485–496. doi: 10.1152/jn.00076.2011

Manna, S. K., Patterson, A. D., Yang, Q., Krausz, W. K., Idle, J. R., Fornace, A. J., et al. (2011). UPLC-MS-based urine metabolomics reveals indole-3-lactic acid and phenyllactic acid as conserved biomarkers for alcohol-induced liver disease in the para-null mouse model. *J. Proteome Res.* 10, 4120–4133. doi: 10.1021/pr200310s

Ministry of Agriculture and Rural Affairs of China (2021). *China Fisheries statistical yearbook, volume 14: Freshwater aquaculture output in each province* (Beijing: China Agriculture Press).

Miyazaki, T., Kuzuya, Y., Yasumoto, S., Yasuda, M., and Kobayashi, T. (2008). Histopathological and ultrastructural features of koi herpesvirus (KHV)-infected carp (*Cyprinus carpio*), and the morphology and morphogenesis of KHV. *Dis. Aquat. Organisms.* 80, 1–11. doi: 10.3354/dao01929

Murray, H. M., Leggiadro, C. T., and Douglas, S. E. (2017). Immunocytochemical localization of pleurocidin to the cytoplasmic granules of eosinophilic granular cells from the winter flounder gill. *J. Fish Biol.* 70, 336–345. doi: 10.1111/j.1095-8649.2007.01452.x

Natividad, J. M., Agus, A., Planchais, J., Lamas, B., Jarry, A. C., Martin, R., et al. (2018). Impaired aryl hydrocarbon receptor ligand production by the gut microbiota is a key factor in metabolic syndrome. *Cell Metab.* 28, 737–749. doi: 10.1016/j.cmet.2018.07.001

Ng, K. M., Ferreyra, J. A., Higginbottom, S. K., Lynch, J. B., Kashyap, P. C., Gopinath, S., et al. (2013). Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature* 502, 96–99. doi: 10.1038/nature12503

Njoroge, J. W., Gruber, C., and Sperandio, V. (2013). The interacting cra and kdpe regulators are involved in the expression of multiple virulence factors in enterohemorrhagic *Escherichia coli*. *J. Bacteriology* 195, 2499–2508. doi: 10.1128/JB.02252-12

Njoroge, J. W., Nguyen, Y., Curtis, M. M., Moreira, C. G., and Sperandio, V. (2012). Virulence meets metabolism: *Cra* and *kdpe* gene regulation in enterohemorrhagic *Escherichia coli*. *Mbio* 3, e00280–e00212. doi: 10.1128/mBio.00280-12

Oberg, M., Bergander, L., Håkansson, H., Rannug, U., and Rannug, A. (2005). Identification of the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity. *Toxicological Sci.* 85, 935–943. doi: 10.1016/j.inoche.2007.11.024

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., and O'hara, R. B. (2011). *R package version 1.17-6. the comprehensive r archive network website* (Vegan: Community Ecology Package).

Onarheim, A. M., Wiik, R., Burghardt, J., and Stackebrandt, E. (1994). Characterization and identification of two *Vibrio* species indigenous to the intestine of fish in cold sea water; description of *Vibrio iliopiscarius* sp. nov. *Systematic Appl. Microbiol.* 17, 370–379. doi: 10.1016/S0723-2020(11)80053-6

Pacheco, A. R., Curtis, M. M., Ritchie, J. M., Munera, D., Waldor, M. K., Moreira, C. G., et al. (2012). Fucose sensing regulates bacterial intestinal colonization. *Nature* 492, 113–117. doi: 10.1038/nature11623

Paine, A. J. (1976). Induction of benzo[a]pyrene mono-oxygenase in liver cell culture by the photochemical generation of active oxygen species. evidence for the involvement of singlet oxygen and the formation of a stable inducing intermediate. *Biochem. J.* 158, 109–117. doi: 10.1042/bj1580109

Paine, A. J., and Francis, J. E. (1980). The induction of benzo[a]pyrene-3-mono-oxygenase by singlet oxygen in liver cell culture is mediated by oxidation products of histidine. *Chemico-Biological Interactions.* 30, 343–353. doi: 10.1016/0009-2797(80)90057-5

Perdew, G. H., and Babbs, C. F. (1991). Production of ah receptor ligands in rat fecal suspensions containing tryptophan or indole-3-carbinol. *Nutr. Cancer.* 16, 209–218. doi: 10.1080/01635589109514159

Platten, M., Nollen, E. A. A., Röhrig, U. F., Fallarino, F., and Opitz, C. A. (2019). Tryptophan metabolism as a common therapeutic target in cancer, neurodegeneration and beyond. *Nat. Rev. Drug Discovery.* 18, 379–401. doi: 10.1038/s41573-019-0016-5

Prasch, A. L., Hiroki, T., Carney, S. A., Wu, D., Takeo, H., Stegeman, J. J., et al. (2003). Aryl hydrocarbon receptor 2 mediates 2,3,7,8-tetrachlorodibenzo-p-dioxin developmental toxicity in zebrafish. *Toxicological Sci.* 1, 138–150. doi: 10.1093/toxsci/kfg202

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). Fast tree 2-approximately maximum-likelihood trees for large alignments. *PloS One* 5, e9490.

Qiao, G., Chen, P., Sun, Q. R., ZhaLivakng, M. M., Zhang, J. L., Li, Z., et al. (2020). Poly-beta-hydroxybutyrate (PHB) in bioflocs alters intestinal microbial community structure, immune-related gene expression and early cyprinid herpesvirus 2 replication in gibel carp (*Carassius auratus gibelio*). *Fish Shellfish Immunol.* 97, 72–82. doi: 10.1016/j. fsi.2019.12.045

Qiu, J., Heller, J., Guo, X., Chen, Z. M., Fish, K., Fu, Y. X., et al. (2012). The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* 36, 92–104. doi: 10.1016/j.immuni.2011.11.011

Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., et al. (2008). Control of t(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* 453, 65–71. doi: 10.1038/nature06880

Ran, C., Li, Y., Ma, X. F., Xie, Y. D., Xie, M. X., Zhang, Y. T., et al. (2021). Interactions between commensal bacterial and viral infection: insights for viral disease control in farmed animals. *Sci. China-Life Sci.* 64, 1–12. doi: 10.1007/S11427-020-1721-5

Rannug, A., Rannug, U., Rosenkranz, H. S., Wingvist, L., Westerholm, R., Agurell, E., et al. (1987). Certain photooxidized derivatives of tryptophan bind with very high affinity to the ah receptor and are likely to be endogenous signal substances. *J. Biol. Chem.* 262, 15422–15427. doi: 10.1016/S0021-9258(18)47743-5

Ran, C., Qin, C. B., Xie, M. X., Zhang, J. X., Li, J., Xie, Y. D., et al. (2018). *Aeromonas veronii* and aerolysin are important for the pathogenesis of motile aeromonad septicemia in cyprinid fish. *Environ. Microbiol.* 20, 3442–3456. doi: 10.1111/1462-2920.14390

Rong, S., Li, T. T., Luo, D., Li, J. B., Yin, L. Y., Li, H., et al. (2017). Changes in the intestinal microbiota of gibel carp (*Carassius gibelio*) associated with cyprinid herpesvirus 2 (CyHV-2) infection. *Curr. Microbiol.* 74, 1130–1136. doi: 10.1007/s00284-017-1294-y

Rosser, E. C., Piper, C., Matei, D. E., Blair, P. A., and Mauri, C. (2020). Microbiota-derived metabolites suppress arthritis by amplifying aryl-hydrocarbon receptor activation in regulatory b cells. *Cell Metab.* 31 (4), 837-851.e10. doi: 10.1016/j.cmet.2020.03.003

Shan, F., Liu, Y. J., Jiang, H. Y., and Tong, F. (2017). *In vitro* and *in vivo* protein release and anti-ischemic/reperfusion injury properties of bone morphogenetic protein-2-loaded glycyrrhetinic acid-poly (ethylene glycol)-b-poly(l-lysine) nanoparticles. *Int. J. Nanomedicine.* 12, 7613–7625. doi: 10.2147/IJN.S146546

Shapira, M. (2016). Gut microbiotas and host evolution: scaling up symbiosis. *Trends Ecol. Evolution.* 31, 539–549. doi: 10.1016/j.tree.2016.03.006

Somamoto, T., Miura, Y. H., Nakanishi, T., and Nakao, M. (2015). Local and systemic adaptive immune responses toward viral infection *via* gills in ginbuna crucian carp. *Dev. Comp. Immunol.* 52, 81–87. doi: 10.1016/j.dci.2015.04.016

Sun, M., Ma, N., He, T., Johnston, L. J., and Ma, X. (2019). Tryptophan (trp) modulates gut homeostasis *via* aryl hydrocarbon receptor (AhR). *Crit. Rev. Food Sci. Nutr.* 1-9. doi: 10.1080/10408398.2019.1598334

Takafumi, L., and Yukio, M. (2015). Effect of booster shot and investigation of vaccination efficacy period against herpesviral haematopoietic necrosis (HVHN) in goldfish (*Carassius auratus*). *Veterinary Microbiol.* 175, 139–144. doi: 10.1016/j.vetmic.2014.11.005

Tochio, T., Kadota, Y., Tanaka, T., and Koga, Y. (2018). 1-kestose, the smallest fructooligosaccharide component, which efficiently stimulates *Faecalibacterium prausnitzii* as well as bifidobacteria in humans. *Foods* 7, 140. doi: 10.3390/foods7090140

Trikha, P., and Lee, D. A. (2019). The role of ahr in transcriptional regulation of immune cell development and function. *Biochim. Biophys. Acta (BBA) - Rev. Cancer.* 1873, 188335. doi: 10.1016/j.bbcan.2019.188335

Wang, L., He, J. G., Liang, L. G., Zheng, X., Jia, P., Shi, X. J., et al. (2012). Mass mortality caused by cyprinid herpesvirus 2 (CyHV-2) in Prussian carp (*Carassius gibelio*) in China. *Bull. Eur. Assoc. Fish Pathologists.* 32, 164–173.

Wang, A. R., Ran, C., Ringoe, E., and Zhou, Z. G. (2018). Progress in fish gastrointestinal microbiota research. *Rev. Aquaculture.* 10, 626–640. doi: 10.1111/raq.12191

Whitfield-Cargile, C. M., Cohen, N. D., Chapkin, R. S., Weeks, B. R., Davidson, L. A., Goldsby, J. S., et al. (2016). The microbiota-derived metabolite indole decreases mucosal inflammation and injury in a murine model of NSAID enteropathy. *Gut Microbes* 7, 246–261. doi: 10.1080/19490976.2016.1156827

Willem, M. D. V., Herbert, T., Matthias, V. H., and Patrice, D. C. (2022). Gut microbiome and health: mechanistic insights. *Gut* 71 (5), 1020–1032. doi: 10.1136/gutjnl-2021-326789

Worley, B., and Powers, R. (2013). Multivariate analysis in metabolomics. *Curr. Microbiol.* 1, 92–107. doi: 10.2174/2213235X11301010092

Wu, T., Ding, Z. F., Ren, M., An, L., Xiao, Z. Y., Liu, P., et al. (2013). The histo- and ultra-pathological studies on a fatal disease of Prussian carp (*Carassius gibelio*) in mainland China associated with cyprinid herpesvirus 2 (CyHV-2). *Aquaculture* 412-413, 8–13.

Wyant, G. A., and Moslehi, J. (2022). Expanding the therapeutic world of tryptophan metabolism. *Circulation* 24, 1799–1820. doi: 10.1161/CIRCULATIONAHA.122.059812

Xu, L. J., Podok, P., Xie, J., and Li, L. Q. (2014). Comparative analysis of differential gene expression in kidney tissues of moribund and surviving crucian carp (*Carassius auratus gibelio*) in response to cyprinid herpesvirus 2 infection. *Arch. Virology.* 159 (8), 1961–1974. doi: 10.1007/s00705-014-2011-9

Yan, Y. Y., Huo, X. C., Ai, T. S., and Su, J. G. (2020). β-glucan and anisodamine can enhance the immersion immune efficacy of inactivated cyprinid herpesvirus 2 vaccine in *Carassius auratus gibelio*. *Fish Shellfish Immunol.* 98, 285–295. doi: 10.1016/j.fsi. 2020.01.025

Yao, Z. F., Huang, J., Zhang, J. M., Nie, H. H., Zheng, X. H., Li, L. J., et al. (2016). Investigation of viral load in healthy fish naturally infected with cyprinid herpesvirus II (CyHV-2). *Hubei Agric. Sci.* 4, 980–983. doi: 10.14088/j.cnki.issn0439-8114. 2016.04.041

Zhang, M. L., Sun, Y. H., Liu, Y. K., Qiao, F., Chen, L. Q., Liu, W. T., et al. (2016). Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. *Aquaculture* 454, 72–80. doi: 10.1016/j.aquaculture.2015.12.014

Zhu, M., Dai, Y. P., Tong, X. Y., Zhang, Y. X., Zhou, Y., Cheng, J. L., et al. (2022). Circ-udg derived from cyprinid herpesvirus 2 promotes viral replication. *Microbiol. Spectrum.* 10, 2165–0497. doi: 10.1128/spectrum.00943-22

Zhu, M., Liu, B., Cao, G. L., Hu, X. L., Wei, Y. H., Yi, J. T., et al. (2015). Identification and rapid diagnosis of the pathogen responsible for haemorrhagic disease of the gill of allogynogenetic crucian carp. *J. Virological Methods* 219, 67–74. doi: 10.1016/j.jviromet.2015.03.019

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Repeated and multiple fecal microbiota transplantations plus partial enteral nutrition as the first-line treatment in active pediatric Crohn's disease

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Background: Most studies have reported fecal microbiota transplantation (FMT) as an effective secondary option for Crohn's disease (CD). However, there is little data on FMT as a first-line treatment for CD. In our study we explore the rates of clinical and endoscopic remission and mucosal healing after FMT plus partial enteral nutrition (PEN), as a first-line treatment for active CD in children.

Methods: We retrospectively enrolled pediatric CD patients who underwent PEN or PEN plus FMT treatment at diagnosis from November 2016 to July 2019 at the Pediatric Department, Tongji Hospital. The two groups were defined as FMT group (repeated and multiple doses of FMT plus PEN) or PEN group (PEN alone). All the patients received PEN intervention. At baseline and week 8- 10, the FMT group was administered multiple doses of FMT to help induce and maintain remission. All patients were evaluated at week 8- 10 and 18-22 *via* clinical and relevant laboratory parameters and endoscopic results. The clinical and endoscopic remission and mucosal healing rates were compared between the two groups at different time points after the therapy.

Results: Twenty-five newly diagnosed active CD patients were included in the study, containing 7 females and 18 males with a median age of 11. 1 $\pm$ 2.3 years. 13 and 12 patients were assigned to the PEN and FMT groups, respectively. At week 8-10, clinical remission was obtained in 83.3% and 53.8% of the FMT and PEN groups, respectively (p=0.202). The endoscopic remission rates were 72.7% for FMT and 25.0% for PEN (p=0.039), whereas the mucosal healing rates were 27.2% for FMT and 0% for PEN (p=0.093). At week 18-22, clinical remission was achieved in 72.7% and 20.0% of patients in the FMT and PEN groups, respectively (p=0.03). Theendoscopic remission rates were 66.6% and 12.5% in the FMT and PEN groups, respectively (p=0.05), whereas the mucosal healing rates were 55.5% and 0% in FMT and PEN groups, respectively (p=0.029).

Conclusion: This study demonstrate that FMT plus PEN can be used as a first-line treatment for active CD in children.

KEYWORDS

fecal microbiota transplantation, partial enteral nutrition, Crohn's disease, first-line treatment, pediatric

Introduction

Crohn's disease (CD) is an acute and chronic nonspecific inflammatory gastrointestinal disorder. In recent years, with the westernization of diet and lifestyle, as well as the widespread use of antibacterial agents and heavy social pressure, CD has become increasingly common worldwide; in China, and the incidence is becoming increasingly in younger (Wang et al., 2013; Ng et al., 2017). Compared to adults, the course of CD in children is more rapid, the disease changes faster, and the intestinal tract is more extensively involved, which seriously affects growth and development (Kuenzig et al., 2022).

Currently, the main treatment methods for CD include exclusive enteral nutrition (EEN), oral corticosteroids, immunity inhibitors, and biologicals. Corticosteroids are critical in the induction of active CD remission, but are unable to sustain long-term treatment with serious adverse reactions. Immunity inhibitors, such as methotrexate and azathioprine, which are used for CD maintenance in children, have significant side effects. Biological agents, such as infliximab and adalimumab are relatively effective in treating CD in children. However, long-term potential adverse events, such as infection and high cost, greatly limit their widespread use. Multiple studies have confirmed that EEN has a significant effect on inducing remission in mild-to-moderate pediatric CD (Buchanan et al., 2009; Frivolt et al., 2014; Moriczi et al., 2020), but foods should be strictly avoided making EEN unsuitable as an effective means of maintaining remission. Partial enteral nutrition (PEN) is a more patient-friendly and tolerant treatment (Johnson et al., 2006), and PEN alone is generally considered partially effective, but PEN in combination with medication has been shown to be beneficial in inducing and maintaining remission (Takagi et al., 2006). Although its etiology is not fully understood, gut imbalance and dysregulation of immunological responses plays a critical role in the development of CD (Gevers et al., 2014). As the best way to regulate intestinal flora, fecal microbiota transplantation (FMT) is recommended as a guideline for therapy of recurrent Clostridium difficile infections (Cammarota et al., 2017).

Published evidences have demonstrated that FMT is one of the effective ways to treat CD (Goyal et al., 2018; Green et al., 2020; Sokol et al., 2020). Most of these studies have addressed FMT as an alternative for the first-line treatment for CD. Data have shown that the therapeutic efficacy of FMT declines over time, and the timing of the second FMT helps maintain the long-term benefit of active CD (Cui et al., 2021). To date, no studies have evaluated FMT coupled with PEN as a first-line treatment in inducing and sustaining remission in CD with children. We conducted a retrospective study to evaluate the clinical remission and mucosal healing *via* repeated and multiple doses of FMT plus PEN (80%), as the first-line treatment in pediatric patients with CD. The study served as a pilot study to NCT05321758 which was registered as a clinical trial.

Methods

Ethics

The present study was approved by the Medical Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (TJ-C20220313). Written informed consent for FMT treatment was obtained from parents or legal guardians of all pediatric subjects.

Study design

We retrospectively reviewed the medical charts of children with mild-to-moderate active CD who were hospitalized in the Department of Pediatrics in Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology from November 2016 to July 2019. The diagnosis of CD was rested on history, clinical symptoms, endoscopy, and histological evidence.

The inclusion criteria met the following: (i) age of 6–14 years with no genetic diseases; (ii) all newly diagnosed with mild-to-moderate CD with early PEN (80%) and/or FMT treatment; (iii) in addition to the PEN and FMT treatment, other treatments should not be added. Exclusion criteria included: (i) children who were treated with PEN (80%) for less than eight weeks or with other drugs during that time, including corticosteroids, methotrexate, thiopurines, and anti-TNF agents; and (ii) incomplete data for patients.

All children with CD received PEN (80% of total calories as a pooled diet, Peptamen, Nestle, Vevey, and swiss) intervention at diagnosis to help induce and maintain clinical remission, and the FMT group received multiple FMT interventions at baseline and week 8- 10 in addition to PEN treatment (Figure 1). The volume of formula per day was calculated according to the estimated energy requirement (EER) × 120% (basal rate plus an additional 20% caloric needs for weight gain). The EER was calculated based on the recommendations of the Chinese Dietary Reference Intake. Another 20% of the calories comes from a regular diet with no allergens and limited animal protein.

Patients treated with FMT coupled with PEN were defined as the FMT group, and those treated with PEN alone served as the PEN group. Patients with mild-to-moderate CD, defined by the Pediatric Crohn's Disease Activity Index (PCDAI) of >10 and ≤40, and Simple Endoscopic Score for CD (SES-CD) of >3, were enrolled in the study. The Paris classification was used to assess the anatomical location and behavior of the disease (Levine et al., 2011). Clinical remission was defined as a PCDAI score of ≤10 (Grover et al., 2016). Endoscopic remission was defined as SES-CD ≤2 and PCDAI score ≤ 10 (Vuitton et al., 2016). Mucosal healing was defined as SES-CD=0 and PCDAI score ≤ 10 (Peyrin-Biroulet et al., 2015).

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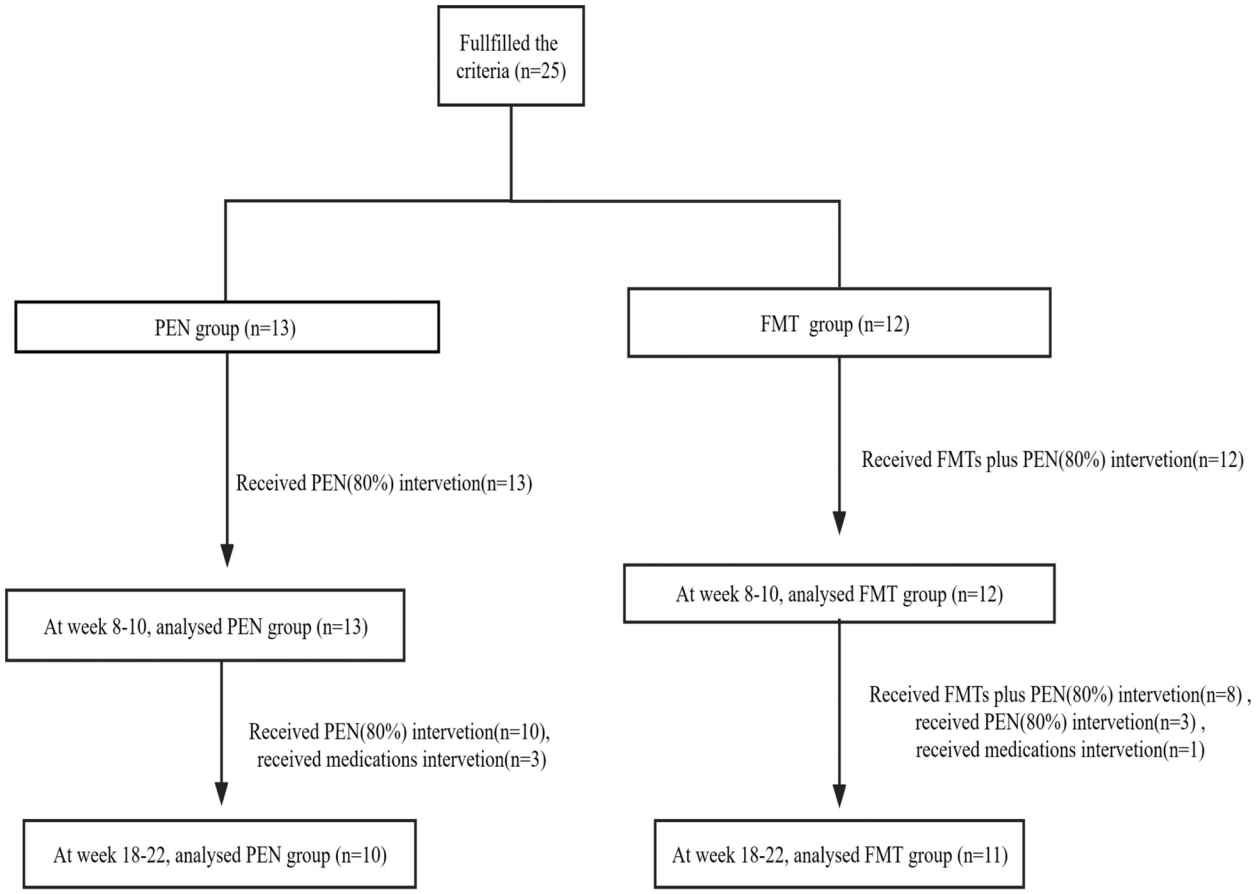

FIGURE 1
Flowchart of patient inclusion. FMT, fecal microbiota transplantation; PEN, Partial enteral nutrition.

The time point at which CD was diagnosed was the baseline. Colonoscopy was conducted by trained pediatric endoscopists at baseline and at two assessment points (week 8- 10 and 18-22) after the therapy, and SES-CD scores were calculated jointly by two pediatric attending physicians. If the participants required additional medication within 18 weeks, they were considered clinically invalid and were not included in the next evaluation point.

Donor screening

12 healthy donors were recruited in the study with one recipient and one donor. Eligible donors, including children with similar age (e.g., relatives and trusted friends), were recruited according to the following criteria (Kelly et al., 2015; Cammarota et al., 2017; Liu et al., 2017): (i) no history of infectious conditions (e.g., tuberculosis); (ii) no history of metabolic syndromes (e.g., diabetes); (iii) no gastrointestinal illnesses and functional disorders, including chronic fatigue and irritable bowel syndrome (IBS); (iv) no allergic diseases (e.g., eczema); (v) no antibiotic taken in the past three months; (vi) no autoimmune diseases; (vii) no drug abuse history; and (viii) on a regular diet during donation of the material.

To minimize the risk of disease transmission, the donors underwent rigorous serological and stool tests within 10 days before the FMT donation. Clinical tests, including complete blood count, biochemistry, hepatitis virus, HIV, and common intestinal pathogens, should be normal to qualify as donors (Supplementary Table 1). They had to rescreen and re-evaluate any abnormalities in the symptoms and signs. Fecal 16S RNA or macrogene sequencing was performed if necessary.

FMT procedure

12 patients received therapy with FMT plus PEN (80%). The guardians of the 12 patients refused immunological interventions, considering the adverse effects of corticosteroids and immunosuppressants, and they agreed to FMT as first-line therapy. The number of FMT infusions was grouped into single (1 day) or multiple infusions (2-10 days continuously). No bowel preparation (cleanup or laxative administration) was performed before the FMT. The donor feces were collected 1 h pre-FMT, attenuated, and mingled with sterile normal saline (1 mg of feces was attenuated with 5ml of saline). Samples were filtered through sterile gauze, and a 100mL fresh fecal microbiota suspension was

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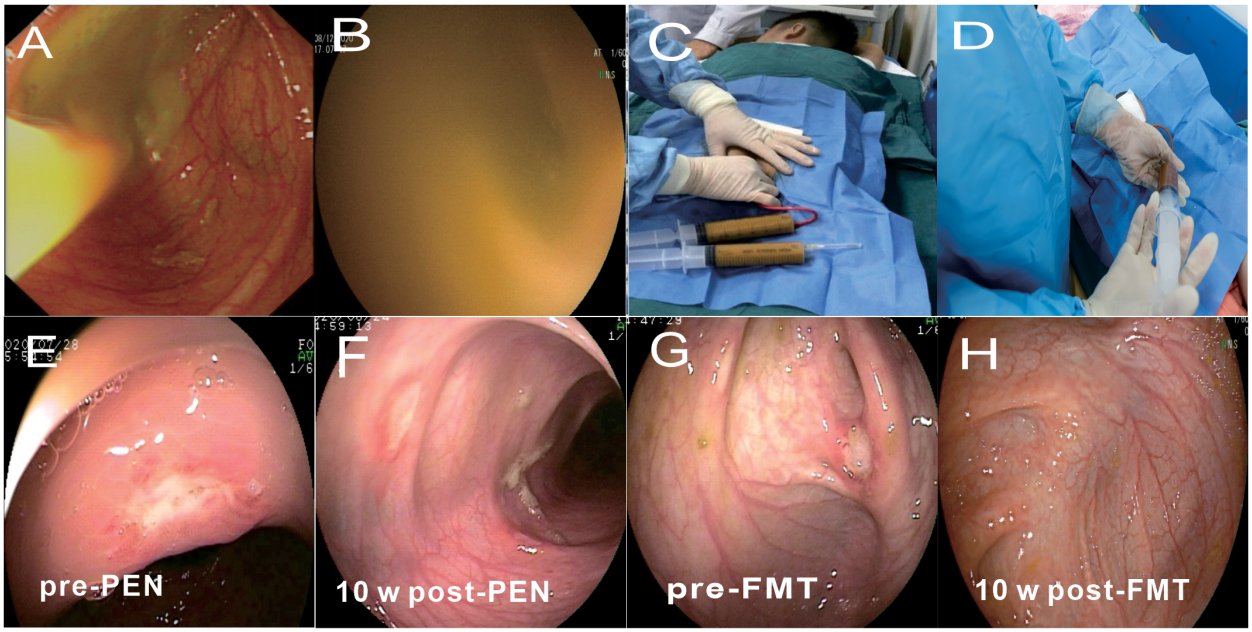

FIGURE 2
Delivery route of fecal microbiota transplantation (FMT) and related clinical response. **(A)** Endoscopic images show microbiota suspension being infused. **(B)** Endoscopic images showed no visible particles when the colonoscope was inserted into the infused microbiota suspension at the end of the ileum. c,d Fecal microbiota transplantation (FMT) were performed by retention enema (**C**, Retention enema, **D**, Infused microbiota suspension under the retention enema). **(E, F)**The change of endoscopic appearance pre-PEN and 10 weeks post-PEN. **(G, H)** The change of endoscopic appearance pre-FMT and 10 weeks post-FMT.

prepared for the FMT. The fecal suspension was poured into a sterile cup for the FMT procedure within 1 h. The routes of administration included colonoscopy and enema. Fecal microbiota transplantation (FMT) were performed by colonoscopy (Figures 2A, B). Fecal microbiota transplantation (FMT) were performed by retention enema (Figures 2C, D). After infusion, the patients were asked to hold a fixed position (>25° semi-reclining or hip-up position) for at least 4 h. The FMT procedure followed a uniform standard for each patient. All the patients in the FMT group received fresh fecal suspensions.

Microbiota analysis

Fecal microbiota was analyzed for two patients before and after FMT. DNA extracted from feces collected from the healthy donors, and patients pre-FMT (one day before FMT), at week 8 and week 18 of after FMT using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). 16S rRNA gene (V3-V4 region) were amplified using PCR primers as described previously (Liu et al., 2017). Diversity and changes of fecal microbiota were analysed on the QIIME2 v.2020.2 platform.

Data collection

The data of twenty-five pediatric patients used in this study were extracted from medical records. To aid in evaluating clinical efficacy and safety, laboratory data were collected at baseline (at week 0), at week 8–10 and 18–22, including laboratory inflammation and biochemical indicators, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), hemoglobin (Hb), leukocyte, serum albumin, platelet, vitamin D, fecal calprotectin, PCDAI, SES-CD score, and FMT-related adverse events (AEs).

Statistical analysis

Continuous variables are presented as mean (standard deviation) or median (range). Noncontinuous parameters are depicted as frequencies and percentages. According to the results acquired, nonparametric test (Mann Whitney U test) or a parametric test (T test) were used to evaluate differences between groups. Univariate analysis between the two groups was conducted using the Chi-square test or Fisher exact test for categorical data. The IBM SPSS Statistics 25 was used for the statistical analyses. Statistical significance was set at $p<0.05$ in a two -side test. GraphPad Prism version 8 was used to draw graphics.

Results

Patient characteristics

From November 2016 to July 2019, twenty-five pediatric patients were analysed in this study, containing seven females and eighteen males with a median age of 11. 1 ± 2.3 years. All patients

TABLE 1 The baseline characteristics of the study population at the time of diagnosis.

Parameters	FMT group	PEN group	P value
Total number	12	13	-
Age(year), M± SD (range)	10.69 ± 2.39	11.56 ± 2. 16	0.319
Sex, female, n (%)	3(0.25)	4(0.31)	1
Disease duration before PEN(mo)	0.068 ± 0.009	0.074 ± 0.015	0.325
Hemoglobin (g/L)	109.6 ± 21.9	107.7± 13.5	0.868
White cell count (109/L)	11.7± 1.6	13.9± 1.5	0.336
Platelet (109/L)	416 ± 43	383 ± 25	0.525
Vitamin D	17.4 ± 4.0	16.7 ± 5.5	0.525
ESR	31.5 ± 22.4	33.5± 18. 1	0.813
CRP (mg/L)	33.9 ± 34.7	33.5 ± 25.3	0.644
Albumin (g/L)	35.9 ± 5.3	35.4 ± 4.9	0.781
Disease location			0.595
L1 Terminal ileum	5 (41.7%)	6 (46.2)	
L2 Colon	3 (0.25%)	6 (46.2)	
L3 Ileocolonic	4 (0.33%)	2 (15.3%)	
+L4 (upper GI tract)	2 (16.7%)	1 (7.7%)	
Disease behaviour			1
B1 non-stricturing or non-penetrating	10 (83.3%)	10 (76.9%)	
B2 stricturing	2 (16.7%)	3 (23. 1%)	
B3 penetrating	0	0	
Perianal involvement	2 (16.7%)	2 (15.3%)	1
PCDAI score	23.2 ± 6.7	23.5 ± 5.6	0.909
SES-CD score	6.75± 1.05	6.54± 1.05	0.62

CRP, C-reactive protein; ESR,erythrocyte sedimentation rate; PCDAI, Pediatric CD Activity Index; SES-CD, Simple Endoscopic Score for CD.

enrolled in the current study were newly diagnosed CD and treated with PEN (80%) for more than eight weeks. Of those, 13 patients belonged to the PEN and 12 in the FMT group, respectively. There were no statistically remarkable differences in the baseline demographics between the two groups (Table 1).

The median time from CD diagnosis (at baseline) to PEN initiation (80%) was 0.07 ± 0.01 months. Twenty-two patients (88%) received PEN orally at all time, but three (12%) patients required nasogastric feeding for the first five days. The guardians of the FMT group all consented to FMT as their first-line treatment. The median time from CD diagnosis to FMT initiation was 0.31 ± 0.09 months in the FMT group. The data of the children in the FMT group are shown in Table 2.

Patients who received multiple doses of FMT usually once a day consecutively, underwent the first colonoscopy, and the others through an enema. In the PEN group, 13 patients were treated with PEN (80%) alone for more than 8 weeks, 10 of them were treated with PEN (80%) alone for more than 18 weeks, and another 3 patients were switched to biologics at weeks 8-10. In the FMT group, 12 patients received one round of FMT plus PEN (80%) therapy for more than 8 weeks, 8 of them received two rounds of FMT plus PEN (80%) therapy for more than 18 weeks, 3 of them received one round of FMT plus PEN (80%) therapy for more than 18 weeks, and another 1 patient switched to biologics at weeks 8- 10 (Figure 1). Of the 25 patients, only three had a PEN (80%) treatment duration longer than 24 weeks.

Evaluation of induction remission (at week 8-10)

One patient in each group did not undergo endoscopic examination at week 8- 10 after the treatment. The induced clinical and endoscopic remissions and mucosal healing rates in the FMT group were remarkably higher than those in the PEN group (Table 3).

Clinical remission was observed in 10 (83.3%) and 7 (53.8%) patients in the FMT and PEN groups, respectively (p=0.202). The mean PCDAI score was decreased from 23.2 ± 6.7 at baseline to 7.6 ± 7. 1 in the FMT group, and from 23.5 ± 5.6 at baseline to 16.7 ± 11. 1 in the PEN group. Endoscopic remission was observed in 8 of 11 of the patients (72.7%) in the FMT group and 3 (25.0%) in the PEN group. Figures 2E-H shows the endoscopic appearance before and after treatment in FMT group and PEN group. Ulcers were still visible in the PEN group, but disappeared in the FMT group after treatment. This showed a better endoscopic remission rate in the FMT group.

Mucosal healing was observed in 3 of 11 patients (27.2%) in the FMT group and 0 (0%) in the PEN group. The mean SES-CD score was reduced from 6.75 ± 1.05 at baseline to 2.36 ± 1.9 in the FMT group, and from 6.54 ± 1.05 at baseline to 4.83 ± 2.8 in the PEN group.

At week 8-10, there were significant statistical differences in PCDAI and SES-CD scores in both groups (both P<.05) (Figure 3). CD-related parameters such as ESR, CRP and serum albumin were evaluated, and both groups showed improvement from baseline. Compared with PEN group, FMT group showed more significant improvement, faster decline of ESR and CRP, faster increase of albumin, and better clinical efficacy. There were remarkable differences between the two groups in ESR, CRP and serum albumin (all P<.05) (Figure 3).

Evaluation of maintaining remission (at week 18-22)

Three patients in the PEN group and one patient in the FMT group were switched to biologics after disease flare, leaving 11 patients in the FMT group and 10 patients in the PEN group to be analyzed at week 18-22. To help maintain remission, eight patients received multiple FMT doses again at week 8- 10 in the FMT group (Table 2).

TABLE 2 Factors related to FMT in patients treated with FMT.

Patient	CDI	EBV	CMV	Median time from CD diagnosis to FMT initiation (mo)	Times of FMT (baseline)	Times of FMT (week 8- 10)	Activity Index (PCDAI)			SES-CD			Fecal calprotectin (μg/g)			Patient-donor relationship	Donor age (yr)
							baseline	week 8 – 10	Week 18 – 22	baseline	week 8 – 10	Week 18 – 22	baseline	week 8 – 10	Week 18 – 22		
P1	–	–	–	0.5	1	/	22	30	/	7	9	/	/	/	/	relative	9
P2	–	–	–	0.2	8	7	20	2	0	6	2	2	/	/	/	friends	9
P3	–	–	–	0.3	1	/	30	5	15	8	2	0	/	/	/	friends	15
P4	–	–	–	0.5	9	10	37	5	5	9	2	0	3330	480	128	relative	12.7
P5	–	–	–	0.2	3	2	32	5	5	7	0	0	1890	166	108	relative	9
P6	–	–	–	0.3	10	/	20	15	25	7	2	7	1093	160	966	friends	13
P7	–	–	–	0.3	9	7	17	0	27	7	0	8	1980		736	relative	10
P8	–	–	–	0.3	7	7	27	5	10	7	3	3	1880	120	710	relative	14
P9	–	–	–	0.3	10	7	20	5	2	6	2	0	/	/	/	friends	12
P10	–	–	–	0.3	4	/	22	5	5	6	0	/	/	/	/	relative	9
P11	–	–	–	0.3	9	4	17	10	10	6	4	/	/	/	/	relative	12
P12	–	–	–	0.3	9	9	15	5	0	5	/	0	/	/	/	relative	7

FMT, fecal microbiota transplantation; CDI, C. difficile infection; EBV, EB virus; CMV, cytomegalovirus; PCDAI, Pediatric CD Activity Index.

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TABLE 3 Comparison of clinical and endoscopic outcomes between the two groups at different time points.

Times	Outcome	FMT group	PEN group	P value
8- 10w	Clinical remission (PCDAI ≤ 10) Endoscopic remission (SES-CD ≤ 2) Mucosal healing (MH) (SES-CD = 0) Clinical remission (PCDAI ≤ 10)	10/12(83.3%) 8/11(72.7%) 3/11(27.2%) 8/11(72.7%)	7/13(53.8%) 3/12(25.0%) 0 2/10(20.0%)	0.202 0.039 0.093 0.03
18-22w	Endoscopic remission (SES-CD ≤ 2) Mucosal healing (MH) (SES-CD = 0)	6/9(66.6%) 5/9(55.5%)	1/8(12.5%) 0	0.05 0.029

FMT, fecal microbiota transplantation; PEN, Partial enteral nutrition; PCDAI, Pediatric CD Activity Index; SES-CD, Simple Endoscopic Score for CD.

The remission rate was remarkably better in the FMT group than in the PEN group (Table 3). In the FMT group, eight (72.7%) patients maintained clinical remission and seven (87.5%) of those received a second round of FMT treatment (Table 2). Only two (20.0%) patients maintained clinical remission in the PEN group. The mean PCDAI score was 9.45 ± 10.3 in the FMT group and 22. 1 ± 10.6 in the PEN group (p=0.09). The mean SES-CD score was 2.2 ± 1.06 in the FMT group, and 5. 1 ± 0.8 in the PEN group (p=0.032) (Figure 3). Endoscopic remission was observed in 6 of 9 (66.6%) patients in the FMT group and in 1 of 8 (12.5%) patients in the PEN group. Mucosal healing was observed in 5 of 9 (55.5%) patients in the FMT group and 0 (0%) patients in the PEN group. Two patients in each group did not undergo an endoscopic examination.

The mean values of ESR, CRP, PCDAI and SES-CD score in the PEN group were rebound between week 8- 10 and week 18-22. The mean PCDAI score was increased from 16.7 ± 11. 1 to 22. 1 ± 10.6, and the SES-CD score was increased from 4.83 ± 2.8 to 5. 1 ± 0.8, ESR and CRP also showed similar changes. However, These parameters of FMT group were relatively stable. There were also significant differences in ESR and SES-CD score between the two

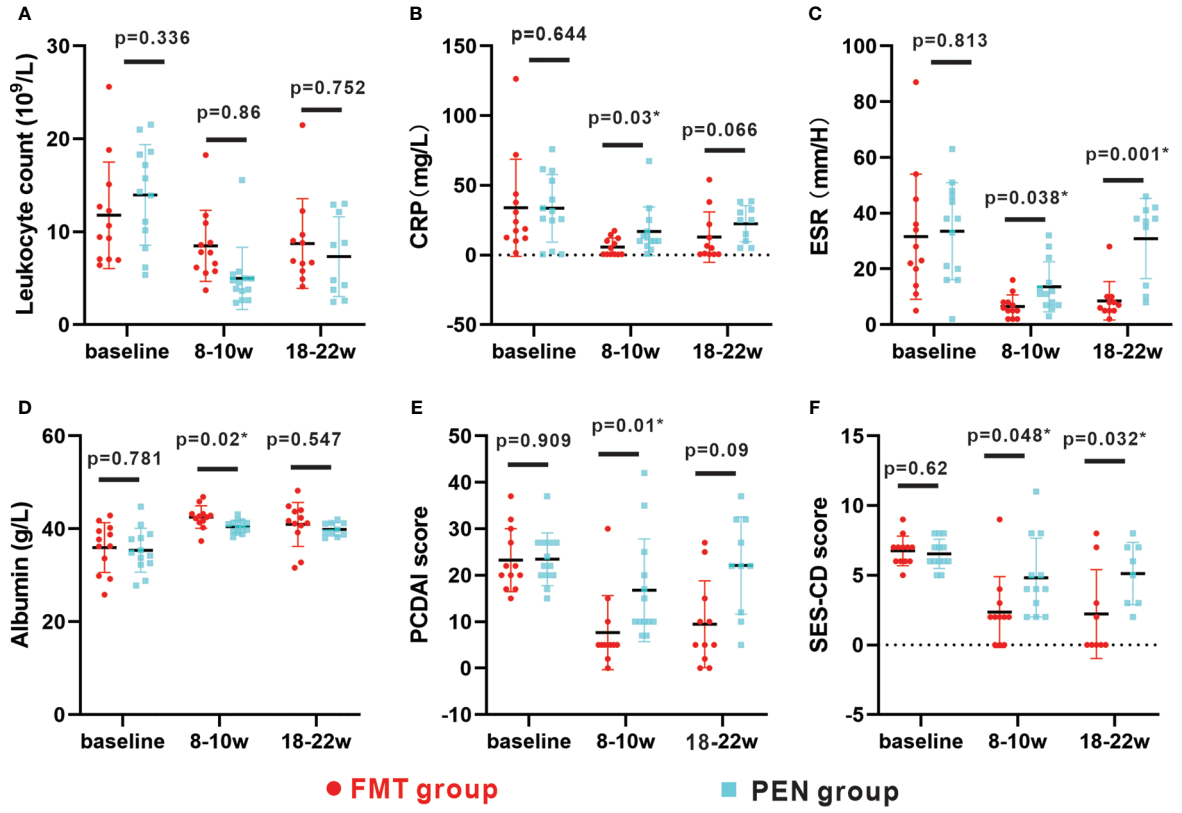

FIGURE 3
Changes in parameters between baseline, 8-10w and 18-22w in two groups. **(A–F)** Changes in leukocyte count, albumin, CRP, ESR, PCDAI score and SES-CD score between baseline, 8- 10w and 18-22w in the FMT group and PEN group respectively. The distribution of values within each group at each timing is illustrated by mean and SD. FMT, fecal microbiota transplantation; PEN, Partial enteral nutrition; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PCDAI, Pediatric CD Activity IndexSES-CD, Simple Endoscopic Score for CD.

groups at week 18–22 (both P<.05) (Figure 3).These findings suggest that the PEN group is not effective in maintaining sustained remission and that FMT can help maintain clinical remission.

Fecal calprotectin (a cutoff <200 μg/g) of 5 patients in FMT group was significantly elevated, ranging between 1903 to 3330μg/g at baseline. Fecal calprotectin decreased significantly for between week 0 and week 8- 10. Fecal calprotectin of 2 children continued to decline between week 8- 10 and week 18-22, while fecal calprotectin increased in the other 2 children, which was considered to be related to disease flare (Table 2).

Safety

A total of 21 FMT-related AEs occurred in 7 patients. Most AEs (90.5%) occurred within two days post-FMT. The AEs included abdominal pain(n=6), abdominal distension (n=5), nausea or vomiting (n=3), fever(n=2), constipation(n=2), diarrhea(n=2) and purpura (n= 1) (Table 4). One patient presented with abdominal pain and purpura in both lower limbs 12 hours after FMT. The diagnosis was allergic purpura. Abdominal pain improved one day after corticosteroid administration, which was considered to be caused by an immune disorder induced by bacterial flora. One patient developed fever with elevated blood routine and CRP levels 28 hour after the FMT. Inflammatory markers and body temperature returned to normal two days after antibiotic administration. The other AEs were self-limiting and symptom-free within 48 h.

Microbial composition changes

16s rDNA sequencing analysis showed that species richness and diversity of fecal microbial in 2 patients with Crohn's disease were significantly lower than those of healthy donors. FMT treatment can increase the species richness and diversity of patients. The bacterial population of the recipient was close to that of the donor after FMT treatment (Figure 4). After FMT treatment, the relative abundance of bacterial genera *Bacteroides*, *Eubacterium*, *Parasutterella*, *Butyricicoccus* were significantly increased and *Clostridioides*, *Ruminococcus*, *Blautia* were decreased in 2 children (Supplement 2).

Discussion

To the best of our knowledge, this is the first study to assess the effects of endoscopic remission and mucosal healing with repeated and multiple doses of FMT plus PEN as the first-line treatment in pediatric CD. The study indicated that within a small sample size of pediatric CD patients, repeated and multiple FMTs plus PEN can not only help induce and maintain remission but also contribute to mucosal healing.

Over the past decade, the therapeutic endpoint of CD has shifted from clinical remission to mucosal healing (Peyrin-Biroulet et al., 2015). The benefits of mucosal healing have been supported by clinical evidence to improve the long-term outcomes of CD patients, including reduced hormone use and incidence of risks, such as relapse and hospitalization (Ruemmele et al., 2015;

TABLE 4 The adverse events of FMT.

Pt	Related AEs	Time from FMT	Causality	SAEs	Clinical treatment and outcome	Cured
1	abdominal pain abdominal distension nausea or vomiting	8h 8h 6h	Probable Probable Probable	× × ×	Self-improvement Self-improvement Self-improvement	√ √ √
2	abdominal pain purpura	6h 15h 6h 12h	Probable Probable Probable P b bl	× √ ×	Self-improvement Improvement after corticosteroid therapy Self-improvement Improvement after antibiotic	√ √ √
	abdominal pain diarrhea	8h 4h	ro a e Probable Probable	× ×	therapy Self-improvement Self-improvement	√ √ √
4	abdominal pain constipation abdominal distension	6h 52h 6h	Probable Probable Probable	× × ×	Self-improvement Self-improvement Self-improvement	√ √ √
5	abdominal pain nausea or vomiting fever	6h 3h 15h	Probable Probable Probable	× × ×	Self-improvement Self-improvement Self-improvement	√ √ √
6	abdominal pain diarrhea abdominal distension nausea or vomiting	8h 4h 8h 2h	Probable Probable Probable Probable	× × × ×	Self-improvement Self-improvement Self-improvement Self-improvement	√ √ √ √
7	Constipation abdominal distension	56h 6h	Probable Probable	× ×	Self-improvement Self-improvement	√ √

AEs, adverse events; SAEs, serious adverse events; FMT, fecal microbiota transplantation; h, hour.

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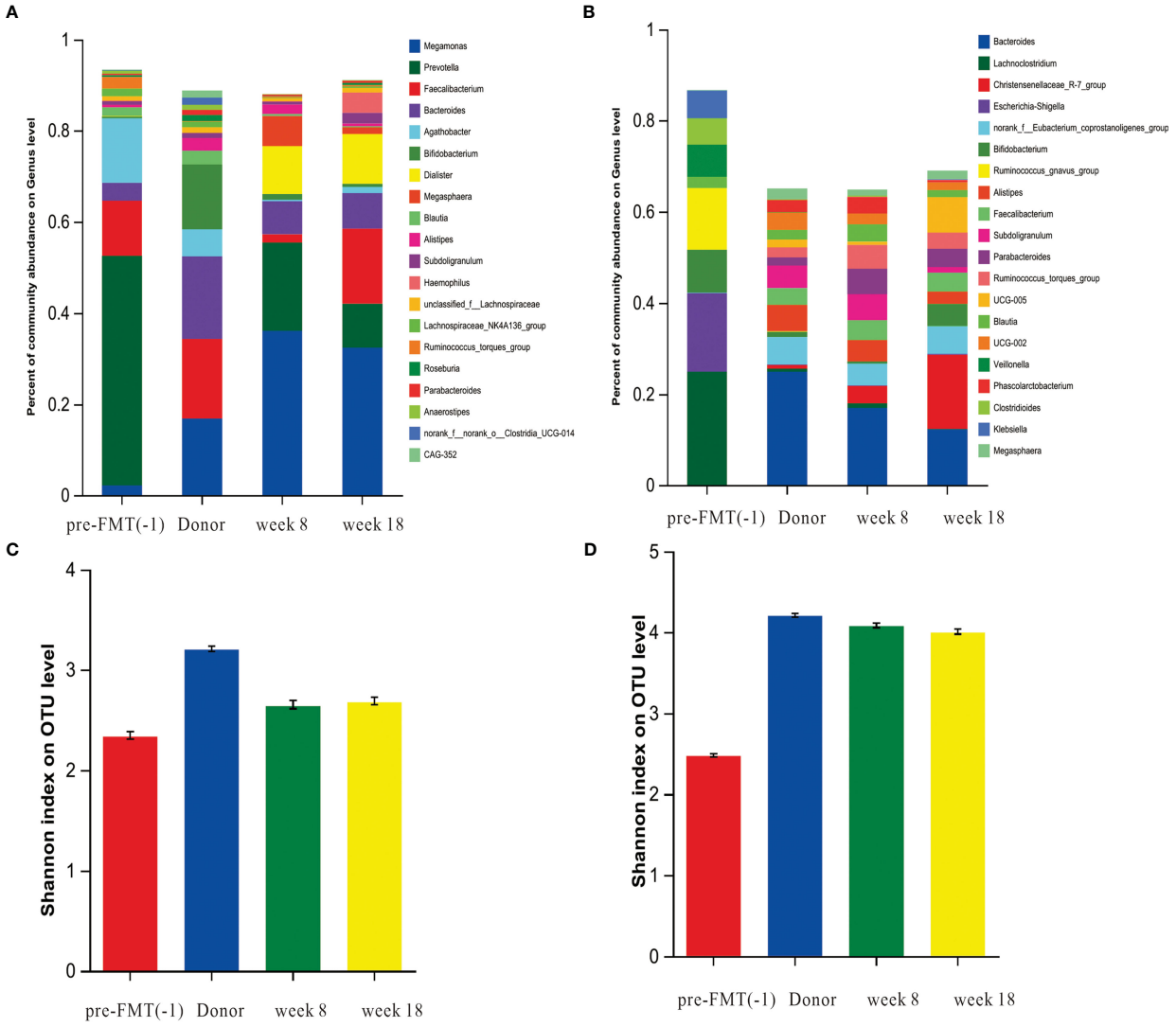

FIGURE 4
The dynamic changes in gut microbiota before and after FMT. DNA extracted from feces collected from the healthy donor, and recipient pre-FMT (-1), week 8, week 18 of after FMT was subjected to 16S rRNA gene (V3-V4 region) libraries construction and sequencing. **(A)** Species composition of feces samples of patient 5 at at genus level. **(B)**, Species composition of feces samples of patient 2 at genus level. **(C)** The Alpha diversity of patient 5 was measured by Shannon indexes. **(D)** The Alpha diversity of patient 2 was measured by Shannon indexes.

Taylor et al., 2020). Previous FMT-related studies mainly focused on clinical remission (Goyal et al., 2018; Sokol et al., 2020; Cui et al., 2021), but paid little attention to mucosal healing. Our study showed that the mucosal healing rate in the FMT group were significantly higher than those in the PEN group in both the induction and maintenance stages, indicating that FMT treatment can promote mucosal healing. This is a very novel clinical result, which may be related to the ability of FMT can remodel gut microbiota and restore microbial diversity. Compared to that of donors, the gut microbiota diversity of pediatric CD patients was significantly decreased, and the abundances of *Eubacterium*, *Clostridia_ UCG-014*, *Oscillibacter*, *Coprostanoligenes* were lower, while the abundance of *Clostridioides* was higher in CD patients. After FMT, the species richness and diversity of our two patients showed significant improvement. FMT changed the relative abundances of several bacterial genera in 2 patients, including the relative abundance of *Bacteroides*, *Eubacterium*, *Parasutterella*, *Butyricicoccus* were significantly increased and *Clostridioides*, *Ruminococcus*, *Blautia* were decreased. We found that after FMT, the microbial community of children with CD was close to that of donors. This is why the endoscopic remission rate decreased from 72% to 66% but the mucosal healing rate increased from 27.2% to 55% at week 18-22.

Partial enteral nutrition uses the same liquid formulation as EEN, which significantly improves compliance and taste compared to EEN. Studies have suggested that PEN is beneficial in inducing and maintaining remission in patients with CD and more patient-friendly and tolerant treatment (Verburgt et al., 2021). Our study also confirms this view, but the effective duration is relatively short. In our study, ESR, CRP, SES-CD and PCDAI scores improved

significantly between baseline and weeks 8- 10 in both groups, but FMT improved more. Between weeks 8- 10 and 18-22, these parameters remained largely unchanged in the FMT group and increased in the PEN group, after FMT treatment, fecal calprotectin also decreased significantly. All of this suggests that the FMT group was more successful in controlling inflammation. This indicates that the PEN treatment alone is poor in maintaining remission in CD patient, whereas FMT plus PEN is effective.

From the available evidence, to date, there is deficient evidence to recommend FMT as the first-line treatment for active pediatric CD patients. Knowledge on the application of FMT in pediatric CD are limited. Most of these studies have reported FMT as a secondary treatment option for refractory CD (Hourigan et al., 2015; Suskind et al., 2015; Goyal et al., 2018; Karolewska-Bochenek et al., 2018). Alka Goyal et al. (2018) reported that after a single FMT treatment, 57% of 21 children IBD patients who were refractory to conventional treatment showed clinical responses at 1 month. In a study by Suskind et al. (2015), 9 adolescents with CD were treated with a single FMT after refractory to conventional treatment, and 5 achieved clinical remission at 6 and 12 weeks. Karolewska-Bochenek K et al (Karolewska-Bochenek et al., 2018). reported that a two-week course of FMT was clinically effective in children with IBD refractory to standard therapy. Our study is the first paper to explore the first-line treatment of CD in children with FMT. Our data suggest that FMT combined with PEN therapy not only helps to induce remission in children with active CD, but also maintains clinical remission. This finding demonstrates that FMT may be a potential first-line treatment regimen for active pediatric CD. Given the drawbacks of current CD treatments, this should be a meaningful practice and exploration.

Several studies have demonstrated that a single FMT is safe and effective in patients with CD; meanwhile, multiple doses of FMT have been shown to be more effective than a single dose (He et al., 2017; Baunwall et al., 2020). Our findings are consistent with these results. Among the 12 patients in the FMT group, only two received a single FMT, and one (50%) was in clinical remission at week 8- 10. The other 10 patients received multiple FMTs, of which nine (90%) showed clinical remission at at week 8- 10. This indicates that the clinical remission rate of multiple FMTs is significantly higher than that of single FMT.

The safety of FMT in pediatrics CD is relatively good. The adverse events (AEs) mainly include nausea, vomiting and abdominal pain, and most of them are mild (Suskind et al., 2015; Goyal et al., 2018). Our recent study reports the short term safety of FMT in children, most AEs (88.5%) occurred within 2 days post-FMT, 91.4% of the AEs were self-limiting (Zou et al., 2022). This current study also shown that the short-term safety of FMT was very good. Although 7 out of 12 patients had FMT-related AEs, accompanied by two serious adverse events, most of the symptoms were mild. These symptoms may be related to poor intestinal mucosal barriers and the intestinal environment in children with CD.

The number of donor strains decreased within 1.5-3 months after FMT, and the theoretical effect of FMT decreased with a decrease in donor strains (Li et al., 2016). A recent study found that after three months of FMT treatment for IBS, the clinical response rate was 65% in the FMT group and 43% in the placebo group. However, the efficacy of FMT decreased significantly after 12 months and was similar to that of the placebo group (Johnsen et al., 2018). Therefore, we recommend periodic FMT to help maintain efficacy. To date, there have been several studies of repeated FMTs (Costello et al., 2019; Schierová et al., 2020; Wang et al., 2020), but few studies of periodic FMTs. Our study confirmed that periodic FMT within 10 weeks could significantly maintain long-term efficacy and help sustain mucosal healing in pediatric CD. In the FMT group of the study, at week 8- 10, eight patients received periodic FMTs in the FMT group, of which seven (87.5%) maintained clinical remission, six (75%) underwent endoscopic remission, and five (62.5%) had mucosal healing; meanwhile, three patients were treated with PEN (80.0%) only and without periodic FMT treatment, of which one (33.3%) was in clinical remission and 0 had mucosal healing. The results demonstrated that periodic FMT could be a safe, feasible, and effective treatment for children with active CD. To our knowledge, this is the first time that periodic FMTs has been used in the treatment of CD in children.

Our study has some limitations. This was not a randomized clinical trial that would have provided more important evidence. Additionally, we primarily focused on the clinical efficacy of repeated FMT, with relatively little attention paid to the variation in gut flora. Finally, the frequency of FMT required to maintain long-term clinical efficacy in pediatric CD requires a larger sample size.

In conclusion, this retrospective study demonstrated that repeated and multiple doses of FMT plus PEN had a beneficial effect in inducing and maintaining clinical remission and mucosal healing in newly diagnosed children with active CD. Therefore, FMT can be a potential first-line treatment regimen for active pediatric CD. Further prospective studies are required to verify these findings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

SS and ZH designed the research. BZ performed the literature search and data extraction and drafted of the manuscript, SL, XL and CD after FMT and follow-up. JH and MR collected the data. BZ provided scientific statistic analysis methods. SS and ZH conducted FMT. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1083236/full#supplementary-material

References

Baunwall, S. M. D., Lee, M. M., Eriksen, M. K., Mullish, B. H., Marchesi, J. R., Dahlerup, J. F., et al. (2020). Faecal microbiota transplantation for recurrent clostridioides difficile infection: An updated systematic review and meta-analysis. *EClinicalMedicine* 29-30, 100642. doi: 10.1016/j.eclinm.2020.100642

Buchanan, E., Gaunt, W. W., Cardigan, T., Garrick, V., McGrogan, P., and Russell, R. K. (2009). The use of exclusive enteral nutrition for induction of remission in children with crohn's disease demonstrates that disease phenotype does not influence clinical remission. *Aliment Pharmacol. Ther.* 30 (5), 501–507. doi: 10.1111/j.1365-2036.2009.04067

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., Kump, P., Satokari, R., et al. (2017). European Consensus conference on faecal microbiota transplantation in clinical practice. *Gut.* 66 (4), 569–580. doi: 10.1136/gutjnl-2016-313017

Costello, S. P., Hughes, P. A., Waters, O., Bryant, R. V., Vincent, A. D., Blatchford, P., et al. (2019). Effect of fecal microbiota transplantation on 8-week remission in patients with ulcerative colitis: A randomized clinical trial. *JAMA.* 321 (2), 156–164. doi: 10.1001/jama.2018.20046

Cui, J., Lin, Z., Tian, H., Yang, B., Zhao, D., Ye, C., et al. (2021). Long-term follow-up results of fecal microbiota transplantation for irritable bowel syndrome: A single-center, retrospective study. *Front. Med. (Lausanne).* 8. doi: 10.3389/fmed.2021.710452

Frivolt, K., Schwerd, T., Werkstetter, K. J., Schwarzer, A., Schatz, S. B., Bufler, P., et al. (2014). Repeated exclusive enteral nutrition in the treatment of paediatric crohn's disease: predictors of efficacy and outcome. *Aliment Pharmacol. Ther.* 39 (12), 1398–1407. doi: 10.1111/apt.12770

Gevers, D., Kugathasan, S., Denson, L. A., et al. (2014). The treatment-naive microbiome in new-onset crohn's disease. *Cell Host Microbe* 15 (3), 382–392. doi: 10.1016/j.chom.2014.02.005.

Goyal, A., Yeh, A., Bush, B. R., Firek, B. A., Siebold, L. M., Rogers, M. B., et al. (2018). Safety, clinical response, and microbiome findings following fecal microbiota transplant in children with inflammatory bowel disease. *Inflammation Bowel Dis.* 24 (2), 410–421. doi: 10.1093/ibd/izx035

Green, J. E., Davis, J. A., Berk, M., Hair, C., Loughman, A., Castle, D., et al. (2020). 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* 12 (1), 1–25. doi: 10.1080/19490976.2020.1854640

Grover, Z., Burgess, C., Muir, R., Reilly, C., and Lewindon, P. J. (2016). Early mucosal healing with exclusive enteral nutrition is associated with improved outcomes in newly diagnosed children with luminal crohn's disease. *J. Crohns Colitis.* 10 (10), 1159–1164. doi: 10.1093/ecco-jcc/jjw075

He, Z., Li, P., Zhu, J., et al. (2017). Multiple fresh fecal microbiota transplants induces and maintains clinical remission in crohn's disease complicated with inflammatory mass. *Sci. Rep.* 7 (1), 4753. doi: 10.1038/s41598-017-04984-z

Hourigan, S. K., Chen, L. A., Grigoryan, Z., Laroche, G., Weidner, M., Sears, C. L., et al. (2015). Microbiome changes associated with sustained eradication of clostridium difficile after single faecal microbiota transplantation in children with and without inflammatory bowel disease. *Aliment Pharmacol. Ther.* 42 (6), 741–752. doi: 10.1111/apt.13326

Johnsen, P. H., Hilpüsch, F., Cavanagh, J. P., Leikanger, I. S., Kolstad, C., Valle, P. C., et al. (2018). Faecal microbiota transplantation versus placebo for moderate-to-severe irritable bowel syndrome: a double-blind, randomised, placebo-controlled, parallel-group, single-centre trial. *Lancet Gastroenterol. Hepatol.* 3 (1), 17–24. doi: 10.1016/S2468-1253(17)30338-2

Johnson, T., Macdonald, S., Hill, S. M., Thomas, A., and Murphy, M. S. (2006). Treatment of active crohn's disease in children using partial enteral nutrition with liquid formula: a randomised controlled trial. *Gut.* 55 (3), 356–361. doi: 10.1136/gut.2004.062554

Karolewska-Bochenek, K., Grzesiowski, P., Banaszkiewicz, A., Gawronska, A., Otowska, M., Dziekiewicz, M., et al. (2018). A two-week fecal microbiota transplantation course in pediatric patients with inflammatory bowel disease. *Adv. Exp. Med. Biol.* 1047, 81–87. doi: 10.1007/5584_2017_123

Kelly, C. R., Kahn, S., Kashyap, P., Laine, L., Rubin, D., Atreja, A., et al. (2015). Update on fecal microbiota transplantation 2015: Indications, methodologies, mechanisms, and outlook. *Gastroenterology.* 149 (1), 223–237. doi: 10.1053/j.gastro.2015.05.008

Kuenzig, M. E., Fung, S. G., Marderfeld, L., Mak, J. W.Y., Kaplan, G. G., Ng, S. C., et al. (2022). Twenty-first century trends in the global epidemiology of pediatric-onset inflammatory bowel disease: Systematic review. *Gastroenterology.* 162 (4), 1147–1159.e4. doi: 10.1053/j.gastro.2021.12.282

Levine, A., Griffiths, A., Markowitz, J., Wilson, D. C., Turner, D., Russell, R. K., et al. (2011). Pediatric modification of the Montreal classification for inflammatory bowel disease: the Paris classification. *Inflammation Bowel Dis.* 17 (6), 1314–1321. doi: 10.1002/ibd.21493

Li, S. S., Zhu, A., Benes, V., Costea, P. I., Hercog, R., Hildebrand, F., et al. (2016). Durable coexistence of donor and recipient strains after fecal microbiota transplantation. *Science.* 352 (6285), 586–589. doi: 10.1126/science.aad8852

Liu, S. X., Li, Y. H., Dai, W. K., Li, X. S., Qiu, C. Z., Ruan, M. L., et al. (2017). Fecal microbiota transplantation induces remission of infantile allergic colitis through gut microbiota re-establishment. *World J. Gastroenterol.* 23 (48), 8570–8581. doi: 10.3748/wjg.v23.i48.8570

Moriczi, M., Pujol-Muncunill, G., Martín-Masot, R., Jiménez Treviño, S., Segarra Cantón, O., Ochoa Sangrador, C., et al. (2020). Predictors of response to exclusive enteral nutrition in newly diagnosed crohn ´ s disease in children: PRESENCE study from SEGHNP. *Nutrients.* 12 (4), 1012. doi: 10.3390/nu12041012

Ng, S. C., Shi, H. Y., Hamidi, N., Underwood, F. E., Tang, W., Benchimol, E. I., et al. (2017). Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies [published correction appears in lancet. *Lancet.* 390 (10114), 2769–2778. doi: 10.1016/S0140-6736(17)32448-0

Peyrin-Biroulet, L., Sandborn, W., Sands, B. E., Reinisch, W., Bemelman, W., Bryant, R. V., et al. (2015). Selecting therapeutic targets in inflammatory bowel disease (STRIDE): Determining therapeutic goals for treat-to-Target. *Am. J. Gastroenterol.* 110 (9), 1324–1338. doi: 10.1038/ajg.2015.233

Ruemmele, F. M., Hyams, J. S., Otley, A., Griffiths, A., Kolho, K. L., Dias, J. A., et al. (2015). Outcome measures for clinical trials in paediatric IBD: an evidence-based, expert-driven practical statement paper of the paediatric ECCO committee. *Gut.* 64 (3), 438–446. doi: 10.1136/gutjnl-2014-307008

Schierová, D., Březina, J., Mrázek, J., Fliegerová, K. O., Kvasnová, S., Bajer, L., et al. (2020). Gut microbiome changes in patients with active left-sided ulcerative colitis after fecal microbiome transplantation and topical 5-aminosalicylic acid therapy. *Cells.* 9 (10), 2283. doi: 10.3390/cells9102283

Sokol, H., Landman, C., Seksik, P., Berard, L., Montil, M., Nion-Larmurier, I., et al. (2020). Fecal microbiota transplantation to maintain remission in crohn's disease: a pilot randomized controlled study. *Microbiome.* 8 (1), 12. doi: 10.1186/s40168-020-0792-5

Suskind, D. L., Brittnacher, M. J., Wahbeh, G., Shaffer, M. L., Hayden, H. S., Qin, X., et al. (2015). Fecal microbial transplant effect on clinical outcomes and fecal microbiome in active crohn's disease. *Inflamm Bowel Dis.* 21 (3), 556–563. doi: 10.1097/MIB.0000000000000307

Takagi, S., Utsunomiya, K., Kuriyama, S., Yokoyama, H., Takahashi, S., Iwabuchi, M., et al. (2006). Effectiveness of an 'half elemental diet' as maintenance therapy for crohn's disease: A randomized-controlled trial. *Aliment Pharmacol. Ther.* 24 (9), 1333–1340. doi: 10.1111/j.1365-2036.2006.03120.x

Taylor, H., Serrano-Contreras, J. I., McDonald, J. A. K., Epstein, J., Fell, J. M., Seoane, R. C., et al. (2020). Multiomic features associated with mucosal healing and inflammation in paediatric crohn's disease. *Aliment Pharmacol. Ther.* 52 (9), 1491–1502. doi: 10.1111/apt.16086

Verburgt, C. M., Ghiboub, M., Benninga, M. A., de Jonge, W. J., and Van Limbergen, J. E. (2021). Nutritional therapy strategies in pediatric crohn's disease. *Nutrients.* 13 (1), 212. doi: 10.3390/nu13010212

Vuitton, L., Marteau, P., Sandborn, W. J., Levesque, B. G., Feagan, B., Vermeire, S., et al. (2016). IOIBD technical review on endoscopic indices for crohn's disease clinical trials. *Gut.* 65 (9), 1447–1455. doi: 10.1136/gutjnl-2015-309903

Wang, Y., Ren, R., Sun, G., Peng, L., Tian, Y., and Yang, Y. (2020). Pilot study of cytokine changes evaluation after fecal microbiota transplantation in patients with ulcerative colitis. *Int. Immunopharmacol.* 85, 106661. doi: 10.1016/j.intimp.2020.10666

Wang, X. Q., Zhang, Y., Xu, C. D., Jiang, L. R., Huang, Y., Du, H. M., et al. (2013). Inflammatory bowel disease in Chinese children: a multicenter analysis over a decade from shanghai. *Inflammation Bowel Dis.* 19 (2), 423–428. doi: 10.1097/MIB.0b013e318286f9f2

Zou, B., Liu, S.-X., Li, X.-S., He, J. Y., Dong, C., Ruan, M. L., et al. (2022). Long-term safety and efficacy of fecal microbiota transplantation in 74 children: A single-center retrospective study. *Front. Pediatr.* 10. doi: 10.3389/fped.2022.964154

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Effect of metformin on sepsis-associated acute lung injury and gut microbiota in aged rats with sepsis

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Background: Recent studies reported the association between the changes in gut microbiota and sepsis, but there is unclear for the gut microbes on aged sepsis is associated acute lung injury (SALI), and metformin treatment for the change in gut microbiota. This study aimed to investigate the effect of metformin on gut microbiota and SALI in aged rats with sepsis. It also explored the therapeutic mechanism and the effect of metformin on aged rats with SALI.

Methods: Aged 20-21 months SD rats were categorized into three groups: sham-operated rats (AgS group), rats with cecal ligation and puncture (CLP)-induced sepsis (AgCLP group), and rats treated with metformin (100 mg/kg) orally 1 h after CLP treatment (AgMET group). We collected feces from rats and analyzed them by 16S rRNA sequencing. Further, the lung samples were collected for histological analysis and quantitative real-time PCR (qPCR) assay and so on.

Results: This study showed that some pathological changes occurring in the lungs of aged rats, such as hemorrhage, edema, and inflammation, improved after metformin treatment; the number of hepatocyte death increased in the AgCLP group, and decreased in the AgMET group. Moreover, metformin relieved SALI inflammation and damage. Importantly, the gut microbiota composition among the three groups in aged SALI rats was different. In particular, the proportion of E. coli and K. pneumoniae was higher in AgCLP group rats than AgS group rats and AgMET group rats; while metformin could increase the proportion of Firmicutes, Lactobacillus, Ruminococcus_1 and Lactobacillus_johnsonii in aged SALI rats. Moreover, Prevotella_9, Klebsiella and Escherichia_Shigella were correlated positively with the inflammatory factor IL-1 in the lung tissues; Firmicutes was correlated negatively with the inflammatory factor IL-1 and IL-6 in the lung tissues.

Conclusions: Our findings suggested that metformin could improve SALI and gut microbiota in aged rats, which could provide a potential therapeutic treatment for SALI in aged sepsis.

KEYWORDS

gut microbiota, metformin, sepsis, sepsis-associated acute lung injury (SALI), aged rats

Introduction

Sepsis refers to life-threatening organ dysfunction caused by the dysregulation of the host's response to infection (Singer et al., 2016), and it's mortality as high as 33%, which affects millions of people worldwide (Angus et al., 2001; Dellinger, 2003; Buchman et al., 2020). Furthermore, studies showed more than 60% septic patients are elderly people (>65 years), and an exponential increase in the incidence and mortality of sepsis in the elderly patients (Milbrandt et al., 2010; Mankowski et al., 2020). The lung is the first organ involved in the progress of sepsis. According to epidemiological data, about 50% of septic patients complicated with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) in the intensive care unit(ICU) (Sevransky et al., 2009; Aziz et al., 2018). As high mortality of the sepsis-related lung injury (SALI) patients and a poor prognosis in SALI suggest a lack of clinically feasible therapeutic methods. Therefore, studying the pathogenesis of SALI to search a new effective therapy method is necessary.

The gut microbiota is the the chief regulator in maintaining homeostasis of the host (Honda and Littman, 2016). The gut microbial composition changes with age (Yatsunenko et al., 2012), as such from infant to adult shows from dominant Bififidobacterium to Bacteroidetes and Clostridia genus, and to age shows the Centenarians' gut microbiota with pathogenic microbiota increasing, which different from the infant and adults (Mariat et al., 2009; Claesson et al., 2012; Rampelli et al., 2013). Furthermore, the above gut microbial composition varies indicates the decreased short-chain fatty acid (SCFA) production, leading to the intestinal inflammation and the mucosa reduction and gut permeability increasing. More importantly, the Centenarians' gut microbiota, Specifically, the relative abundance of the Faecalibacterium, Eubacteriaceae, Lactobacillus and Clostridia decreased and the relative abundance of the Proteobacteria and Bacilli is reduction, resulting the productive SCFA gut microbiota is decreasing, and yet changed pathogen is increasing in the elderly (Rampelli et al., 2013). Additionally, the previous study showed that chronic mild inflammation in the elderly, which might lead to an abnormal increase in intestinal wall permeability (Buford et al., 2018). Therefore, maintaining the balance of the gut microbiota and improving the gut permeability may be effective measures for treating age-related SALI.

Metformin is the first-line treatment in patients with type 2 diabetes (Flory and Lipska, 2019). Studies confirmed that metformin had an anti-inflammatory effect on the expression of inflammatory factors (Cameron et al., 2016; Wu et al., 2018). Furthermore, some studies showed that metformin delayed aging by reducing the production of reactive oxygen species (Valencia et al., 2017). Metformin is taken orally and absorbed mainly in the small intestine (Buse et al., 2016; Honda and Littman, 2016). Metformin also affects the resident intestinal microbiota and can correct intestinal damage by augmenting the intestinal microbiota (Lee et al., 2018; Maniar et al., 2018; Brandt et al., 2019). This study was performed to investigate the effect of metformin on the intestinal microbiota of senile rats with sepsis and assess whether metformin could be an effective drug for treating SALI.

Materials and methods

Animals

Twenty-four male Sprague–Dawley (SD) rats (6–8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and housed until 20–21 months. All rats were housed in a specific pathogen-free animal laboratory. The rats were fed a standard diet and purified water under controlled laboratory conditions (12-h light/dark cycle). They were randomly divided into three groups: sham-operated rats (AgS group, n=4), rats with cecal ligation and puncture (CLP)-induced sepsis (AgCLP group, n=12), and rats treated with oral metformin (100 mg/kg) 1 h after AgCLP treatment (AgMET group, n=8). The AgCLP model was used in this study, and anesthesia was provided by injecting hydantoin (10%, 3–4 mL/g) into the peritoneal cavity. Microsurgery was performed in the midline of the abdomen and involved ligating one half of the free end of the cecum, puncturing the cecum with a 21-gauge needle in two locations at the ligature site, and applying gentle pressure until the feces were extruded. The bowel was then placed back into the abdominal cavity, and the incision was closed. The procedure was performed by the same person to minimize the impact of different ligation and puncture sites on the results. After closing the incision, the rats were injected subcutaneously with normal saline (1 mL/100 g) at 37°C and placed back in the cage to rewarm for 1 h. In the AgS group, the rats underwent only open surgery and did not undergo ligation or puncture of the cecum. The rats in the metformin group were given metformin (25 mg/kg) intragastrically 1 h after the surgery. All rats were sacrificed 24 h after CLP treatment. Only five aged rats with SALI survived in the AgCLP group, and four aged rats with SALI survived in the AgMET group after 24 h of CLP treatment. This animal experiment was approved by the Life Science Ethics Review Committee of Zhengzhou University.

Histological analysis

The tissues from rat lungs were analyzed using hematoxylin and eosin (H&E) staining. The lung and kidney tissues were fixed in 4% paraformaldehyde for 24h and embedded in paraffin. The tissues were then stained and observed under a light microscope. The degree of alveolar congestion, hemorrhage, infiltration and aggregation of neutrophils or leucocytes, and alveolar wall thickness was observed. The lung sections were scored using the aforementioned indicators, with a maximum score of 16.

Terminal deoxynucleotide transferase d-UTP nick-end labeling assay

The lung tissues were paraffin-embedded and fixed, and the proportion of apoptotic cells was determined using a terminal deoxynucleotide transferase d-UTP nick-end labeling (TUNEL) assay and fluorescence microscopy.

16S rRNA gene sequencing for gut microbiota analysis

First, extraction of genome DNA: we used the CTAB/SDS method to extract the genome DNA, and its' concentration and purity was monitored on 1% agarose gels. And then we diluted the DNA to 1ng/μL by using sterile water based on the concentration. Next, the primers 341F (5'-CCTAyGGRBGCasCAG-3') and 806R (5'-GGA CTA CNN GGG TAT CTA AT-3') were used to amplify the 16S rRNA genes of 16SV3–V4 regions with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs) is used to performed all PCR reactions. Then, PCR Products quantification and qualification analysis. Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400-450bp(16S) and ITS (100-400bp) were chosen for further experiments. PCR products was mixed in equidensity ratios. Then, the Qiagen Gel Extraction Kit was used to purify the mixture PCR products (Qiagen, Germany).Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina NovaSeq 6000 platform and 250 bp paired-end reads were generated. According to 97% similarity, we used the Usearch (version 11.0.667) with no ambiguous bases to cluster. The Mothur v1.42.1 and the vegan

package in R-package were used to calculated the Alpha diversity and beta diversity, respectively. The PICRUSt2 software package (https://github.com/picrust/picrust2) used to calculate the pathway enrichment.

Quantification of mRNA using qRT–PCR

We used TRIzol reagent (TaKaRa, Tokyo, Japan) to extract total RNA from the lungs. The concentration and purity of RNA were quantified using ultraviolet spectroscopy. The corresponding cDNA was synthesized by reverse transcription of mRNA using a TaqMan reverse transcription kit (UE, Suzhou, China). All qRT-PCR was used 40 cycles for amplification, and the results were analyzed by the $2^{-\Delta\Delta}$CT method. The gene expression was normalized using reduced glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The gene primers were chemokine (C-C motif) ligand 7 (CCL7) forward primer: CTTCTGTGTGTGCTGCTCAAC, reverse primer: CTATGGCCTCCTCAACCCAC; interleukin (IL)-6 forward primer: AGAGACTTCCAGCCAGTTGC, reverse primer: AGTCTCCTCTCCGGACTTGT; CCL3 forward primer: TGCTGTTCTTCTCTGCACCA, reverse primer: CAGGTCCTTTGGGGTCAGC; IL-1β forward primer: GCAACTGTTCCTGAACTCAACT, reverse primer: ATCTTTTGGGGTCCGTCAACT; chemokine (C-X-C motif) ligand 1 (CXCL1) forward primer: CGCTCGCTTCTCTGTGCA, reverse primer: TTCTGAACCATGGGGGCTTC; and GAPDH forward primer: TGTGAACGGATTTGGCCGTA, reverse primer: GATGGTGATGGGTTTCCCGT.

Statistical analysis

The GraphPad Prism (Version 6.0; GraphPad Software Inc., USA) R-package were used to statistical analyses. The quantitative data was assessed by mean ± standard deviation. The unidirectional or bidirectional analysis of variance (ANOVA) was used for multiple groups, and an unpaired-sample Student *t* test was used for the statistical analysis in two groups. Furthermore, the Mothur v1.42.1 and the vegan package in R-package were used to calculated the Alpha diversity and beta diversity, respectively. The PICRUSt2 software package (https://github.com/picrust/picrust2) used to calculate the pathway enrichment. P value <0.05 indicated a statistically significant difference.

Results

Metformin alleviated the inflammation and lung injury in aged rats with SALI

A previous study (DeFronzo et al., 2016) reported that metformin may induce kidney failure result of lactic acidosis. Hence, the histological analysis to check whether metformin could induce kidney injury. The results showed that metformin did not cause kidney damage, confirming that the dose of metformin was safe to administer (Figure S1). We next performed H&E and TUNEL assays on lung tissues to assess the effect of metformin on SALI in aged rats. The results showed that lung tissue destruction, inflammatory infiltration, and alveolar wall thickening in the aged rats in the AgCLP group compared with the rats in the AgS group. However, lung tissue destruction and inflammatory infiltration were significantly improved in the AgMET group (Figures 1A, B, $P < 0.05$). The effect of metformin on apoptotic cells of lung tissues in aged rats with sepsis was determined by assessing the percentage of apoptotic cells through the TUNEL staining. The apoptotic cells of lung tissues significantly increased in aged rats with sepsis compared with that in AgS rats, and metformin treatment attenuated sepsis-induced apoptotic cells of lung tissues (Figures 1C, D, $P < 0.05$). Further, the mRNA expression of the inflammatory factors *CCL3*, *CCL7*, *CXCL1*, *IL-1*, and *IL-6* substantially increased in aged rats with SALI compared with the AgS group. However, metformin reversed the expression of these inflammatory factors induced by sepsis (Figures 1E, I, $P < 0.05$). Metformin improved the inflammatory response in rats with sepsis, which was consistent with our previously reported results (Liang et al., 2022). Hence, these data suggested that metformin attenuated lung injury and inflammation in aged rats with SALI.

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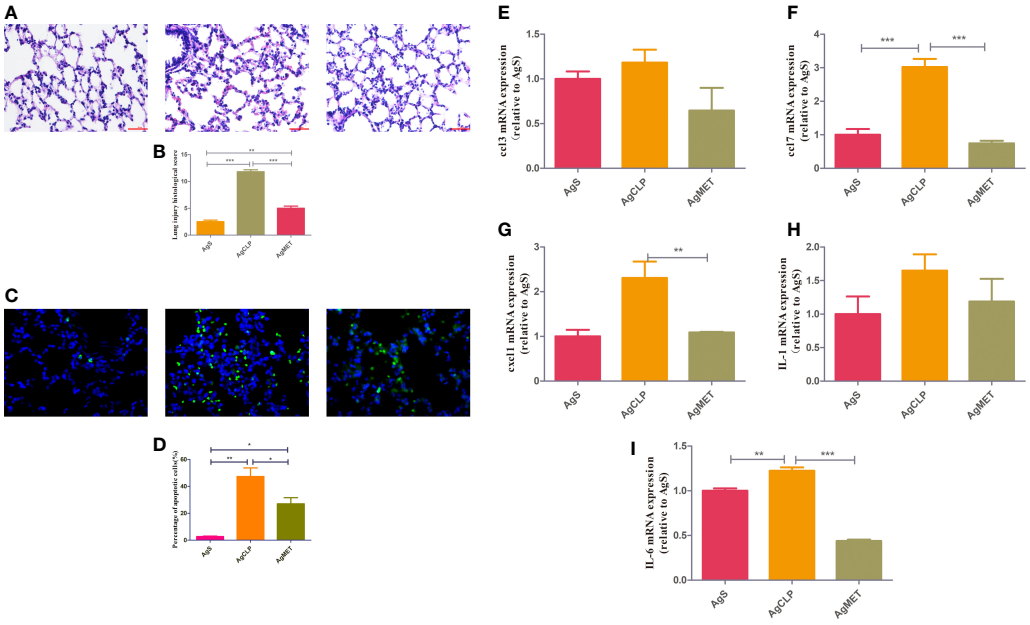

FIGURE 1
Metformin reduces SALI in aged rats with sepsis. **(A)** H&E staining showed edema and an increased amount of hemorrhage in the lung tissue of rats in the AgCLP group compared with the control group. These changes could be reversed after metformin treatment. **(B)** The level of lung injury was assessed semi-quantitatively based on the lung injury score ($P < 0.05$). **(C)** TUNEL results showed an increased apoptosis in the AgCLP group and a decreased apoptosis in the AgMET group. **(D)** Three sections were taken from each group, and the number of apoptotic cells was measured ($P < 0.05$). **(E-I)** ccl3, ccl7, cxcl1, IL-1 and IL-6 expression increased in AgCLP group and metformin could decreased these factors' expression. *p<0.05; **p<0.01; ***p<0.001.

Effects of metformin on the intestinal microbiota in aged rats

16S rRNA metagenomic analysis was used to assess the composition of gut microbiota, evaluating the effect of metformin on gut microbiota in aged rats with SALI. Alpha diversity (ACE, Chao1, Shannon, and Simpson indexes) can reflect the abundance of bacteria in the community. We used ACE and Chao1 to assess the abundance of microbiota. The results showed that metformin could improve the SALI induced the decreased Alpha diversity (Figure 2A). Next, we analyzed the β-diversity between bacterial populations. β-diversity assessed the differences of gut microbiota between multiple samples and the changes of microbiome under different factors. The results of β-diversity showed that compared with the AgCLP group, the gut microbiota in AgMET group was similar to that in the AgS group (Figure 2B).

We explored the effect of metformin on the abundance of gut microbiota. The results showed that compared with the AgS and AgCLP groups, the AgMET group had a higher *Firmicutes/Bacteroidetes* ratio (Figure S2). At the genus level, the abundance of *Lactobacillus* slightly increased, while the abundance of *Ruminococcus-1* decreased in the AgCLP group compared with the AgS group, and increased after metformin treatment. Furthermore, the increased relative abundance of *Prevotella-9* in the AgCLP group compared with the AgS group, which related with inflammation, while metformin could reverse this change. At the species level, the abundance of *Lactobacillus johnsonii* slightly increased, and *Escherichia coli* and *K. pneumoniae* increased in the AgCLP group compared with the AgS and AgMET groups (Figures 3A, B). In the AgCLP group, the increase in the abundance of these opportunistic pathogens increased the inflammatory response of the host, which was consistent with our previous findings (Liang et al., 2022). Furthermore, the abundance of *Romboutsia* and *Ruminococcus-1* decreased and the abundance of *Proteobacteria*, *Escherichia coli*, and *K. pneumoniae* increased in AgCLP group compared with that in the AgMET group, indicating a decrease in the abundance of intestinal microbiota associated with SCFA production. In addition, we analyzed the correlations between inflammatory factors and gut microbiota (Figure 4), the results show that *Klebsiella pneumoniae, Escherichia coli, Prevotella-9* and *Proteobacteria* were positively correlated with iL-6, while Romboutsia, Latobacillus, Latobacillus_johnsonii,Firmicutes were negatively correlated with IL-1 and IL-6. Therefore, metformin administration could improve the gut microbiota disorder in aged rats with SALI.

Discussion

This study assessed the effect of metformin for alleviating inflammation, lung injury and gut microbiota disorder in aged rats with sepsis. Metformin treatment reversed the pathological changes including lung tissue damage, hemorrhage, and edema in aged rats with CLP-induced sepsis. Meanwhile, significant apoptotic cells of lung tissues in the AgCLP group but with a considerable improvement in the AgMET group. The gut microbiota composition in the AgMET group varied from such as an increasing relative abundance of some opportunistic pathogen

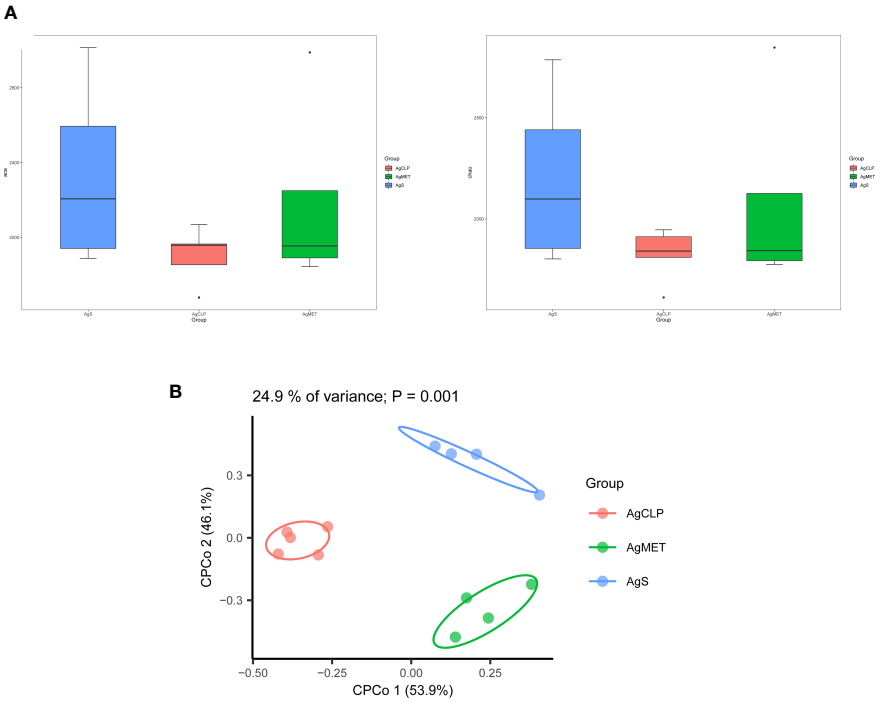

FIGURE 2
Effect of metformin on intestinal microbiota diversity in aged rats with sepsis. **(A)**16S rRNA metagenomic analysis for α-diversity (ACE, Chao1); **(B)** β-diversity reflected microbial richness in groups and between groups.

such as *E. coli* and *K. pneumoniae*, relating with LPS production and inflammation to anti-inflammation, which exerted the protection of aged rats with SALI. These results showed that metformin is a therapeutic alternative for treating aged SALI.

Metformin is a clinical first-line hypoglycemic drug as its specific antihyperglycemic properties with excellent safety profile. Metformin also has other functions such as anti-aging and anti-tumor effects (Shafiee et al., 2014; Pryor et al., 2019). Furthermore, enhanced the lifespan of *Caenorhabditis* by affecting the metabolism of microbial folate and methionine by modifying the gut microbiota (Cabreiro et al., 2013). Metformin could inhibit IκB kinase/nuclear factor-κB activation to suppress the expression of senescence-related factors (Moiseeva et al., 2013). It was later discovered that fecal microbial transplantation decreased the expression of IL-18 (Lee et al., 2019). Metformin improved the intestinal barrier function by modulating the gut microbiota, thereby increasing the number of mucus-producing goblet cells (Hur and Lee, 2015). Additionally, metformin inhibited apoptosis *via* the phosphoinositide 3-kinase/Akt signaling pathway, which was found to be effective in brain injury caused by sepsis (Tang et al., 2017). These studies also supported our findings.

The *Firmicutes* and *Bacteroidetes* is the main gut microbiota in human, accounting for 75.9% and 10.83%, respectively (Pan et al., 2018), which was consistent with the results of this study. We found that the abundance of *Firmicutes* decreased in the AgCLP group compared with the AgS group, and this change was reversed after metformin treatment. *Firmicutes* are mainly Gram-negative bacteria, consisting of specialized anaerobes or parthenogenic anaerobes, of which *Faecalibacterium* is involved in the formation of butyric acid, while *Dialister* is engaged in the final phase of propionic acid production (Nava et al., 2011; Tanca et al., 2017). The abundance of *Firmicutes* decreased noticeably in type 2 diabetes. In this study, AgCLP caused variations in the abundance of intestinal microbiota, such as a decline in the abundance of lactic acid–producing bacteria and probiotics and an increase in the abundance of opportunistic pathogenic bacteria associated with inflammation, such as *E. coli* and *K. pneumoniae*. Metformin treatment reversed these changes, resulting in an increase in the abundance of *L. johnsonii* and a decline in the abundance of *E. coli* and *K. pneumoniae*. *Bacteroides thetaiotaomicron* and *L. johnsonii* reduced the infiltration of intestinal inflammatory cells, alleviated edema, disrupted the cell wall mannans of *Candida albicans*, and inhibited the development of *C. albicans* (Charlet et al., 2020). In sepsis, intestinal barrier dysfunction and increased permeability contribute to the pathological transfer of intestinal bacteria or endotoxins, worsening sepsis (Hassoun et al., 2001; Meng et al., 2017). Metformin further enhances intestinal barrier function by increasing the number of villi through the modulation of intestinal microbiota, such as *Firmicutes* and lactic acid–producing bacteria. *Prevotella* interacts with the immune system and enhances mucosal inflammation mediated by TH17, stimulating epithelial cells to produce inflammatory factors such as IL-8 and IL-6 (Zeng et al., 2019), which agreed with our results.

This study had some limitations. First, the sample size in the experimental groups was small, and this findings should be confirmed by other similar studies, in the other hand, in the next study, we could be further verify the findings. Second, we did not include the metformin-alone group, reducing the rigor of the study.

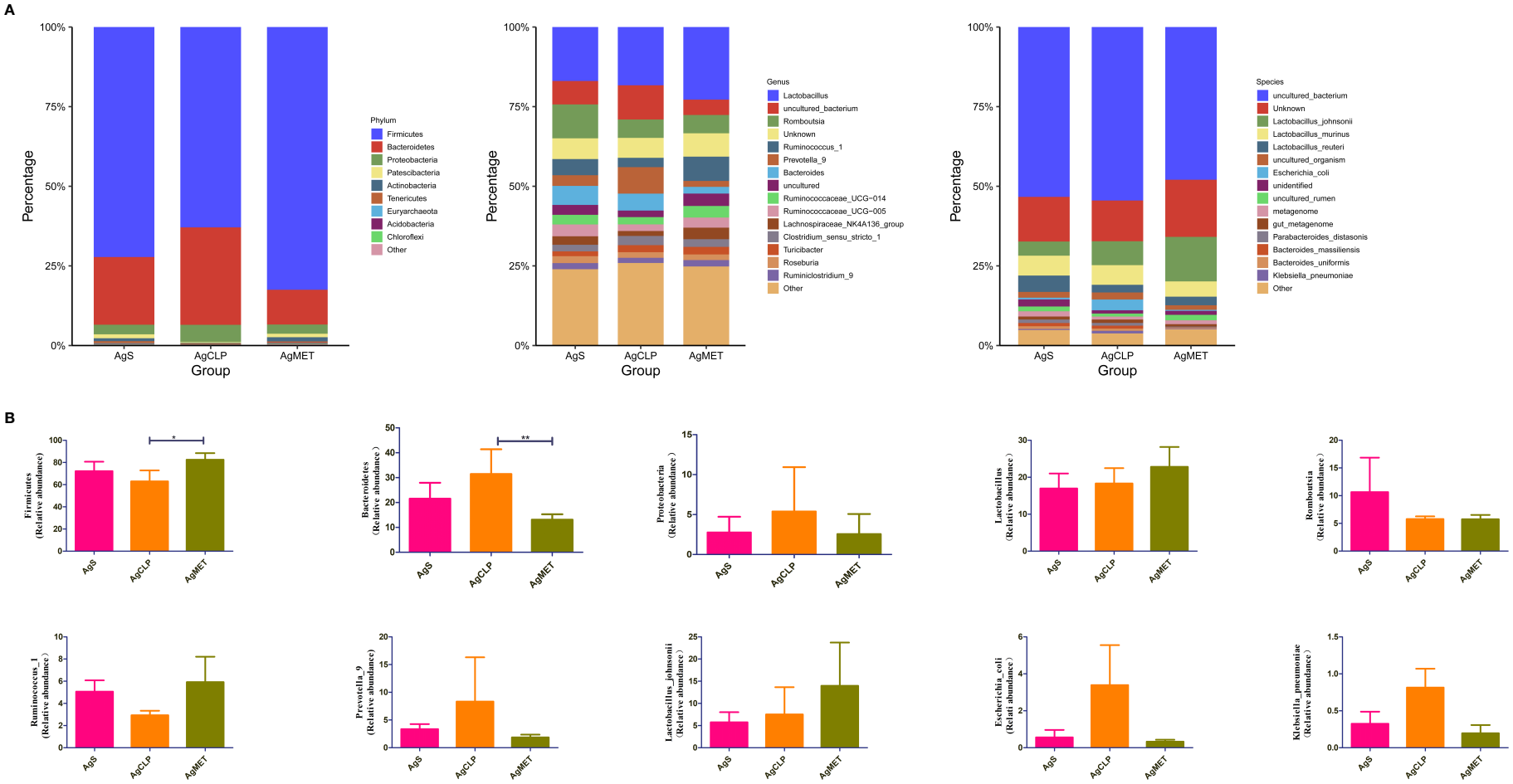

FIGURE 3
Effects of metformin on intestinal microbiota. **(A)** Three figures show the differences between the microbiota in the AgMET, AgS, and AgCLP groups at the phylum, genus, and species levels, respectively. The abundance of opportunistic pathogenic bacteria increased in the AgCLP group. **(B)** Histogram showing the differential microbiota, highlighting the changes in intestinal microbiota between the experimental groups. *p<0.05; **p<0.01.

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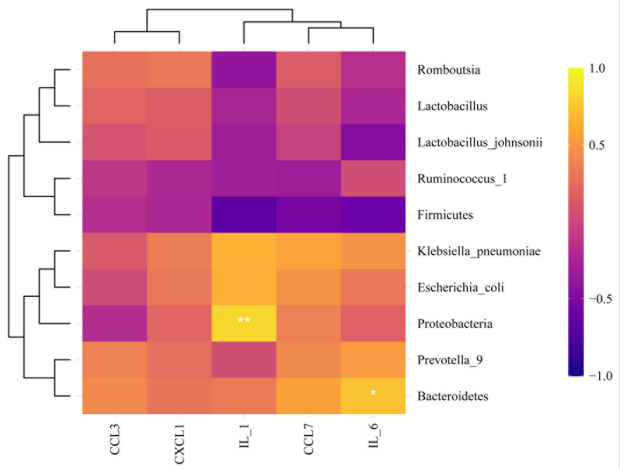

FIGURE 4
Correlation analysis. Correlation analysis between microbiota and inflammatory parameters.

However, after metformin treatment, the kidney injury had no difference between AgCLP group and AgMET group, which suggested the dose of metformin is safe. More importantly, our previous study (Liang et al., 2022) search the effect of metformin alone group on septic aged rats.

Conclusions

This study indicated that metformin could relieve inflammation, lung injury and gut microbiota in aged rats with SALI. More importantly, metformin reversed the imbalance of gut microbiota such as as increasing relative abundance of opportunistic pathogen such as *E. coli* and *K. pneumoniae*. in aged rats with sepsis, which could provide a potential treatment for aged SALI.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA938428.

Ethics statement

The animal study was reviewed and approved by the Life Science Ethics Review Committee of the Affiliated Hospital of Qingdao University.

Author contributions

RZ and YW designed the study. SW, YN, BD, YL and ZL performed the experiments and collected the animal sample, and they also conducted the data analysis. YW wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1139436/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Metformin alleviated sepsis-associated acute kidney injury.

SUPPLEMENTARY FIGURE 2
Firmicutes/*Bacteroidetes* ratio ($P < 0.05$).

References

Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., Carcillo, J., and Pinsky, M. R. (2001). Epidemiology of severe sepsis in the united states: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* 29 (7), 1303–1310. doi: 10.1097/00003246-200107000-00002

Aziz, M., Ode, Y., Zhou, M., Ochani, M., Holodick, N. E., Rothstein, T. L., et al. (2018). B-1a cells protect mice from sepsis-induced acute lung injury. *Mol. Med.* 24 (1), 26. doi: 10.1186/s10020-018-0029-2

Brandt, A., Hernández-Arriaga, A., Kehm, R., Sánchez, V., Jin, C. J., Nier, A., et al. (2019). Metformin attenuates the onset of non-alcoholic fatty liver disease and affects intestinal microbiota and barrier in small intestine. *Sci. Rep.* 9 (1), 6668. doi: 10.1038/s41598-019-43228-0

Buchman, T. G., Simpson, S. Q., Sciarretta, K. L., Finne, K. P., Sowers, N., Collier, M., et al. (2020). Sepsis among medicare benefificiaries: 1. *burdens sepsis 2012-2018. Crit. Care Med.* 48 (3), 276–288. doi: 10.1097/CCM.0000000000004224

Buford, T. W., Carter, C. S., VanDerPol, W. J., et al. (2018). Composition and richness of the serum microbiome diffffer by age and link to systemic inflflammation. *GeroScience.* 40 (3), 257–268. doi: 10.1007/s11357-018-0026-y

Buse, J. B., DeFronzo, R. A., Rosenstock, J., Kim, T., Burns, C., Skare, S., et al. (2016). The primary glucose-lowering effect of metformin resides in the gut, not the circulation: Results from short-term pharmacokinetic and 12-week dose-ranging studies. *Diabetes Care* 39 (2), 198–205. doi: 10.2337/dc15-0488

Cabreiro, F., Au, C., Leung, K.-Y., Vergara-Irigaray, N., Cochemé, H. M., Noori, T., et al. (2013). Metformin retards aging in c. elegans by altering microbial folate and methionine metabolism. *Cell.* 153 (1), 228–239. doi: 10.1016/j.cell.2013.02.035

Cameron, A. R., Morrison, V. L., Levin, D., Mohan, M., Forteath, C., Beall, C., et al. (2016). Anti-inflammatory effects of metformin irrespective of diabetes status. *Circ. Res.* 119 (5), 652–665. doi: 10.1161/CIRCRESAHA.116.308445

Charlet, R., Bortolus, C., Sendid, B., and Jawhara, S. (2020). Bacteroides thetaiotaomicron and lactobacillus johnsonii modulate intestinal inflammation and eliminate fungi *via* enzymatic hydrolysis of the fungal cell wall. *Sci. Rep.* 10 (1), 11510. doi: 10.1038/s41598-020-68214-9

Claesson, M. J., Jeffffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., et al. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature.* 488 (7410), 178–184. doi: 10.1038/nature11319

DeFronzo, R., Fleming, G. A., Chen, K., and Bicsak, T. A. (2016). Metformin associated lactic acidosis: current perspectives on causes and risk. *Metabolism.* 65 (2), 20–29. doi: 10.1016/j.metabol.2015.10.014

Dellinger, R. P. (2003). Cardiovascular management of septic shock. *Crit. Care Med.* 31 (3), 946–955. doi: 10.1097/01.CCM.0000057403.73299.A6

Flory, J., and Lipska, K. (2019). Metformin in 2019. *JAMA.* 321 (19), 1926–1927. doi: 10.1001/jama.2019.3805

Hassoun, H. T., Kone, B. C., Mercer, D. W., Moody, F. G., Weisbrodt, N. W., and Moore, F. A. (2001). Post-injury multiple organ failure: the role of the gut. *Shock.* 15 (1), 1–10. doi: 10.1097/00024382-200115010-00001

Honda, K., and Littman, D. R. (2016). The microbiota in adaptive immune homeostasis and disease. *Nature* 535 (7610), 75–84. doi: 10.1038/nature18848

Hur, K. Y., and Lee, M. S. (2015). Gut microbiota and metabolic disorders. *Diabetes Metab. J.* 39 (3), 198–203. doi: 10.4093/dmj.2015.39.3.198

Lee, H., Kim, J., An, J., Lee, S., Choi, D., Kong, H., et al. (2019). Downregulation of IL-18 expression in the gut by metformin-induced gut microbiota modulation. *Immune Netw.* 19 (4), e28. doi: 10.4110/in.2019.19.e28

Lee, H., Lee, Y., Kim, J., An, J., Lee, S., Kong, H., et al. (2018). Modulation of the gut microbiota by metformin improves metabolic profiles in aged obese mice. *Gut Microbes* 9 (2), 155–165. doi: 10.1080/19490976.2017.1405209

Liang, H., Song, H., Zhang, X., Song, G., Wang, Y., Ding, X., et al. (2022). Metformin attenuated sepsis-related liver injury by modulating gut microbiota. *Emerg. Microbes Infect.* 11 (1), 815–828. doi: 10.1080/22221751.2022.2045876

Maniar, K., Singh, V., Moideen, A., Bhattacharyya, R., Chakrabarti, A., and Banerhee, D. (2018). - inhalational supplementation of metformin butyrate: A strategy for prevention and. *BioMed. Pharmacother.* 107, 495–506. doi: 10.1016/jbiopha201808021

Mankowski, R. T., Anton, S. D., Ghita, G. L., Brumback, B., Cox, M. C., Mohr, A. M., et al. (2020). Older sepsis survivors suffer persistent disability burden and poor long-term survival. *J. Am. Geriatr. Soc* 68 (9), 1962–1969. doi: 10.1111/jgs.16435

Mariat, D., Firmesse, O., Levenez, F., Guimarães, V., Sokol, H., Doré, J., et al. (2009). The fifirmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* 9 (123). doi: 10.1186/1471-2180-9-123

Meng, M., Klingensmith, N. J., and Coopersmith, C. M. (2017). New insights into the gut as the driver of critical illness and organ failure. *Curr. Opin. Crit. Care* 23 (2), 143–148. doi: 10.1097/MCC.0000000000000386

Milbrandt, E. B., Eldadah, B., Nayfield, S., Hadley, E., and Angus, D. C. (2010). Toward an integrated research agenda for critical illness in aging. *Am. J. Respir. Crit. Care Med.* 182 (8), 995–1003. doi: 10.1164/rccm.200904-0630CP

Moiseeva, O., Deschênes-Simard, X., St-Germain, E., Igelmann, S., Huot, G., Cadar, A. E., et al. (2013). Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-κB activation. *Aging Cell.* 12 (3), 489–498. doi: 10.1111/acel.12075

Nava, G. M., Friedrichsen, H. J., and Stappenbeck, T. S. (2011). Spatial organization of intestinal microbiota in the mouse ascending colon. *ISME J.* 5 (4), 627–638. doi: 10.1038/ismej.2010.161

Pan, H., Guo, R., Zhu, J., Wang, Q., Ju, Y., Xie, Y., et al. (2018). A gene catalogue of the sprague-dawley rat gut metagenome. *Gigascience* 7 (5), giy055. doi: 10.1093/gigascience/giy055

Pryor, R., Norvaisas, P., Marinos, G., Best, L., Thingholm, L. B., Quintaneiro, L. M., et al. (2019). Host microbe-drug-nutrient screen identifiies bacterial effffectors of metformin therapy. *Cell.* 178 (6), 1299–312.e29. doi: 10.1016/j.cell.2019.08.003

Rampelli, S., Candela, M., Turroni, S., Biagi, E., Collino, S., Franceschi, C., et al. (2013). Functional metagenomic profifiling of intestinal microbiome in extreme ageing. *Aging.* 5 (12), 902–912. doi: 10.18632/aging.100623

Sevransky, J. E., Martin, G. S., Shanholtz, C., Mendez-Tellez, P. A., Pronovost, P., Brower, R., et al. (2009). Mortality in sepsis versus non-sepsis induced acute lung injury. *Crit. Care* 13 (5), R150. doi: 10.1186/cc8048

Shafiee, M. N., Khan, G., Ariffin, R., Abu, J., Chapman, C., Deen, S., et al. (2014). Preventing endometrial cancer risk in polycystic ovarian syndrome (PCOS) women: could metformin help? *Gynecol Oncol.* 132 (1), 248–253. doi: 10.1016/j.ygyno.2013.10.028

Singer, M., Deutschman, C. S., Seymour, C. W., Shankar-Hari, M., Annane, D., Bauer, M., et al. (2016). The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA.* 315 (8), 801–810. doi: 10.1001/jama.2016.0287

Tanca, A., Abbondio, M., Palomba, A., Fraumene, C., Manghina, V., Cucca, F., et al. (2017). Potential and active functions in the gut microbiota of a healthy human cohort. *Microbiome.* 5 (1), 79. doi: 10.1186/s40168-017-0293-3

Tang, G., Yang, H., Chen, J., Shi, M., Ge, L., Ge, X., et al. (2017). Metformin ameliorates sepsis-induced brain injury by inhibiting apoptosis, oxidative stress and neuroinflammation *via* the PI3K/Akt signaling pathway. *Oncotarget.* 8 (58), 97977–97989. doi: 10.18632/oncotarget.20105

Valencia, W. M., Palacio, A., Tamariz, L., and Florez, H. (2017). Metformin and ageing: improving ageing outcomes beyond glycaemic control. *Diabetologia.* 60 (9), 1630–1638. doi: 10.1007/s00125-017-4349-5

Wu, K., Tian, R., Huang, J., Yang, Y., Dai, J., Jiang, R., et al. (2018). Metformin alleviated endotoxemia-induced acute lung injury *via* restoring AMPK-dependent suppression of mTOR. *Chem. Biol. Interact.* 291, 1–6. doi: 10.1016/j.cbi.2018.05.018

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature.* 486 (7402), 222–227. doi: 10.1038/nature11053

Zeng, Q., Li, D., He, Y., Li, Y., Yang, Z., Zhao, X., et al. (2019). Discrepant gut microbiota markers for the classification of obesity-related metabolic abnormalities. *Sci. Rep.* 9 (1), 13424. doi: 10.1038/s41598-019-49462-w

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The gut microbiome: A line of defense against tuberculosis development

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The tuberculosis (TB) burden remains a significant global public health concern, especially in less developed countries. While pulmonary tuberculosis (PTB) is the most common form of the disease, extrapulmonary tuberculosis, particularly intestinal TB (ITB), which is mostly secondary to PTB, is also a significant issue. With the development of sequencing technologies, recent studies have investigated the potential role of the gut microbiome in TB development. In this review, we summarized studies investigating the gut microbiome in both PTB and ITB patients (secondary to PTB) compared with healthy controls. Both PTB and ITB patients show reduced gut microbiome diversity characterized by reduced *Firmicutes* and elevated opportunistic pathogens colonization; *Bacteroides* and *Prevotella* were reported with opposite alteration in PTB and ITB patients. The alteration reported in TB patients may lead to a disequilibrium in metabolites such as short-chain fatty acid (SCFA) production, which may recast the lung microbiome and immunity *via* the "gut-lung axis". These findings may also shed light on the colonization of *Mycobacterium tuberculosis* in the gastrointestinal tract and the development of ITB in PTB patients. The findings highlight the crucial role of the gut microbiome in TB, particularly in ITB development, and suggest that probiotics and postbiotics might be useful supplements in shaping a balanced gut microbiome during TB treatment.

KEYWORDS

gut microbiome, *Mycobacterium tuberculosis*, *Firmicutes*, *Bacteroidetes*, short-chain fatty acids, tuberculosis

1 Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is one of the leading infectious disease killers worldwide (Avoi and Liaw, 2021). According to the latest WHO report, it is estimated that a quarter of the global population is infected with *M. tuberculosis*. Even though only about 5-10% of infected people develop active TB, in 2020 alone, the incidence of TB was about 127 cases per 100,000 people, and approximately 1.3 million HIV-negative people died of TB (WHO, 2021). Furthermore, most TB cases were

reported in less developed regions, especially in South-East Asia, Africa, and the Western Pacific regions (WHO, 2021). However, the incidence might be underestimated as in some areas, especially in sub-Saharan Africa, the diagnosis of TB is still a challenge, and it is estimated that approximately 50% of TB cases remain undiagnosed (Mnyambwa et al., 2021; Jayasooriya et al., 2022). In the year 2015, all WHO members adopted the WHO's End TB strategy which aims to reduce the absolute number of TB deaths by 95% and the incidence rate by 90% by 2035 compared to the 2015 baseline. Six years have passed, and the incidence of TB has only dropped by 10%. With only 13 years left, the situation is still challenging.

TB is transmitted by cough-generated aerosols from patients, and it primarily affects the lungs, causing pulmonary tuberculosis (PTB) (Tan et al., 2020). However, it can also involve other parts of the body. TB that affects areas outside the lungs is called extrapulmonary tuberculosis. Approximately 1-3% of total TB cases (Sheer and Coyle, 2003; Cho et al., 2018) and 10% of all extrapulmonary tuberculosis cases involve the gastrointestinal tract, causing intestinal tuberculosis (ITB) (Abu-Zidan and Sheek-Hussein, 2019; Maulahela et al., 2022). Swallowing of sputum in PTB patients has a certain chance of causing ITB (Gan et al., 2016). This is because *M. tuberculosis* is more resistant to the gastric acid barrier due to its special cell wall structure (Vandal et al., 2009). However, not all PTB patients develop ITB, as they might benefit from the protective effect of the intestinal barrier.

The intestinal barrier is a highly complex system, including the outer mucus layer, the epithelial layer, the underlying lamina propria, and components such as commensal microbiota, antimicrobial peptides, secretory immunoglobulin A, and immune cells (König et al., 2016; Vancamelbeke and Vermeire, 2017). Intestinal microbiota with a complex and dynamic microbial community is of vital importance to human health (Chen et al., 2021). It can not only regulate host physiological processes such as digestion, nutrient absorption, and metabolism, but also modulate host immunity in protection against pathogens and toxins (Wang et al., 2017; Comberiati et al., 2021). It is of great importance in gut homeostasis and colonization resistance to exogenous pathogens (Ducarmon et al., 2019), and dysbiosis in microbiome composition can result in susceptibility to infections and disease development (Budden et al., 2017). It is reported that altered microbiota composition can cause increased epithelial permeability and disruption in the mucus layer, resulting in susceptibility to *Clostridioides difficile* (Bien et al., 2013) and *Citrobacter rodentium* infection (Wlodarska et al., 2011). A recent study in patients with COVID-19 observed significant gut dysbiosis with enrichment of opportunistic pathogens (Zuo et al., 2020). Therefore, the gut microbiome of the host might also be crucial in preventing TB infection or decelerating the disease progression (Hu et al., 2019b).

With the universal application of Next-Generation Sequencing and bioinformatic analysis, there are increasing studies investigating the association between *M. tuberculosis* infection and alteration of gut microbiota. Here, we reviewed all the previous reports on the intestinal microbiome in active TB patients (including PTB and ITB) without any treatment, summarized their main findings, and tried to deduce the reasons for ITB development in PTB patients.

2 Alteration of gut microbiome in active TB patients

M. tuberculosis infection is known to cause dysregulation of the immune system, resulting in dysregulation of the gut microbiome (Osei Sekyere et al., 2020). In this review, we included studies referring to the alterations in the gut microbiome of TB patients (Luo et al., 2017; Maji et al., 2018; Huang et al., 2019; Hu et al., 2019a; Hu et al., 2019b; Li et al., 2019; Namasivayam et al., 2020; Cao et al., 2021; He et al., 2021; Naidoo et al., 2021; Shi et al., 2021; Ding et al., 2022; Wang S. et al., 2022; Wang Y. et al., 2022; Yang et al., 2022; Ye et al., 2022; Yoon et al., 2022). All patients included in the study were without antibiotic treatment, as the antibiotics can result in dysbiosis and mask the results caused by *M. tuberculosis* infection (Hu et al., 2019a; Namasivayam et al., 2020). The main findings are summarized in Table 1 and Figure 1. The study design and sequencing techniques used in these studies are also included.

Most of the studies found a decreased alpha-diversity in TB patients (Maji et al., 2018; Hu et al., 2019a; Hu et al., 2019b; Li et al., 2019; Namasivayam et al., 2020; Cao et al., 2021; He et al., 2021; Shi et al., 2021; Ding et al., 2022; Wang S. et al., 2022; Wang Y. et al., 2022; Yang et al., 2022; Ye et al., 2022; Yoon et al., 2022), with only one exception reporting increased diversity in both newly diagnosed PTB and recurrent PTB patients (Luo et al., 2017). However, it should be noted that the study by Luo et al. reported a significant difference in the age structure between the healthy control group and the two TB patient groups (Luo et al., 2017), which might have contributed to the observed enhancement in gut microbiome diversity. In a mouse model challenged with *M. tuberculosis*, dysbiosis resembling that observed in TB patients was observed in TB patients was reported (Winglee et al., 2014). The authors found a rapid initial post-infection reduction in alpha-diversity of the gut microbiome followed by slight recovery of diversity until death (Winglee et al., 2014). They proposed that the change in gut microbiome was due to the crosstalk between microbiota and immune system activation, while the recovery of diversity indicated the attainment of balance.

The dysbiosis observed in the gut microbiome of TB patients at the taxonomic level was mainly in the following aspects.

2.1 *Firmicutes*

Firmicutes, which play a role in nutrition and metabolism (Stojanov et al., 2020), are the most abundant microbiome in the healthy human colon, comprising 64% of the gut microbiome (Piccioni et al., 2022). The imbalance in the ratio of *Firmicutes*/*Bacteroides* was also reported to indicate disrupted intestinal homeostasis, pathogen invasion, or unhealthy conditions

TABLE 1 Studies investigating the alteration of gut microbiome in pulmonary tuberculosis patients or intestinal tuberculosis patients without antibiotics comparing with the healthy controls.

Study design		Change in diversity	Change in microbiota composition	Sequencing technology	Literature
Patients	Controls				
Stool samples from active PTB patients (n=29)	Stool samples from healthy controls (n=22)	decreased alpha-deversity	*Bifidobacterium* and *Prevotella* decreased in patients	16S rRNA gene amplicon (Illumina) sequencing	(Cao et al., 2021)
			Bacteroidetes increased in patients		
Stool samples from PTB patients (n=10)	Stool samples from healthy controls (n=20)	decreased alpha-deversity	*Bacteroidetes*, *Clostridales*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotella*, *Romboutsia*, *Dialister*, *Gemmiger*, *Collinsella* and *Roseburia* decreased in patients;	16S rRNA gene amplicon (Illumina) sequencing	(Ding et al., 2022)
			Proteobacteria, *Actinobacteria*, *Bifidobacteriales*, *Coriobacteriales*, *Rhizobiales*, *Bifidobacteriaceae*, *Coriobacteriaceae*, *Caulobacteraceae*, *Phyllobacteriaceae*, *Burkholderiaceae*, *Granulicatella*, *Solobacterium*, *Erysipelotrichaceae unclassified* and *Actinomyces* increased in patients		
Colon biopsy samples from ITB patients (n=6)	Colon biopsy samples from healthy controls (n=4)	no significant difference	*Firmicutes*, *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Collinsella*, *Dorea*, *Oscillibacter*, *Ruminococcus* decreased in patients;	16S rRNA gene amplicon (Illumina) sequencing	(He et al., 2021)
			Proteobacteria, *Enterobacteriaceae*, *Lactobacillus*, *Pseudomonas*, *Klebsiella*, *Mycobacterium* increased *in patients*		
Stool samples from PTB patients (n=30)	Stool samples from healthy controls (n=52)	decreased alpha-deversity	*Roseburia hominis*, *Roseburia inulinivorans*, *Roseburia intestinalis*, *Eubacterium rectale*, *Coprococcus comes*, *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Ruminococcus obeum*, *Akkermansia muciniphila*, *Haemophilus parainfluenzae* decreased in patients;	Shotgun metagenomic Illumina sequencing	(Hu et al., 2019a)
			unclassified *Coprobacillus bacterium*, *Clostridium bolteae* increased in patients		
Stool samples from active PTB patients (n=28), latent PTB (n=10)	Stool samples from healthy controls (n=13)	minor decreased alpha-deversity	*Bacteroides* slightly increased in patients	16S rRNA gene amplicon (Illumina) sequencing	(Hu et al., 2019b)
Stool samples from active PTB patients (n=25), latent PTB (n=32)	Stool samples from healthy controls (n=23)	not reported	*Firmicutes*/*Bacteroidetes* ratio decreased in patients;	16S rRNA gene amplicon (Illumina) sequencing	(Huang et al., 2019)
			Bacteroidetes increased in patients		
Stool samples from PTB patients (n=18)	Stool samples from healthy controls (n=18)	decreased alpha-deversity	*Bifidobacteriaceae*, *Ruminococcaceae*, *Bacteroidaceae*, *Faecalibacterium*, *Faecalibacterium prausnitzii* decreased in patients;	16S rRNA gene amplicon (454) pyrosequencing	(Li et al., 2019)
			Prevotellaceae, *Enterococcus* increased in patients		
Stool samples from new PTB patients (n=19), recurrent PTB (n=18)	Stool samples from healthy controls (n=20) but with younger age structure and more female	increased alpha-diversity	*Bacteroidetes* and *Coprococcus* depletion in RTB and NTB;	16S rRNA gene amplicon (Illumina) sequencing	(Luo et al., 2017)
			Firmicutes decreased in RTB, *Roseburia* decreased in NTB, *Lachnospira* and *Prevotella* decreased in both NTB and RTB patients;		
			Actinobacteria, *Proteobacteria*, *Streptococcus* increased in both NTB and RTB patients, *Escherchia* and *Collinsella* increased in RTB		
Stool samples from PTB patients (n=6)	Stool samples from healthy blood relatives of each patient (n=6)	decreased alpha-deversity	*Bifidobacterium* decreased and *Prevotella* depletion in patients;	16S rRNA gene amplicon (Illumina) sequencing; faecal whole genome shotgun sequencing (Illumina)	(Maji et al., 2018)
			Faecalibacterium, *Coprococcus*, *Phascolarctobacterium*, *Pseudobutyrivibrio*, *Bacteroides*, *Eubacterium rectale*, *Phascolarctobacterium succinatutens*, *Roseburia inulinivorans*, *Faecalibacterium prausnitzii*, *Shigella sonnei*, *Escherichia Coli*, *Streptococcus pneumoniae*, *Streptococcus vestibularis* were increased in patients		

(Continued)

TABLE 1 Continued

Study design		Change in diversity	Change in microbiota composition	Sequencing technology	Literature
Patients	Controls				
Stool samples from PTB patients (n=58) and symptomatic controls (n=47)	Stool samples from close contacts PTB cases (n=73) and close contacts of symptomatic controls (n=82)	inconclusive	*Erysipelotrichaceae*, *Anaerostipes* and *Blautia* increased in patients	16S rRNA gene amplicon (Illumina) sequencing	(Naidoo et al., 2021)
Stool samples from new *M. tuberculosis* PTB patients (n=21)	Stool samples from healthy controls (n=10)	decreased alpha-deversity	*Bacteroidetes*, *Actinobacteria*, *Veillonellaceae*, *Succinivibrionaceae* and *Crocinitomicaceae* decreased in patients	16S rRNA gene amplicon (Illumina) sequencing	(Namasivayam et al., 2020)
Stool samples from PTB patients with antibiotics (n=39) and PTB patients without antibiotics (n=55)	Stool samples from TB negative controls (n=62)	decreased alpha-deversity	*Lachnospiraceae*, *Lachnoclostridium*, *Anaeroglobus* decreased in PTB patients without antibiotics; *Enterococcus*, *Clostridiales* and *Rothia* increased in patients	16S rRNA gene amplicon (454) pyrosequencing	(Shi et al., 2021)
Stool samples from new PTB patients (n=83)	Stool samples from healthy controls (n=31)	decreased alpha-deversity	*Firmicutes*, *Actinobacteria*, *Clostridales*, *Bifidobacteriales*, *Bifidobacteriaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Marinifilaceae*, *Eggerhellaceae*, *Barnesiellaceae*, *Blautia*, *Roseburia*, *Bifidobacterium*, undifined *Ruminococcaceae*, *Fusicatenibacter*, *Romboutsia* decreased in patients; *Bacteroidetes*, *Bacteroidales*, *Bacteroidaceae*, *Tannerellaceae*, *Fusobacteriaceae*, *Erysipelotrichaceae*, *Prevotellaceae*, *Bacteroides*, *Parabacteroides*, *Fusobacterium*, *Lachnoclostridium*, *Bacteroides vulgatus* increased in patients	16S rRNA gene amplicon (454) pyrosequencing	(Wang S. et al., 2022)
Stool samples from new PTB patients (n=56) and latent PTB (n=36)	Stool samples from healthy controls (n=50)	decreased alpha-deversity	*Firmicutes*, *Tenericutes*, *Roseburia* decreased in patients; *Actinobacteria*, *Bifidobacterium* increased in patients	16S rRNA gene amplicon (Illumina) sequencing	(Wang Y. et al., 2022)
Stool samples from new PTB patients (n=55)	Stool samples from healthy controls (n=50) with slightly younger median age	decreased alpha-deversity	*Bacteroidetes* and *Bacteroides fragilis* decreased in patients	RT-qPCR for targeting certain phylum, family or species	(Yang et al., 2022)
Stool samples from PTB patients (n=69)	Stool samples from healthy controls (n=10)	decreased alpha-deversity	*Bacteroidetes, Proteobacteria, Fusobacteria, Bacteroidaceae, Tannerllaceae, Bacteroides, Veillonella* increased in patients *Firmicutes, Actinobacteria, Bifidobacteriaceae, Butyricioccaceae, Ruminococcaceae, Faecalibacterium, Bifidobacterium, Agathobacter* decreased in patients	16S rRNA gene amplicon (515, 806) pyrosequencing	(Ye et al., 2022)
Stool samples from ITB patients (n=11)	Stool samples from healthy controls (n=63)	decreased alpha-deversity	*Proteobacteria*, *Megasphaera*, *Veillonellales* decreased in patients *Verrucomicrobia*, *Rhizobiales*, *Blautia* increased in patients	16S rRNA gene amplicon (Illumina) sequencing	(Yoon et al., 2022)

(Stojanov et al., 2020). The significant reduction in the phylum *Firmicutes* in TB patients was observed by several independent groups (Hu et al., 2019a; He et al., 2021; Wang S. et al., 2022; Wang Y. et al., 2022; Ye et al., 2022). The relationship between reduced *Firmicutes* and *M. tuberculosis* infection might be regarded as reciprocal causation. On one hand, the imbalanced microbiome composition caused by *Firmicutes* reduction might cause susceptibility to *M. tuberculosis* infection or the activation of TB in latent TB infection. On the other hand, the reduction of *Firmicutes* might also be triggered by the dysregulated immune system caused by *M. tuberculosis* infection.

Precisely, within *Firmicutes*, *Clostridiales* and *Veillonellales* were found to be decreased by some studies (Ding et al., 2022; Wang S. et al., 2022; Yoon et al., 2022). Meanwhile, many observations support the reduction of families *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae* within *Clostridiales* (Maji et al., 2018; Li et al., 2019; He et al., 2021; Shi et al., 2021; Ding et al., 2022; Wang S. et al., 2022; Ye et al., 2022) and the reduction of *Veillonellaceae* within *Veillonellales* (Maji et al., 2018; Namasivayam et al., 2020). More interesting findings were observed at the genus level. Some of the most common genera in *Firmicutes* such as *Faecalibacterium*, *Ruminococcus*, *Blautia*, *Roseburia*, *Lachnospira*, *Eubacterium*, *Coprococcus*, and *Dorea* were all observed to be decreased (Luo et al., 2017; Hu et al., 2019a; Hu et al., 2019b; Li et al., 2019; He et al., 2021; Shi et al., 2021; Ding et al., 2022; Wang S. et al., 2022; Wang Y. et al., 2022; Yang et al., 2022; Ye et al., 2022; Yoon et al., 2022), whereas *Granulicatella*, *Lactobacillus*, *Enterococcus*, and

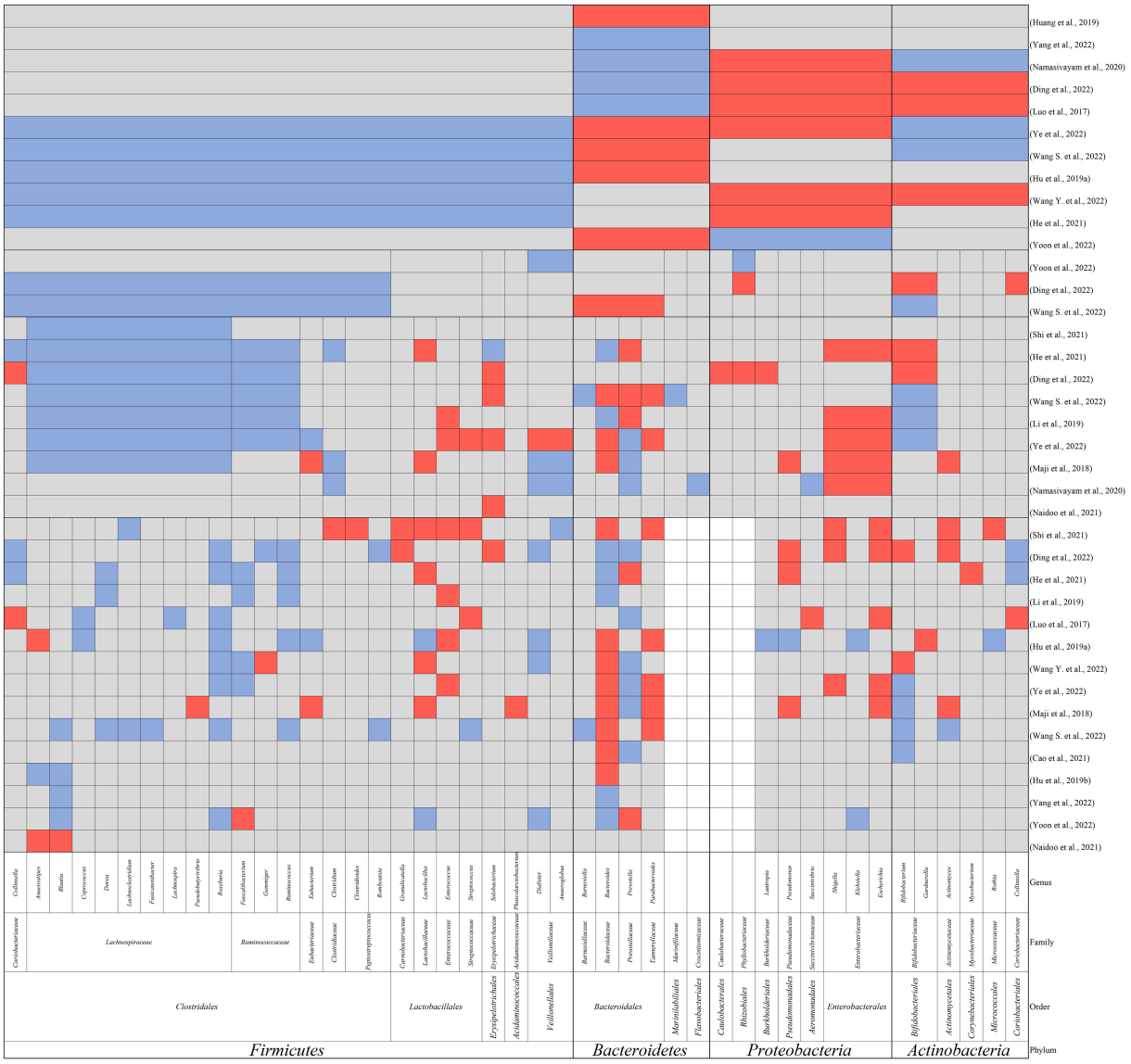

FIGURE 1
The main findings in alteration of gut microbiomes in TB patients compared to healthy controls at the phylum, order, family, and genus level. Red: elevation; blue: reduction; grey: not reported; white: no reported genus within the family.

Streptococcus were observed to be increased in patients (Luo et al., 2017; Maji et al., 2018; Hu et al., 2019a; Li et al., 2019; He et al., 2021; Shi et al., 2021; Ding et al., 2022; Wang Y. et al., 2022; Ye et al., 2022).

As mentioned earlier, the reduced genera primarily belong to the two most abundant families in *Firmicutes*, *Lachnospiraceae* and *Ruminococcaceae*. They are obligate anaerobic and butyrate-producing bacteria (Sorbara et al., 2020; Liu et al., 2021). Butyrate is a short-chain fatty acid (SCFA) that is an essential regulator for the maintenance of intestinal homeostasis (Parada Venegas et al., 2019). Butyrate can interact with G-coupled receptors such as GPR43, GPR41, and GPR109a (Hodgkinson et al., 2023), leading to increased regulatory T cells (Tregs) and dendritic cell precursors, improved epithelial barrier function, as well as the increased expression of anti-inflammatory cytokines such as IL-10 (Liu et al., 2018). Additionally, butyrate can also inhibit HDAC activity to decompact chromatin and upregulate gene expression, inducing Tregs and the antimicrobial activity in intestinal macrophages (Schulthess et al., 2019). In addition, Phenylbutyrate (PBA), a derivative of butyrate, has been found to induce the expression of antimicrobial peptides in lung epithelial cells (Steinmann et al., 2009) and directly restrict the growth of *M. tuberculosis in vitro* or even within macrophages (Coussens et al., 2015). In clinical trials for TB patients, PBA in combination with vitamin D has also been shown to increase the clearance of *M. tuberculosis* by inducing the antimicrobial peptide LL-37 (Mily et al., 2013; Mily et al., 2015), while also ameliorating inflammation and improving symptom relief (Bekele et al., 2018; Rekha et al., 2018). LL-37 was reported to disrupt the cell wall of intra- and extracellular *M. tuberculosis* (Deshpande et al., 2020) and also activate the autophagy of macrophages (Rekha et al., 2015).

Therefore, a decreased butyrate level would result in elevated pro-inflammatory responses, reduced antimicrobial activity, and impaired epithelial barrier function (Chen et al., 2019b).

Conversely, the increased genera in patients all belong to the order *Lactobacillales*, a group of lactic acid-producing bacteria. Lactic acid bacteria are generally regarded as beneficial microorganisms that support the host's gut homeostasis and enhance the epithelial barrier (Ren et al., 2020). However, it is also reported that lactic acid bacteria can induce Th1 and suppress Th2 responses during *M. tuberculosis* infection (Ghadimi et al., 2010). Meanwhile, it is also worth noting that some of the bacteria in *Enterococcus*, *Streptococcus*, and *Granulicatella* are opportunistic pathogens. The disrupted epithelial barrier caused by reduced butyrate can facilitate the colonization of these opportunistic pathogens.

2.2 *Bacteroidetes*

Bacteroidetes are the second most abundant microbiota in the healthy human colon, comprising 23% of the gut microbiota (Sánchez-Tapia et al., 2019). Similar to *Firmicutes*, alterations in *Bacteroidetes* are also important in metabolism and energy balance (Chen et al., 2019a). However, unlike *Firmicutes*, *Bacteroidetes* are the main producer of the other two members of SCFAs, namely acetate and propionate (Feng et al., 2018).

Despite the contradictory findings in the alteration of *Bacteroidetes*, the most predominant findings were related to the three most abundant genera in *Bacteroidetes*, namely *Bacteroides*, *Prevotella*, and *Parabacteroides* (Rinninella et al., 2019; Zafar and Saier, 2021). In most studies, *Bacteroides* and *Parabacteroides* were reported to be increased in TB patients while *Prevotella* was reported to be decreased (Maji et al., 2018; Hu et al., 2019a; Hu et al., 2019b; Shi et al., 2021; Wang S. et al., 2022; Wang Y. et al., 2022; Ye et al., 2022).

Both *Bacteroides* and *Parabacteroides* are acetate-producing bacteria. Like butyrate, acetate can enhance antimicrobial peptides such as defensins, and also increase the epithelial barrier repairment by inducing the production of IL-22 (Fachi et al., 2020). Defensin, such as defensin-1, was found to inhibit the intracellular growth of mycobacterium inside granulomas (Sharma et al., 2017). Moreover, acetate was also reported to increase phagocytosis and bacterial killing by macrophages and neutrophils (Galvão et al., 2018). In addition, *Bacteroides* was also one of the major sources of propionate in the gut microbiota (Louis and Flint, 2017). Propionate was also shown to have antimicrobial activity. Propionate produced by *Bacteroides* was reported to limit the colonization of many bacteria such as *Salmonella* (Jacobson et al., 2018) and *E.coli* (Ormsby et al., 2020) by regulating intracellular pH. However, it should not be neglected that acetate may also suppress CD4+ T cell activation and Th1 and Th17 response while propionate may suppress antigen-specific CD8+ T cell activation by alleviating the IL-12 production by dendritic cells (Nastasi et al., 2017). These effects may also increase the susceptibility of the host to infections (Ahn et al., 2017; Piccinni et al., 2019).

In contrast, studies have shown that *Prevotella* can augment Th17-mediated mucosal inflammation (Kempski et al., 2017) and increase epithelial permeability to bacterial products (Larsen, 2017). This might be because *Prevotella* can activate TLR2-signaling and induce the secretion of IL-6, IL-8, and CCL20 by epithelial cells (Tamanai-Shacoori et al., 2022), as well as the secretion of IL-1β, IL-6, and IL-23 by dendritic cells (Kwok et al., 2012). These cytokines can induce Th17 immune response and neutrophil recruitment, increasing infection severity and tissue damage (Larsen, 2017; Shen and Chen, 2018). Therefore, reduced *Prevotella* as well as increased *Bacteroides* and *Parabacteroides* might simultaneously exert an anti-inflammatory effect.

Intriguingly, in the context of ITB, there seems to be minor differences compared with PTB patients. The most significant observation would be the opposite trends with decreased *Bacteroides* and increased *Prevotella* in ITB patients (He et al., 2021; Yoon et al., 2022). As the major sources of both acetate and propionate, decreased *Bacteroides* together with downregulated *Firmicutes* in ITB patients would result in a dramatic depletion of SCFA production. Based on the critical role that SCFAs play in epithelial barrier function, antimicrobial protein production, and immunomodulation, this depletion would cause excessive immune responses, increased inflammatory lesions, and antimicrobial peptide production. It might also increase the invasion and colonization of *M. tuberculosis* and other opportunistic pathogens in the gut.

Moreover, the increased *Prevotella* would also increase the Th17 response inducing neutrophil accumulation and granuloma formation after *M. tuberculosis* infection (Seiler et al., 2003). However, when exposed to excessive IL-17 produced by Th17 cells, longer survival of neutrophils can cause increased neutrophil infiltration and the formation of pathological lesions (Torrado and Cooper, 2010). This is also in line with the observation of elevated IL-17 expression in ITB patients (Pugazhendhi et al., 2013).

2.3 *Proteobacteria* and *Actinobacteria*

At the phylum level, *Proteobacteria* were observed to be increased in TB patients (Luo et al., 2017; Namasivayam et al., 2020; He et al., 2021; Ding et al., 2022; Wang Y. et al., 2022), while conflicting trends were reported for *Actinobacteria* (Luo et al., 2017; Namasivayam et al., 2020; Ding et al., 2022; Wang S. et al., 2022; Wang Y. et al., 2022). However, at the genus level, *Pseudomonas (*Maji et al., 2018; He et al., 2021; Shi et al., 2021*)*, *Shigella* (Shi et al., 2021; Ding et al., 2022)and *Escherichia* from *Proteobacteria* (Luo et al., 2017; Shi et al., 2021; Ding et al., 2022)and *Actinomyces* from *Actinobacteria* (Maji et al., 2018; Shi et al., 2021; Ding et al., 2022)were all reported to be increased in patients. These bacteria are all common opportunistic pathogens and are always associated with the disruption of mucosal barriers (Pujic et al., 2015). An imbalanced SCFA constitution alters the gut environment resulting in dysregulated immune response and

breakdown of the epithelial barrier, causing the colonization of opportunistic pathogens.

3 Microbiome-immune crosstalk during *M. tuberculosis* infection

The gut microbiome and lung microbiome are not separate groups within an organism. They are tightly related by the so-called "gut-lung axis", which means that the metabolites produced by the gut microbiome can reach the systemic circulation and shape the lung microbiome and the immune response in the lung, and vice versa (Enaud et al., 2020). Among the metabolites of the microbiome, SCFAs are the most extensively studied. SCFAs including acetate, propionate, and butyrate have been shown to have a modulatory role in the immune system and epithelial function.

In PTB patients, compared with healthy controls (Figure 2A), the main findings are the loss of *Firmicutes* such as *Lachnospiraceae* and *Ruminococcaceae*, and the enrichment of *Bacteroidetes* (Figure 2B). In the murine model challenged with *M. tuberculosis*, the authors also observed a post-infection reduction of butyrate-producing *Lachnospiraceae* and *Ruminococcaceae* and enrichment of acetate/propionate-producing *Bacteroides*, similar to the observations in humans (Winglee et al., 2014). Furthermore, two studies on the relationship between *Helicobacter hepaticus* and *M. tuberculosis infection* found that infection by *Helicobacter hepaticus* resulting in similar dysbiosis with increased *Bacteroidaceae* and decreased *Clostridiales*, *Ruminococcaceae*, *Lachnospiraceae*, and *Prevotellaceae* could cause hyperactivated immune response, overexpressed pro-inflammatory cytokines, and increased susceptibility to *M. tuberculosis*, resulting in severe lung damage (Arnold et al., 2015; Majlessi et al., 2017). These observations in patients and murine models may lead to the potential altered SCFA composition with decreased butyrate but increased acetate and propionate. A fecal metabolomic study also revealed slightly increased acetate and a significant decrease in butyrate in PTB patients (Wang S. et al., 2022).

Acetate, butyrate, and propionate are all SCFAs that can exert anti-inflammatory effects by binding to GPR41 and GPR43. However, butyrate is the only SCFA known to bind to GPR109A (Liu et al., 2018). *In vivo* experiments using *Gpr109a*$^{-/-}$ mice failed to ameliorate the inflammatory response and epithelial barrier dysfunction after sodium butyrate administration (Chen et al., 2018), indicating the importance of GPR109A in anti-inflammatory response and epithelial barrier construction. Another experiment using *Gpr109a*$^{-/-}$ mice observed dysregulated immune responses and increased M1 macrophage polarization (Zhang Z. et al., 2022). Increased acetate and propionate may remedy the loss of butyrate in GPR41 and GPR43 activation but may not rescue the loss of GPR109A activation. The loss of butyrate in the gut microbiome and further in the circulation by the "gut-lung axis" results in dysbiosis in the lung microbiome (Hu et al., 2020; Vázquez-Pérez et al., 2020; Xiao et al., 2022; Zhang M. et al., 2022), as well as the disruption of the lung epithelial barrier and upregulation of pro-inflammatory cytokines in the systemic circulation such as IFN-γ, TNF, and IL-17A (Machado et al., 2021). These pro-inflammatory cytokines and the opening up of tight junctions in the lung epithelial barrier can facilitate the migration of immune cells such as neutrophils and macrophages (Akdis, 2021). Macrophages and neutrophils are the first-line innate immune defense against *M. tuberculosis* by phagocytosis (Roca et al., 2019). Moreover, immune cells such as macrophages and dendritic cells can present antigens to T and B cells and augment adaptive immune responses. After infection, CD4+ T cells can not only further strengthen the innate immunity but also promote the function and survival of CD8+ T cells (Lu et al., 2021), whilst CD8+ T cells can directly kill *M. tuberculosis* by their cytolytic function (Lin and Flynn, 2015). Antibody opsonization was also shown to promote the phagocytosis of macrophages (Chandra et al., 2022).

However, when the SCFA level in circulation is sustainably reduced due to an imbalanced microbiome in TB, as observed in ITB patients with decreased Bacteroides (Figure 2C), the resulting depletion of IL-10 production and anti-inflammatory response can provoke the persistence of an overactivated pro-inflammatory response. Meanwhile, excessive TNF production was found to

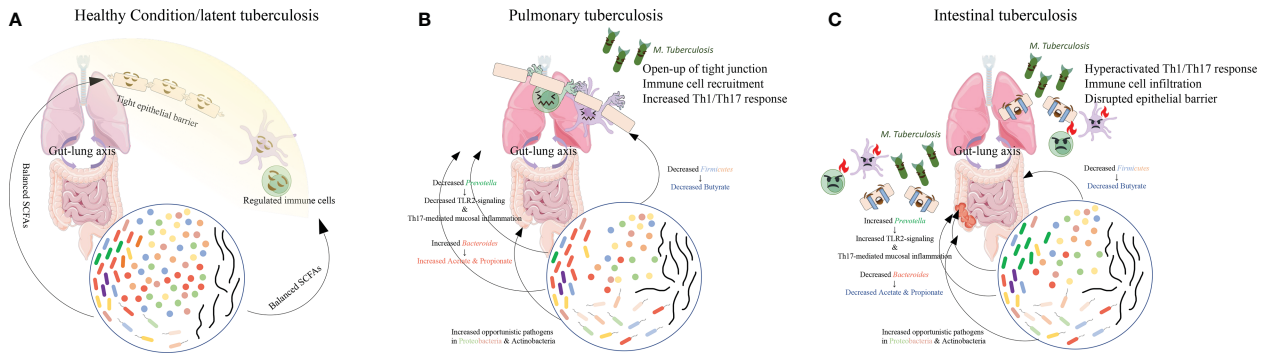

FIGURE 2
The main findings in gut microbiome composition in PTB and ITB patients. Compared with the healthy conditions **(A)**, in PTB patients **(B)**, reduced *Firmicutes* and *Prevotella* and increased *Bacteroides* altered the proportion of each SCFA, causing immune cell recruitment and mildly increased immune response. However, when *Bacteroides* decreased and *Prevotella* increased **(C)**, decreased SCFAs production resulted in drastic activation of immune response and disruption of epithelial barrier, facilitating the colonization of *M. tuberculosis* in the intestine and the development of ITB.

induce necroptosis of granuloma macrophages by activating the RIP1-RIP3 necroptosome (Stutz et al., 2018), which can facilitate bacterial replication and activation (Roca et al., 2019). Moreover, the increased *Prevotella* in ITB patients' gut microbiota could further induce Th17 responses and aggravate neutrophil infiltration and pathological lesions in both lung and gut. The upregulated pro-inflammatory cytokine production may contribute to the overactivation of neutrophils and lead to impairment of mycobacterial controls within granulomas and thus exacerbate disease (Moreira-Teixeira et al., 2020). The observation of higher levels of neutrophils in the circulation of active TB patients also indicates the detrimental role of an overactivated immune response (Moideen et al., 2018). The uncontrolled replication and invasion of *M. tuberculosis* might facilitate its colonization in the gut and cause intestinal TB.

4 Perspectives and conclusions

The treatment of TB requires long-term multidrug treatment with a mixture of broad-spectrum and mycobacterial-specific antibiotics, especially for multidrug-resistant TB. However, it has also been reported that anti-TB medications can result in further dysbiosis of the intestinal microbiome in TB patients (Namasivayam et al., 2017; Wipperman et al., 2017; Hu et al., 2019b; Yoon et al., 2022). Intestinal microbiome disruption can also, in turn, limit the efficiency of treatment (Negi et al., 2020). A study of *M. tuberculosis* infection in mice pre-treated with isoniazid and pyrazinamide for 8 weeks also showed a higher lung bacterial burden. Besides, alleviated TNF and IL-1β production, decreased MHCII expression, and defective *M. tuberculosis* control were found in the alveolar macrophages of the mice. This phenotype can be partially reversed by fecal transplantation (Khan et al., 2019). Moreover, in our review, the current findings in TB patients also indicate a correlation between severely imbalanced gut microbiome with the development of ITB in PTB patients. Therefore, a balanced gut microbiome is crucial during *M. tuberculosis* infection. To achieve this goal, probiotics and postbiotics as potential routine supplements during TB treatment could be a one-stone-two-birds strategy.

Probiotics, such as *Bacteroides fragilis* and *Lactobacillus plantarum*, have already been considered novel probiotics in TB treatment (Liu et al., 2021; Eribo et al., 2022). *B. fragilis* has been reported to exert anti-inflammatory function by decreasing excessive IFN-γ and inducing IL-10 secretion in mice through its metabolite PSA (polysaccharide) (Johnson et al., 2015; Johnson et al., 2018). The study by Negi et al., also reported increased MHCII expression on lung dendritic cells and a lower *M. tuberculosis* burden in the lung of mice after treatment with *Lactobacillus plantarum*. Another *in vitro* study using *Lacticaseibacillus rhamnosus* PMC203 found a direct restriction in *M. tuberculosis* growth and increased killing ability in infected RAW 264.7 cells (Rahim et al., 2022).

Postbiotics, such as indole propionic acid, can inhibit *M. tuberculosis* by targeting tryptophan synthesis (Negatu et al., 2019). PBA as a derivative of probiotics (butyrate) has also been tested in clinical trials and observed to provide significant relief of symptoms (Bekele et al., 2018; Rekha et al., 2018). However, the usage and concentration of probiotics and postbiotics must be individualized in the context of the patients. For example, different concentrations of SCFAs might have distinct functions (Ashique et al., 2022). Another example is the usage of SCFAs, which might be helpful in normal TB patients, but detrimental in people with HIV co-infection (Machado et al., 2021)

As mentioned above, studies have shown that the gut microbiome alteration in general TB patients (PTB) is characterized by dysbiosis, which is defined as reduced butyrate-producing *Firmicutes* and *Prevotella* (*Bacteroidetes*), and increased lactic acid-producing *Firmicutes*, *Bacteroides*, *Parabacteroides*, and opportunistic pathogens in *Proteobacteria* and *Actinobacteria*. The most significant consequence of this alteration, given the abundance of Firmicutes and Bacteroidetes in the human gut microbiome, is the change in the composition of SCFAs, with reduced butyrate and increased acetate and propionate metabolite production. When acetate and propionate production is further decreased by the reduction of *Bacteroides*, there might be an increased susceptibility to *M. tuberculosis* infection in the gut, causing ITB. Therefore, the gut microbiome may act as the defense line in preventing ITB development. Probiotics and postbiotics could become potential supplements in TB treatment and ITB prevention.

Author contributions

ZY and JC designed the study. ZY and XS wrote the manuscript. AW and CH made contributions to the revision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

References

Abu-Zidan, F. M., and Sheek-Hussein, M. (2019). Diagnosis of abdominal tuberculosis: lessons learned over 30 years: pectoral assay. *World J. Emergency Surg.* 14, 1–7. doi: 10.1186/s13017-018-0220-3

Ahn, Y.-H., Jeon, S.-B., Chang, C. Y., Goh, E.-A., Kim, S. S., Kim, H. J., et al. (2017). Glatiramer acetate attenuates the activation of CD4+ T cells by modulating STAT1 and –3 signaling in glia. *Sci. Rep.* 7, 40484. doi: 10.1038/srep40484

Akdis, C. A. (2021). Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? *Nat. Rev. Immunol.* 21, 739–751. doi: 10.1038/s41577-021-00538-7

Arnold, I. C., Hutchings, C., Kondova, I., Hey, A., Powrie, F., Beverley, P., et al. (2015). Helicobacter hepaticus infection in BALB/c mice abolishes subunit-vaccine-induced protection against m. tuberculosis. *Vaccine* 33, 1808–1814. doi: 10.1016/j.vaccine.2015.02.041

Ashique, S., De Rubis, G., Sirohi, E., Mishra, N., Rihan, M., Garg, A., et al. (2022). Short chain fatty acids: Fundamental mediators of the gut-lung axis and their involvement in pulmonary diseases. *Chem. Biol. Interact.* 368, 110231. doi: 10.1016/j.cbi.2022.110231

Avoi, R., and Liaw, Y. C. (2021). Tuberculosis death epidemiology and its associated risk factors in sabah, Malaysia. *Int. J. Environ. Res. Public Health* 18, 9740. doi: 10.3390/ijerph18189740

Bekele, A., Gebreselassie, N., Ashenafi, S., Kassa, E., Aseffa, G., Amogne, W., et al. (2018). Daily adjunctive therapy with vitamin D3 and phenylbutyrate supports clinical recovery from pulmonary tuberculosis: a randomized controlled trial in Ethiopia. *J. Internal Med.* 284, 292–306. doi: 10.1111/joim.12767

Bien, J., Palagani, V., and Bozko, P. (2013). The intestinal microbiota dysbiosis and clostridium difficile infection: is there a relationship with inflammatory bowel disease? *Therap Adv. Gastroenterol.* 6, 53–68. doi: 10.1177/1756283X12454590

Budden, K. F., Gellatly, S. L., Wood, D. L. A., Cooper, M. A., Morrison, M., Hugenholtz, P., et al. (2017). Emerging pathogenic links between microbiota and the gut–lung axis. *Nat. Rev. Microbiol.* 15, 55–63. doi: 10.1038/nrmicro.2016.142

Cao, D., Liu, W., Lyu, N., Li, B., Song, W., Yang, Y., et al. (2021). Gut mycobiota dysbiosis in pulmonary tuberculosis patients undergoing anti-tuberculosis treatment. *Microbiol. Spectr.* 9, e00615–e00621. doi: 10.1128/spectrum.00615-21

Chandra, P., Grigsby, S. J., and Philips, J. A. (2022). Immune evasion and provocation by mycobacterium tuberculosis. *Nat. Rev. Microbiol.* 20, 750–766. doi: 10.1038/s41579-022-00763-4

Chen, L., Li, H., Li, J., Chen, Y., and Yang, Y. (2019a). Lactobacillus rhamnosus GG treatment improves intestinal permeability and modulates microbiota dysbiosis in an experimental model of sepsis. *Int. J. Mol. Med.* 43(3), 1139–1148. doi: 10.3892/ijmm.2019.4050

Chen, G., Ran, X., Li, B., Li, Y., He, D., Huang, B., et al. (2018). Sodium butyrate inhibits inflammation and maintains epithelium barrier integrity in a TNBS-induced inflammatory bowel disease mice model. *EBioMedicine* 30, 317–325. doi: 10.1016/j.ebiom.2018.03.030

Chen, L., Sun, M., Wu, W., Yang, W., Huang, X., Xiao, Y., et al. (2019b). Microbiota metabolite butyrate differentially regulates Th1 and Th17 cells' differentiation and function in induction of colitis. *Inflammation Bowel Dis.* 25(9), 1450–1461. doi: 10.1093/ibd/izz046

Chen, Y., Zhou, J., and Wang, L. (2021). Role and mechanism of gut microbiota in human disease. *Front. Cell Infect. Microbiol.* 11, 625913. doi: 10.3389/fcimb.2021.625913

Cho, J.-K., Choi, Y. M., Lee, S. S., Park, H. K., Cha, R. R., Kim, W. S., et al. (2018). Clinical features and outcomes of abdominal tuberculosis in southeastern Korea: 12 years of experience. *BMC Infect. Dis.* 18, 1–8. doi: 10.1186/s12879-018-3635-2

Comberiati, P., Di Cicco, M., Paravati, F., Pelosi, U., Di Gangi, A., Arasi, S., et al. (2021). The role of gut and lung microbiota in susceptibility to tuberculosis. *Int. J. Environ. Res. Public Health* 18, 12220. doi: 10.3390/ijerph182212220

Coussens, A. K., Wilkinson, R. J., and Martineau, A. R. (2015). Phenylbutyrate is bacteriostatic against mycobacterium tuberculosis and regulates the macrophage response to infection, synergistically with 25-Hydroxy-Vitamin D3. *PloS Pathog.* 11, e1005007. doi: 10.1371/journal.ppat.1005007

Deshpande, D., Grieshober, M., Wondany, F., Gerbl, F., Noschka, R., Michaelis, J., et al. (2020). Super-resolution microscopy reveals a direct interaction of intracellular mycobacterium tuberculosis with the antimicrobial peptide LL-37. *Int. J. Mol. Sci.* 21 (18), 6741. doi: 10.3390/ijms21186741

Ding, X., Zhou, J., Chai, Y., Yan, Z., Liu, X., Dong, Y., et al. (2022). A metagenomic study of the gut microbiome in PTB'S disease. *Microbes Infect.* 24, 104893. doi: 10.1016/j.micinf.2021.104893

Ducarmon, Q. R., Zwittink, R. D., Hornung, B. V. H., Van Schaik, W., Young, V. B., and Kuijper, E. J. (2019). Gut microbiota and colonization resistance against bacterial enteric infection. *Microbiol. Mol. Biol. Rev.* 83(3), e00007–19. doi: 10.1128/MMBR.00007-19

Enaud, R., Prevel, R., Ciarlo, E., Beaufils, F., Wieërs, G., Guery, B., et al. (2020). The gut-lung axis in health and respiratory diseases: A place for inter-organ and inter-kingdom crosstalks. *Front. Cell Infect. Microbiol.* 10, 9. doi: 10.3389/fcimb.2020.00009

Eribo, O. A., Du Plessis, N., and Chegou, N. N. (2022). The intestinal commensal, bacteroides fragilis, modulates host responses to viral infection and therapy: Lessons for exploration during mycobacterium tuberculosis infection. *Infect. Immun.* 90, e0032121. doi: 10.1128/IAI.00321-21

Fachi, J. L., Sécca, C., Rodrigues, P. B., Mato, F. C. P. D., Di Luccia, B., Felipe, J. D. S., et al. (2020). Acetate coordinates neutrophil and ILC3 responses against c. difficile through FFAR2. *J. Exp. Med.* 217(3), jem.20190489. doi: 10.1084/jem.20190489

Feng, W., Ao, H., and Peng, C. (2018). Gut microbiota, short-chain fatty acids, and herbal medicines. *Front. Pharmacol.* 9, 1354. doi: 10.3389/fphar.2018.01354

Galvão, I., Tavares, L. P., Corrêa, R. O., Fachi, J. L., Rocha, V. M., Rungue, M., et al. (2018). The metabolic sensor GPR43 receptor plays a role in the control of klebsiella pneumoniae infection in the lung. *Front. Immunol.* 9, 142. doi: 10.3389/fimmu.2018.00142

Gan, H., Mely, M., Zhao, J., and Zhu, L. (2016). An analysis of the clinical, endoscopic, and pathologic features of intestinal tuberculosis. *J. Clin. Gastroenterol.* 50, 470–475. doi: 10.1097/MCG.0000000000000514

Ghadimi, D., De Vrese, M., Heller, K. J., and Schrezenmeir, J. (2010). Lactic acid bacteria enhance autophagic ability of mononuclear phagocytes by increasing Th1 autophagy-promoting cytokine (IFN-γ) and nitric oxide (NO) levels and reducing Th2 autophagy-restraining cytokines (IL-4 and IL-13) in response to mycobacterium tuberculosis antigen. *Int. Immunopharmacol.* 10, 694–706. doi: 10.1016/j.intimp.2010.03.014

He, C., Wang, H., Yu, C., Peng, C., Shu, X., Liao, W., et al. (2021). Alterations of gut microbiota in patients with intestinal tuberculosis that different from crohn's disease. *Front. Bioeng Biotechnol.* 9, 673691. doi: 10.3389/fbioe.2021.673691

Hodgkinson, K., El Abbar, F., Dobranowski, P., Manoogian, J., Butcher, J., Figeys, D., et al. (2023). Butyrate's role in human health and the current progress towards its clinical application to treat gastrointestinal disease. *Clin. Nutr.* 42, 61–75. doi: 10.1016/j.clnu.2022.10.024

Hu, Y., Cheng, M., Liu, B., Dong, J., Sun, L., Yang, J., et al. (2020). Metagenomic analysis of the lung microbiome in pulmonary tuberculosis - a pilot study. *Emerg. Microbes Infect.* 9(1), 1444–1452. doi: 10.1080/22221751.2020.1783188

Hu, Y., Feng, Y., Wu, J., Liu, F., Zhang, Z., Hao, Y., et al. (2019a). The gut microbiome signatures discriminate healthy from pulmonary tuberculosis patients. *Front. Cell Infect. Microbiol.* 9, 90. doi: 10.3389/fcimb.2019.00090

Hu, Y., Yang, Q., Liu, B., Dong, J., Sun, L., Zhu, Y., et al. (2019b). Gut microbiota associated with pulmonary tuberculosis and dysbiosis caused by anti-tuberculosis drugs. *J. Infect.* 78, 317–322. doi: 10.1016/j.jinf.2018.08.006

Huang, S. F., Yang, Y. Y., Chou, K. T., Fung, C. P., Wang, F. D., and Su, W. J. (2019). Systemic proinflammation after mycobacterium tuberculosis infection was correlated to the gut microbiome in HIV-uninfected humans. *Eur. J. Clin. Invest.* 49(5):e13068. doi: 10.1111/eci.13068

Jacobson, A., Lam, L., Rajendram, M., Tamburini, F., Honeycutt, J., Pham, T., et al. (2018). A gut commensal-produced metabolite mediates colonization resistance to salmonella infection. *Cell Host Microbe* 24, 296–307.e297. doi: 10.1016/j.chom.2018.07.002

Jayasooriya, S., Dimambro-Denson, F., Beecroft, C., Balen, J., Awokola, B., Mitchell, C., et al. (2022). Patients with presumed tuberculosis in sub-Saharan Africa that are not diagnosed with tuberculosis: a systematic review and meta-analysis. *Thorax.* 78(1), 50–60. doi: 10.1136/thoraxjnl-2021-217663

Johnson, J. L., Jones, M. B., and Cobb, B. A. (2015). Bacterial capsular polysaccharide prevents the onset of asthma through T-cell activation. *Glycobiology* 25, 368–375. doi: 10.1093/glycob/cwu117

Johnson, J. L., Jones, M. B., and Cobb, B. A. (2018). Polysaccharide-experienced effector T cells induce IL-10 in FoxP3+ regulatory T cells to prevent pulmonary inflammation. *Glycobiology* 28, 50–58. doi: 10.1093/glycob/cwx093

Kempski, J., Brockmann, L., Gagliani, N., and Huber, S. (2017). TH17 cell and epithelial cell crosstalk during inflammatory bowel disease and carcinogenesis. *Front. Immunol.* 8. doi: 10.3389/fimmu.2017.01373

Khan, N., Mendonca, L., Dhariwal, A., Fontes, G., Menzies, D., Xia, J., et al. (2019). Intestinal dysbiosis compromises alveolar macrophage immunity to mycobacterium tuberculosis. *Mucosal Immunol.* 12, 772–783. doi: 10.1038/s41385-019-0147-3

König, J., Wells, J., Cani, P. D., García-Ródenas, C. L., Macdonald, T., Mercenier, A., et al. (2016). Human intestinal barrier function in health and disease. *Clin. Transl. Gastroenterol.* 7, e196. doi: 10.1038/ctg.2016.54

Kwok, S. K., Cho, M. L., Her, Y. M., Oh, H. J., Park, M. K., Lee, S. Y., et al. (2012). TLR2 ligation induces the production of IL-23/IL-17 *via* IL-6, STAT3 and NF-kB pathway in patients with primary sjogren's syndrome. *Arthritis Res. Ther.* 14, R64. doi: 10.1186/ar3780

Larsen, J. M. (2017). The immune response to prevotella bacteria in chronic inflammatory disease. *Immunology* 151, 363–374. doi: 10.1111/imm.12760

Li, W., Zhu, Y., Liao, Q., Wang, Z., and Wan, C. (2019). Characterization of gut microbiota in children with pulmonary tuberculosis. *BMC Pediatr.* 19, 445. doi: 10.1186/s12887-019-1782-2

Lin, P. L., and Flynn, J. L. (2015). CD8 T cells and mycobacterium tuberculosis infection. *Semin. Immunopathol.* 37, 239–249. doi: 10.1007/s00281-015-0490-8

Liu, H., Wang, J., He, T., Becker, S., Zhang, G., Li, D., et al. (2018). Butyrate: A double-edged sword for health? *Adv. Nutr.* 9, 21–29. doi: 10.1093/advances/nmx009

Liu, Y., Wang, J., and Wu, C. (2021). Microbiota and tuberculosis: A potential role of probiotics, and postbiotics. *Front. Nutr.* 8, 626254. doi: 10.3389/fnut.2021.626254

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

Lu, Y. J., Barreira-Silva, P., Boyce, S., Powers, J., Cavallo, K., and Behar, S. M. (2021). CD4 T cell help prevents CD8 T cell exhaustion and promotes control of mycobacterium tuberculosis infection. *Cell Rep.* 36, 109696. doi: 10.1016/j.celrep.2021.109696

Luo, M., Liu, Y., Wu, P., Luo, D. X., Sun, Q., Zheng, H., et al. (2017). Alternation of gut microbiota in patients with pulmonary tuberculosis. *Front. Physiol.* 8, 822. doi: 10.3389/fphys.2017.00822

Machado, M. G., Sencio, V., and Trottein, F. (2021). Short-chain fatty acids as a potential treatment for infections: a closer look at the lungs. *Infect. Immun.* 89, e0018821. doi: 10.1128/IAI.00188-21

Maji, A., Misra, R., Dhakan, D. B., Gupta, V., Mahato, N. K., Saxena, R., et al. (2018). Gut microbiome contributes to impairment of immunity in pulmonary tuberculosis patients by alteration of butyrate and propionate producers. *Environ. Microbiol.* 20, 402–419. doi: 10.1111/1462-2920.14015

Majlessi, L., Sayes, F., Bureau, J. F., Pawlik, A., Michel, V., Jouvion, G., et al. (2017). Colonization with helicobacter is concomitant with modified gut microbiota and drastic failure of the immune control of mycobacterium tuberculosis. *Mucosal Immunol.* 10, 1178–1189. doi: 10.1038/mi.2016.140

Maulahela, H., Simadibrata, M., Nelwan, E. J., Rahadiani, N., Renesteen, E., Suwarti, S. W. T., et al. (2022). Recent advances in the diagnosis of intestinal tuberculosis. *BMC Gastroenterol.* 22, 89. doi: 10.1186/s12876-022-02171-7

Mily, A., Rekha, R. S., Kamal, S. M., Akhtar, E., Sarker, P., Rahim, Z., et al. (2013). Oral intake of phenylbutyrate with or without vitamin D3 upregulates the cathelicidin LL-37 in human macrophages: a dose finding study for treatment of tuberculosis. *BMC Pulm Med.* 13, 23. doi: 10.1186/1471-2466-13-23

Mily, A., Rekha, R. S., Kamal, S. M., Arifuzzaman, A. S., Rahim, Z., Khan, L., et al. (2015). Significant effects of oral phenylbutyrate and vitamin D3 adjunctive therapy in pulmonary tuberculosis: A randomized controlled trial. *PloS One* 10, e0138340. doi: 10.1371/journal.pone.0138340

Mnyambwa, N. P., Philbert, D., Kimaro, G., Wandiga, S., Kirenga, B., Mmbaga, B. T., et al. (2021). Gaps related to screening and diagnosis of tuberculosis in care cascade in selected health facilities in East Africa countries: A retrospective study. *J. Clin. Tuberculosis Other Mycobacterial Dis.* 25, 100278. doi: 10.1016/j.jctube.2021.100278

Moideen, K., Kumar, N. P., Nair, D., Banurekha, V. V., Bethunaickan, R., and Babu, S. (2018). Heightened systemic levels of neutrophil and eosinophil granular proteins in pulmonary tuberculosis and reversal following treatment. *Infect. Immun.* 86(6), e00008–18. doi: 10.1128/IAI.00008-18

Moreira-Teixeira, L., Stimpson, P. J., Stavropoulos, E., Hadebe, S., Chakravarty, P., Ioannou, M., et al. (2020). Type I IFN exacerbates disease in tuberculosis-susceptible mice by inducing neutrophil-mediated lung inflammation and NETosis. *Nat. Commun.* 11, 5566. doi: 10.1038/s41467-020-19412-6

Naidoo, C. C., Nyawo, G. R., Sulaiman, I., Wu, B. G., Turner, C. T., Bu, K., et al. (2021). Anaerobe-enriched gut microbiota predicts pro-inflammatory responses in pulmonary tuberculosis. *EBioMedicine* 67, 103374. doi: 10.1016/j.ebiom.2021.103374

Namasivayam, S., Diarra, B., Diabate, S., Sarro, Y. D. S., Kone, A., Kone, B., et al. (2020). Patients infected with mycobacterium africanum versus mycobacterium tuberculosis possess distinct intestinal microbiota. *PloS Negl. Trop. Dis.* 14, e0008230. doi: 10.1371/journal.pntd.0008230

Namasivayam, S., Maiga, M., Yuan, W., Thovarai, V., Costa, D. L., Mittereder, L. R., et al. (2017). Longitudinal profiling reveals a persistent intestinal dysbiosis triggered by conventional anti-tuberculosis therapy. *Microbiome* 5, 71. doi: 10.1186/s40168-017-0286-2

Nastasi, C., Fredholm, S., Willerslev-Olsen, A., Hansen, M., Bonefeld, C. M., Geisler, C., et al. (2017). Butyrate and propionate inhibit antigen-specific CD8+ T cell activation by suppressing IL-12 production by antigen-presenting cells. *Sci. Rep.* 7, 14516. doi: 10.1038/s41598-017-15099-w

Negatu, D. A., Yamada, Y., Xi, Y., Go, M. L., Zimmerman, M., Ganapathy, U., et al. (2019). Gut microbiota metabolite indole propionic acid targets tryptophan biosynthesis in *Mycobacterium tuberculosis*. *mBio* 10, e02781–e02718. doi: 10.1128/mBio.02781-18

Negi, S., Pahari, S., Bashir, H., and Agrewala, J. N. (2020). Intestinal microbiota disruption limits the isoniazid mediated clearance of mycobacterium tuberculosis in mice. *Eur. J. Immunol.* 50, 1976–1987. doi: 10.1002/eji.202048556

Ormsby, M. J., Johnson, S. A., Carpena, N., Meikle, L. M., Goldstone, R. J., Mcintosh, A., et al. (2020). Propionic acid promotes the virulent phenotype of crohn's disease-associated adherent-invasive escherichia coli. *Cell Rep.* 30, 2297–2305.e2295. doi: 10.1016/j.celrep.2020.01.078

Osei Sekyere, J., Maningi, N. E., and Fourie, P. B. (2020). Mycobacterium tuberculosis, antimicrobials, immunity, and lung-gut microbiota crosstalk: current updates and emerging advances. *Ann. N Y Acad. Sci.* 1467, 21–47. doi: 10.1111/nyas.14300

Parada Venegas, D, De la Fuente, MK, Landskron, G, González, MJ, Quera, R, Dijkstra, G, et al. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Front Immunol* (2019) 10:277. doi: 10.3389/fimmu.2019.00277. Erratum in: Front Immunol. 2019 Jun 28;10:1486.

Piccinni, M. P., Lombardelli, L., Logiodice, F., Kullolli, O., Maggi, E., and Barkley, M. S. (2019). Medroxyprogesterone acetate decreases Th1, Th17, and increases Th22 responses *via* AHR signaling which could affect susceptibility to infections and inflammatory disease. *Front. Immunol.* 10, 642. doi: 10.3389/fimmu.2019.00642

Piccioni, A., Rosa, F., Manca, F., Pignataro, G., Zanza, C., Savioli, G., et al. (2022). Gut microbiota and clostridium difficile: What we know and the new frontiers. *Int. J. Mol. Sci.* 23, 13323. doi: 10.3390/ijms232113323

Pugazhendhi, S., Jayakanthan, K., Pulimood, A. B., and Ramakrishna, B. S. (2013). Cytokine gene expression in intestinal tuberculosis and crohn's disease. *Int. J. Tuberc Lung Dis.* 17, 662–668. doi: 10.5588/ijtld.12.0600

Pujic, P., Beaman, B. L., Ravalison, M., Boiron, P., and Rodríguez-Nava, V. (2015). "Chapter 40 - nocardia and actinomyces," in *Molecular medical microbiology*, *2nd ed.* Eds. Y.-W. Tang, M. Sussman, D. Liu, I. Poxton and J. Schwartzman (Boston: Academic Press), 731–752.

Rahim, M. A., Seo, H., Kim, S., Tajdozian, H., Barman, I., Lee, Y., et al. (2022). *In vitro* anti-tuberculosis effect of probiotic lacticaseibacillus rhamnosus PMC203 isolated from vaginal microbiota. *Sci. Rep.* 12, 8290. doi: 10.1038/s41598-022-12413-z

Rekha, R. S., Mily, A., Sultana, T., Haq, A., Ahmed, S., Mostafa Kamal, S. M., et al. (2018). Immune responses in the treatment of drug-sensitive pulmonary tuberculosis with phenylbutyrate and vitamin D3 as host directed therapy. *BMC Infect. Dis.* 18, 303. doi: 10.1186/s12879-018-3203-9

Rekha, R. S., Rao Muvva, S. S., Wan, M., Raqib, R., Bergman, P., Brighenti, S., et al. (2015). Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of mycobacterium tuberculosis in human macrophages. *Autophagy* 11, 1688–1699. doi: 10.1080/15548627.2015.1075110

Ren, C., Zhang, Q., De Haan, B. J., Faas, M. M., Zhang, H., and De Vos, P. (2020). Protective effects of lactic acid bacteria on gut epithelial barrier dysfunction are toll like receptor 2 and protein kinase c dependent. *Food Funct.* 11, 1230–1234. doi: 10.1039/C9FO02933H

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G., Gasbarrini, A., et al. (2019). What is the healthy gut microbiota composition? a changing ecosystem across age, environment, diet, and diseases. *Microorganisms* 7, 14. doi: 10.3390/microorganisms7010014

Roca, F. J., Whitworth, L. J., Redmond, S., Jones, A. A., and Ramakrishnan, L. (2019). TNF induces pathogenic programmed macrophage necrosis in tuberculosis through a mitochondrial-Lysosomal-Endoplasmic reticulum circuit. *Cell* 178, 1344–1361.e1311. doi: 10.1016/j.cell.2019.08.004

Sánchez-Tapia, M., Tovar, A. R., and Torres, N. (2019). Diet as regulator of gut microbiota and its role in health and disease. *Arch. Med. Res.* 50, 259–268. doi: 10.1016/j.arcmed.2019.09.004

Schulthess, J., Pandey, S., Capitani, M., Rue-Albrecht, K. C., Arnold, I., Franchini, F., et al. (2019). The short chain fatty acid butyrate imprints an antimicrobial program in macrophages. *Immunity* 50, 432–445.e437. doi: 10.1016/j.immuni.2018.12.018

Seiler, P., Aichele, P., Bandermann, S., Hauser, A. E., Lu, B., Gerard, N. P., et al. (2003). Early granuloma formation after aerosol mycobacterium tuberculosis infection is regulated by neutrophils *via* CXCR3-signaling chemokines. *Eur. J. Immunol.* 33, 2676–2686. doi: 10.1002/eji.200323956

Sharma, R., Saikia, U. N., Sharma, S., and Verma, I. (2017). Activity of human beta defensin-1 and its motif against active and dormant mycobacterium tuberculosis. *Appl. Microbiol. Biotechnol.* 101, 7239–7248. doi: 10.1007/s00253-017-8466-3

Sheer, T. A., and Coyle, W. J. (2003). Gastrointestinal tuberculosis. *Curr. Gastroenterol. Rep.* 5, 273–278. doi: 10.1007/s11894-003-0063-1

Shen, H., and Chen, Z. W. (2018). The crucial roles of Th17-related cytokines/signal pathways in m. tuberculosis infection. *Cell. Mol. Immunol.* 15, 216–225. doi: 10.1038/cmi.2017.128

Shi, W., Hu, Y., Ning, Z., Xia, F., Wu, M., Hu, Y. O. O., et al. (2021). Alterations of gut microbiota in patients with active pulmonary tuberculosis in China: a pilot study. *Int. J. Infect. Dis.* 111, 313–321. doi: 10.1016/j.ijid.2021.08.064

Sorbara, MT, Littmann, ER, Fontana, E, Moody, TU, Kohout, CE, Gjonbalaj, M, et al. Functional and Genomic Variation between Human-Derived Isolates of Lachnospiraceae Reveals Inter- and Intra-Species Diversity. *Cell Host Microbe* (2020) 28(1):134–146.e4. doi: 10.1016/j.chom.2020.05.005

Steinmann, J., Halldórsson, S., Agerberth, B., and Gudmundsson, G. H. (2009). Phenylbutyrate induces antimicrobial peptide expression. *Antimicrob. Agents Chemother.* 53, 5127–5133. doi: 10.1128/AAC.00818-09

Stojanov, S., Berlec, A., and Štrukelj, B. (2020). The influence of probiotics on the Firmicutes/Bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. *Microorganisms* 8, 1715. doi: 10.3390/microorganisms8111715

Stutz, M. D., Ojaimi, S., Allison, C., Preston, S., Arandjelovic, P., Hildebrand, J. M., et al. (2018). Necroptotic signaling is primed in mycobacterium tuberculosis-infected macrophages, but its pathophysiological consequence in disease is restricted. *Cell Death Differentiation* 25, 951–965. doi: 10.1038/s41418-017-0031-1

Tamanai-Shacoori, Z., Le Gall-David, S., Moussouni, F., Sweidan, A., Polard, E., Bousarghin, L., et al. (2022). SARS-CoV-2 and prevotella spp.: friend or foe? a systematic literature review. *J. Med. Microbiol.* 71(5). doi: 10.1099/jmm.0.001520

Tan, Z. M., Lai, G. P., Pandey, M., Srichana, T., Pichika, M. R., Gorain, B., et al. (2020). Novel approaches for the treatment of pulmonary tuberculosis. *Pharmaceutics* 12(12), 1196. doi: 10.3390/pharmaceutics12121196

Torrado, E., and Cooper, A. M. (2010). IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev.* 21, 455–462. doi: 10.1016/j.cytogfr.2010.10.004

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

Vandal, O. H., Nathan, C. F., and Ehrt, S. (2009). Acid resistance in mycobacterium tuberculosis. *J. Bacteriol* 191, 4714–4721. doi: 10.1128/JB.00305-09

Vázquez-Pérez, J. A., Carrillo, C. O., Iñiguez-García, M. A., Romero-Espinoza, I., Márquez-García, J. E., Falcón, L. I., et al. (2020). Alveolar microbiota profile in patients with human pulmonary tuberculosis and interstitial pneumonia. *Microb. Pathog.* 139, 103851. doi: 10.1016/j.micpath.2019.103851

Wang, Y., Deng, Y., Liu, N., Chen, Y., Jiang, Y., Teng, Z., et al. (2022). Alterations in the gut microbiome of individuals with tuberculosis of different disease states. *Front. Cell Infect. Microbiol.* 12, 836987. doi: 10.3389/fcimb.2022.836987

Wang, S., Yang, L., Hu, H., Lv, L., Ji, Z., Zhao, Y., et al. (2022). Characteristic gut microbiota and metabolic changes in patients with pulmonary tuberculosis. *Microb. Biotechnol.* 15, 262–275. doi: 10.1111/1751-7915.13761

Wang, B., Yao, M., Lv, L., Ling, Z., and Li, L. (2017). The human microbiota in health and disease. *Engineering* 3, 71–82. doi: 10.1016/J.ENG.2017.01.008

WHO (2021). *Global tuberculosis report 2021*. World Health Organization. https://www.who.int/publications/i/item/9789240037021

Winglee, K., Eloe-Fadrosh, E., Gupta, S., Guo, H., Fraser, C., and Bishai, W. (2014). Aerosol mycobacterium tuberculosis infection causes rapid loss of diversity in gut microbiota. *PloS One* 9, e97048. doi: 10.1371/journal.pone.0097048

Wipperman, M. F., Fitzgerald, D. W., Juste, M.a.J., Taur, Y., Namasivayam, S., et al. (2017). Antibiotic treatment for tuberculosis induces a profound dysbiosis of the microbiome that persists long after therapy is completed. *Sci. Rep.* 7, 10767. doi: 10.1038/s41598-017-10346-6

Wlodarska, M., Willing, B., Keeney, K. M., Menendez, A., Bergstrom, K. S., Gill, N., et al. (2011). Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated citrobacter rodentium-induced colitis. *Infect. Immun.* 79, 1536–1545. doi: 10.1128/IAI.01104-10

Xiao, G., Cai, Z., Guo, Q., Ye, T., Tang, Y., Guan, P., et al. (2022). Insights into the unique lung microbiota profile of pulmonary tuberculosis patients using metagenomic next-generation sequencing. *Microbiol. Spectr.* 10, e0190121. doi: 10.1128/spectrum.01901-21

Yang, F., Yang, Y., Chen, L., Zhang, Z., Liu, L., Zhang, C., et al. (2022). The gut microbiota mediates protective immunity against tuberculosis *via* modulation of lncRNA. *Gut Microbes* 14, 2029997. doi: 10.1080/19490976.2022.2029997

Ye, S., Wang, L., Li, S., Ding, Q., Wang, Y., Wan, X., et al. (2022). The correlation between dysfunctional intestinal flora and pathology feature of patients with pulmonary tuberculosis. *Front. Cell Infect. Microbiol.* 12, 1090889. doi: 10.3389/fcimb.2022.1090889

Yoon, H., Park, Y. S., Shin, C. M., Kim, N., and Lee, D. H. (2022). Gut microbiome in probable intestinal tuberculosis and changes following anti-tuberculosis treatment. *Yonsei Med. J.* 63, 34–41. doi: 10.3349/ymj.2022.63.1.34

Zafar, H., and Saier, M. H.Jr. (2021). Gut bacteroides species in health and disease. *Gut Microbes* 13, 1–20. doi: 10.1080/19490976.2020.1848158

Zhang, Z., Li, J., Zhang, M., Li, B., Pan, X., Dong, X., et al. (2022). GPR109a regulates phenotypic and functional alterations in macrophages and the progression of type 1 diabetes. *Mol. Nutr. Food Res.* 66, e2200300. doi: 10.1002/mnfr.202200300

Zhang, M., Shen, L., Zhou, X., and Chen, H. (2022). The microbiota of human lung of pulmonary tuberculosis and the alteration caused by anti-tuberculosis drugs. *Curr. Microbiol.* 79, 321. doi: 10.1007/s00284-022-03019-9

Zuo, T., Zhang, F., Lui, G. C. Y., Yeoh, Y. K., Li, A. Y. L., Zhan, H., et al. (2020). Alterations in gut microbiota of patients with COVID-19 during time of hospitalization. *Gastroenterology* 159, 944–955.e948. doi: 10.1053/j.gastro.2020.05.048

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Intestinal bacteria—a powerful weapon for fungal infections treatment

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The morbidity and mortality of invasive fungal infections are rising gradually. In recent years, fungi have quietly evolved stronger defense capabilities and increased resistance to antibiotics, posing huge challenges to maintaining physical health. Therefore, developing new drugs and strategies to combat these invasive fungi is crucial. There are a large number of microorganisms in the intestinal tract of mammals, collectively referred to as intestinal microbiota. At the same time, these native microorganisms co-evolve with their hosts in symbiotic relationship. Recent researches have shown that some probiotics and intestinal symbiotic bacteria can inhibit the invasion and colonization of fungi. In this paper, we review the mechanism of some intestinal bacteria affecting the growth and invasion of fungi by targeting the virulence factors, quorum sensing system, secreting active metabolites or regulating the host anti-fungal immune response, so as to provide new strategies for resisting invasive fungal infection.

KEYWORDS

fungal resistance, probiotics, antifungal, toxicity factor, active metabolites

1 Introduction

Invasive fungal infections (IFI) are an increasingly serious public health problem, which can cause prominent invasive lesions with serious symptoms in the host, such as life-threatening bloodstream infection and organ dysfunction (Bongomin et al., 2017). The most common clinical pathogenic fungi are *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. With the increasing number of immunosuppressed people caused by organ transplantation, cancer treatment, HIV and so on, the morbidity and mortality of IFI are rising gradually (Kato et al., 2018; Mayer and Kronstad, 2019). IFI are easy to relapse and difficult to cure, killing more than 1.5 million people worldwide every year, almost as many as tuberculosis (Bongomin et al., 2017). Moreover, IFI can lead to intestinal fungal dysbiosis, which in turn has a certain effect on metabolic diseases and intestinal disorders diseases such as gastric cancer, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease (CeD), colorectal cancers etc. (Table 1) (Ramirez-Garcia et al., 2016; Li et al., 2019).

TABLE 1 Detrimental roles of fungi in the human gastrointestinal tract on metabolic diseases and intestinal disorders diseases.

Diseases	Related fungi	Related mechanism	Reference
Inflammatory bowel disease (IBD)	*Candida albicans* ↑ *Candida tropicalis* ↑ *Saccharomyces cerevisiae* ↓	Production of anti-glycan antibody such as anti-*S. cerevisiae* antibodies (ASCA) can be induce during colonization or infection of *C. albicans*	(Sendid et al., 2009)
Irritable bowel syndrome (IBS)	*Candida albicans* ↑ *Saccharomyces cerevisiae* ↑	After fungal stimulation, Dectin-1/Sy pathway drives visceral allergic reaction and mast cells release more histamine.	(Pimentel and Lembo, 2020)
Colorectal cancers	*Candida albicans* ↑ *Candida tropicalis* ↑	*C. albicans* mediates the up-regulation of macrophage glycolytic pathway, and up-regulates the expression and secretion of IL-7, and then induces type 3 intrinsic lymphocytes (ILC3) to express high levels of IL-22 through AhR and STAT3 pathways, which aggravates the progression of colorectal cancer.	(Wong and Yu, 2019)
Celiac disease (CeD)	*Candida albicans* ↑	*C. albicans* virulence factor, hyphal wall protein 1 (HWP1), is the same as or highly homologous to celiac-related-α and γ gliadin in t-cell epitope and acts as a substrate for transglutaminase (TG), assisting in the production of autoreactive antibodies	(Chibbar and Dieleman, 2019)

"↑"indicates an increase in abundance; "↓"indicates a decrease in abundance.

However, the current antifungal drugs are limited and outdated. Only three types of antifungal drugs are the first line drugs to treat human systemic fungal infections (Table 2). Amphotericin B, the first discovered polyene anti-fungal agent, binds to the ergosterol of cell membrane and "strips" it out, thereby destroying the structure of the fungal cell membrane. While the high toxicity limits its clinical application. Later, azoles, novel anti-fungal agents with lower side-effect, were developed, that can block the ergosterol synthesis by inhibiting lanosterol 14-α-demethylase (encoded by *ERG11*) (Perfect, 2017). However, due to the agricultural use of azoles to protect crops from fungal infection, azoles resistance gradually developed in the 1990s (Verweij et al., 2020). The third-generation antifungal drug echinocandins (such as caspofungin) work by blocking the biosynthesis of β-(1,3) d-glucan (encoded by *FKS1*), an essential component of fungal cell walls. Unfortunately, echinocandin-resistant *Candida* species have emerged in both laboratory and clinical settings (Medici and Del Poeta, 2015).

Furthermore, the widespread use of clinically antifungal agents has accelerated fungal resistance rates (Fairlamb et al., 2016). *Candida auris* is a superbug that first reported in Japan in 2009 (Wang et al., 2020). Although the exact mode of transmission is unclear, *C. auris* can rapidly affect patients and colonize the skin persistently (Schelenz et al., 2016; Lockhart et al., 2017; Vallabhaneni et al., 2017). Like other fungi, it can cause superficial and invasive candidiasis as well as bloodstream infections (Mane et al., 2016; Chowdhary et al., 2017). In addition, *C. auris* has multi-drug resistance to two or more classes of drugs and a few (about 4%) to all classes of antifungal drugs (Sanguinetti et al., 2015). Echinocandins are currently commonly used to treat *C. auris* infections, but caspofungin has been proved to be ineffective against *C. auris* biofilms (Harriott et al., 2010; Pahwa et al., 2014).

Meanwhile, COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged at the end of 2019 and since then has spread worldwide (Gorbalenya et al., 2020; Zhou et al., 2020). When the patient's condition worsens, hospitalization is required and eventually intubation may be required and the patient is transferred to the intensive care unit (Zhu et al., 2020). The respiratory failure associated with novel coronavirus infection is the main driver of death in this population; However, some observations indicate that patients with COVID-19 may also have a higher risk of secondary infections (Rawson et al., 2020). For example, patients can further develop fungal infections (eg, IFI) at an advanced stage of the disease, especially in severely ill or immunocompromised patients (Guo et al., 2019). In China,

TABLE 2 First-line antifungal drugs and their limitations.

First-line anti-fungal drugs	Action target	Limitations	Reference
Amphotericin B	Fungal ergosterol	Side effects such as fever, heart inflammation and kidney problems are even life-threatening	(Sabra and Branch, 1990)
Azole drugs	Lanosterol 14-α-demethylase	Inhibit cytochrome P450 (a drug metabolizing liver enzyme needed for detoxification of human blood); Liver toxicity caused by combination with other drugs	(Marx-Stoelting et al., 2020)
Echinocandins	β-(1,3) D-glucan	Be administered once a day, and each infusion needs to last for one hour; Low bioavailability; Ocular toxicity	(Patil and Majumdar, 2017)

Chen et al. performed fungal culture on admission to all 99 patients and identified 5 cases (5%, 5/99) of fungal infection, including 1 case of *Aspergillus flavus*, 1 case of *Candida glabrata* and 3 cases of *C. albicans* (Chen et al., 2020). Yang et al. found that the presence of *A. flavus*, *A. fumigatus* and *C. albicans* in all 52 critical patients (3/52, 5.8%) (Yang et al., 2020). Clinical abuse of broad-spectrum antibacterial drugs is conducive to the selection of pathogens unaffected by continuous antibacterial therapy, such as *Clostridium difficile* and *C. albicans*, which may increase the susceptibility of secondary infection of fungal pathogens, increasing the morbidity and drug resistance. Therefore, there is an urgent need to exploit new treatment strategies and novel antifungal agents to manage IFI (Antinori et al., 2020).

The mammalian gut harbors a large number of microorganisms, including bacteria, fungi, archaea, protozoa, and viruses, collectively known as the microbiome. The intestinal microbial community is a dense and diverse ecosystem, whose main functions include metabolizing indigestible carbon sources, providing nutrients for the host, regulating the immune system, and preventing the invasion and colonization of pathogenic microorganisms. Thus, the destruction of the intestinal ecosystem facilitates the invasion of pathogens. For example, antibiotic treatment can kill pathogenic bacteria and cure the infection, but also inhibit the growth of other beneficial or colonizing bacteria of the same type, making it vulnerable to new pathogenic microorganisms (Kumamoto, 2016). Researches have shown that germ-free (GF) mice are more susceptible to infection than conventionally reared mice, and become less susceptible when fecal microbiota from conventional mice are transplanted with GF mice (Hooper et al., 2012).

Invasive fungi have a symbiotic relationship with bacteria in the host body to form a dynamic balance of micro-ecology, which plays an important role in the maintenance of human health (Limon et al., 2017). In the microecology, there is a mutual competition between fungi and bacteria (Zuo et al., 2018). In recent years, researches on some probiotics and intestinal commensal bacteria have shown that they can affect the colonization of invasive fungi in the intestinal tract. For example, intestinal commensal bacteria, *Enterococcus faecalis* and *Staphylococcus*, can inhibit the growth and biofilm formation of *C. albicans*. Biofilm is an important virulence factor of fungi, which endows fungi with drug resistance (Peters et al., 2012). In conclusion, the researches on probiotics and intestinal bacteria can provide new insights for clinical antifungal treatment. This review describes the antifungal activity of probiotics and intestinal commensal bacteria against the invasion and colonization of clinical common fungi, providing new strategies for the treatment of fungal infections.

2 The antifungal effect of probiotics

In the human body, the total amount of intestinal microorganisms are equivalent to the number of cells. Probiotics refer to "live microorganisms confer a health benefit on the host when administered in sufficient quantities" (Hill et al., 2014). The interaction between probiotics and intestinal microorganisms enables probiotics to alleviate the occurrence and development of some diseases. For example, some clinical randomized controlled trials (RCTs) have proved that probiotics can significantly alleviate the symptoms and signs related to COVID-19 such as diarrhea, headache, fever, cough, dyspnea, fatigue, myalgia and anosmia, reduce the viral load of nasopharynx, the duration of pulmonary infiltrates and the symptoms of digestive system and non-digestive system, and reduce the risk of respiratory failure and mortality (Gutiérrez-Castrellón et al., 2022). Probiotics are present not only in the intestine but also in other parts of body, such as mouth, respiratory tract, skin and genital tract. In recent years, more and more researches have shown that probiotics can exert their probiotic effects in intestinal health, immune development, nutrition metabolism, emotional management, liver diseases, oral diseases, gynecological diseases and skin health. What we need to pay special attention to is that some probiotics can inhibit the colonization of pathogenic bacteria in the intestine, strengthen the intestinal barrier, regulate the intestinal flora, synthesize active metabolites, etc. (Taverniti et al., 2021).

Clinical researches have shown that probiotics can regulate cytokine secretion, thus affecting the non-specific and specific immunity. Castrellón et al. conducted a single-center, four-blind, randomized trial on COVID-19 patients, and found that probiotic adjuvant therapy significantly increased the production of specific IgM and IgG responses to SARS-CoV-2, reduced the level of D-dimer, and reduced the risk of venous thromboembolism (such as pulmonary embolism), thus reducing the severity and mortality of COVID-19 patients (Gutiérrez-Castrellón et al., 2022). In addition, COVID-19 can cause intestinal ecological imbalance, altered intestinal permeability, and microbial translocation. However, Wu et al. found that in patients treated with probiotic adjuvant therapy COVID-19, partial recovery of intestinal ecological imbalance is evidenced by increased microbial diversity tests, and inflammatory markers such as TNF-α, IL-1β, IL-4 and IL-12P70 are also reduced (Wu et al., 2021).

Human immunodeficiency virus (HIV) infection results in altered intestinal microbiota and increased intestinal permeability, accompanied by related microbial translocation, immune activation and inflammation (González-Hernández et al., 2012). Researches have shown that probiotics can affect the immune response of people with HIV. They can avoid bacterial overgrowth and translocation by stimulating the secretion of polymeric IgA, stimulate the production of regulatory T cells by anti-inflammatory cytokines, and weaken inflammation, thus improving the immune function of HIV patients (Trois et al., 2008). In another trial, levels of D- dimer and inflammatory markers IL-6 and CRP decreased after 8 weeks of probiotic intervention with in HIV-infected subjects receiving stable antiretroviral therapy (ART), suggesting that probiotics play a beneficial role by weakening inflammation (Stiksrud et al., 2015). In addition, *Candida* colonization has been reported in more than 50% of HIV patients and symptomatic candidiasis has been reported in approximately 10% of patients. Researches have shown that the use of probiotics can reduce the number of pathogenic microorganisms (*C. albicans*, *E. coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Clostridium perfringens*)

in HIV patients and tend to restore a normal range of microbial landscape (Wilson et al., 2013).

At present, the generally recognized probiotics mainly include *Lactobacillus*, Non-pathogenic *Escherichia coli*, *Bacillus*, *Enterococcus* and yeast (such as *Saccharomyces boulardii*), most of which are derived from the intestinal tract or fermented food (Zhang et al., 2022). In recent years, due to various hot researches on the intestinal microorganisms, probiotics have been proposed to antagonize opportunistic fungi and regulate the intestinal flora. On the one hand, probiotics can inhibit the invasion and colonization of pathogenic fungi by targeting their virulence factors. The researches have also revealed that the quorum sensing system is related to fungal virulence, and can be used as the target of anti-virulence treatment. On the other hand, probiotics can accumulate in the intestine and produce a variety of active metabolites (such as lactic acid, short-chain fatty acids, tryptophan, tryptophan metabolite, hydrogen peroxide, and bacteriocin, etc.) that inhibit fungal colonization (Mathur et al., 2020).

2.1 Virulence factor is a new antifungal target

Fungi have different pathogenic mechanisms. For example, the first step of *C. albicans* to cause infection is adhesion (Jin et al., 2005). After being swallowed by the phagocytes, yeast cells produce germ tubes and grow hypha, penetrating and destroying the host cell membrane. *C. albicans* can also produce various enzymes such as hydrolase and protease to invade and destroy tissues (Watamoto et al., 2009). *A. fumigatus* can produce some mycotoxins that damage local tissues (Rementeria et al., 2005). *C. neoformans* produces substances such as capsule, melanin, and mannitol to help the bacteria invade the host (Alspaugh, 2015). Additionally, fungi form biofilms against antifungal drugs. Therefore, researches in recent years are devoted to inhibiting or even killing fungi by targeting their virulence factors.

Researches have shown that two new food-derived yeasts, *Saccharomyces cerevisiae* and *Issatchenkia occidentalis*, can inhibit the virulence characteristics of *candida* adhesion, filamentation and biofilm formation, indicating that food-derived yeasts can be used as an alternative antifungal therapy (Kunyeit et al., 2019). In addition, the cell-free supernatant of *L. rhamnosus* was found to reduce the virulence of *C. albicans* and inhibit the formation of its germination tubes. *L. rhamnosus* can also secrete 1- acetyl-β-carboline (1-ABC), which inhibits the transformation of *C. albicans* into mycelium and biofilm formation by inhibiting the DYRK1 family kinase of fungi, thus inhibiting its pathogenicity (Figure 1) (Leão et al., 2015). The antifungal mixture produced by *Lactobacillus crispatus* inhibits the growth and formation of *C. albicans* hyphae (Wang et al., 2017a). *Lactobacillus acidophilus* inhibits *C. albicans* biofilm formation and alleviates host candidiasis symptoms (Vilela et al., 2015). That is, *Lactobacillus* has inhibitory effects on fungal growth, biofilm formation, mycelium development, dental canal germination and virulence. Therefore, *Lactobacillus* can be used as a probiotic as a therapeutic adjuvant for mycosis to improve antifungal effects.

Bacillus is a common Gram-positive bacterium that is resistant to external harmful factors and widely distributed in soil, water, air, and animal intestinal tracts. In some ways, it can act as a probiotic to maintain the balance of microorganisms in the human body. In the human intestinal tract, co-parasitism between *Bacillus* and *C. albicans* can inhibit the invasion of *C. albicans*. Rautela's research shows that lipopeptide secreted by *Bacillus* can inhibit the formation of fungal biofilm by reducing the expression of specific biofilm-forming genes, such as *HWP1* and *ALS3* (Rautela et al., 2014) (Table 3, Figure 1). Recent researches have shown that *Bacillus safensis* could inhibit fungal melanin exfoliation and melanosis through contact, thereby reducing *C. albicans* virulence and inhibiting biofilm formation (Mayer and Kronstad, 2017). *B. safensis* can also produce chitinase, which can damage fungal cell walls and inhibit fungal virulence factors. It is a vital active substance. Meanwhile, the virulence of other fungi (such as *C. neoformans*) could be significantly inhibited by *Bacillus* (Farrer et al., 2016) (Table 3). In summary, *Bacillus* reduces the fungal virulence by inhibiting the generation of hyphae, which may be a new antifungal target.

A key regulatory hub for virulence is quorum sensing (QS). QS is a signaling transmission mechanism between microorganisms, through the secretion and release of some specific signal molecules, the perception of their concentration changes to monitor the population density, regulate the physiological function of the population, so as to adapt to the surrounding environment. It is also called "intercellular communication" or "self-induction". The signaling molecules produced during the regulation are also called autoinducers (AI) (Galloway et al., 2011). Researchers found that QS regulates biological behaviors of bacteria, such as fluorescence production, toxin production, and the production of extracellular enzymes of pathogenic bacteria, especially biofilm formation (Miller and Bassler, 2001; Hooshangi and Bentley, 2008; Yang and Defoirdt, 2015). For example, *S. aureus* requires the Agr quorum sensing system to achieve intestinal colonization, and the lipopeptide fencarinin produced by *Bacillus* (mainly *Bacillus subtilis*) has a similar structure to AIP (auto-inducing peptide), which is a key factor in Agr quorum sensing and can interfere with Agr signal transduction, thereby inhibiting Agr quorum sensing and ultimately preventing *S. aureus* from colonizing the intestinal tract to eliminate this notorious multi-antibiotic-resistant bacterium (Piewngam et al., 2018). Moreover, quorum sensing systems exist not only in bacteria but also in fungi.

Farnesol in *C. albicans* is the first discovered quorum sensing molecule (QSM) (Hornby et al., 2001). Researches have shown that farnesol can inhibit *C. albicans* biofilm formation, can inhibit the transformation of *C. albicans* from yeast type to mycelium type, and can also induce the expression of antioxidant genes to resist oxidative stress (Langford et al., 2009). The Ras1 cyclic AMP protein kinase A pathway positively regulates *C. albicans* mycelial growth, and farnesol inhibits mycelial growth by inhibiting elements in this pathway (Davis-Hanna et al., 2008). 2-phenylethanol (2-PE) is a QSM in *S. cerevisiae*. Researches have shown that exogenous 2-PE could stimulate biofilm formation at low cell concentrations. In addition, 2-PE is synthesized mainly by the Ehrlich pathway, involving *ARO8p* and *ARO9p*, which are

TABLE 3 Substances secreted by bacteria and their influence on fungi.

	Invasive fungi	Produced by bacteria	Mechanism of action	Functional effect	References
Pseudomonas aeruginosa	*Candida albicans*	Phenazine	Oxidative activity, intracellular ROS production increased, mitochondrial membrane hyperpolarized, affect *C. albicans* respiration, apoptosis.	Inhibit metabolism and biofilm formation	(Gibson et al., 2009; Tupe et al., 2015)
		Lipopolysaccharide	LPS up-regulated the expression of transcription factor EFG1 in *C.albicans* biofilm, increased glycolysis and inhibited *Candida* hyphae-specific genes (HSGs)	Alter gene expression during biofilm formation and inhibit hyphal formation	(Bandara et al., 2013)
		Secretory lysophospholipase C	Degraded phosphatidylcholine (phospholipids abundant in eukaryotes)	The death of the fungal cell	(Hogan and Kolter, 2002)
		Acyl homoserine lactones (AHLs)	Inhibit the Ras1–cAMP–PKA pathway for hyphal growth in *C. albicans*	Inhibit virulence	(Davis-Hanna et al., 2008)
	Cryptococcus neoformans	Pyocyanine、2-heptyl-3,4-dihydroxyquinoline (PQS)	*Pseudomonas aeruginosa* contacts *C.neoformans*, and antifungal molecules play a role	Inhibit growth	(Rella et al., 2012)
	Aspergillus fumigatus	Pyocyanine	Chelated iron, deprived of *A.fumigatus* necessary nutrition for growth and metabolism	Inhibit virulence	(Mowat et al., 2010)
Staphylococcus	*Candida albicans*	–	–	Inhibit biofilm formation	(Adam et al., 2002)
	Cryptococcus neoformans	–	*Staphylococcus aureus* attached to *cryptococcus capsulatus*, mitochondrial pathway was enhanced, and cells died.	Apoptotic cell death	(Ikeda et al., 2007)
	Aspergillus fumigatus	–	Fungal growth may be significantly limited by intercellular contact and synthesis of bacterial products	Inhibit growth, conidia, hypha and biofilm formation	(Ramírez-Granillo et al., 2021)
Bacillus	*Candida albicans*	Lipopeptide	Biosurfactant properties, reduced the mRNA expression of hypha-specific genes *HWP1* and *ALS3*	Inhibit biofilm formation	(Ceresa et al., 2016; Janek et al., 2020)
	Cryptococcus neoformans	Chitinase Laccase	Reduce the structural stability of cell wall to inhibit capsule formation; Inhibit melanin formation	Inhibit biofilm formation and virulence factor production	(Farrer et al., 2018; Upadhya et al., 2018)
	Aspergillus fumigatus	–	Inhibit the gene expression related to hyphae	Inhibit hyphae formation	(Benoit et al., 2015)
Enterococcus faecalis	*Candida albicans*	Bacteriocin Entv	Regulate that Frs system of *Candida*, secreting GelE, SprE and some extracellular protease	Inhibit the formation of hyphae and biofilm, inhibit virulence	(Cruz et al., 2013)
Lactobacillus	*Candida albicans*	Short-chain fatty acid Lactic acid	Inhibit the expression of *C. albicans HWP1* gene, reduce the adhesion and the stability of biofilm structure	Inhibit virulence and hyphae formation	(Noverr and Huffnagle, 2004; Jang et al., 2019)
	Cryptococcus neoformans	Sodium butyrate	Inhibit the formation of capsule and melanin	Inhibit growth and enhance macrophages	(Hoberg et al., 1983)
Escherichia coli	*Candida albicans*	Lipopolysaccharide	–	Modulate biofilm formation	(Bandara et al., 2010)
	Aspergillus fumigatus	Cytosolic proteins	Inhibit the development of conidia and cause hyphal atrophy	Inhibit growth	(Balhara et al., 2014)
Acinetobacter baumannii	*Candida albicans*	Outermembrane protein A(OmpA)	OmpA Attaches *A. baumannii* to *C.albicans* Filament	Inhibit hyphae and biofilm formation	(Brossard and Campagnari, 2012)
Serratia marcescens	*Candida albicans*	Glycolipid	Biosurfactant properties, anti-adhesion	Inhibit biofilm formation	(Dusane et al., 2011)

"-" No detailed reports at the time of review

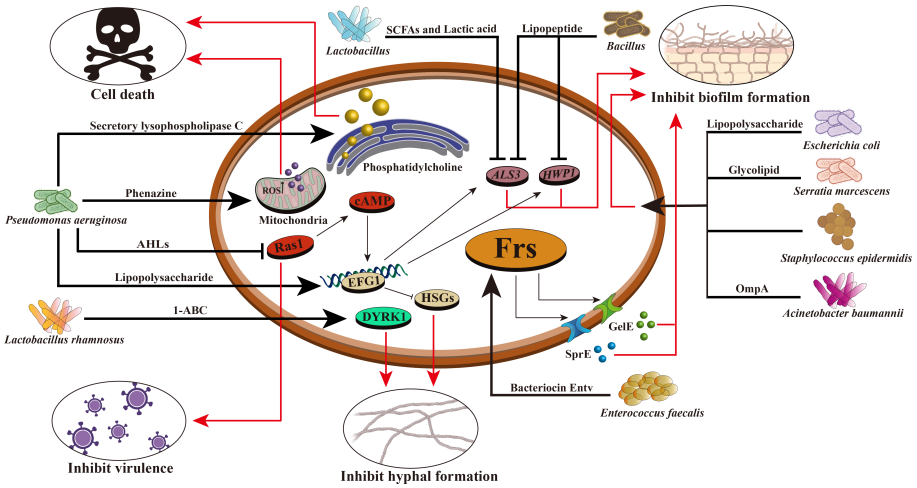

FIGURE 1
Mechanism of intestinal bacteria against *C. albicans*. Intestinal bacteria prevent the proliferation and colonization of *C. albicans* through various mechanisms. For example, *Pseudomonas aeruginosa* can produce phenazine, intracellular ROS production increased, mitochondrial membrane hyperpolarization, leading to the death of *C. albicans*; It can secrete lipopolysaccharide and improve EFG1 transcription in the hypoxia condition of biofilm environment, thereby stimulating glycolysis and inhibiting *Candida* hyphae-specific genes (HSGs), changing gene expression in biofilm formation and inhibiting hypha formation; Producing secretory lysophospholipase C, which degrades phosphatidylcholine and leads to the death of *C. albicans*; AHLs are produced that inhibit the *C. albicans* Ras1–cAMP–PKA pathway and reduce its virulence. *Enterococcus faecalis* can produce bacteriocin Entv, which can regulate the Frs system of *C. albicans*, secrete GelE, SprE and some extracellular proteases, inhibit the formation of *C. albicans* biofilm and hyphae, and reduce its toxicity. *Acinetobacter baumannii* can secrete OmpA and make it adhere to *C. albicans* to inhibit the formation of *C. albicans* hyphae and biofilm. The glycolipid substances secreted by *Serratia marcescens* have the characteristics of biosurfactant and can inhibit the formation of *C. albicans* biofilm. *Bacillus* can produce lipopeptide and also has the characteristics of biosurfactant, which can reduce the expression of *HWP1* and *ALS3* of *C. albicans* and inhibit its biofilm formation. *Lactic acid bacteria* can produce short-chain fatty acids and lactic acid, reduce the expression of *HWP1*, and inhibit the hyphal formation of *C. albicans*. *Lactobacillus rhamnosus* can secrete 1- acetyl-β-carboline (1-ABC) to inhibit the formation of *C. albicans* biofilm by inhibiting the fungal DYRK1 family kinase. *Staphylococcus epidermidis* can inhibit the biofilm formation of *C. albicans*, but its inhibition mechanism is still unclear.

essential for the formation of biofilm. This research screened the mutants *ΔARO8* and *ΔARO9*, and found that the deletions of these two genes reduced the 2-PE content, ethanol yield, extracellular polysaccharide content, the expression of *FLOp* participating in cell adhesion in the early stage of fermentation, and decreased the formation of biofilm (Wang et al., 2017b; Zhang et al., 2021). These findings suggest that the production of 2-PE could promote the formation of *S. cerevisiae* biofilm, so and thus the formation of biofilm could be inhibited by reducing the production of 2-PE. Other fungi also have quorum sensing signal molecules, such as *C. neoformans*, with an 11-amino acid polypeptide signaling molecule that promotes fungal growth on solid media (De Sordi and Mühlschlegel, 2009). All these indicate that the quorum sensing system plays an important role in fungi, but the signaling molecules and mechanisms of most fungi are still unclear. However, in the future antifungal therapy, we can study the mechanism of QS in fungi to understand the pathogenic mechanism, and use the quorum sensing system as a target for fungal anti-virulence therapy to develop new antifungal drugs.

2.2 Probiotics produce active metabolites to inhibit fungi

Among a variety of active metabolites produced by probiotics, bacteriocin is a 20-60 amino acid long polypeptide or protein with antibacterial activity synthesized by bacteria through the ribosomal pathway during the metabolic process. It is safe, nontoxic and has strong antibacterial activity, and is an important active metabolite (Donia and Fischbach, 2015). At present, it is generally believed that bacteriocin can destroy the cell membrane structure, form pores in the cell membrane, leak out intracellular substances, inhibit the function of the proton pump, cause the disorder of membrane potential and pH gradient, and then lead to the inhibition of DNA, RNA, protein and polysaccharide synthesis. It causes the loss of intracellular nutrients and eventually leads to bacterial cell death, thereby inhibiting the growth of intestinal pathogens and regulating the balance of intestinal flora (Moll et al., 1999). For example, *E. faecalis* can secrete a bacteriocin, Entv, which can inhibit the virulence, hyphal and biofilm formation of *C. albicans*, and thus inhibit its colonization and invasion of the intestinal tract (Table 3, Figure 1) (Graham et al., 2017). *L. rhamnosus* L60 and *Lactobacillus fermentum* L23 have potential probiotic activities, and their secondary active metabolite, bacteriocin, can inhibit the biosynthesis of aflatoxin B1 (AFB1) and the growth of *A. flavus* (Gerbaldo et al., 2012). Moreover, bacteriocin has a unique antibacterial mechanism, which is not easy to cause drug resistance; It has low immunogenicity and it is not easy to generate immune reaction in human body; It can be decomposed by protease in human body, so it is less toxic to the human body and will not destroy normal intestinal ecology (Barbosa et al., 2015). Therefore, probiotics that secrete bacteriocins that have specific antibacterial effects without disrupting the normal intestinal flora may be more desirable antibacterial agents than antibiotics.

According to related reports, lactic acid is considered as an important antibacterial substance that can lower the pH of fermentation broth, creating an acidic environment, which is not conducive to the growth of pathogenic bacteria. In addition, lactic acid can also enter the cell membrane and cannot be excreted, acidifying the cytoplasm of bacteria, interfering with intracellular functions and leading to cell death, thereby achieving a strong antibacterial effect (Tachedjian et al., 2017). Vulvovaginal candidiasis (VCC) is one of the major causes of female genital infections, and its main pathogenic factor is *C. albicans*. Clinically, fluconazole is the first choice for the treatment of VCC, but the increasing resistance of *Candida* strains to this treatment has prompted the search for alternative therapies for VCC (Rodríguez-Cerdeira et al., 2019). It is well known that lactic acid bacteria can ferment sugar to produce large amounts of lactic acid and obtain energy. For example, researches have confirmed that *Lactobacillus casei* can inhibit the growth of *C. albicans* causing VCC, and reduce the formation of *C. albicans* biofilm and hyphae, reducing their drug resistance and preventing invasive systemic infection (Paniágua et al., 2021). DRutz et al. also found a protective effect of oral administration of *L. acidophilus* on candidal vaginitis (Drutz, 1992). There are also researches that show that after women are infected with HIV, lactic acid produced by lactobacillus can acidify the vagina and reduce the burden of HIV, thus reducing the risk of HIV transmission among women like men and reducing the colonization of some opportunistic pathogenic *Candida* (Tachedjian et al., 2017).

Besides, short-chain fatty acids (SCFAs: such as acetate, propionate and butyrate) produced by probiotics can change the pH environment of the intestine. For example, butyrate can reduce the expression of several virulent genes of *Salmonella enterica* and *Salmonella typhimurium* and inhibit the growth of enterohemorrhagic *Escherichia coli* (EHEC) (Shin et al., 2002). SCFAs secreted by *Lactobacillus* can inhibit the morphological transformation of *C. albicans* and reduce its pathogenicity (Liang et al., 2016). They can affect the formation of hyphae and pseudohyphae by affecting the expression level of the *HWP1* gene of *C. albicans*, reduce adhesion activity, and lose structural stability of biofilm (Matsuda et al., 2018; Rossoni et al., 2018). Furthermore, *Lactobacillus* can produce butyrate, which can inhibit filamentation of *C. albicans*, block the formation of capsule and melanin of *C. neoformans*, and enhance the effector function of macrophages (Nguyen et al., 2011). In addition, SCFAs can be used as hosts to reduce oxygen concentration and create an environment that is not conducive to fungal growth (Rivera-Chávez et al., 2016). Probiotics can also inhibit pathogen adhesion to intestinal epithelium by producing bacteriocins and SCFAs; improve the barrier function of intestinal mucosa by increasing mucus layer and producing tight junction proteins; stimulate the production of secretory IgA to improve intestinal immunity, and then relieve metabolic diseases and intestinal disorders diseases such as IBD, IBS and colorectal cancers (Sendid et al., 2009; Eslami et al., 2019; Simon et al., 2021).

Indole, a metabolite of tryptophan, is usually produced by intestinal bacteria containing tryptophanase, and is a specific intestinal commensal bacterial signal (Beaumont et al., 2018). Indole is important for innate immunity, inhibition of inflammation, elimination of free radicals and maintaining the integrity of intestinal immune barrier (Roager and Licht, 2018). Researches have shown that commensal *E. coli* can produce indole to inhibit the chemotaxis of pathogenic *E. coli*, and inhibit the adhesion of pathogenic bacteria to the intestinal epithelium by increasing the expression of genes involved in intestinal epithelial function, such as actin cytoskeleton and adhesive junction (Bansal et al., 2007). In addition, indole can be used as a ligand for aryl hydrocarbon receptor (AhR) to mediate intestinal immune regulation, which is beneficial to immune homeostasis and affects host physiological function. *Bifidobacterium infantis* has been reported to produce indole -3- lactic acid, which activates the AhRs of the intestinal epithelium by increasing its upregulation of CYP1A1 protein expression. The activation of AhR leads to the transcription of lL-22. In the intestinal tract, lL-22 can regulate the release of antimicrobial peptides, further increase the expression of antimicrobial peptides, and reduce the colonization of *C. albicans* in the gastrointestinal tract (Liu et al., 2020). Another research also showed that the cell-free supernatant of *Lactobacillus paracasei* contained metabolites such as lactic acid, SCFAs, indoleacetic acid and 2- hydroxy -3- methylbutyric acid, etc., which had good antifungal activity (Honoré et al., 2016). After these metabolites secreted by probiotics, including probiotics, regulate the intestinal flora, the changes of microbial metabolites in the intestinal tract will eventually affect the intestinal barrier, and the permeability of the intestinal barrier will be reduced, and our general inflammation will also be reduced, thus greatly reducing our infection rate and prevalence rate. These active metabolites are considered to be a molecule derived from natural resources, which are less susceptible to drug resistance than the high cost and unpredictability of developing new antifungal agents, is a feasible and promising approach.

Whether the probiotics themselves are used to inhibit the virulence factors of fungi, or anti-virulence treatment by targeting the quorum sensing system of fungi, or the active metabolites secreted by probiotics to inhibit the invasion and colonization of fungi in the intestine, all these have shown that probiotics can promote human health. Therefore, the potential of probiotics inspires more people to explore the untapped microorganisms in the healthy human gut to fight fungi.

3 Using intestinal commensal bacteria to inhibit the invasion of pathogenic fungi

At present, it has been confirmed that in addition to some recognized probiotics, some intestinal commensal bacteria can also inhibit the invasion of fungi through a certain mechanism, including Gram-positive and Gram-negative bacteria, such as *Staphylococcus*, *Pseudomonas aeruginosa* and *E. coli*, etc.

Staphylococcus is a common Gram-positive coccus that can exist on the human body surface, mouth and throat and inside the intestine, mainly causing nosocomial cross-infection. Human infection with *Staphylococcus* can cause severe diseases such as

pneumonia, sepsis, and toxic shock syndrome, and even lead to death. But researches have found that *Staphylococcus* can inhibit the growth of *C. albicans*, when they are dominant in the niche. Culture supernatant of *S. epidermidis* has been proved to inhibit *C. albicans* biofilm formation, but the mechanism of inhibition is still unclear (Bhattacharyya et al., 2014). When *S. aureus* and *C. neoformans* are co-cultured, they can contact with each other to inhibit the growth of *C. neoformans*. When the two organisms are not in contact, inhibition of *C. neoformans* disappears (Saito and Ikeda, 2005). The mechanism is that *S. aureus* enhances the mitochondrial pathway of *C. neoformans* by attaching to the capsule of *C. neoformans*, resulting in the death of *C. neoformans* (Ikeda, 2011). *S. aureus* has been found to significantly inhibit the formation and development of *A. fumigatus* biofilm *in vitro*, regardless of the amount of bacterial inoculum with *A. fumigatus* (Ramírez Granillo et al., 2015). In general, *C. albicans* and *C. neoformans* can be inhibited by *S. aureus* when they are in contact with *S. aureus* (Saito and Ikeda, 2005).

In addition to Gram-positive bacteria, some Gram-negative bacteria can also inhibit the virulence factors of invasive fungus or directly inhibit their growth. For example, *P. aeruginosa*, a Gram-negative bacterium found in various water, air, normal human skin, respiratory tract, and intestinal tract, and is also an opportunistic pathogen. Human infection can cause postoperative wound infection, abscess, bacteremia and other symptoms, and even lead to death. Clinically, mixed infections of *P. aeruginosa* with *C. albicans*, *A. fumigatus*, and *C. neoformans* have been found in patients with pulmonary fibrosis (Nazik et al., 2017). But some experiments show that *P. aeruginosa* can inhibit *C. albicans* infection. *P. aeruginosa* can produce phenazine to inhibit the metabolic process and *C. albicans* biofilm formation (Morales et al., 2013). Therefore, the combination of azoles and phenazine can enhance the efficacy of candidiasis and provide a new idea for clinical treatment of *Candida* infections (Nishanth Kumar et al., 2014). In addition, *P. aeruginosa* can secrete lipopolysaccharide, which can inhibit the formation of *C. albicans* hyphae and biofilm formation by affecting the expression of key genes during biofilm formation (Bandara et al., 2013). It is recognized that Fe^{2+} and Cu^{2+} are indispensable nutrients for the growth of *C. albicans*. However, *P. aeruginosa* can inhibit fungal growth by inhibiting the fungal absorption processes of Fe^{2+} and Cu^{2+} (Robinett et al., 2019). Similarly, *P. aeruginosa* produces pyocyanin that inhibits the growth of *C. neoformans* when contacts with *C. neoformans* (Rella et al., 2012). The pyocyanin can also inhibit *A. fumigatus* growth, resulting in a deficiency of metal iron ions in the bacterium (Sass et al., 2018). Based on the current researches, we found that pyoverdine is the key substance for inhibiting the growth of fungi, and its inhibitory effect is regulated by the metabolism of metal ions, such as Fe^{2+} and Cu^{2+} (Hunsaker and Franz, 2019).

Besides *P. aeruginosa*, there are some Gram-negative bacteria that can inhibit the growth of invasive fungi. For example, *E. coli* can secrete lipopolysaccharide to inhibit the formation of *Candida* biofilm (Bandara et al., 2009). In addition, a specific protein produced by a variety of *Escherichia* bacteria can inhibit the development of conidia and cause hyphal atrophy, which in turn inhibits the growth of *A. fumigatus* (Yadav et al., 2005). *Acinetobacter baumannii* shows antifungal activity by binding to *C. albicans* hyphae and inducing apoptosis to prevent its biofilm formation through outer membrane protein (OmpA) (Gaddy et al., 2009).

Some intestinal commensal bacteria can also defend fungi by regulating the host anti-fungal immune response. For example, Villena et al. in a mouse model infected with *Candida* have found that supplementation of *L. casei* diet to mice can reduce mortality, increase phagocytosis and fungicidal activity of intestinal macrophages, and increase the number and function of neutrophils to prevent colonization and invasion of *Candida* (Villena et al., 2011). Researches have confirmed that *Bacteroides fragilis* produces the molecular polysaccharide A(PSA), which can eliminate fungi and maintain the steady state of the intestinal environment by inducing anti-inflammatory regulatory T cells (Treg cells) through TLR2 stimulation (Round et al., 2011). To sum up, intestinal commensal bacteria are essential to maintain the intestinal microecological homeostasis, and at the same time, some intestinal commensal bacteria can be used to prevent and control IFI. Although this approach still has great limitations, it is a vital step in developing new antifungal strategies.

4 Conclusion and prospect

In recent years, IFI have increased, together with the increase in drug resistance and the limited variety of antifungal drugs, which have posed an unprecedented threat to public health. Therefore, we need new strategies to fight IFI. There is a great deal of microbial symbiosis in our gut, including bacteria, fungi, viruses, archaea, bacteriophage and protozoa. Microbes settle down immediately after the birth of mammals, and many microbes that reside in the intestinal tract adapt to the intestinal environment and compete with other microbes for the nutritional niche of the host. Moreover, more and more researches have focused on the use of intestinal bacteria against fungi, and they have been confirmed that some probiotics and intestinal commensal bacteria can inhibit fungal growth, invasion, and gut colonization.

Besides some of the traditional probiotics mentioned above, "next-generation probiotics" are beginning to emerge as new preventive and therapeutic tools, such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* (O'Toole et al., 2017). Researches have shown that oral administration of live *F. prausnitzii* or its supernatant significantly reduces the severity of TNBS colitis, in part because it secretes metabolites that prevent NF-κB activation and IL-8 production (Sokol et al., 2008). Although there are few researches on the next generation of probiotics against pathogenic fungi, but Faecalibacterium can produce high levels of butyrate, and whether it inhibits fungi like other intestinal probiotics needs to be explored.

However, the use of intestinal bacteria against IFI also has certain limitations. For example, the mechanism by which some intestinal symbionts affect fungal colonization is still unclear and needs to be further studied, which is also a promising direction for future researches on intestinal bacteria. Whether or how they can be applied clinically for antifungal therapy is also a problem worthy of deep thinking. In addition, the protection provided by

microorganisms is highly dependent on the host's diet, and probiotic-derived metabolites need to be consumed specific nutrients before they are produced. For example, SCFAs-butyrate and propionate are derived from the fermentation of fibers in the colon. Classical western-style diet usually lacks fruits and vegetables (sources of fiber and flavonoids), resulting in decreased abundance of intestinal bacteria and reorganization of intestinal microflora (Wastyk et al., 2023). At the same time, the intestinal flora deprived of dietary fiber damages the colonic mucus barrier, thus increasing pathogen susceptibility (Desai et al., 2016). All in all, the intestinal flora and the human body are mutually beneficial and inseparable. It is an important place for probiotics and some intestinal commensal bacteria to exert an antifungal effect. In combination with the rapidly developing omics methods, we can explore the antifungal probiotics and commensal bacteria in the intestine. In-depth study of their antifungal mechanism will provide new ideas and treatment strategies for "fungal inhibition by bacteria" in the future against pathogenic fungi.

Author contributions

Conceptualization, YL and ZZ; methodology, YL, LC, and ZZ; software, LC and SM; validation, LC, CC, and ZH; investigation, SM; writing—original draft preparation, LC; writing—review and editing, LC, CC, and ZH; supervision, YL and ZZ; funding acquisition, YL and ZZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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References

Adam, B., Baillie, G. S., and Douglas, L. J. (2002). Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J. Med. Microbiol.* 51 (4), 344–349. doi: 10.1099/0022-1317-51-4-344

Alspaugh, J. A. (2015). Virulence mechanisms and *Cryptococcus neoformans* pathogenesis. *Fungal Genet. Biol.* 78, 55–58. doi: 10.1016/j.fgb.2014.09.004

Antinori, S., Bonazzetti, C., Gubertini, G., Capetti, A., Pagani, C., Morena, V., et al. (2020). Tocilizumab for cytokine storm syndrome in COVID-19 pneumonia: an increased risk for candidemia? *Autoimmun. Rev.* 19 (7), 102564. doi: 10.1016/j.autrev.2020.102564

Balhara, M., Ruhil, S., Kumar, M., Dhankhar, S., and Chhillar, A. K. (2014). Inhibition of conidiophore development in *Aspergillus fumigatus* by an *Escherichia coli* DH5α strain, a promising antifungal candidate against aspergillosis. *J. Mycol. Med.* 24 (1), 1–12. doi: 10.1016/j.mycmed.2013.07.055

Bandara, H. M., BP, K. C., Watt, R. M., Jin, L. J., and Samaranayake, L. P. (2013). *Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development. *Mol. Oral. Microbiol.* 28 (1), 54–69. doi: 10.1111/omi.12006

Bandara, H., Lam, O. L. T., Watt, R. M., Jin, L. J., and Samaranayake, L. P. (2010). Bacterial lipopolysaccharides variably modulate *in vitro* biofilm formation of *Candida* species. *J. Med. Microbiol.* 59 (Pt 10), 1225–1234. doi: 10.1099/jmm.0.021832-0

Bandara, H., Yau, J. Y. Y., Watt, R. M., Jin, L. J., and Samaranayake, L. P. (2009). *Escherichia coli* and its lipopolysaccharide modulate *in vitro candida* biofilm formation. *J. Med. Microbiol.* 58 (Pt 12), 1623–1631. doi: 10.1099/jmm.0.012989-0

Bansal, T., Englert, D., Lee, J., Hegde, M., Wood, T. K., and Jayaraman, A. (2007). Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization, and gene expression. *Infect. Immun.* 75 (9), 4597–4607. doi: 10.1128/iai.00630-07

Barbosa, J., Caetano, T., and Mendo, S. (2015). Class I and class II lanthipeptides produced by *Bacillus* spp. *J. Nat. Prod.* 78 (11), 2850–2866. doi: 10.1021/np500424y

Beaumont, M., Neyrinck, A. M., Olivares, M., Rodriguez, J., de Rocca Serra, A., Roumain, M., et al. (2018). The gut microbiota metabolite indole alleviates liver inflammation in mice. *FASEB J.* 32 (12), fj201800544. doi: 10.1096/fj.201800544

Benoit, I., van den Esker, M. H., Patyshakuliyeva, A., Mattern, D. J., Blei, F., Zhou, M., et al. (2015). *Bacillus subtilis* attachment to *Aspergillus* niger hyphae results in mutually altered metabolism. *Environ. Microbiol.* 17 (6), 2099–2113. doi: 10.1111/1462-2920.12564

Bhattacharyya, S., Gupta, P., Banerjee, G., Jain, A., and Singh, M. (2014). Inhibition of biofilm formation and lipase in *Candida albicans* by culture filtrate of *Staphylococcus epidermidis in vitro*. *Int. J. Appl. Basic Med. Res.* 4 (Suppl 1), S27–S30. doi: 10.4103/2229-516x.140721

Bongomin, F., Gago, S., Oladele, R. O., and Denning, D. W. (2017). Global and multi-national prevalence of fungal diseases-estimate precision. *J. Fungi (Basel)* 3 (4), 57–62. doi: 10.3390/jof3040057

Brossard, K. A., and Campagnari, A. A. (2012). The *Acinetobacter baumannii* biofilm-associated protein plays a role in adherence to human epithelial cells. *Infect. Immun.* 80 (1), 228–233. doi: 10.1128/iai.05913-11

Ceresa, C., Rinaldi, M., Chiono, V., Carmagnola, I., Allegrone, G., and Fracchia, L. (2016). Lipopeptides from *Bacillus subtilis* AC7 inhibit adhesion and biofilm formation of *Candida albicans* on silicone. *Antonie Van Leeuwenhoek* 109 (10), 1375–1388. doi: 10.1007/s10482-016-0736-z

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., et al. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in wuhan, China: a descriptive study. *Lancet* 395 (10223), 507–513. doi: 10.1016/s0140-6736(20)30211-7

Chibbar, R., and Dieleman, L. A. (2019). The gut microbiota in celiac disease and probiotics. *Nutrients* 11 (10), 2375–2386. doi: 10.3390/nu11102375

Chowdhary, A., Sharma, C., and Meis, J. F. (2017). *Candida auris*: a rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. *PloS Pathog.* 13 (5), e1006290. doi: 10.1371/journal.ppat.1006290

Cruz, M. R., Graham, C. E., Gagliano, B. C., Lorenz, M. C., and Garsin, D. A. (2013). *Enterococcus faecalis* inhibits hyphal morphogenesis and virulence of *Candida albicans*. *Infect. Immun.* 81 (1), 189–200. doi: 10.1128/iai.00914-12

Davis-Hanna, A., Piispanen, A. E., Stateva, L. I., and Hogan, D. A. (2008). Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. *Mol. Microbiol.* 67 (1), 47–62. doi: 10.1111/j.1365-2958.2007.06013.x

Desai, M. S., Seekatz, A. M., Koropatkin, N. M., Kamada, N., Hickey, C. A., Wolter, M., et al. (2016). A dietary fiber-deprived gut microbiota degrades the colonic mucus

barrier and enhances pathogen susceptibility. *Cell* 167 (5), 1339–1353.e1321. doi: 10.1016/j.cell.2016.10.043

De Sordi, L., and Mühlschlegel, F. A. (2009). Quorum sensing and fungal-bacterial interactions in *Candida albicans*: a communicative network regulating microbial coexistence and virulence. *FEMS Yeast Res.* 9 (7), 990–999. doi: 10.1111/j.1567-1364.2009.00573.x

Donia, M. S., and Fischbach, M. A. (2015). HUMAN MICROBIOTA. small molecules from the human microbiota. *Science* 349 (6246), 1254766. doi: 10.1126/science.1254766

Drutz, D. J. (1992). *Lactobacillus* prophylaxis for *Candida vaginitis*. *Ann. Intern. Med.* 116 (5), 419–420. doi: 10.7326/0003-4819-116-5-419

Dusane, D. H., Pawar, V. S., Nancharaiah, Y. V., Venugopalan, V. P., Kumar, A. R., and Zinjarde, S. S. (2011). Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of serratia marcescens. *Biofouling* 27 (6), 645–654. doi: 10.1080/08927014.2011.594883

Eslami, M., Yousefi, B., Kokhaei, P., Hemati, M., Nejad, Z. R., Arabkari, V., et al. (2019). Importance of probiotics in the prevention and treatment of colorectal cancer. *J. Cell Physiol.* 234 (10), 17127–17143. doi: 10.1002/jcp.28473

Fairlamb, A. H., Gow, N. A., Matthews, K. R., and Waters, A. P. (2016). Drug resistance in eukaryotic microorganisms. *Nat. Microbiol.* 1 (7), 16092. doi: 10.1038/nmicrobiol.2016.92

Farrer, R. A., Ford, C. B., Rhodes, J., Delorey, T., May, R. C., Fisher, M. C., et al. (2018). Transcriptional heterogeneity of *Cryptococcus gattii* VGII compared with non-VGII lineages underpins key pathogenicity pathways. *mSphere* 3 (5), 25–38. doi: 10.1128/mSphere.00445-18

Farrer, R. A., Voelz, K., Henk, D. A., Johnston, S. A., Fisher, M. C., May, R. C., et al. (2016). Microevolutionary traits and comparative population genomics of the emerging pathogenic fungus cryptococcus gattii. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 371 (1709). doi: 10.1098/rstb.2016.0021

Gaddy, J. A., Tomaras, A. P., and Actis, L. A. (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* 77 (8), 3150–3160. doi: 10.1128/iai.00096-09

Galloway, W. R., Hodgkinson, J. T., Bowden, S. D., Welch, M., and Spring, D. R. (2011). Quorum sensing in gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem. Rev.* 111 (1), 28–67. doi: 10.1021/cr100109t

Gerbaldo, G. A., Barberis, C., Pascual, L., Dalcero, A., and Barberis, L. (2012). Antifungal activity of two *Lactobacillus* strains with potential probiotic properties. *FEMS Microbiol. Lett.* 332 (1), 27–33. doi: 10.1111/j.1574-6968.2012.02570.x

Gibson, J., Sood, A., and Hogan, D. A. (2009). *Pseudomonas aeruginosa-Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. *Appl. Environ. Microbiol.* 75 (2), 504–513. doi: 10.1128/aem.01037-08

González-Hernández, L. A., Jave-Suarez, L. F., Fafutis-Morris, M., Montes-Salcedo, K. E., Valle-Gutierrez, L. G., Campos-Loza, A. E., et al. (2012). Synbiotic therapy decreases microbial translocation and inflammation and improves immunological status in HIV-infected patients: a double-blind randomized controlled pilot trial. *Nutr. J.* 11, 90. doi: 10.1186/1475-2891-11-90

Gorbalenya, A. E., Baker, S. C., Baric, R. S., de Groot, R. J., Drosten, C., Gulyaeva, A. A., et al (2020). The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5 (4), 536–544. doi: 10.1038/s41564-020-0695-z

Graham, C. E., Cruz, M. R., Garsin, D. A., and Lorenz, M. C. (2017). *Enterococcus faecalis* bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* 114 (17), 4507–4512. doi: 10.1073/pnas.1620432114

Guo, L., Wei, D., Zhang, X., Wu, Y., Li, Q., Zhou, M., et al. (2019). Clinical features predicting mortality risk in patients with viral pneumonia: the MuLBSTA score. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.02752

Gutiérrez-Castrellón, P., Gandara-Martí, T., Abreu, Y. A. A. T., Nieto-Rufino, C. D., López-Orduña, E., Jiménez-Escobar, I., et al. (2022). Probiotic improves symptomatic and viral clearance in Covid19 outpatients: a randomized, quadruple-blinded, placebo-controlled trial. *Gut. Microbes* 14 (1), 2018899. doi: 10.1080/19490976.2021.2018899

Harriott, M. M., Lilly, E. A., Rodriguez, T. E., Fidel, P. L., and Noverr, M. C. (2010). *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiol. (Reading)* 156 (Pt 12), 3635–3644. doi: 10.1099/mic.0.039354-0

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11 (8), 506–514. doi: 10.1038/nrgastro.2014.66

Hoberg, K. A., Cihlar, R. L., and Calderone, R. A. (1983). Inhibitory effect of cerulenin and sodium butyrate on germination of *Candida albicans*. *Antimicrob. Agents Chemother.* 24 (3), 401–408. doi: 10.1128/aac.24.3.401

Hogan, D. A., and Kolter, R. (2002). *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* 296 (5576), 2229–2232. doi: 10.1126/science.1070784

Honoré, A. H., Aunsbjerg, S. D., Ebrahimi, P., Thorsen, M., Benfeldt, C., Knøchel, S., et al. (2016). Metabolic footprinting for investigation of antifungal properties of *Lactobacillus paracasei*. *Anal. Bioanal. Chem.* 408 (1), 83–96. doi: 10.1007/s00216-015-9103-6

Hooper, L. V., Littman, D. R., and Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. *Science* 336 (6086), 1268–1273. doi: 10.1126/science.1223490

Hooshangi, S., and Bentley, W. E. (2008). From unicellular properties to multicellular behavior: bacteria quorum sensing circuitry and applications. *Curr. Opin. Biotechnol.* 19 (6), 550–555. doi: 10.1016/j.copbio.2008.10.007

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., et al. (2001). Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* 67 (7), 2982–2992. doi: 10.1128/aem.67.7.2982-2992.2001

Hunsaker, E. W., and Franz, K. J. (2019). Copper potentiates azole antifungal activity in a way that does not involve complex formation. *Dalton Trans.* 48 (26), 9654–9662. doi: 10.1039/c9dt00642g

Ikeda, R. (2011). Possible participation of the Rho/Rho-associated coiled-coil-forming kinase pathway in the cell death of *Cryptococcus neoformans* caused by *Staphylococcus aureus* adherence. *Microbiol. Immunol.* 55 (8), 552–557. doi: 10.1111/j.1348-0421.2011.00356.x

Ikeda, R., Saito, F., Matsuo, M., Kurokawa, K., Sekimizu, K., Yamaguchi, M., et al. (2007). Contribution of the mannan backbone of cryptococcal glucuronoxylomannan and a glycolytic enzyme of *Staphylococcus aureus* to contact-mediated killing of *Cryptococcus neoformans*. *J. Bacteriol.* 189 (13), 4815–4826. doi: 10.1128/jb.00412-07

Janek, T., Drzymała, K., and Dobrowolski, A. (2020). *In vitro* efficacy of the lipopeptide biosurfactant surfactin-C(15) and its complexes with divalent counterions to inhibit *Candida albicans* biofilm and hyphal formation. *Biofouling* 36 (2), 210–221. doi: 10.1080/08927014.2020.1752370

Jang, S. J., Lee, K., Kwon, B., You, H. J., and Ko, G. (2019). *Vaginal lactobacilli* inhibit growth and hyphae formation of *Candida albicans*. *Sci. Rep.* 9 (1), 8121. doi: 10.1038/s41598-019-44579-4

Jin, Y., Samaranayake, Y. H., Yip, H. K., and Samaranayake, L. P. (2005). Characterization of switch phenotypes in *Candida albicans* biofilms. *Mycopathologia* 160 (3), 191–200. doi: 10.1007/s11046-005-6331-x

Kato, H., Yoshimura, Y., Suido, Y., Ide, K., Sugiyama, Y., Matsuno, K., et al. (2018). Prevalence of, and risk factors for, hematogenous fungal endophthalmitis in patients with *Candida* bloodstream infection. *Infection* 46 (5), 635–640. doi: 10.1007/s15010-018-1163-z

Kumamoto, C. A. (2016). The fungal mycobiota: small numbers, Large impacts. *Cell Host Microbe* 19 (6), 750–751. doi: 10.1016/j.chom.2016.05.018

Kunyeit, L., Kurrey, N. K., Anu-Appaiah, K. A., and Rao, R. P. (2019). Probiotic yeasts inhibit virulence of non-albicans *Candida* species. *mBio* 10 (5), 36–48. doi: 10.1128/mBio.02307-19

Langford, M. L., Atkin, A. L., and Nickerson, K. W. (2009). Cellular interactions of farnesol, a quorum-sensing molecule produced by *Candida albicans*. *Future Microbiol.* 4 (10), 1353–1362. doi: 10.2217/fmb.09.98

Leão, M. V., Gonçalves e Silva, C. R., Santos, S. S., and Leite, P. G. (2015). [*Lactobacillus rhamnosus* may change the virulence of *Candida albicans*]. *Rev. Bras. Ginecol. Obstet.* 37 (9), 417–420. doi: 10.1590/so100-720320150005217

Li, X. V., Leonardi, I., and Iliev, I. D. (2019). Gut mycobiota in immunity and inflammatory disease. *Immunity* 50 (6), 1365–1379. doi: 10.1016/j.immuni.2019.05.023

Liang, W., Guan, G., Dai, Y., Cao, C., Tao, L., Du, H., et al. (2016). Lactic acid bacteria differentially regulate filamentation in two heritable cell types of the human fungal pathogen *Candida albicans*. *Mol. Microbiol.* 102 (3), 506–519. doi: 10.1111/mmi.13475

Limon, J. J., Skalski, J. H., and Underhill, D. M. (2017). Commensal fungi in health and disease. *Cell Host Microbe* 22 (2), 156–165. doi: 10.1016/j.chom.2017.07.002

Liu, Q., Yu, Z., Tian, F., Zhao, J., Zhang, H., Zhai, Q., et al. (2020). Surface components and metabolites of probiotics for regulation of intestinal epithelial barrier. *Microb. Cell Fact* 19 (1), 23. doi: 10.1186/s12934-020-1289-4

Lockhart, S. R., Etienne, K. A., Vallabhaneni, S., Farooqi, J., Chowdhary, A., Govender, N. P., et al. (2017). Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. *Clin. Infect. Dis.* 64 (2), 134–140. doi: 10.1093/cid/ciw691

Mane, A., Vidhate, P., Kusro, C., Waman, V., Saxena, V., Kulkarni-Kale, U., et al. (2016). Molecular mechanisms associated with fluconazole resistance in clinical *Candida albicans* isolates from India. *Mycoses* 59 (2), 93–100. doi: 10.1111/myc.12439

Marx-Stoelting, P., Knebel, C., and Braeuning, A. (2020). The connection of azole fungicides with xeno-sensing nuclear receptors, drug metabolism and hepatotoxicity. *Cells* 9 (5), 1192–1210. doi: 10.3390/cells9051192

Mathur, H., Beresford, T. P., and Cotter, P. D. (2020). Health benefits of *Lactic acid bacteria* (LAB) fermentates. *Nutrients* 12 (6), 1679–1682. doi: 10.3390/nu12061679

Matsuda, Y., Cho, O., Sugita, T., Ogishima, D., and Takeda, S. (2018). Culture supernatants of *Lactobacillus gasseri* and *L. crispatus* inhibit *Candida albicans* biofilm formation and adhesion to HeLa cells. *Mycopathologia* 183 (4), 691–700. doi: 10.1007/s11046-018-0259-4

Mayer, F. L., and Kronstad, J. W. (2017). Disarming fungal pathogens: *Bacillus safensis* inhibits virulence factor production and biofilm formation by *Cryptococcus neoformans* and *Candida albicans*. *mBio* 8 (5), 335–346. doi: 10.1128/mBio.01537-17

Mayer, F. L., and Kronstad, J. W. (2019). The spectrum of interactions between *Cryptococcus neoformans* and bacteria. *J. Fungi (Basel)* 5 (2), 31–45. doi: 10.3390/jof5020031

Medici, N. P., and Del Poeta, M. (2015). New insights on the development of fungal vaccines: from immunity to recent challenges. *Mem. Inst. Oswaldo Cruz* 110 (8), 966–973. doi: 10.1590/0074-02760150335

Miller, M. B., and Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol.* 55, 165–199. doi: 10.1146/annurev.micro.55.1.165

Moll, G. N., Konings, W. N., and Driessen, A. J. (1999). Bacteriocins: mechanism of membrane insertion and pore formation. *Antonie Van Leeuwenhoek* 76 (1-4), 185–198. doi: 10.1023/A:1002002718501

Morales, D. K., Grahl, N., Okegbe, C., Dietrich, L. E., Jacobs, N. J., and Hogan, D. A. (2013). Control of *Candida albicans* metabolism and biofilm formation by *Pseudomonas aeruginosa* phenazines. *mBio* 4 (1), e00526-00512. doi: 10.1128/mBio.00526-12

Mowat, E., Rajendran, R., Williams, C., McCulloch, E., Jones, B., Lang, S., et al. (2010). *Pseudomonas aeruginosa* and their small diffusible extracellular molecules inhibit *Aspergillus fumigatus* biofilm formation. *FEMS Microbiol. Lett.* 313 (2), 96–102. doi: 10.1111/j.1574-6968.2010.02130.x

Nazik, H., Moss, R. B., Karna, V., Clemons, K. V., Banaei, N., Cohen, K., et al. (2017). Are cystic fibrosis *Aspergillus fumigatus* isolates different? intermicrobial interactions with *Pseudomonas*. *Mycopathologia* 182 (3-4), 315–318. doi: 10.1007/s11046-016-0087-3

Nguyen, L. N., Lopes, L. C., Cordero, R. J., and Nosanchuk, J. D. (2011). Sodium butyrate inhibits pathogenic yeast growth and enhances the functions of macrophages. *J. Antimicrob. Chemother.* 66 (11), 2573–2580. doi: 10.1093/jac/dkr358

Nishanth Kumar, S., Nisha, G. V., Sudaresan, A., Venugopal, V. V., Sree Kumar, M. M., Lankalapalli, R. S., et al. (2014). Synergistic activity of phenazines isolated from *Pseudomonas aeruginosa* in combination with azoles against *Candida* species. *Med. Mycol.* 52 (5), 482–490. doi: 10.1093/mmy/myu012

Noverr, M. C., and Huffnagle, G. B. (2004). Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect. Immun.* 72 (11), 6206–6210. doi: 10.1128/iai.72.11.6206-6210.2004

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

Pahwa, N., Kumar, R., Nirkhiwale, S., and Bandi, A. (2014). Species distribution and drug susceptibility of candida in clinical isolates from a tertiary care centre at indore. *Indian J. Med. Microbiol.* 32 (1), 44–48. doi: 10.4103/0255-0857.124300

Paniágua, A. L., Correia, A. F., Pereira, L. C., de Alencar, B. M., Silva, F. B. A., Almeida, R. M., et al. (2021). Inhibitory effects of *Lactobacillus casei* shirota against both *Candida auris* and candida spp. isolates that cause vulvovaginal candidiasis and are resistant to antifungals. *BMC Complement Med. Ther.* 21 (1), 237. doi: 10.1186/s12906-021-03405-z

Patil, A., and Majumdar, S. (2017). Echinocandins in ocular therapeutics. *J. Ocul. Pharmacol. Ther.* 33 (5), 340–352. doi: 10.1089/jop.2016.0186

Perfect, J. R. (2017). The antifungal pipeline: a reality check. *Nat. Rev. Drug Discovery* 16 (9), 603–616. doi: 10.1038/nrd.2017.46

Peters, B. M., Jabra-Rizk, M. A., O'May, G. A., Costerton, J. W., and Shirtliff, M. E. (2012). Polymicrobial interactions: impact on pathogenesis and human disease. *Clin. Microbiol. Rev.* 25 (1), 193–213. doi: 10.1128/cmr.00013-11

Piewngam, P., Zheng, Y., Nguyen, T. H., Dickey, S. W., Joo, H. S., Villaruz, A. E., et al. (2018). Pathogen elimination by probiotic *Bacillus via* signalling interference. *Nature* 562 (7728), 532–537. doi: 10.1038/s41586-018-0616-y

Pimentel, M., and Lembo, A. (2020). Microbiome and its role in irritable bowel syndrome. *Dig. Dis. Sci.* 65 (3), 829–839. doi: 10.1007/s10620-020-06109-5

Ramirez-Garcia, A., Rementeria, A., Aguirre-Urizar, J. M., Moragues, M. D., Antoran, A., Pellon, A., et al. (2016). *Candida albicans* and cancer: can this yeast induce cancer development or progression? *Crit. Rev. Microbiol.* 42 (2), 181–193. doi: 10.3109/1040841x.2014.913004

Ramírez-Granillo, A., Bautista-Hernández, L. A., Bautista-De Lucío, V. M., Magaña-Guerrero, F. S., Domínguez-López, A., Córdova-Alcántara, I. M., et al. (2021). Microbial warfare on three fronts: mixed biofilm of *Aspergillus fumigatus* and *Staphylococcus aureus* on primary cultures of human limbo-corneal fibroblasts. *Front. Cell Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.646054

Ramírez Granillo, A., Canales, M. G., Espíndola, M. E., Martínez Rivera, M. A., de Lucio, V. M., and Tovar, A. V. (2015). Antibiosis interaction of *Staphylococccus aureus* on *Aspergillus fumigatus* assessed *in vitro* by mixed biofilm formation. *BMC Microbiol.* 15, 33. doi: 10.1186/s12866-015-0363-2

Rautela, R., Singh, A. K., Shukla, A., and Cameotra, S. S. (2014). Lipopeptides from *Bacillus* strain AR2 inhibits biofilm formation by *Candida albicans*. *Antonie Van Leeuwenhoek* 105 (5), 809–821. doi: 10.1007/s10482-014-0135-2

Rawson, T. M., Moore, L. S. P., Zhu, N., Ranganathan, N., Skolimowska, K., Gilchrist, M., et al. (2020). Bacterial and fungal coinfection in individuals with coronavirus: a rapid review to support COVID-19 antimicrobial prescribing. *Clin. Infect. Dis.* 71 (9), 2459–2468. doi: 10.1093/cid/ciaa530

Rella, A., Yang, M. W., Gruber, J., Montagna, M. T., Luberto, C., Zhang, Y. M., et al. (2012). *Pseudomonas aeruginosa* inhibits the growth of *Cryptococcus* species. *Mycopathologia* 173 (5-6), 451–461. doi: 10.1007/s11046-011-9494-7

Rementeria, A., López-Molina, N., Ludwig, A., Vivanco, A. B., Bikandi, J., Pontón, J., et al. (2005). Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev. Iberoam Micol.* 22 (1), 1–23. doi: 10.1016/s1130-1406(05)70001-2

Rivera-Chávez, F., Zhang, L. F., Faber, F., Lopez, C. A., Byndloss, M. X., Olsan, E. E., et al. (2016). Depletion of butyrate-producing *Clostridia* from the gut microbiota drives an aerobic luminal expansion of *Salmonella*. *Cell Host Microbe* 19 (4), 443–454. doi: 10.1016/j.chom.2016.03.004

Roager, H. M., and Licht, T. R. (2018). Microbial tryptophan catabolites in health and disease. *Nat. Commun.* 9 (1), 3294. doi: 10.1038/s41467-018-05470-4

Robinett, N. G., Culbertson, E. M., Peterson, R. L., Sanchez, H., Andes, D. R., Nett, J. E., et al. (2019). Exploiting the vulnerable active site of a copper-only superoxide dismutase to disrupt fungal pathogenesis. *J. Biol. Chem.* 294 (8), 2700–2713. doi: 10.1074/jbc.RA118.007095

Rodríguez-Cerdeira, C., Gregorio, M. C., Molares-Vila, A., López-Barcenas, A., Fabbrocini, G., Bardhi, B., et al. (2019). Biofilms and vulvovaginal candidiasis. *Colloids Surf. B. Biointerfaces* 174, 110–125. doi: 10.1016/j.colsurfb.2018.11.011

Rossoni, R. D., de Barros, P. P., de Alvarenga, J. A., Ribeiro, F. C., Velloso, M. D. S., Fuchs, B. B., et al. (2018). Antifungal activity of clinical *Lactobacillus* strains against *Candida albicans* biofilms: identification of potential probiotic candidates to prevent oral candidiasis. *Biofouling* 34 (2), 212–225. doi: 10.1080/08927014.2018.1425402

Round, J. L., Lee, S. M., Li, J., Tran, G., Jabri, B., Chatila, T. A., et al. (2011). The toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 332 (6032), 974–977. doi: 10.1126/science.1206095

Sabra, R., and Branch, R. A. (1990). Amphotericin b nephrotoxicity. *Drug Saf.* 5 (2), 94–108. doi: 10.2165/00002018-199005020-00003

Saito, F., and Ikeda, R. (2005). Killing of *cryptococcus neoformans* by *Staphylococcus aureus*: the role of cryptococcal capsular polysaccharide in the fungal-bacteria interaction. *Med. Mycol.* 43 (7), 603–612. doi: 10.1080/13693780500078417

Sanguinetti, M., Posteraro, B., and Lass-Flörl, C. (2015). Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses* 58 (Suppl 2), 2–13. doi: 10.1111/myc.12330

Sass, G., Nazik, H., Penner, J., Shah, H., Ansari, S. R., Clemons, K. V., et al. (2018). Studies of *Pseudomonas aeruginosa* mutants indicate pyoverdine as the central factor in inhibition of *Aspergillus fumigatus* biofilm. *J. Bacteriol.* 200 (1), 345–358. doi: 10.1128/jb.00345-17

Schelenz, S., Hagen, F., Rhodes, J. L., Abdolrasouli, A., Chowdhary, A., Hall, A., et al. (2016). First hospital outbreak of the globally emerging *Candida auris* in a European hospital. *Antimicrob. Resist. Infect. Control* 5, 35. doi: 10.1186/s13756-016-0132-5

Sendid, B., Jouault, T., Vitse, A., Fradin, C., Colombel, J. F., and Poulain, D. (2009). [Anti-glycan antibodies establish an unexpected link between *C. albicans* and crohn disease]. *Med. Sci. (Paris)* 25 (5), 473–481. doi: 10.1051/medsci/2009255473

Shin, R., Suzuki, M., and Morishita, Y. (2002). Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic *Escherichia coli* O157:H7. *J. Med. Microbiol.* 51 (3), 201–206. doi: 10.1099/0022-1317-51-3-201

Simon, E., Călinoiu, L. F., Mitrea, L., and Vodnar, D. C. (2021). Probiotics, prebiotics, and synbiotics: implications and beneficial effects against irritable bowel syndrome. *Nutrients* 13 (6), 2112–2125. doi: 10.3390/nu13062112

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L. G., Gratadoux, J. J., et al. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of crohn disease patients. *Proc. Natl. Acad. Sci. U.S.A.* 105 (43), 16731–16736. doi: 10.1073/pnas.0804812105

Stiksrud, B., Nowak, P., Nwosu, F. C., Kvale, D., Thalme, A., Sonnerborg, A., et al. (2015). Reduced levels of d-dimer and changes in gut microbiota composition after probiotic intervention in HIV-infected individuals on stable ART. *J. Acquir. Immune Defic. Syndr.* 70 (4), 329–337. doi: 10.1097/qai.0000000000000784

Tachedjian, G., Aldunate, M., Bradshaw, C. S., and Cone, R. A. (2017). The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Res. Microbiol.* 168 (9-10), 782–792. doi: 10.1016/j.resmic.2017.04.001

Taverniti, V., Cesari, V., Gargari, G., Rossi, U., Biddau, C., Lecchi, C., et al. (2021). Probiotics modulate mouse gut microbiota and influence intestinal immune and serotonergic gene expression in a site-specific fashion. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.706135

Trois, L., Cardoso, E. M., and Miura, E. (2008). Use of probiotics in HIV-infected children: a randomized double-blind controlled study. *J. Trop. Pediatr.* 54 (1), 19–24. doi: 10.1093/tropej/fmm066

Tupe, S. G., Kulkarni, R. R., Shirazi, F., Sant, D. G., Joshi, S. P., and Deshpande, M. V. (2015). Possible mechanism of antifungal phenazine-1-carboxamide from *Pseudomonas* sp. against dimorphic fungi benjaminiella poitrasii and human pathogen candida albicans. *J. Appl. Microbiol.* 118 (1), 39–48. doi: 10.1111/jam.12675

Upadhya, R., Baker, L. G., Lam, W. C., Specht, C. A., Donlin, M. J., and Lodge, J. K. (2018). *Cryptococcus neoformans* Cda1 and its chitin deacetylase activity are required for fungal pathogenesis. *mBio* 9 (6), 287–298. doi: 10.1128/mBio.02087-18

Vallabhaneni, S., Kallen, A., Tsay, S., Chow, N., Welsh, R., Kerins, J., et al. (2017). Investigation of the first seven reported cases of *Candida auris*, a globally emerging invasive, multidrug-resistant fungus-united states, may 2013-august 2016. *Am. J. Transplant.* 17 (1), 296–299. doi: 10.1111/ajt.14121

Verweij, P. E., Lucas, J. A., Arendrup, M. C., Bowyer, P., Brinkmann, A. J. F., Denning, D. W., et al. (2020). The one health problem of azole resistance in *Aspergillus fumigatus*: current insights and future research agenda. *Fungal Biol. Rev.* 34 (4), 202–214. doi: 10.1016/j.fbr.2020.10.003

Vilela, S. F., Barbosa, J. O., Rossoni, R. D., Santos, J. D., Prata, M. C., Anbinder, A. L., et al. (2015). *Lactobacillus acidophilus* ATCC 4356 inhibits biofilm formation by *C.*

albicans and attenuates the experimental candidiasis in *Galleria mellonella*. *Virulence* 6 (1), 29–39. doi: 10.4161/21505594.2014.981486

Villena, J., Salva, S., Agüero, G., and Alvarez, S. (2011). Immunomodulatory and protective effect of probiotic *Lactobacillus casei* against *Candida albicans* infection in malnourished mice. *Microbiol. Immunol.* 55 (6), 434–445. doi: 10.1111/j.1348-0421.2011.00334.x

Wang, Z., Bai, X., Guo, X., and He, X. (2017b). Regulation of crucial enzymes and transcription factors on 2-phenylethanol biosynthesis *via* Ehrlich pathway in *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol.* 44 (1), 129–139. doi: 10.1007/s10295-016-1852-5

Wang, S., Wang, Q., Yang, E., Yan, L., Li, T., and Zhuang, H. (2017a). Antimicrobial compounds produced by vaginal *Lactobacillus crispatus* are able to strongly inhibit *Candida albicans* growth, hyphal formation and regulate virulence-related gene expressions. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00564

Wang, F., Xin, C., Liu, J., Ran, Z., Zhao, C., and Song, Z. (2020). Interactions between invasive fungi and symbiotic bacteria. *World J. Microbiol. Biotechnol.* 36 (9), 137. doi: 10.1007/s11274-020-02913-3

Wastyk, H. C., Perelman, D., Topf, M., Fragiadakis, G. K., Robinson, J. L., Sonnenburg, J. L., et al. (2023). Randomized controlled trial demonstrates response to a probiotic intervention for metabolic syndrome that may correspond to diet. *Gut. Microbes* 15 (1), 2178794. doi: 10.1080/19490976.2023.2178794

Watamoto, T., Samaranayake, L. P., Jayatilake, J. A., Egusa, H., Yatani, H., and Seneviratne, C. J. (2009). Effect of filamentation and mode of growth on antifungal susceptibility of *Candida albicans*. *Int. J. Antimicrob. Agents* 34 (4), 333–339. doi: 10.1016/j.ijantimicag.2009.03.008

Wilson, N. L., Moneyham, L. D., and Alexandrov, A. W. (2013). A systematic review of probiotics as a potential intervention to restore gut health in HIV infection. *J. Assoc. Nurses AIDS Care* 24 (2), 98–111. doi: 10.1016/j.jana.2012.04.004

Wong, S. H., and Yu, J. (2019). Gut microbiota in colorectal cancer: mechanisms of action and clinical applications. *Nat. Rev. Gastroenterol. Hepatol.* 16 (11), 690–704. doi: 10.1038/s41575-019-0209-8

Wu, C., Xu, Q., Cao, Z., Pan, D., Zhu, Y., Wang, S., et al. (2021). The volatile and heterogeneous gut microbiota shifts of COVID-19 patients over the course of a probiotics-assisted therapy. *Clin. Transl. Med.* 11 (12), e643. doi: 10.1002/ctm2.643

Yadav, V., Mandhan, R., Dabur, R., Chhillar, A. K., Gupta, J., and Sharma, G. L. (2005). A fraction from *Escherichia coli* with anti-*Aspergillus* properties. *J. Med. Microbiol.* 54 (Pt 4), 375–379. doi: 10.1099/jmm.0.45748-0

Yang, Q., and Defoirdt, T. (2015). Quorum sensing positively regulates flagellar motility in pathogenic vibrio harveyi. *Environ. Microbiol.* 17 (4), 960–968. doi: 10.1111/1462-2920.12420

Yang, X., Yu, Y., Xu, J., Shu, H., Xia, J., Liu, H., et al. (2020). Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir. Med.* 8 (5), 475–481. doi: 10.1016/s2213-2600(20)30079-5

Zhang, W., Lai, S., Zhou, Z., Yang, J., Liu, H., Zhong, Z., et al. (2022). Screening and evaluation of lactic acid bacteria with probiotic potential from local Holstein raw milk. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.918774

Zhang, D., Wang, F., Yu, Y., Ding, S., Chen, T., Sun, W., et al. (2021). Effect of quorum-sensing molecule 2-phenylethanol and ARO genes on *Saccharomyces cerevisiae* biofilm. *Appl. Microbiol. Biotechnol.* 105 (9), 3635–3648. doi: 10.1007/s00253-021-11280-4

Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579 (7798), 270–273. doi: 10.1038/s41586-020-2012-7

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China 2019. *N. Engl. J. Med.* 382 (8), 727–733. doi: 10.1056/NEJMoa2001017

Zuo, T., Wong, S. H., Cheung, C. P., Lam, K., Lui, R., Cheung, K., et al. (2018). Gut fungal dysbiosis correlates with reduced efficacy of fecal microbiota transplantation in *Clostridium difficile* infection. *Nat. Commun.* 9 (1), 3663. doi: 10.1038/s41467-018-06103-6

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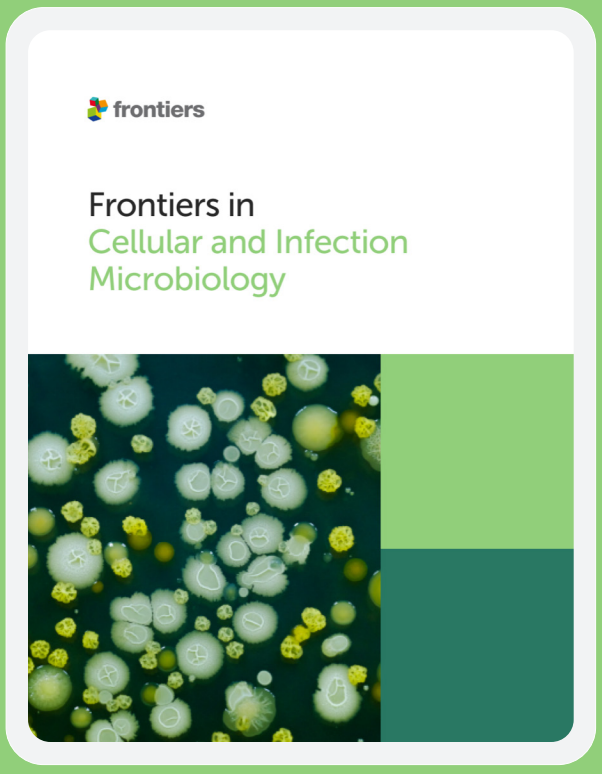

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