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New techniques in microbiome research – host-microbiome interactions using 'meta-omics' techniques, volume II

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New techniques in microbiome research – host-microbiome interactions using 'meta-omics' techniques, volume II

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Editorial: New techniques in microbiome research - volume II: Host-microbiome interactions using 'meta-omics' techniques

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Editorial on the Research Topic
New techniques in microbiome research - volume II: Host-microbiome interactions using 'meta-omics' techniques

Recent advancements in meta-omics techniques have significantly enhanced our understanding of the microbiome and its association with various human diseases. By integrating data from metagenomics, metatranscriptomics, metaproteomics, metabolomics, and spatial resolution, researchers can comprehensively analyze microbial communities and their interactions with the human host. A central theme of this Research Topic is the role of the microbiome in shaping human health and disease outcomes. Key studies included this Research Topic explore the diversity of microbial species, their functional contributions, and the downstream impacts on human health and diseases.

Key developments of meta-omics tools in unraveling the mechanisms through which microbiota affect human health and diseases

Comprehensive microbiome profiling

High-throughput sequencing technologies have enabled detailed characterization of the human microbiome, revealing its complexity and diversity. For instance, the Human Microbiome Project has cataloged thousands of microbial species, providing a foundational understanding of microbial composition in healthy and diseased states (Turnbaugh et al., 2007).

Integration of multi-omics data

Combining various omics datasets offers a holistic view of microbiome function. Metagenomics provides insights into microbial gene content, metatranscriptomics reveals gene expression patterns, metaproteomics identifies active proteins, and metabolomics quantifies metabolic products. This integrative approach has been pivotal in linking specific microbial functions to human health and disease (Zhang et al., 2019).

Biomarker discovery and their association with human diseases

Meta-omics analyses have identified correlations between microbiome composition and diseases. Metagenomic analyses have shown that specific microbial taxa and their functional capacities are strongly associated with metabolic health. For instance, certain gut bacteria have been associated with the development of type 2 diabetes (Muller et al., 2021), highlighting potential targets for therapeutic interventions (Fan and Pedersen, 2021). The ability to assess microbial genes and their metabolic outputs through metabolomics has provided new insights into how microbial-derived metabolites, such as short-chain fatty acids, influence human metabolic pathways, offering potential avenues for early diagnosis and targeted therapy.

Therapeutic interventions

Understanding microbiome-disease associations has led to the exploration of microbiome-based therapies. Probiotic and prebiotic interventions, as well as fecal microbiota transplantation (FMT), are being investigated for their potential to restore healthy microbiome composition and mitigate disease progression. FMT has gained traction as a treatment for recurrent *Clostridioides difficile* (*C. difficile*) infections and has been explored for its potential role in other conditions such as IBD (Ianiro et al., 2021).

Microbiome and cancer

The impact of the microbiome on cancer has been one of the most intriguing areas of research. Studies suggest that the microbiota can influence cancer progression, immune response, and even the efficacy of cancer therapies. For example, research has shown that microbial species residing in the tumor microenvironment can modulate immune checkpoints and affect the response to immunotherapy. Studies have shown that certain microbial species, such as *Fusobacterium nucleatum*, can exacerbate colorectal cancer by promoting inflammation and genomic instability (Kostic et al., 2013).

Microbiome and neurological disorders

The gut-brain axis has become a focal point of research into neurodegenerative diseases. Meta-omics approaches have shown that gut dysbiosis can impact neurological health by altering microbial metabolite production, which in turn affects neuroinflammation and brain function (Cryan et al., 2019). This emerging evidence suggests that gut microbiota may play a crucial role in the development of diseases such as Parkinson's and Alzheimer's, offering new opportunities for microbiome-based therapies.

Microbiome and infection

The microbiome profoundly impacts human susceptibility to infections and immune defense. Dysbiosis in human body such as the gut, vaginal, and skin microbiomes is associated with an increased risk of infections (Elkafas et al., 2022). Similarly, dysbiosis in the gut microbiome compromises immune function, leading to a greater risk of gastrointestinal infections, including *C. difficile* infection (Acevedo-Román et al., 2024). Research also highlights the gut-lung axis, showing that gut microbial imbalances can impact respiratory infections, suggesting a systemic role of the microbiome in maintaining immune homeostasis (Acevedo-Román et al., 2024). Moreover, sputum microbiota is associated with an severe and critically ill influenza patients (Gu et al., 2023), In addition, evidence indicates that the gut microbiota can alter SARS-CoV-2 virus load and COVID-19 severity (Zuo et al., 2020).

Microbiome and women health

The microbiome plays a critical role in women's health, particularly in the context of the vaginal and gut microbiomes. The vaginal microbiome, which is typically dominated by Lactobacillus species, is essential in maintaining a healthy environment by producing lactic acid and maintaining an acidic pH. Disruptions in this balance can result in bacterial vaginosis, yeast infections, and increased susceptibility to sexually transmitted infections (STIs) (Cocomazzi et al., 2023). In cases of bacterial vaginosis and other vaginal dysbiosis, an increase in pathogens like *Gardnerella vaginalis* and *Prevotella* has been linked to adverse reproductive outcomes, such as infertility and miscarriage (Cocomazzi et al., 2023) (Gu et al., 2022).

In addition, oral microbiome changes are associated with the menstrual cycle. Different microbiome profiles were observed during the follicular phase, the early and late luteal phases. Alpha diversity and beta diversity analyses revealed distinct microbial profiles across the four menstrual phases. Probiotic lactobacilli were used in the treatment of vaginal infections (Cohen et al., 2020). The urinary microbiota signatures are associated with different types of urinary diversion.

Furthermore, the gut microbiome also has significant implications for hormonal regulation and immune responses, which can impact conditions like polycystic ovary syndrome (PCOS) and pregnancy outcomes. Studies have shown that alterations in the gut and vaginal microbiota are associated with insulin resistance, hormonal imbalances, and inflammation in

women with PCOS, contributing to symptoms like infertility and metabolic disturbances (Cocomazzi et al., 2023) (Gu et al., 2022).

Key studies featured in this Research Topic

The aim of this Research Topic is to offer a platform for articles that expand our understanding of host-microbiome interactions using 'meta-omics' techniques. This Research Topic includes 4 reviews and 14 original research articles, highlighting critical associations between the microbiome and various human diseases. These contributions have made significant strides in advancing our knowledge of the complex interplay between the microbiome and disease states, including the microbiome and cancers, (Cheng et al., Li et al., Cai et al., Zhang et al.); Single-cell analysis and spatial resolution of the gut microbiome (Madhu et al.); Microbiome and Parkinson's disease (Jia et al.), depression (Li et al.), and insomnia (Wang et al., Li et al.); Oral microbiome changes associated with the menstrual cycle (Yamazaki et al., 2023), vaginal infection (Liu et al.), and urinary diversion (Liu et al.); Microbiome and influenza infection (Gu et al.); Microbiome in *C. difficile* Infection (Vázquez-Cuesta et al.) and split-dose bowel preparations (Zou et al.); Microbiome in fatty liver disease (Niu et al.) and gallstones and cholesterol metabolism (Zhang et al.); and assessing efficacy of clinical disinfectants for pathogenic fungi (Li et al.).

The microbiome and cancers

A review on respiratory tract microbiota and lung cancer investigates the bacterial communities inhabiting different regions of the respiratory system (Cheng et al.). The emerging evidence suggests that gut microbiota could play a key role in mediating oncogenesis through various mechanisms, potentially opening new avenues for therapeutic interventions (Li et al.). In addition, new colorectal cancer (CRC)-associated species were found, such as *Porphyromonas endodontalis*, *Ruminococcus torques*, and *Odoribacter splanchnicus*. Additionally, certain stage-specific bacterial taxa were identified, which may aid in diagnosing colorectal polyps (BP) and the four distinct CRC stages (Cai et al.). Furthermore, specific bacterial species and metabolic pathways may influence the occurrence of a series of immune-related adverse events (irAEs) in gastric, esophageal, and colon cancers. Notably, *Ruminococcus callidus* and *Bacteroides xylanisolvens* were enriched in patients without severe irAEs. Several microbial metabolic pathways, including citrulline and arginine biosynthesis, were associated with irAE development (Zhang et al.).

Single-cell analysis and spatial resolution of the gut microbiome

The authors reviewed innovative microbial single cell sequencing techniques, highlighting their broad applications in addressing critical questions regarding microbiome composition and spatial heterogeneity. These advancements offer deeper insights into the functional roles and interactions of individual microbial cells within complex communities (Madhu et al.).

Microbiome and neurologic disorders

The microbiota-gut-brain axis plays a pivotal role in regulating neuroprotective functions in the host. Patients with Parkinson's disease (PD) often exhibit gut microbiota dysbiosis (Jia et al.). Moreover, the gut microbiome and its associated metabolic pathways appear to play a significant role in the long-term development of depression (Li et al.). In addition, the identification of bacterial classes like Negativicutes and Selenomonadales provides a new direction for therapeutic strategies to manage sleep disorders via microbiota modulation (Wang et al.). In another study, ten gut microbiome (GM) taxa were found to have causal associations with insomnia (Li et al.).

Microbiome and women's health

Oral microbiome changes associated with the menstrual cycle

During the follicular phase, the relative abundance of the Streptococcus genus was significantly higher compared to the early and late luteal phases, while the Prevotella 7 and Prevotella 6 genera exhibited significantly lower abundance during the follicular phase than in the luteal phases. Alpha diversity was notably lower in the follicular phase, and beta diversity analyses revealed distinct microbial profiles across the four menstrual phases (Yamazaki et al.).

Use of probiotic lactobacilli in the treatment of vaginal infections

Probiotic Lactobacilli play a crucial role in maintaining the balance of the vaginal microenvironment. A review explores their therapeutic potential in treating female vaginal infections. Probiotic lactobacilli contribute to restoring and preserving vaginal health by competing with pathogenic bacteria, enhancing the local immune response, and producing antimicrobial substances (Liu et al.).

The urinary microbiota signatures and urinary diversion

The urinary microbiota signatures associated with different types of urinary diversion was studied. Urinary microbial landscapes of radical cystectomy and urinary diversion (UD) patients were analyzed (Liu et al.).

Microbiome and respiratory infection

Sputum microbiota characteristics and severe and critically ill influenza patients

Bacteroidetes showed significant depletion in the critically ill cohort. The sputum microbiomes in the severe influenza group were marked by an overrepresentation of *Neisseria*, *Porphyromonas*, *Actinobacillus*, *Alloprevotella*, *Nanosynbacter lyticus* TM7x, and *Clostridia* UCG-014. Notably, *Alloprevotella* exhibited an inverse correlation with *influenza* cycle threshold (Ct) values. Additionally, C-reactive protein (CRP) levels demonstrated a positive correlation with the presence of *Haemophilus* and *Porphyromonas* (Gu et al.).

Microbiome and *Clostridioides difficile* infection and split-dose bowel preparations

The gut microbiome and *Clostridioides difficile* infection

Distinct microbiome patterns were identified among healthy individuals, colonized patients, those with *Clostridioides difficile* infection (CDI), recurrent *Clostridioides difficile* infection (R-CDI), and patients with non-*Clostridioides difficile* infection (NOCDI) diarrhea. Potential microbiome biomarkers were discovered that may be valuable in distinguishing true CDI infections from other conditions, improving diagnostic accuracy and guiding treatment strategies (Vázquez-Cuesta et al.).

Gut microbiota in children with split-dose bowel preparations

In pediatric patients undergoing split-dose PEG bowel preparation and colonoscopy, gut microbiota showed significant alterations at the genus, species, and functional pathway levels. However, no significant changes were observed at the phylum level (Zou et al.).

Microbiome in fatty liver disease, gallstones, and cholesterol metabolism

Correlations between specific oral and gut fungal species with clinical parameters were identified from patients with Metabolic Dysfunction-Associated Fatty Liver Disease (MAFLD) patients (Niu et al.). One study reveals significant differences in microbial profiles between cholesterol and pigment gallstone patients (Zhang et al.).

Disinfectants for pathogenic fungi

A combination of NaClO and H_2O_2 has shown potential as a more effective disinfectant, particularly against fungal pathogens, offering an alternative solution for more efficient microbial control (Li et al.).

Conclusion

In conclusion, the integration of meta-omics techniques has revolutionized our ability to explore the microbiome's contributions to human diseases, providing a more detailed understanding of microbial functions and their impact on human health and diseases. As these techniques continue to evolve, they hold immense promise for identifying novel diagnostic biomarkers and therapeutic targets across a wide range of diseases.

Author contributions

TL: Writing – original draft.

Conflict of interest

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

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ORIGINAL RESEARCH
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Assessing Efficacy of Clinical Disinfectants for Pathogenic Fungi by Single-Cell Raman Microspectroscopy

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Disinfectants are crucial for root canal therapy (RCT), as metabolism of canal-inhabiting microbes can cause refractory infections. To develop effective yet patient- and environment-friendly disinfectant formulations, we quantitatively assessed the metabolism-inhibiting effects of intracanal disinfectants *via* D_2O-probed Single-Cell Raman Spectra (SCRS), using *Candida albicans* (*C. albicans*) as a pathogen model. For chlorhexidine gluconate (CHX), sodium hypochlorite (NaClO), and hydrogen peroxide (H_2O_2), at their MIC of 4, 168, and 60 μg/ml, respectively, despite the complete growth halt, metabolic activity of individual fungal cells was reduced on average by 0.4%, 93.9%, and 94.1% at 8 h, revealing a "nongrowing but metabolically active" (NGMA) state that may underlie potential refractory infections, particularly for CHX. In contrast, at their Metabolic Activity-based Minimum Inhibitory Concentrations (MIC-MA) of 8, 336, and 120 μg/ml, respectively, metabolic activity of all cells was completely halted throughout 8 h exposure. Moreover, combined use of NaClO+H_2O_2 (combination at 0.5× MIC-MA each) outperforms solo uses of CHX, NaClO, H_2O_2, or other binary combinations. Furthermore, dynamics of SCRS revealed distinct fungicidal mechanisms of CHX, NaClO, H_2O_2, and their pairwise combinations. MIC-MA is advantageous in critically assessing antifungal efficacy, and NaClO+H_2O_2 can potentially serve as a more efficient disinfectant formula for fungal pathogens.

Keywords: heavy water, single-cell technology, *Candida albicans*, chlorhexidine gluconate, sodium hypochlorite, hydrogen peroxide

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INTRODUCTION

Microbial infections in the pulp and periapical tissues could cause pulpitis, apical periodontitis, or even persistent inflammatory reaction. In these endodontic infections, pathogenic fungi such as *Candida albicans* (*C. albicans*) are the most frequently isolated eukaryotes (Siqueira and Sen, 2004; Kumar et al., 2015). In particular, *C. albicans* can readily form biofilms (Alshanta et al., 2019) colonize dentinal walls, and penetrate into dentinal tubules (Siqueira et al., 2002), resulting in persistent infections (Mergoni et al., 2018). Therefore, one key goal of root canal treatment (RCT) is to control and prevent microbial infections in the intracanal areas.

During RCT, mechanical debridement by hand and rotary instruments can leave 35% or more surface area of canals untouched (Tomson and Simon, 2016). Therefore, the use of liquid intracanal disinfectants such as chlorhexidine gluconate (CHX), sodium hypochlorite (NaClO), and hydrogen peroxide (H_2O_2), to ensure thoroughness of the intricate debridement of intracanal and accessory canals, is a vital step of RCT and key to the final endodontic treatment success (Ahmed and Dummer, 2018). An ideal intracanal disinfectant should be effective in inhibiting the metabolism of all microbial members, capable of dissolving pulp tissue remnant and smear layer, and, moreover, nontoxic or nonallergic (Galler, 2016). However, frequently, these desirable features are mutually exclusive: e.g., the more potent pathogen-inhibitory effect can be linked to more severe side effects to host tissues (Gomes-Filho et al., 2008). Therefore, the choice of type, concentration, duration, or combination of intracanal disinfectants is often based on empirical notion, due to the inability to rapidly assess the interaction between disinfectants and microbial cells (Chong and Pitt Ford, 1992; Rahimi et al., 2014). Therefore, there is an urgent need for method development to tackle this challenge.

Current methods that assess efficacy of disinfectants and other antimicrobials can be broadly classified as "growth-based" or "non-growth-based". Growth-based methods typically examine the sensitivity of cell growth curve to antimicrobial *via* dilution and diffusion, and quantitative parameters such as "minimum inhibitory concentration" [MIC, i.e., the lowest drug concentration under which microbial growth is entirely inhibited (Brauner et al., 2016)] are then derived. As growth inhibition does not necessarily correlate with metabolic inhibition or cell death, the growth-based methods are usually unable to distinguish between bactericidal and bacteriostatic effects and consequentially fail to detect "non-growing but metabolically active" (NGMA) cells (Tao et al., 2017), which are responsible for many latent or recurring infections (due to their ability to resume growth after removal of antimicrobials) and eventually lead to treatment failure (Manina and McKinney, 2013). Moreover, these methods can be time-consuming (frequently exceeding 24 h for fast-growing pathogens as extended duration of drug exposure to detect growth changes is required).

Rather than assessing the efficacy of antimicrobials based on "growth" inhibition, we recently introduced D_2O-probed Single-cell Raman Microspectroscopy (D_2O-Ramanometry), which can serve as a quantitative yet universal method to detect and measure metabolic-activity change of cells in response to drug treatments at the single-cell resolution (Teng et al., 2016; Tao et al., 2017; Bauer et al., 2020). Specifically, we proposed "Minimum Inhibitory Concentration based on Metabolic Activity" (MIC-MA), defined as the minimal dose under which the median ΔC-D-ratio at 8 h of drug exposure is ≤0 and the Standard Deviation (SD) of the ΔC-D ratio among individual cells is ≤0.005, to evaluate the metabolism-inhibiting efficacy of antimicrobials (Tao et al., 2017). However, this method and concept have not been tested in pathogenic fungi or for disinfectants; thus, it is unclear whether and how such metabolic-activity-based fungus–disinfectant interaction, including its inter-cellular heterogeneity, can be quantitatively accessed and screened for rational development of efficient disinfecting formula.

To tackle this challenge, here employing *C. albicans* as a model of fungal pathogen, we quantitatively assessed the metabolism-inhibiting effects of clinical intracanal disinfectants *via* D_2O-probed Single-Cell Raman Spectra (SCRS), aiming to demonstrate application of the method in screening new formula of intracanal disinfectants of reliable antimicrobial efficacy.

RESULTS

Tracking D_2O Incorporation in *C. albicans via* Single-Cell Raman Spectra

All living cells consume H_2O in the metabolism process and the H_2O intake rate is proportional to the level of cellular metabolic activity (Berry et al., 2015). Thus, metabolic inhibiting effect of an antimicrobial to a cell can be quantified based on the H_2O intake rate of the cell under drug exposure, which is measured *via* the extent of Raman shift at the C-D (carbon-deuterium vibration) band in 2,040 to 2,300 cm^{-1} in the SCRS of a cell to which D_2O is fed (Berry et al., 2015). However, high levels of D_2O, which accelerate cellular D_2O intake and thus allow faster assays, can be cytotoxic or cytostatic to cells (Takeda et al., 1998). Therefore, we started by selecting a proper concentration of D_2O, *via* comparison among the growth curves of *C. albicans* under various D_2O levels (**Figure 1A**). Compared with the control group (D_2O-free conditions), *C. albicans* growth was not significantly inhibited by a D_2O level below 30% during 10 h of culture ($p > 0.05$; **Figure 1A**). After overnight culture, which corresponds to the stationary phase of *C. albicans*, intensity of the C-D band (2,040–2,300 cm^{-1}) increased along with the elevation of D_2O level in the medium (**Figure 1B**). The corresponding C-D ratio, defined as ratio of the integrated spectral intensity of the C-D band (2,040–2,300 cm^{-1}) compared to the sum of the C-D band and the predominant C-H band (2,800–3,100 cm^{-1}) (Tao et al., 2017), showed strong positive correlation with media D_2O level ($R^2 = 0.997$, $p < 0.01$; **Figure 1C**), consistent with the positive link between substrate level and cellular substrate intake rate (i.e., metabolic activity). Therefore, 30% D_2O was chosen for evaluating *C. albicans*' metabolic response to the intracanal disinfectants *via* SCRS.

Under 30% D_2O (in the media), the C-D peak emerged and increased along with duration of D_2O incubation (**Figure 1D**). The corresponding C-D ratio started growing almost immediately after D_2O introduction, yet in contrast, the growth of OD_{600} was not detectable until ~4 h afterwards (**Figure 1E**). These results support detecting D_2O incorporation *via* SCRS under these conditions and

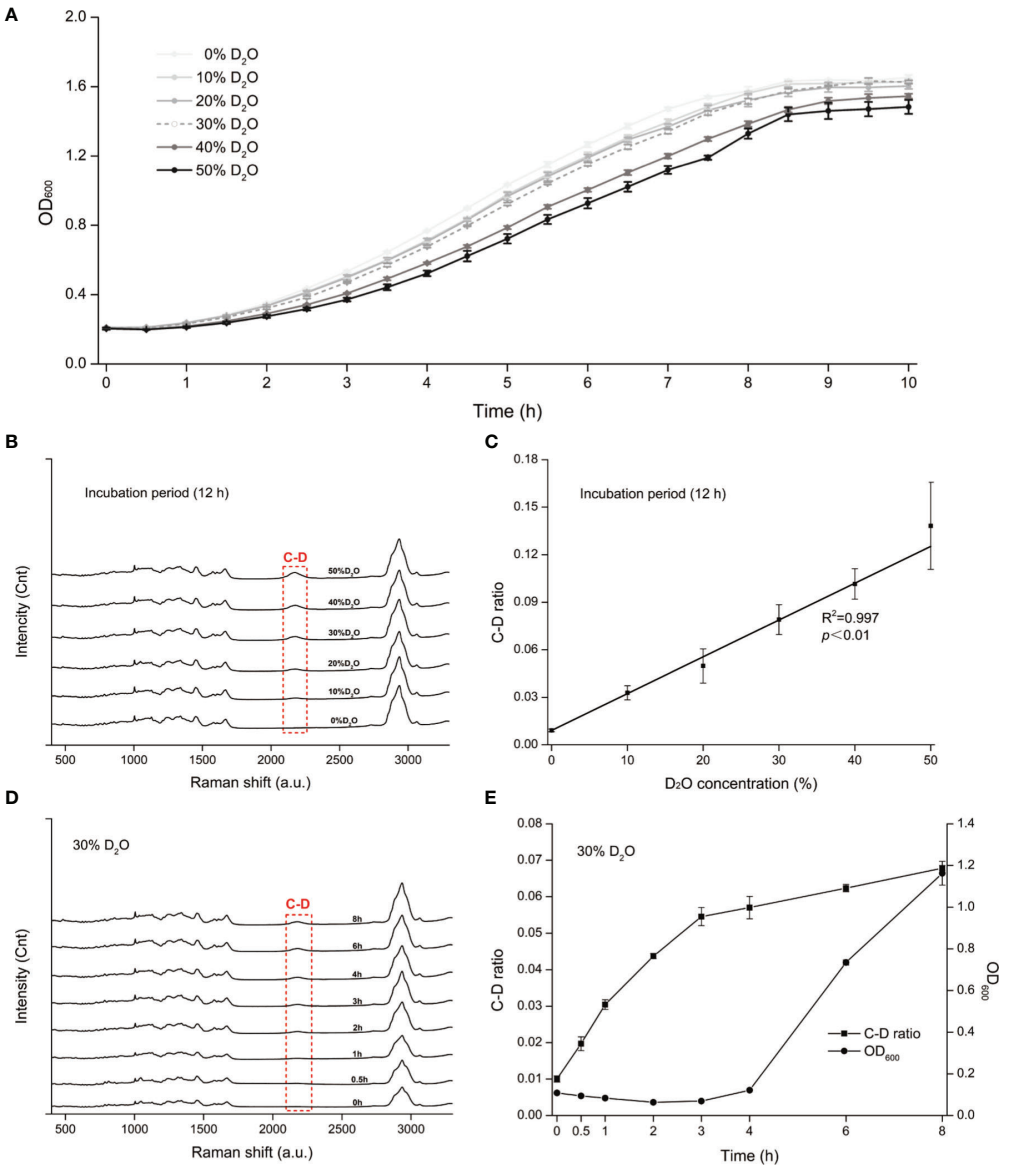

FIGURE 1 | D_2O-Labeled Single-Cell Raman Microspectroscopy of *Candida albicans* (*C. albicans*). **(A)** Temporal change of OD_{600} for the *C. albicans*, under various D_2O doses. **(B)** Change of SCRS under increasing levels of D_2O. *C. albicans* was grown in the medium supplemented with various levels of D_2O overnight to reach the stationary phase, followed by SCRS acquisition. **(C)** Correlation between the C-D ratio and the D_2O concentration for *C. albicans*. The experimental strains were grown respectively under a series of D_2O levels and then cultured for 12 h, followed by SCRS measurement. **(D)** Temporal change of SCRS for *C. albicans* under 30% D_2O. Graduate emergence of the C-D peak in SCRS was apparent under drug-free cultures. **(E)** Temporal change of C-D ratio and OD_{600} of *C. albicans* under 30%. The CD ratio curve is distinct from the OD_{600}-based growth curve. Error bars indicate standard deviation among three biological replicates.

can serve as a proxy for assessing the metabolic activity of *C. albicans*, and suggest that D_2O-probed SCRS can be more sensitive and faster than OD_{600} in detecting *C. albicans* growth, which also incurs metabolic change of individual cells.

MIC and MIC-MA of CHX, NaClO, and H_2O_2 for *C. albicans*

To assess the metabolic susceptibility of *C. albicans* to each of the three intracanal disinfectants, we determined the corresponding MIC and MIC-MA of CHX, NaClO, and H_2O_2, *via* broth dilution and D_2O-probed SCRS, respectively (**Figures 2A–F**; Materials and Methods). In contrast to MIC-MA, which assesses metabolic activity of individual cells, MIC evaluates drug efficacy based on growth inhibition of the whole bacterial population (Materials and Methods).

For CHX, the MIC and MIC-MA are 4 and 8 μg/ml, respectively (**Tables S1** and **1**). At the MIC of CHX, although fungal growth was entirely inhibited, the temporal dynamics of C-D ratio showed that

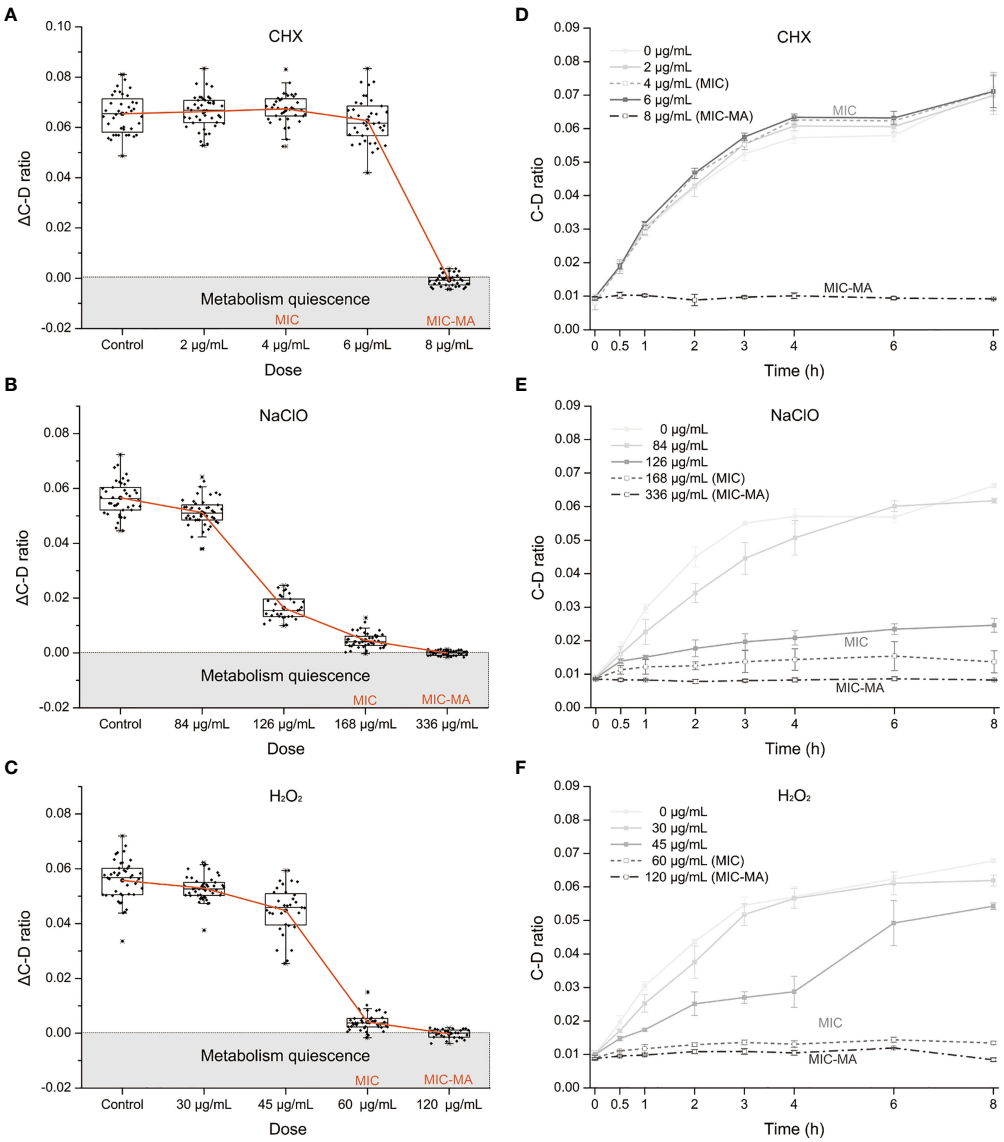

FIGURE 2 | The MIC-MA for each of the three intracanal disinfectants. **(A–C)** Dose effects of CHX, NaClO, and H_2O_2 on the ΔC-D-ratio of *C. albicans* cells. **(D–F)** Temporal dynamics of the C-D ratio of *C. albicans* under increasing doses of CHX, NaClO, and H_2O_2.

it was not lower than the drug-free control and eventually reached nearly an equivalent level after 8 h treatment ($p > 0.05$; **Figure 2D**). Thus, metabolic activity of *C. albicans* cells was still quite active under the MIC of CHX (4 μg/ml; **Figures 2A, D**). For the 0.5× MIC and 1.5× MIC groups, the trend within 8 h is similar to the MIC group ($p > 0.05$; **Figure 2D**). Notably, at the MIC-MA of CHX, the C-D ratio was maintained at the baseline level within 8 h from the start of drug exposure to the end of observation period (**Figure 2D**), suggesting that the metabolism of *C. albicans* was completely inhibited (ΔC-D-ratios < 0 at 8 h; **Figure 2A**).

TABLE 1 | Comparison of MIC and MIC-MA for the three antimicrobial disinfectants tested.

Disinfectant (μg/ml)	MIC	MIC-MA
CHX	4	8
NaClO	168	336
H_2O_2	60	120

For NaClO and H_2O_2, the MICs are 168 and 60 μg/ml, respectively (**Tables S1**, **1**), and its MIC-MA are 336 and 120 μg/ml, respectively, in which the averaged ΔC-D-ratios was <0 during an 8-h period (**Figures 2B, C**). The temporal dynamics of the C-D ratio showed a drug-dose-dependent effect that is reproducible (**Figures 2E, F**). At their 0.5× MICs of NaClO (84 μg/ml) and H_2O_2 (30 μg/ml), to a certain extent, the

increment of C-D ratio was inhibited as compared to the drug-free control ($p < 0.05$). However, at their MICs of NaClO (168 μg/ml) and H_2O_2 (60 μg/ml), the increase of C-D ratio was much lower than the drug-free control ($p < 0.05$), and the C-D ratio was maintained at a low level but failed to reach the baseline level during the whole period. Finally, at the MIC-MAs of NaClO (336 μg/ml) and H_2O_2 (120 μg/ml), the C-D ratio always stayed at the baseline level (**Figures 2B, C, E, F**); thus, the metabolic activity of *C. albicans* was entirely inhibited instantaneously and throughout the 8-h duration.

Comparison Between MIC-MAs and MICs Reveals NGMA *C. albicans* Cells

The MICs of CHX, NaClO, and H_2O_2 for *C. albicans* are 4, 168, and 60 μg/ml, respectively (**Tables S1** and **1**), while the corresponding MIC-MAs are 8, 336, and 120 μg/ml (**Tables S2** and **1**). Notably, under the MIC-MA level for these three intracanal disinfectants, the majority of *C. albicans* cells have entered "metabolism quiescence zone". However, at the MIC of CHX, C-D ratio curve was comparable to the drug-free control, showing the presence of considerable cellular metabolic activity after drug treatment. For NaClO and H_2O_2, under the MIC treatment, the metabolic activity of the fungal cells was inhibited to a significant degree; however, almost all fungal cells still exhibited a relatively low level of metabolic activity even after 8-h drug exposure (**Figure 3A**). Therefore, (i) the MIC-MAs of the above three intracanal disinfectants were twice that of MIC; (ii) at their respective MIC level of CHX, NaClO, and H_2O_2, despite the completely halted growth, metabolic activity of *C. albicans* cells was inhibited by merely 0.4%, 93.9%, and 94.1% at 8 h (i.e., the inhibitory effect of NaClO and H_2O_2 on the metabolic activity of *C. albicans* was much stronger than that of CHX, while no significant difference was found between NaClO and H_2O_2; **Figure 3B**), suggesting the presence of "nongrowing but metabolically active" (NGMA) cells that may underlie refractory infections for each of the treatments (particularly for CHX).

Assessing the Efficacy of Disinfectant Combinations on Inhibiting Fungal Metabolism

The distinct *C. albicans*-inhibitory effects of CHX, NaClO, and H_2O_2 raise the possibility that rational combination of the disinfectants can potentially further improve the efficacy. To probe this hypothesis, we measured the MIC-MA of multiple combinations of different agents and concentrations.

The CHX (0.5× MIC-MA) and H_2O_2 (0.5× MIC-MA) combination was unable to completely inhibit *C. albicans*' metabolic activity, which was equivalent to the effect of using H_2O_2 (0.5× MIC-MA) alone ($p > 0.05$) (**Figure 4A**). The combination of CHX (0.5× MIC-MA) and NaClO (0.5× MIC-MA) was not satisfactory in inhibiting *C. albicans* metabolic activity either (**Figure 4B**). However, the metabolism of *C. albicans* can be completely inhibited by the combination of NaClO (0.5× MIC-MA) and H_2O_2 (0.5× MIC-MA) (**Figure 4C**). Notably, further reduction of the level of the two disinfectants, i.e., the NaClO (0.25× MIC-MA) and H_2O_2 (0.25× MIC-MA) combination, failed to completely inhibit the metabolic activity (**Figure 4C**). Thus, among the various singular or combinatorial recipes tested here, the combination of NaClO and H_2O_2 exhibits the most efficient inhibitory effect on fungal metabolism (**Figure 4D**).

Raman Barcodes for Stress Response Provided Mechanistic Insights into Drug Efficacy

Each SCRS sampled from a drug-responding *C. albicans* population consists of thousands of Raman bands. Thus, by identifying those marker Raman bands that are both specific and shared among the six stress-response programs (Materials and Methods), we derived RBCS [Raman barcode of cellular-response to stress (Teng et al., 2016)], which consists of 48 elementary Raman bands that collectively characterize the temporal pattern of *C. albicans*' response to each of the treatments (**Table S3**). Among them, four bands that represent

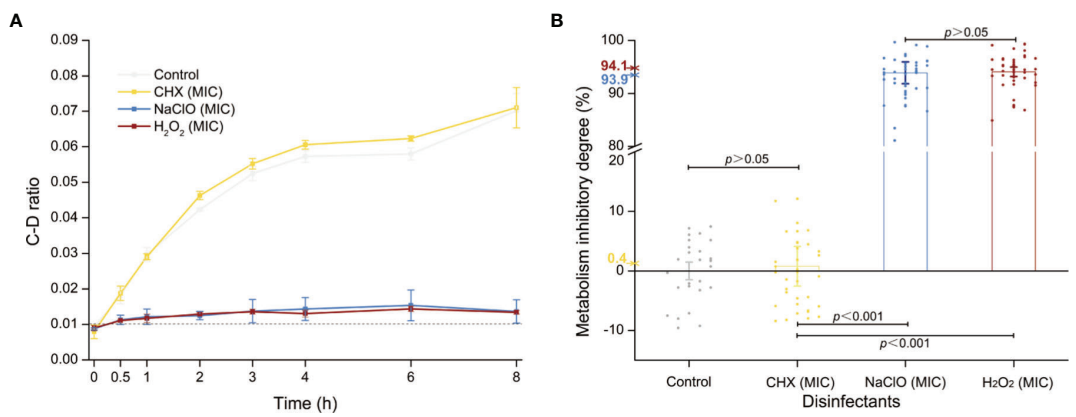

FIGURE 3 | Metabolic activity of *C. albicans* under the MIC of each of the three intracanal disinfectants. **(A)** Temporal dynamics of the C-D ratio of *C. albicans* under the MIC doses. **(B)** The degree of metabolic-activity inhibition of *C. albicans* at 8 h of exposure under the MICs of CHX, NaClO, or H_2O_2. Each dot represents a cell.

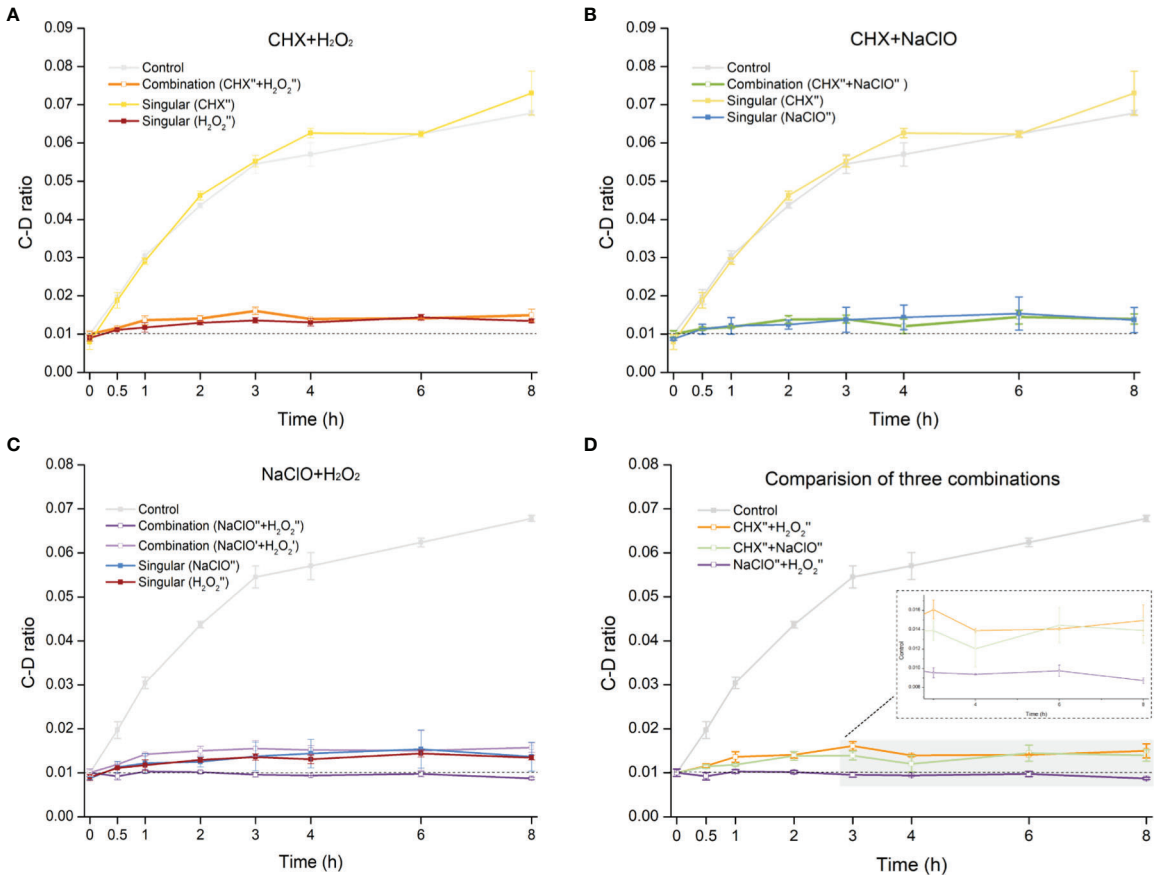

FIGURE 4 | Comparison of metabolic-activity inhibition of *C. albicans* cells under various combinations of the disinfectants. Temporal dynamics of the C-D ratio of *C. albicans* under the singular disinfectant or combination of CHX and H_2O_2 **(A)**, CHX and NaClO **(B)**, or NaClO and H_2O_2 **(C)** were shown. **(D)** NaClO+H_2O_2 combination outperforms the combination of either CHX+H_2O_2 or CHX+NaClO. ′: 0.25× MIC-MA; ″: 0.5× MIC-MA.

carbohydrates (1,048 and 1,147 cm^{-1}) and proteins (758 and 1,005 cm^{-1}), respectively, were shared among the marker bands for all the six stressors; thus, they are part of a general cellular response.

Under CHX, the most prominent change was nucleic acid (1,578 cm^{-1}) (**Figure 5A**). Compared with the control group, the intensity of nucleic acid band was generally elevated upon exposure to CHX. It is possible that the density of nucleic acids gradually decreases with the growth of cells in normal-growing cells, while CHX can alter cell membrane integrity by electrostatic binding with the negatively charged cell wall, which results in the leakage of low-molecular-weight components (Bernardi and Teixeira, 2015). These may have caused the relative increase in intensity of nucleic acid band. Under NaClO or H_2O_2, temporal changes of protein bands for NaClO (1,206 cm^{-1}) (**Figure 5B**) and for H_2O_2 (1,582, 1,572, and 1,561 cm^{-1}) (**Figure 5C**) were the most pronounced. Compared with the control group, the intensity of protein bands gradually reduced along with the duration of exposure. NaClO and H_2O_2 can produce hypochlorous acid (HClO) (Siqueira, 1997) and O_2 (Huang and Pik, 2014) respectively, whose strong oxidizing effect can lead to destruction of proteins and other substances.

As for the combinations, the protein bands of 890 cm^{-1} (**Figure 5D**) for CHX+NaClO and of 1,613 and 1,572 cm^{-1} (**Figure 5E**) for CHX+H_2O_2 were reduced. It is possible that CHX precipitates proteins (Bernardi and Teixeira, 2015) and that the strong oxidizing effect of NaClO or H_2O_2 on protein (Siqueira, 1997; Huang and Pik, 2014) eventually leads to the decrease of protein content or density in cells. Under NaClO+H_2O_2, the intensity of protein (1,561 and 1,104 cm^{-1}) and nucleic acid (898 cm^{-1}) both decreased (**Figure 5F**), indicative of the oxidation effect that results in decreased proteins and nucleic acids (Siqueira, 1997).

DISCUSSION

Proper administration of intracanal disinfectants is vital for both long-term efficacy of antimicrobial RCT and reducing side effects that compromise patient experience. For example, NaClO is one of the most widely used intracanal disinfectants due to its ability to dissolving necrotic tissue and antibacterial activity (Siqueira et al., 1999). However, the proper concentration to administer under a

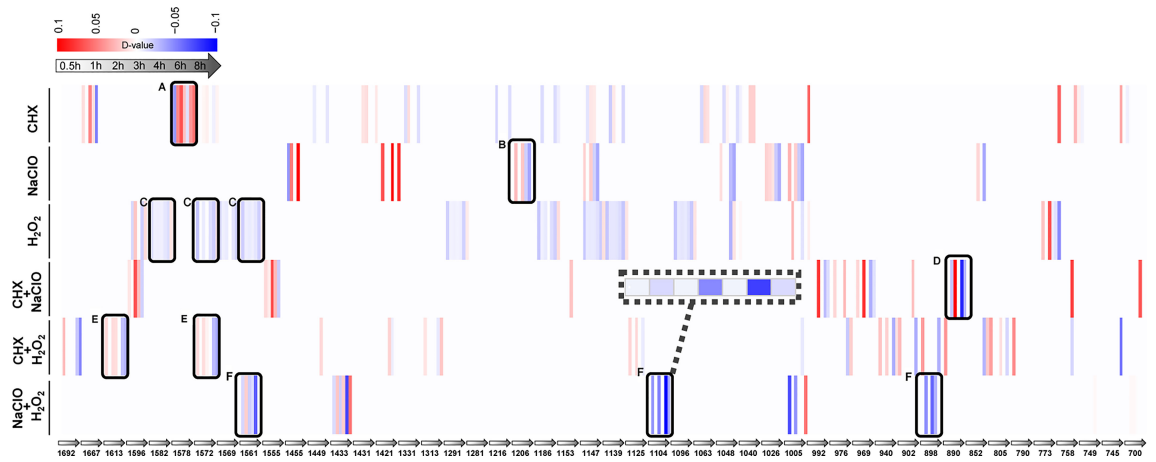

FIGURE 5 | Raman-barcode of cellular-response to stressors (RBCS) under various duration of exposure to the disinfectants. The change trend of marker Raman bands under singular CHX **(A)**, NaClO **(B)**, H_2O_2 **(C)** or combination of CXH and NaClO **(D)**, CHX and H_2O_2 **(E)**, NaClO+H_2O_2 **(F)** were shown. The change in Raman band intensity was calculated as D-value (between stressed and control cells) and shown as blue (decreased intensity) or red (increased intensity; $p < 0.001$, Wilcoxon rank sum test).

clinical setting is controversial, as overly high drug concentrations can reduce biocompatibility and promote irritation of periodontal and periapical tissues (Tanomaru Filho et al., 2002; Gomes-Filho et al., 2008). CHX has also been suggested as an effective antimicrobial agent for RCT; however, cytotoxic effect, allergic reaction, and extrinsic tooth or tissue staining may ensue with usage (Bernardi and Teixeira, 2015). H_2O_2 remains a frequently used agent in RCT despite its potential for serious complications, which include air emphysema or even systemic gas embolus (Akuji and Chambers, 2017). Therefore, it is critical to develop a methodological scheme that can (*i*) quantitatively assess the antimicrobial effect based on halting of pathogen metabolic activity, instead of just growth, for avoiding later or recurring infections (Lopatkin et al., 2019), and (*ii*) rapidly screen new formula of intracanal disinfectants of reliable antimicrobial efficacy.

The susceptibility of fungal pathogens to these three intracanal disinfectants was compared *via* MIC. For *C. albicans* ATCC 64342 (also an oral isolate), Ferguson et al. reported the MICs of CHX, NaClO, and H_2O_2 as <0.63 μg/ml, <10 μg/ml, and 234 μg/ml, respectively (Ferguson et al., 2002). These MICs are comparable to our measurements for *C. albicans* ATCC 10231 here, with the variation likely due to the change of *C. albicans* strains and distinction in medium composition, amount of inoculum, incubation temperature, facilities, technical skills, etc. (Mouton et al., 2018). However, MIC's reliance on growth inhibition can be time-consuming (frequently exceeding 24 h for common pathogens (Berkow et al., 2020), and also results in the inability to detect NGMA cells of *C. albicans* whose metabolic activity may cause reinfections after clinical latency (Manina and McKinney, 2013; Manina et al., 2015; Lempp et al., 2020).

Our research group originally proposed the "MIC-MA" parameter using D_2O-Ramanometry to evaluate the metabolism-inhibiting efficacy of drugs, which tackles the drawbacks of the growth-inhibition-based methods (Tao et al., 2017). Here, we employed MIC-MA to further assess the metabolism-level inhibition of CHX, NaClO, and H_2O_2 on pathogenic fungi at single-cell resolution. Metabolic activity of the *C. albicans* cell population was inhibited by merely 0.4%, 93.9%, and 94.1% at their respective MIC level of CHX, NaClO, and H_2O_2. In contrast, the MIC-MA dose of 8 μg/ml for CHX, 336 μg/ml for NaClO, or 120 μg/ml for H_2O_2 can each completely halt the metabolic activity of *C. albicans*. The MIC-MAs of the above three intracanal disinfectants are approximately twice of MICs. This result is consistent with previous literature that shows that the MIC-MAs of NaF or CHX on four prevalent members of oral microbiota (*Streptococcus mutans*, *Streptococcus gordonii*, *Streptococcus sanguinis*, and *Lactobacillus fermentum*) are 2–3 times that of MICs (Tao et al., 2017). Moreover, the *C. albicans*-metabolism inhibition of CHX features a distinct threshold in the dose effect (**Figure 2A**), yet those of NaClO and H_2O_2 are quite different as semi-linear dose dependency (**Figures 2B, C**). In particular, at its MIC dose when cellular growth is fully arrested, for CHX, the vast majority of the disinfectant-exposed *C. albicans* cells are still alive (metabolically active). Thus, by distinguishing NGMA *C. albicans* cells and quantifying the degree of heterogeneity in metabolic phenotypes (Zhang et al., 2018; Bauer et al., 2020), MIC-MA would be an advantageous parameter to MIC in assessing disinfectant–pathogen interaction.

Based on growth inhibition, Kuruvilla et al. showed that CHX and NaClO combined in the root canal resulted in the greater reduction of microorganisms than either alone (Kuruvilla and Kamath, 1998). In addition, an antibacterial synergistic effect between CHX and H_2O_2 (Heling and Chandler, 1998; Steinberg et al., 1999) and those between NaClO and H_2O_2 were reported (Cerioni et al., 2009). Based on metabolic inhibition, we found that the combined formula of NaClO and H_2O_2 at 0.5× MIC-MA of each can elicit a level of metabolic inhibition of *C. albicans* that is equivalent to solo use of either NaClO or H_2O_2 at their

respective MIC-MA level. Since a lower level of each ingredient can reduce side effect (Tanomaru Filho et al., 2002; Gomes-Filho et al., 2008), this NaClO+H_2O_2 formula should be more efficient. Moreover, no apparent synergistic effect in metabolic inhibition was observed when combining CHX and either NaClO or H_2O_2. Therefore, growth- and metabolic-based assessment can produce linked yet distinct findings.

Moreover, as a signature for the mode of action, RBCS, which is a barcode of temporal pattern of 48 elementary Raman bands, was derived for each of the pairs offungi–drug interaction. Compared to other single-cell stress-response profiling methods such as morphological analysis (Choi et al., 2014), fluorescence imaging-based biosensing (Shintaku et al., 2014), or transcriptomics (Islam et al., 2011), RBCS can be advantageous as (i) it rapidly yields a comprehensive and landscape-like view of molecular events of stress response in a label-free, non-disruptive, and simple manner [without the need for preexisting biomarkers (Xu et al., 2017; He et al., 2021)]; (ii) it can predict global gene expression profiles, and *vice versa*, as the SCRS and transcriptomes (e.g., *via* RNA sequencing) can be connected linearly through a shared low-dimensional subspace (Germond and Ichimura, 2018; Kobayashi-Kirschvink et al., 2018).

Notably, in order to derive a clinically relevant personalized disinfectant level, drug dosages above MIC-MAs, which are much lower than the recommended clinical concentrations (2% CHX, 1%–5.25% NaClO, 3% H_2O_2), should be measured not just for pure culture of *C. albicans* as was here, but also for the microbiota of an infected root canal system, which likely are much more resistant to the disinfectants in the polymicrobial biofilm state (Ricucci and Siqueira, 2010) (Swimberghe et al., 2019). The heterogeneity of drug response thus can be measured *via* SCRS, the metabolite-conversion network can be profiled *via* algorithms such as Intra-Ramanome Correlation Analysis [IRCA (He et al., 2021)], and mechanism can be decoded *via* Raman-activated cell sorting [RACS (He et al., 2019); e.g., RAGE (Xu et al., 2020) and flow-mode RACS (Wang and Xin, 2020)], to establish the links between disinfectant-susceptibility phenotype and genomes or transcriptome at single-cell resolution. Nevertheless, by developing a workflow for single-cell Raman-based interaction assay for *C. albicans* and disinfectants used in RCT, this study paves the way for culture-free, rapid, mechanism-based assessment of personalized disinfecting efficacy and screening of treatment regimens for fungal infections.

MATERIALS AND METHODS

Fungal Strain, Media, and Disinfectants

Candida albicans ATCC 10231 (*C. albicans*), one of the most commonly used fungal strain for drug susceptibility testing (Fidalgo et al., 2010), was obtained from China Center of Industrial Culture Collection. This strain was inoculated on Sabouraud dextrose agar plates at 37°C for 12 h. Grown colonies were picked from the plate and incubated in RPMI 1640 culture medium (pH 7.0 ± 0.1) in an aerobic incubator at 37°C. The 20% Chlorhexidine gluconate (Macklin, Shanghai, China) and 3% hydrogen peroxide (Huanbomiao, Hebei, China) and the 5.25% sodium hypochlorite (Weizhenyuan, Fujian, China) were purchased. All media were stored at 4°C.

Sensitivity of *C. albicans* to D_2O Concentration

C. albicans cells were 1:100 diluted from the stationary-phase culture and exposed to a final concentration of 0%, 10%, 20%, 30%, 40%, or 50% D_2O. To track fungal growth under the D_2O concentrations, the cells were cultivated in a Bioscreen C (Lab systems, Helsinki, Finland). The working volume in the Bioscreen plate was 300 μl/well and the temperature was controlled at 37°C and optical density (OD) was controlled at 600 nm. OD of the samples was automatically read at regular intervals of 30 min, over a 10-h period (before every measurement, the sample was gently shaken for 10 s). In addition, the cells were sampled at various time points for acquisition of SCRS to probe the intake of D_2O by *C. albicans* cells. Three biological replicates were carried out.

Measuring the MICs of Each of the Three Intracanal Disinfectants for *C. albicans*

Several colonies from a 24-h-old *C. albicans* culture grown on Sabouraud dextrose agar plate were transferred to 5 ml of sterile water and vortexed for 15 s until evenly distributed. The measurement of MIC was performed using the broth dilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines. By adjusting cell density, cell suspensions were prepared to reach 0.5 McFarland standard (1×10^6 to 5×10^6 cells/ml). Then, a working suspension was prepared *via* a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 to obtain a final cell density of 5×10^2 to 2.5×10^3 cells/ml. Then, the MIC value was determined by measuring the change of OD_{600} before and after a 24-h disinfectant exposure. Three biological parallels were carried out.

Measuring the MIC-MAs for Each of the Three Intracanal Disinfectants for *C. albicans*

C. albicans cells were incubated at 37°C in media with the various formulas of intracanal disinfectants and 30% D_2O. Samples were collected at 0, 0.5, 1, 2, 3, 4, 6, and 8 h after exposure, respectively, for acquisition of SCRS. The concentrations of disinfectants were initially set as 0, 1/2× MIC, MIC, and 2× MIC. Then, the change of C-D ratio of ~30 individual *C. albicans* cells randomly sampled from the population was profiled before and after drug treatment for 8 h. The approximate MIC-MA value was found when the mean value of C-D ratio at 8 h minus 0 h (ΔC-D ratio) of drug exposure is ≤0 and the SD is ≤0.005.

Calculation of the Metabolism-Inhibiting Degree of the Disinfectants (%)

The C-D ratio values of the control group (i.e., the absence of disinfectants) were employed as a reference to calculate the percentage decrease of the C-D ratio values after exposure to the intracanal disinfectants, so as to quantify the degree of metabolic inhibition. The following formula was used: $1 - x/x_0$,

where x_0 is the C-D ratio of the control group at 8 h, while x is the C-D ratio of the test group 8 h after exposure to a particular formula of the intracanal disinfectants.

Assessment of the Efficacy of Combinations of Intracanal Disinfectants

The combination of disinfectants tested includes MIC and 0.5× MIC of CHX and H_2O_2, MIC and 0.5× MIC of CHX and NaClO, and MIC and 0.5× MIC of NaClO and H_2O_2, respectively. The negative control did not include any disinfectants. Samples were collected at 0, 0.5, 1, 2, 3, 4, 6, and 8 h after exposure respectively for SCRS acquisition. Then, the effect of *C. albicans* metabolism inhibiting was determined by measuring the change of C-D ratio value. Three biological replicates were carried out for each condition.

Raman Microspectroscopy Analysis

Samples pretreatment and SCRS acquisition were performed as we previously described with slight modification (Teng et al., 2016; Tao et al., 2017). In brief, SCRS were obtained using a Clinical Antimicrobial Susceptibility Test Ramanometry instrument (CAST-R; Qingdao Single-Cell Biotech Inc, China) or a modified confocal Raman-fluorescent microscope based on LabRam HR system (Horiba Ltd., U.K.). The acquisition time for each cell was 1 s.

Pre-processing of raw SCRS data was performed using R (version 3.5.1), including background subtraction and area normalization. Spectra were cropped to a spectral region of interest ranging from 600 cm^{-1} to 1800 cm^{-1} for chemometrics analysis.

Raman Barcodes for Stress Response and Chemometrics Analyses

After a series of basic processing of the raw Raman spectra (600 cm^{-1} to 1800 cm^{-1}), in order to get the marker bands, the Random Forest model was firstly used to classify SCRS under different disinfectant treatments *via* default parameters [R package "randomForest", ntree = 5,000, using default mtry of sqrt(p) where p is the number of Raman bands]. The rank lists of Raman bands in the order of "band importance" by Random Forests were determined over 50 iterations of the algorithm. Then, the SCRS datasets were reordered based on the rank list and used as the input data for calculating the minimum number (Nmin) of Raman bands for discriminating between the control and the stressed cells *via* ROC (receiver operating characteristic) analysis, based on the largest AUC (area under the ROC curve). Finally, Top Nmin ranking bands that show significant difference between the control and the disinfectant-exposed cells were designated as the marker bands for each of the disinfectant treatments (Wilcoxon rank sum test; $p < 0.01$).

DATA AVAILABILITY STATEMENT

All the Single-cell Raman Spectra produced in this study are publicly accessible at: http://pub.single-cell.cn/index.php/Publication/view_anti/P_SCRS0101.

AUTHOR CONTRIBUTIONS

FL, LR, PZ, and FY conceived and designed the research. FL carried out the experiments with the supervision of LR, RC, XS, JX, PZ, and FY. FL, LR, RC, XS, and PZ analyzed data. FL drew the figures. FL and LR drafted the manuscript. FL, XS, JX, PZ, and FY critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2022.772378/full#supplementary-material

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Correlation of the gut microbiome and immune-related adverse events in gastrointestinal cancer patients treated with immune checkpoint inhibitors

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Introduction: The wide application of immune checkpoint inhibitors has significantly improved the survival expectation of cancer patients. While immunotherapy brings benefits to patients, it also results in a series of immune-related adverse events (irAEs). Increasing evidence suggests that the gut microbiome is critical for immunotherapy response and the development of irAEs.

Methods: In this prospective study, we recruited 95 patients with advanced/unresectable gastrointestinal cancers treated with immunotherapy and report a comprehensive analysis of the association of the gut microbiome with irAEs. Metagenome sequencing was used to analyze the differences in bacterial composition and metabolic pathways of baseline fecal samples.

Results: In summary, we identified bacterial species and metabolic pathways that might be associated with the occurrence of irAEs in gastric, esophageal, and colon cancers. Ruminococcus callidus and Bacteroides xylanisolvens were enriched in patients without severe irAEs. Several microbial metabolic pathways involved in the urea cycle, including citrulline and arginine biosynthesis, were associated with irAEs. We also found that irAEs in different cancer types and toxicity in specific organs and the endocrine system were associated with different gut microbiota profiles. These findings provide the basis for future mechanistic exploration.

KEYWORDS

immune-related adverse event, gut microbiome, gastrointestinal cancer, metagenome sequencing, probiotic

Introduction

The wide usage of immunotherapy has drastically changed the cancer treatment landscape in recent years. Immunotherapy aims to boost host immune system to fight cancer by blocking immune checkpoints, which significantly improves the long-term survival and life quality of cancer patients (Liu et al., 2021). While immunotherapy can activate the immune system, it may also produce unique therapeutic toxicities known as immune-related adverse events (irAEs). IrAEs are characterized by high incidence, unknown mechanisms, and are difficult to predict. Studies have shown that the overall incidence of irAEs ranges from 54% to 76% (Xu et al., 2018). Although most irAEs tend to be mildly toxic and self-limited, severe irAEs still occur in 5-30% patients, limiting their therapeutic benefit. Therefore, understanding the mechanism of irAEs, developing effective predictive markers, and formulating individualized strategies to prevent and manage irAEs have become urgent issues for physicians.

On the biomarker discovery aspect, recent studies have proposed several potential biomarkers of irAEs, including body composition parameters (Daly et al., 2017), circulating IL-17 (Tarhini et al., 2015), IL-10 (Sun et al., 2008), CD163 (Fujimura et al., 2018), and eosinophil counts (Nakamura et al., 2019; Liu et al., 2021). In addition, a growing body of preclinical and clinical evidence suggests that the gut microbiome is critical to immunotherapy response and may also influence the onset and development of irAEs (Vétizou et al., 2015; Bhatt et al., 2017; Batten et al., 2019; Andrews et al., 2021; Liu et al., 2021; Bredin and Naidoo, 2022; Tan et al., 2022).. Previous studies in melanoma patients have shown that gut bacterial diversity (Batten et al., 2019), specific microbial quantities (such as Bacteroidetes (Dubin et al., 2016; Chaput et al., 2017), *Bacteroides vulgatus* and *Bacteroides dorei* (Usyk et al., 2021)) and related microbial-derived products (such as systemic and intestinal lipopolysaccharide (McCulloch et al., 2022)) are closely associated with the occurrence and/or severity of irAE. In a study of combined CTLA-4 and PD-1 blockade treated cohort, Miles C et al. showed irAEs could be distinguished by the higher abundance of *Bacteroides intestinalis* and *Intestinibacter bartlettii* and further demonstrated in a murine model that *Bacteroides intestinalis* was closely associated with host intestinal IL-1β and immunotherapy-related enterotoxicity (Andrews et al., 2021). One study on non-small cell lung cancer patients also identified microbial biomarkers associated with clinical efficacy and irAE severity, including *Agathobacter, Lactobacillus* and *Raoultella* etc (Hakozaki et al., 2020).

The above-mentioned studies mainly focus on melanoma and lung cancer patients, for whom immunotherapy shows an overall encouraging efficacy. They provide a solid base on the relationship between the gut microbiome and irAEs. For gastrointestinal (GI) cancers, the relationship between the gut microbiota and tumor immune microenvironment is physically closer and metabolically more complex. Several studies have shown that gut microbiota composition is related to the occurrence and development of GI cancers such as colon cancer (Feng et al., 2015; Hashemi Goradel et al., 2019; Akkız, 2021). In 2020, our group further showed a close association between the gut microbiome and immunotherapy efficacy (Peng et al., 2020). Considering the heterogeneity of microbial biomarkers across cancer types and the high incidence and mortality rates of GI cancer in Asia, we believe it is essential to explore and understand how the gut microbiome is involved in the process of irAEs in GI cancer patients. To achieve this goal, we recruited a cohort of GI cancer patients receiving immunotherapy. By analyzing their gut microbiome before treatment using metagenomics, we identified a number of microbes that are closely associated with irAEs and could be potential predictive biomarkers and/or therapeutic targets.

Materials and methods

Patient recruitment and clinical evaluation

A total of 135 patients with advanced/unresectable gastrointestinal cancers (esophageal cancer, gastric cancer, and colon cancer) who were hospitalized and scheduled for immunotherapy in Beijing Cancer Hospital from March 2018 to July 2021 were included in this study. The study was conducted under Institutional Review Board (IRB)–approved protocols (2018KT66) and complied with the declaration of Helsinki. All patients were fully informed about the research content and signed the consent. Final 95 patients were included for analysis because 17 patients were treated with combined immunotherapy and 23 patients failed to provide baseline fecal samples (Figure 1). Baseline fecal samples were defined as fecal samples collected before the start of immunotherapy or within 3 weeks of the first infusion of immunotherapy. All patients received the following two treatment regimens without antibiotic use during the treatment until disease progression or intolerable toxicity: 1) PD-1/PD-L1 inhibitor, repeated every 2 or 3 weeks; 2) combined PD-1/PD-L1 inhibitor and CTLA-4 inhibitor immunotherapy, repeated every 3 or 6 weeks.

Treatment responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST).1.1 standard. Cases of irAEs were graded according to the Common Terminology

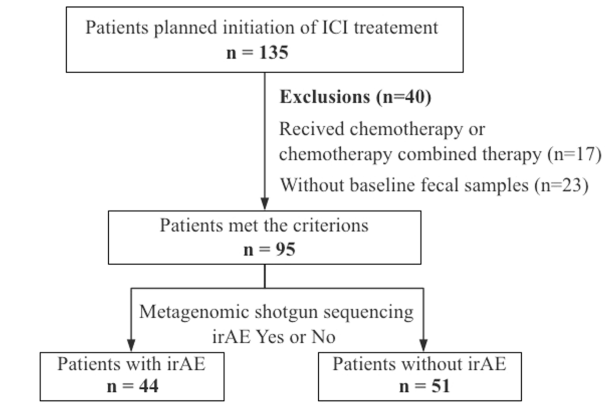

FIGURE 1
Study workflow.

Criteria for Adverse Events 4.03 (CTCAE v4.03), and the irAE scores were reviewed by at least two oncologists. The grade of irAEs was scored from 1 to 5 and was divided into mild (grade 1–2) and severe (grade 3–5) or low (grade 1) and high (grade2-5). IrAEs were limited to adverse events that were definitely or most likely linked to the treatment of immunotherapy. Patients with no irAEs were confirmed at the last clinical visit. Patients' demographic and clinical information were collected and summarized in Table 1, including age, gender, diagnosis, microsatellite status, allergy history, and combined medication history etc.

Fecal DNA extraction and metagenomic shotgun sequencing

Baseline fecal samples were collected using the Wehealthgene® Fecal Microlution™ Collection Kit (Catalog No. ML-001A, Wehealthgene). All fresh fecal samples were stored in sterile containers at -20°C and transported to the sequencing facility. Fecal DNA was extracted according to the protocol provided in the QIAamp PowerFecal DNA Kit (Cat. No. 12830-50, Qiagen). NEBNext Ultra DNA Library Prep Kit was used to construct individual sequencing library for each sample. The pooled library was sequenced by the Illumina NovoSeq 6000 sequencing platform according to manufacturer's protocol on 150 bp paired-end reads (Novo Gene, China).

Metagenome sequences were first quality controlled to remove low-quality reads and contaminated host reads. Specifically, Fastp (version 0.20.0) and KneadData (version 0.6.1) was applied to remove reads either low in quality or read length with trimmomatic-options "ILLUMINACLIP:adapter:2:40:15 SLIDINGWINDOW:4:20 MINLEN:50". The resulting reads were further filtered to remove host reads using Bowtie2 (bowtie2-options –very-sensitive –dovetail; hg19 version of the human genome) (Supplementary Table 1).

For bacterial taxa and functional profiling, HUMAnN2 (Franzosa et al., 2018) (version 2.2.0) was applied. Specifically, previously generated high-quality reads from each sample were classified using a marker-gene based approach through MetaPhlAn2 (Truong et al., 2015) with default parameters (version 2.2.0). The resulting species relative abundance were listed in Supplementary Table 2. Functional profiles were conducted by mapping reads to the pangenomes of species identified by MetaPhlAn2. The coding sequences of proteins were annotated in UniRef 90. Unmapped reads were translated and mapped to UniRef90 by DIAMOND (Buchfink et al., 2015).

TABLE 1 Demographic and clinical information of enrolled patients.

Clinical factor		Total (n=95)	irAE-Yes (n=44)	irAE-No (n=51)	*P* value (fisher test)
Gender	Male	69	33	36	0.653
	Female	26	11	15	
Diagnosis	Esophageal Cancer	27	15	12	0.083
	Gastric Cancer	35	11	24	
	Colorectal Cancer	33	18	15	
Age	< 60	54	22	32	0.221
	>= 60	41	22	19	
BMI	Normal	64	32	32	0.381
	Non-Normal	31	12	19	
Allergy History	Yes	6	4	2	0.411
	No	89	40	49	
Tumor Stage	Stage III	7	2	5	0.445
	Stage IV	88	42	46	
ECOG	<2	91	43	48	0.621
	>=2	4	1	3	
MSI	MSI High	42	21	21	0.542
	Non-MSI High	53	23	30	
Prior Treatment	>= 3	35	20	15	0.136
	<3	60	24	36	
Treatment	Anti-PD-1 Anti-PD-L1 Anti-PD1+anti_CTLA4	69 18 8	29 10 5	40 8 3	0.405

Reads which failed to map to the pangenomes of known species were labeled as "unclassified". Gene families were analyzed to reconstruct metabolic pathway based on the MetaCyc databases (Caspi et al., 2018). The resulting metabolic pathway abundance were listed in Supplementary Table 3.

Statistical analyses

Patients' demographic and clinical information was compared using Fisher's exact test to assess the association between patients' demographic/clinical characteristics and immune-related adverse events. For gut microbial community analysis, alpha diversity (represented by Shannon index and inverse Simpson) and beta diversity (calculated by Bray-Curtis distance) were analyzed. To identify potential confounders, we applied permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2014), a distance-based method that tests for association between microbiome and environmental factors of interest. To identify differential microbial taxa or metabolic pathways between comparison groups, MaAsLin2 (version 1.7.3) (Mallick et al., 2021) was used with the following parameters (min_abundance=0.0, min_prevalence=0.1, min_variance=0.0, normalization="NONE", transform="LOG", analysis_method="LM", correction="BH", standardize=FALSE). Microbial features were considered as significant when FDR corrected P value <0.3. Otherwise, P value less than 0.05 was considered statistically significant. The Wilcoxon rank sum test was applied for specific microbial feature comparison. All statistical analyses and plotting were performed in R (version: 3.6.3).

Results

Patient characteristics

This study included 27 patients with esophageal cancer, 35 with gastric cancer, and 33 with colorectal cancer (N=95, Table 1). There were no statistically significant differences between the non-irAE group and the irAE group regarding the demographic/clinical information, such as gender, age, BMI, allergy history, tumor type, tumor stage, performance status, microsatellite status, number of prior lines of treatment, and immunotherapy drugs (Table 1). Forty-four patients reported immune-related adverse events with varying organ toxicity and severity (Table 2). Among them, the median time to first irAE occurrence was 27 days (min to max days: 1-212), and 35 (79.5%) patients developed irAEs within 12 weeks after receiving immunotherapy.

Gut microbiome composition was correlated with the occurrence of immune-related adverse events

We first evaluated the gut microbiome of the baseline fecal samples in patients with and without irAE at the community level. No significant differences were observed in either alpha (both richness and evenness, data not shown) or beta diversity between the two groups, indicating there might be individual bacterial taxa related to irAE instead of the overall community shift (Figure 2A).

After PERMANOVA confirmed there was no significant correlation between patients' demographic/clinical features with the gut microbiome, we applied differential analysis to identify the specific microbial taxa related to irAE (Figure 2B; Supplementary Table 4). Species such as *Clostridium hathewayi, Ruminococcus torques, Bacteroides massiliensis*, *Paraprevotella clara*, *Parabacteroides distasonis* and *Megamonas* were enriched in patients without irAEs (Figure 3A). Meanwhile, *Bifidobacterium dentium*, *Rothia mucilaginosa* and *Gemella haemolysans* were significantly higher in irAE patients (Figure 3A). Because metagenomics sequencing enables microbial functional level exploration, we thus compared MetaCyc metabolic pathways between patients with and without irAE. We also identified some metabolic pathways that were statistically different between the irAE group and the non-irAE group (Figure 3B). Among them, urea cycle (PWY-4984) and citrulline biosynthesis (CITRULBIO-PWY) were enriched in the non-irAE group, while glycine metabolism either in the super pathway of heme b biosynthesis from glycine (PWY-5920) or tetrapyrrole biosynthesis from glycine (PWY-5189), threonine and methionine biosynthesis (THRESYN-PWY, PWY-724), histidine biosynthesis (HISTSYN-PWY), pyruvate fermentation to acetate and lactate (PWY-5100) and TCA cycle VII acetate producers (PWY-7254) were significantly enriched in participants with irAEs. Notably, the above-mentioned *R. mucilaginosa*, *B. dentium* and *G. haemolysans* contributed to most of the metabolic pathways.

The occurrence of irAE was further divided into mild (grades 1-2) and severe (≥ grade 3), because mild symptoms can mostly be recovered with clinical intervention and severe irAEs (≥ grade 3) may related to serious clinical consequences and the patients have limited survival benefits due to ICI withdrawal. Analysis of the data indicated that *Ruminococcus callidus* and *Bacteroides xylanisolvens* further helped to distinguish the population with grade ≥3 irAEs (Figure 3C).

irAE in different cancer types were associated with different gut microbiota profiles

We further explored the differences in gut microbiota in patients with different cancer types (Supplementary Table 5). In patients with esophageal cancer, regardless of whether the patients had immune-related adverse reactions, there were no significant differences in the alpha diversity or beta diversity of the gut microbiota at baseline (Supplementary Figure 1). *Ruminococcus torques* was enriched in the non-irAE group while *Dialister invisus* and *Eubacterium ventriosum* were enriched in the high irAE group compared to the low-grade irAE group (Figure 4A). At the pathway level, we found ubiquinol-6 biosynthesis (PWY3O-19) and glutamine biosynthesis (PWY-6549) were significantly enriched in the high irAE group (Figure 4B).

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TABLE 2 Patient irAE information.

Organ toxicity (Total occurrences)	irAE	Occurrences	Grade 1	Grade 2	Grade ≥3
Skin toxicity (n=21)	Rash	11	9	1	1
	Pruritus	7	7	0	0
	Hair loss	2	2	0	0
	Hemangioma	1	0	1	0
Blood toxicity (n=14)	Leukopenia	5	5	0	0
	Neutropenia	2	1	0	1
	Anemia	6	4	2	0
	Thrombocytopenia	1	0	1	0
Hepatotoxicity (n=20)	ALT elevation	7	5	0	3
	AST elevation	7	5	1	2
	Bilirubin elevation	4	2	2	0
Gastrointestinal toxicity (n=2)	Diarrhea	2	2	0	0
Endocrine toxicity (n=12)	Hypothyroidism	5	3	2	0
	Hyperthyroidism	5	4	1	0
	Adrenal insufficiency	1	0	0	1
	Hypercalcemia	1	0	0	1
Myotoxicity (n=4)	Myositis	4	2	0	2
Pulmonary toxicity (n=1)	Interstitial pneumonia	1	1	0	0
Renal toxicity (n=8)	Proteinuria	8	5	2	1
Cardiac toxicity (n=1)	Premature atrial beats	1	1	0	0

Among gastric cancer patients with irAEs, the gut microbiome showed reduced but not yet significant alpha diversity along with the irAE severity (Supplementary Figure 2A). Microbial community was significantly different among groups (low grade vs high grade: PERMANOVA p=0.046, Supplementary Figure 2B). Notably, multiple common probiotics species were enriched in the non-irAE group. At the family level, *Lactobacillaceae*, *Bifidobacteriaceae* and *Eubacteriaceae* were enriched in the non-irAE or low-grade irAE group (Supplementary Figure 2C). At the species level, we identified a few probiotic species enriched in no-irAE or low-irAE group, such as *Lactobacillus salivarius*, *Bifidobacterium longum*, *Bifidobacterium dentium*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum* (Figure 4A). In addition, several butyrate producers, such as *Eubacterium rectale*, *Megasphaera elsdenii* etc were also enriched in

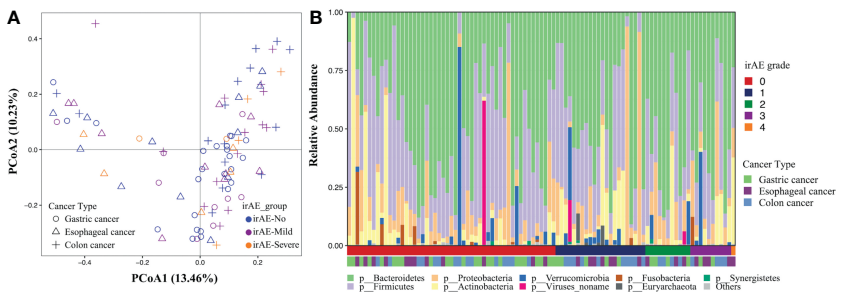

FIGURE 2
Gut microbial composition associated with immune-related adverse events. The analysis was performed on all patients. **(A)** Principal coordinates analysis of the microbial community based on the Bray-Curtis distance calculated at the species level. Each dot represents a stool sample with shape and color indicating cancer types and the severity of irAE. **(B)** Phylogenetic composition of common bacterial taxa at the phylum level, ordered by the irAE grade (the first horizontal bar underneath) and cancer types (the second horizontal bar underneath).

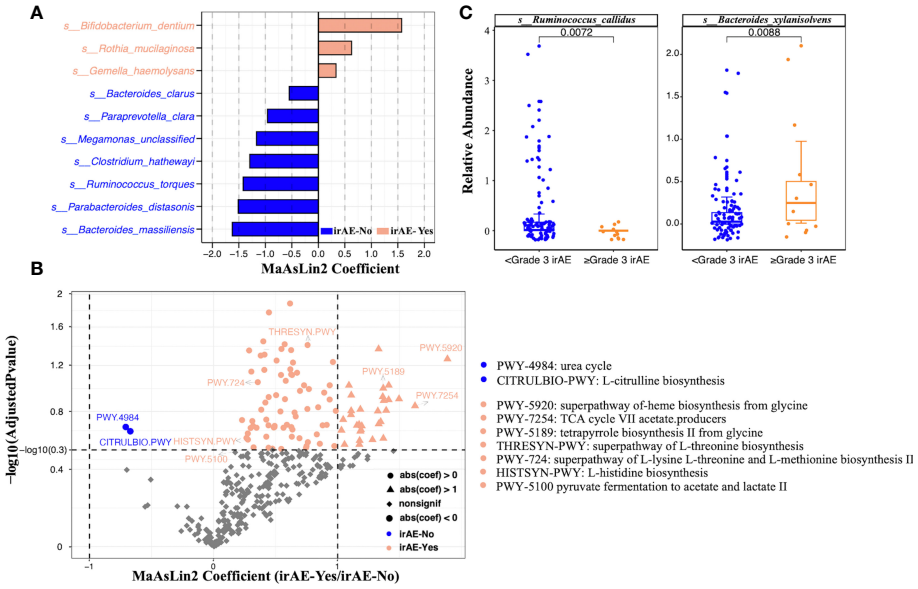

FIGURE 3
Significantly differential species and metabolic pathways between irAE and non-irAE groups. The analysis was performed on all patients. **(A)** Butterfly plot of differential bacterial species between patients with and without irAEs. The x axis shows the effect size represented by MaAsLin2 coefficient (positive number means enrichment in the irAE group; negative number means enrichment in the non-irAE group). **(B)** Volcano plot of microbial metabolic pathways. The x axis shows the effect size represented by MaAsLin2 coefficient (positive number means enrichment in the irAE group; negative number means enrichment in the non-irAE group). The dashed horizontal line shows adjusted P value of 0.3 and the two dashed vertical line showed coefficients of -1 and 1. **(C)** Boxplots showing the relative abundance of *Ruminococcus callidus* and *Bacteroides xylanisolvens* in the mild (<grade3) and severe (≥grade3) irAE groups. Wilcoxon rank sum test was applied, and p values were labeled for each comparison.

these groups (Figure 4A). At the pathway level, isopropanol biosynthesis (PWY-6876) and multiple pathways in menaquinol biosynthesis (PWY-5838, PWY-5840, PWY-5897, PWY-5898, PWY-5899) were enriched in no-irAE or low-irAE group (Figure 4B).

In patients with colon cancer, there were no significant differences in the alpha diversity and beta diversity of the gut microbiota in each group at baseline (Supplementary Figure 3). At the species level, *Bacteroides stercoris* were enriched in the high-grade irAE group (Figure 4A). At the pathway level, arginine biosynthesis (ARGSYNBSUB-PWY, ARGSYN-PWY, PWY-7400), and pyruvate fermentation to acetate and lactate pathway (PWY-5100) were significantly higher in low-grade irAE group (Figure 4B).

Differences in gut microbiota profiles were associated with specific toxicity

Considering that irAEs in different organs may involve different mechanisms, we next compared the relationship between specific toxicity (skin, blood, endocrine, and liver) and the gut microbiota

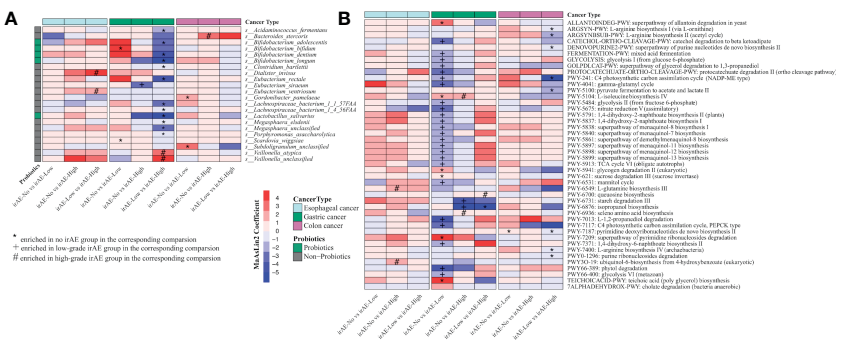

FIGURE 4
Significantly differential species and metabolic pathways in patients with different cancer types stratified by irAE severity. **(A)** Differential species between each comparison (irAE groups: irAE_No, irAE_Low and irAE_High) for esophageal cancer, colon cancer, and gastric cancer patients. Commonly considered probiotic species (green color) based on Probio https://bidd.group/probio/homepage.htm. **(B)** Differential metacyc pathway between each comparison (irAE groups: irAE_No, irAE_Low and irAE_High) for esophageal cancer, colon cancer and gastric cancer patients. MaAsLin2 was used to identify differential species and pathway between irAE groups. Each column indicates a comparison, and the labels (*,#,+) indicate the direction of enrichment. enrichment. Different colors of the rows indicate different cancer types.

composition in different subgroups of irAE patients (Supplementary Table 6; Supplementary Figure 4–7). For skin irAEs, *Methanobrevibacter smithii*, *Bifidobacterium dentium*, *Roseburia intestinalis* and *Faecalibacterium prausnitzii* were enriched in the irAE group, while *Megasphaera micronuciformi*, *Clostridium hathewayi*, *Ruminococcus torques* and *Flavonifractor plautii* were enriched in the non-irAE group (Figure 5A). Out of total 111 metabolic pathways, glycerol degradation pathway (GOLPDLACT-PWY) and urea cycle (PWY-4984) were enriched in the non-irAE group with a major contribution from *F. plautii* (Figure 5B). For hematologic irAEs, *Bacteroides massiliensis* was enriched in the non-irAE group while notably, *Akkermansia* was enriched in gastric cancer patients with hematologic irAEs (Figure 5A; Supplementary Figure 5C). We also found urea cycle (PWY-4984) and citrulline biosynthesis (CITRULBIO-PWY) were significantly enriched in the non-irAE group (Figure 5B; Supplementary Figure 8).

For endocrine irAEs, two *Clostridium* species (*Clostridium clostridioforme* and *Clostridium hathewayi*) were enriched in the non-irAE group, while *Alistipes finegoldii*, *Veillonella atypica* and *Lachnospiraceae* bacterium were increased in the irAE group (Figure 5A). Meanwhile, significant increases of the enterobactin biosynthesis pathway (ENTBACSYN-PWY) and O-antigen biosynthesis pathway (PWY-7328) were observed in the non-irAE group (Supplementary Figure 8). Lastly, for liver irAEs, *Ruminococcus torques*, *Parabacteroides distasonis* and *Clostridium hathewayi* were enriched in the non-irAE group compared to the enrichment of *Bifidobacterium pseudocatenulatum* in irAE patients (Figure 5). The significantly enriched pathways in the non-irAE

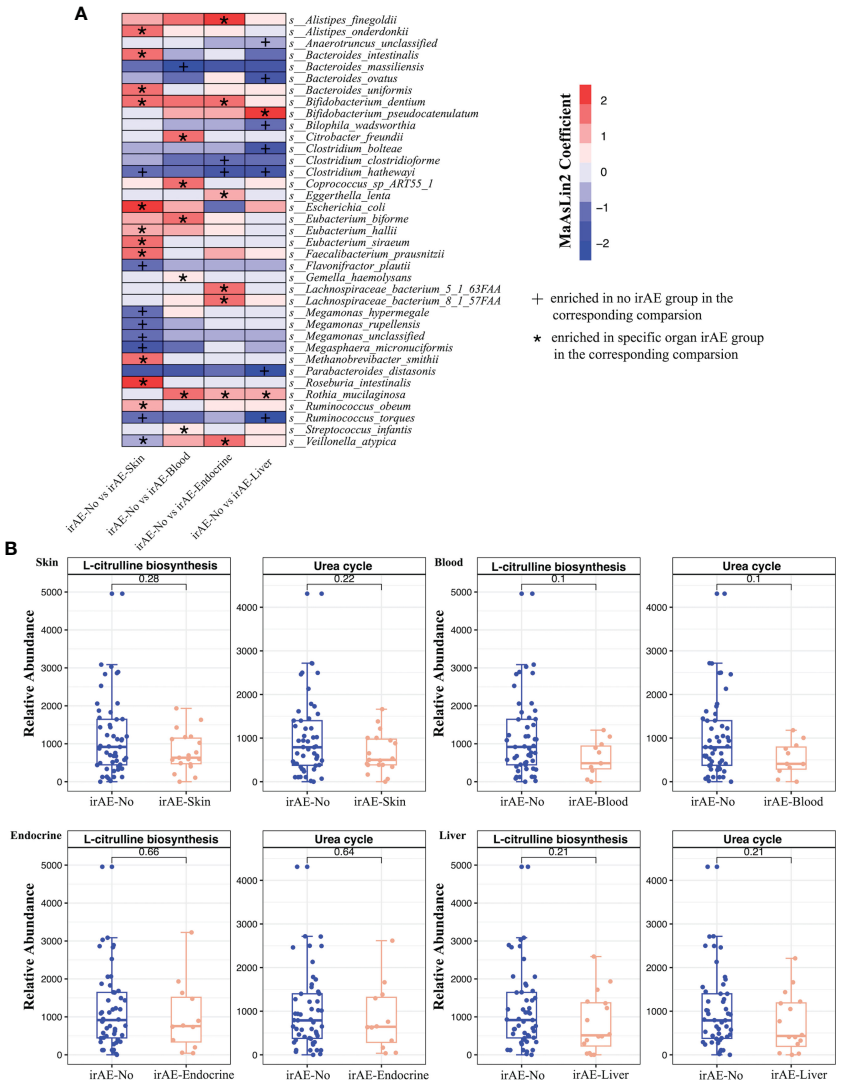

FIGURE 5
(A) Differential species between irAE and non-irAE patients for each comparison group. Each column indicates a comparison for the specific toxicity and the labels (*, +) indicate the direction of enrichment. **(B)** Boxplots showing the relative abundance of microbial metabolic pathways in irAE patients across different toxicities. Wilcoxon rank sum test was applied, and p values were labeled for each comparison.

group included urea cycle (PWY-4984) and citrulline biosynthesis (CITRULBIO-PWY), the same as in hematologic irAEs (Figure 5B; Supplementary Figure 8).

Discussion

In this prospective study, we comprehensively analyzed the association between the gut microbiome and irAEs in 95 gastrointestinal cancer patients treated with immune checkpoint inhibitors and identified bacterial species and metabolic function pathways that were closely associated with irAEs.

Among the bacterial biomarkers related to irAEs, we found that *Ruminococcus callidus* was enriched in people without severe irAEs. A recent study reported that increased abundance of *R. callidus* was associated with favorable response to anti-PD-1 therapy and improved survival in hepatobiliary tumors (Mao et al., 2021). *Ruminococcus* was also reported to be associated with immune-related enteropathy and other immune diseases such as allergy, eczema, asthma, and other diseases (Chaput et al., 2017; Gopalakrishnan et al., 2018; Park et al., 2018), indicating its potential role in immune-modulation. We also found higher quantities of *Lactobacillus* and *Bifidobacterium* in gastric cancer patients with non- or low-grade irAEs. Many species of *Lactobacillus* and *Bifidobacterium* are considered to be probiotics that provide health benefits to the host. In addition to the prophylactic effects, their involvement in immunotherapy is starting to be noticed in recent years. Three studies on non-small cell lung cancer (NSCLC) patients showed an increase of *Lactobacillus* and/or *Bifidobacterium* in non or low-grade irAE cases (Hakozaki et al., 2020; Cascone et al., 2021; Chau et al., 2021).In a study of 70 Japanese NSCLC patients treated with PD-1/PD-L1, Hakozaki T et al. found that baseline samples from patients with no or grade 1 irAEs were enriched in *Lactobacillus* (Hakozaki et al., 2020). In a prospective cohort study from the United States, the researchers compared baseline fecal samples from 33 advanced non-small cell lung cancer patients with 32 healthy controls and analyzed the gut microbiome using 16S rRNA sequencing. *Bifidobacterium* was associated with lower severity of irAEs (Chau et al., 2021). In the NEOSTAR trial, Cascone et al. also observed an association between decreased toxicity to nivolumab and *Bifidobacterium* (Cascone et al., 2021). Similarly, in one preclinical study, Wang et al. found the abundance of *Lactobacillus* was significantly reduced in mice with immune-associated colitis (Satoh et al., 2020). Regarding the potential mechanisms, different species confer beneficial effects through various ways. Tan et al. demonstrated that *Lactobacillus rhamnosus* alleviated immune-related enteritis by regulating Treg cells in the mouse model (Tan et al., 2020). A study on *Lactobacillus reuteri* showed that it can prevent immune enteritis by reducing the number of group 3 innate lymphocytes (ILC3s) (Satoh et al., 2020). Preclinical studies also suggested *Bifidobacterium* supplementation could alleviate colitis in mice receiving anti-CLTA-4 (Wang et al., 2018), and this was potentially mediated by gut microbiome optimization, thereby enhancing the expression of IL-10Ra and IL-10 of intestinal Treg cells and ultimately alleviating immune-related intestinal damages (Sun et al., 2020).

Quite a few studies focusing on irAEs in melanoma patients showed decreased microbial diversity and potential bacterial biomarkers such as *Lachnospiraceae, Streptococcaceae* as well as several *Bacteroides* species (*B. dorei, B. vulgatus, B. intestinalis*) (Dubin et al., 2016; Chaput et al., 2017; Andrews et al., 2021; Liu et al., 2021; Usyk et al., 2021; McCulloch et al., 2022). Andrews et al. further demonstrated in preclinical models that the higher abundance of *B. intestinalis* promoted irAE toxicity through the upregulation of IL-1beta (Andrews et al., 2021). In the current study, however, we did not observe any bacterial diversity associated with irAEs and identified rather different bacterial biomarkers. This could potentially be due to variations in sequencing/analysis methods, cohort characteristics, treatment regimens, etc. because even within melanoma studies, we found multiple inconsistencies between published studies. Moreover, we fully acknowledge the gut microbiome profile is unique to each cancer type, as we have shown that even within gastrointestinal cancers, the gut microbiome of colorectal, gastric, and esophageal cancer patients are significantly different. Thus, gut microbial signatures related to each cancer type or even each specific cohort might be different, and this should be noted in current clinical practices.

Besides the above-mentioned microbial biomarkers, their encoded functions are also of great interest because the underlying mode of action on how gut microbiome impact irAEs might overlap among different cancer types despite the heterogeneous microbial biomarkers. In the current study, we showed pathways involved in the urea cycle, including citrulline and arginine biosynthesis, were associated with irAEs. Previous research showed urea cycle dysregulation and arginine metabolism play an important role in immunotherapy (Kim et al., 2018). Oral supplementation of arginine could significantly increase the efficacy of cyclophosphamide combined with anti-PD1 antibody in a mouse model (Satoh et al., 2020). Specifically, by engineering a probiotic strain to convert ammonia to arginine, Canale et al. showed that the enhanced anti-tumor effect was mediated by arginine and dependent on T cells (Canale et al., 2021). Although the role of urea cycle in irAEs has not been reported, our data indicated its potential connections and warrants further examination. In addition, a recent study demonstrated that a gut ecosystem enriched with beneficial microbial functions and a richer butyrate production pathway was significantly associated with a reduced incidence of irAEs in melanoma patients (Maung et al., 2020). Rectal or oral butyrate-based therapy has shown promising results in intestinal inflammatory diseases (Vernia et al., 2000; Hallert et al., 2003; Di Sabatino et al., 2005). In our study, although we did not find significant enrichment of butyrate-producing pathways in non- or low-grade irAE, a decent number of butyrate producers were identified. For example, significant enrichment of *Eubacterium rectale* and *Megasphaera elsdenii* was observed in non-/low irAE gastric cancer patients.

In this study, we investigated the correlation between gut microbiota and irAEs in 95 gastrointestinal cancer patients. With the help of metagenomic sequencing, we identified bacterial species and metabolic pathways that might be associated with the occurrence of irAEs in gastric, esophageal, and colon cancers and across multiple organs. We believe this work provides a foundation for future mechanism exploration and clinical applications. Despite these exciting findings, we also acknowledge that there are several limitations of this study, including the lack of external validation cohorts as well as experimental validation of the potential mechanisms we hypothesized.

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 sra BioProject: PRJNA910239.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Beijing Cancer Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YZ: Resources, data curation, investigation, methodology, writing–original draft. SC: Resources, data curation, investigation, and manuscript writing. HZ: data curation, data analysis, manuscript writing. ZH, TX, BZ: data curation, investigation, reviewed and revised the manuscript. DD: data curation, data analysis. YL implemented the bioinformatics workflow and technical support. XY, YK, YT: research design, analysis supervise, reviewed, and revised the manuscript. LS, ZP: Conceptualization, supervision, funding acquisition. 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.2023.1099063/full#supplementary-material

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Tumor bacterial markers diagnose the initiation and four stages of colorectal cancer

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Increasing evidence has supported dysbiosis in the faecal microbiome along control-adenoma-carcinoma sequence. In contrast, the data is lacking for *in situ* tumor bacterial community over colorectal cancer (CRC) progression, resulting in the uncertainties of identifying CRC-associated taxa and diagnosing the sequential CRC stages. Through comprehensive collection of benign polyps (BP, *N* = 45) and the tumors (*N* = 50) over the four CRC stages, we explored the dynamics of bacterial communities over CRC progression using amplicons sequencing. Canceration was the primarily factor governing the bacterial community, followed by the CRC stages. Besides confirming known CRC-associated taxa using differential abundance, we identified new CRC driver species based on their keystone features in NetShift, including *Porphyromonas endodontalis*, *Ruminococcus torques* and *Odoribacter splanchnicus*. Tumor environments were less selective for stable core community, resulting in heterogeneity in bacterial communities over CRC progression, as supported by higher average variation degree, lower occupancy and specificity compared with BP. Intriguingly, tumors could recruit beneficial taxa antagonizing CRC-associated pathogens at CRC initiation, a pattern known as "cry-for-help". By distinguishing age- from CRC stage-associated taxa, the top 15 CRC stage-discriminatory taxa contributed an overall 87.4% accuracy in diagnosing BP and each CRC stage, in which no CRC patients were falsely diagnosed as BP. The accuracy of diagnosis model was unbiased by human age and gender. Collectively, our findings provide new CRC-associated taxa and updated interpretations for CRC carcinogenesis from an ecological perspective. Moving beyond stratifying case-control, the CRC-stage discriminatory taxa could add the diagnosis of BP and the four CRC stages, especially the patients with poor pathological feature and un-reproducibility between two observers.

KEYWORDS

colorectal cancer (CRC) stage, CRC-associated taxa, average variation degree, occupancy and specificity, CRC-stage discriminatory taxa, diagnosis model

Introduction

Colorectal cancer (CRC) is the third most prevalent cancer, which ranks the second in terms of mortality globally (Arnold et al., 2017; Cai and Liu, 2021). Although the incidence and mortality rates of CRC are decreasing in recent years, CRC is still one of the most life-threatening cancers and advanced CRC remains an incurable disease at metastatic stages, 5-year survival rate of 13% compared to 90% when diagnosed at an initial stage (Bray et al., 2018; Montalban-Arques and Scharl, 2019). The trend in younger patients, along with the continued burden, highlight the need for early detection of CRC that alleviates the incidence of metastatic CRC (Cheng et al., 2020). In particular, the clinical trials of patients are intimately associated with the CRC stages, thus there is an urgent requirement for accurately diagnosing the stages of CRC.

Over the past decades, the Tumour, Nodes, and Metastasis (TNM) staging system has contributed the cornerstone for the management of CRC patients. However, some problems have occurred with the TNM system, such as increasing complexity of CRC, poor clinical evidence, tumor deposits, and un-reproducibility between different observers (Quirke et al., 2007), which in turn confuse the accuracy of identified CRC stage and subsequent therapy. It is now widely recognized that the microbes contribute indispensable roles in host health and gastrointestinal tumor progression (Marchesi et al., 2016; Xiong, 2018; Guo et al., 2022). In accordance, intensive studies have shown dysbiosis (shift in gut commensal microbiota toward opportunistic pathogens) in the gut microbiota in CRC patients compared with healthy controls. Going forward, CRC-associated taxa have been identified for distinguishing healthy from colorectal adenomas, and CRC individuals (Shah et al., 2018; Wu et al., 2021; Coker et al., 2022). However, few studies have explored the dynamics of microbial communities over CRC progression. As a result, it is uncertain whether microbial taxa are indicative of each CRC stage, rather than just stratify case from control cohorts. However, this information is fundamental to establish CRC stage-dependent clinical trials.

Accumulating evidence depicts that the gut microbiota is an important etiological element in CRC initiation, progression, and metastasis (Kong et al., 2019; Cheng et al., 2020; Mizutani et al., 2020; Li et al., 2022). By this logic, identification of bacteria involved in CRC progression could provide new targets for CRC diagnosis and prevention (Montalban-Arques and Scharl, 2019; Fong et al., 2020). Indeed, case studies have proposed that the occurrence of CRC is attributed to the enrichment of *Fusobacterium nucleatum* (encoding FadA) (Flanagan et al., 2014), *Bacteroides fragilis* and *Escherichia coli* hosting polyketide synthase (pks) islands (Feng et al., 2015), *Clostridium symbiosum* (Xie et al., 2017), or *Parvimonas micra* (Löwenmark et al., 2020), respectively. It should be noted that the identification of "driver" taxa is generally based on their significant enrichment in CRC patients compared to healthy controls, which ignores the CRC stage (Flanagan et al., 2014; Löwenmark et al., 2020). In particular, a "driver" taxon is attributed to strong biotic interactions, rather than its sheer abundances, though this does not rule out that some "driver" taxa are numerically abundant (Dai et al., 2018). Recently, NetShift approach has been developed to quantify major changes in associations of each constituent taxon between healthy and diseased networks, in which the importance of a single species between health states can be calculated based on its topological features (Kuntal et al., 2019). By this logic, taxa that increase in their importance in the network of CRC patients could be the "driver" taxa, moving beyond enriched abundance. Furthermore, according to the "driver-passenger" model, the CRC driver taxa could be superseded by "passenger" bacteria that are better adapted to the conditions in and around carcinoma cells, thereby outcompeting the initial driver species (Feng et al., 2015; Bridges et al., 2019). For these reasons, a systematic analysis of CRC-associated bacteria along CRC progression is required from an ecological prospective, rather than case-control study.

In spite of a growing body of evidence with regard to the dysbiosis in gut microbiota in CRC patients, data on the association between *in situ* tumor microbial dynamics over CRC progression is lacking. The gut microbiotas are significantly varied as over human lifetime (Falony et al., 2016; Greenhalgh et al., 2016; Ghosh et al., 2020), thus a key challenge is to distinguish the alterations in microbial assembly over CRC progression from these as human aged. Additionally, faecal microbiota only partially mirrors mucosal microbiota in CRC, with low correlations between paired faecal and mucosal samples (Flemer et al., 2017). In this regard, the deployment of faecal microbes for mirroring tumor microbiota could bias the identification of CRC-associated bacteria. To overcome above obstacles, we explored the dynamics of bacterial communities in tumor tissues along the four CRC stages, and benign polyps as controls. We attempted to address the following concerns: (1) exploring the dynamics of bacterial communities over CRC progression, (2) identifying CRC-associated bacteria based on biotic interactions, (3) screening biomarkers for diagnosing each CRC stage, irrespective of host age.

Materials and methods

Experimental design and sample collection

Subjects underwent standard colonoscopy examinations at Hwa Mei Hospital in Ningbo City, China, were recruited to the study. The patients were selected based on the following criteria: no complicating diseases (such as chronic bowel disease, diabetes, and hypertension); no family history of CRC and recurrence in CRC patients, no use of antibiotic in the month prior to surgery. Written informed consent was obtained from the volunteers to utilize their tissue samples. All volunteers were categorized into a different group based on the histopathological features in the TNM staging system of malignant tumors after surgery. The samples with uncertain TNM stage (e.g., poor clinical evidence, tumor deposits, un-reproducibility between two observers) were excluded in the analysis. Based on these selective criterions, 95 subjects (aged 21–89 years, 70 males and 25 females) were enrolled in the analysis from 120 volunteers, in which included 45 BPs and 50 tumors over the four CRC stages. The general information (age, gender) and clinical data (body mass index (BMI), carcinoembryonic antigen (CEA), TNM stage) are summarized in Table S1 and Figure S1. All tissue

samples were stored at −80°C until further processing. We want to point out that no tissue samples can be obtained from healthy individuals, thus benign polyps (BPs) were served as controls.

DNA extraction, amplification, and sequencing of the 16S rDNA genes

Tumor or BP tissue (1 gram) were homogenized with four volumes (weight/volume) of phosphate buffer solution (PBS, pH = 7.4) and centrifuged at 4000 rpm for 5 min. To collect microbial biomasses, the supernatant was transferred and centrifuged at 12000 rpm for 10 min at 4°C. DNA was extracted using the FAST DNA Spin kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocols. The concentration and purity of DNA extracts were evaluated by using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The V3–V4 regions of bacterial 16S rDNA genes were amplified by using the primer pair: 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTWT- CTAAT-3') (Takahashi et al., 2014). To minimize PCR induced biases, each sample was amplified in triplicates as follows: 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final elongation step of 72°C for 10 min in 30 μL PCR reaction system. Every triplicate amplicons from each sample were pooled and purified using a PCR fragment purification kit. The concentrations of purified products were detected using a PicoGreen-iT dsDNA Assay Kit (Invitrogen, Carlsbad, USA). Equimolar amounts of amplicons for each sample were pooled, and sequenced on a single run using an Illumina MiSeq platform (Illumina, San Diego, USA), producing 2 × 300 bp paired-end reads.

Processing of Illumina sequencing data

The FASTQ format data were analyzed by the Quantitative Insights into Microbial Ecology 2 (QIIME 2) pipeline (Bolyen et al., 2019). In short, the raw sequences were processed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) that could obtain reads with a single-nucleotide difference (Callahan et al., 2016), known as amplicon sequence variants (ASVs). Primers were screened and removed. Filtered reads were then de-replicated and de-noised using DADA2 with default parameters. Then, paired-end sequences were merged, and chimeras were identified and removed using Usearch (version 11.0.667) and the "uchime2_ref" command. Reads were truncated at the quality control score of 20. Taxonomic assignment of ASVs was performed based on the SILVA v138 16S database (Quast et al., 2012). ASVs classified as Mitochondria, Chloroplast, Archaea, and Eukaryota in origin were removed from the bacterial community. Only ASVs detected with a minimum of three samples were included. Finally, to adjust unequal sequencing depth, all samples were rarefied to the same sequencing depth in downstream analysis. After filtering and rarefaction to 14,221 reads per sample, a total of 3601 ASVs were included in the final analyses.

Diagnosis model of CRC stages

To identify bio-indicators for quantitatively diagnosing the stages of CRC, we created a classification model using random forests (RF), a robust machine-learning algorithm for classification and regression that is suitable for microbial population data (Liaw and Wiener, 2002). Given that the structures of bacterial community were highly temporal dynamics over human ontogeny, we first determined host age-discriminatory lineages (when bacterial taxonomic level is undefined, namely, bacterial phylum, class, order, family, genus, or ASV) across the BP controls. The relative abundances of all lineages in BP were regressed against corresponding host age using default parameters. The 10-fold cross-validation function was implemented to identify the minimal number of top-ranking age-discriminatory lineages, which only contained the most important variables based on the cross-validation curve (Fushiki, 2011). To rule out the ontogenic effects on bacterial community, the age-discriminatory lineages were excluded from the dataset. Then, the relative abundances of all lineages were classified into corresponding stages, that is, BPs and the four CRC stages (Table 1). The RF model was repeated to identify the top CRC stage-discriminatory lineages. After this optimization, the identified CRC stage-discriminatory lineages were employed as dependent variables for diagnosing BP and CRC stages. A consistency between observed and diagnosed category was termed a correct diagnosis; otherwise, the classification was termed as a false diagnosis. To acquire finer taxonomic information for the CRC stage-discriminatory ASVs, we manually identified their species classification by aligning their representative sequences in the basic local alignment searching tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

The following analyses were performed in R 3.6.3, unless otherwise stated (http://www.R-project.org/). Alpha diversity of bacterial community was compared among CRC stages using one-way analysis of variance (ANOVA). Canonical analysis of principal coordinates (CAP) and non-parametric multivariate analysis of variance (NPMANOVA) were used to assess the differences in bacterial communities along CRC stages based on Bray-Curtis distances (Anderson and Willis, 2003). Statistical differences in beta-diversity between health status, age, and gender were calculated using perMANOVA with the adonis2 function in vegan package (Oksanen et al., 2018). Bacterial community stability was evaluated by average variation degree (AVD). A lower AVD value indicates higher stability (Xun et al., 2021). An UpSet plot was used to display the number of shared and

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unique bacterial ASVs among groups (Lex et al., 2014). The 500 most abundant ASVs were selected from the bacterial communities in each group, specificity and occupancy were calculated as described previously (Gweon et al., 2021). Specificity is the mean abundance of species (S) in the samples within a group; and occupancy is the relative frequency of occurrence of S in the samples within a group. The two metrics (specificity and occupancy) were used as the axes in SPEC-OCCU biplot (Dufrêne and Legendre, 1997; Gweon et al., 2021). CRC driver taxa were screened based on their "Neighbor Shift (NESH) Score"

TABLE 1 The predicted accuracy based on profiles of colorectal cancer (CRC) stage-discriminatory lineages at bacterial phylum, class, order, family, genus or ASV level, respectively.

Taxonomy	Observed	Predicted					Overall accuracy
		T1	T2	T3	T4	BP	
Phylum	T1	**3**	1	2	3	0	(59/95) 62.1%
	T2	0	**1**	8	0	4	
	T3	1	3	**12**	1	5	
	T4	1	3	2	**0**	0	
	BP	0	0	2	0	**43**	
Class	T1	**2**	2	1	0	4	(61/95) 64.2%
	T2	1	**2**	6	0	4	
	T3	2	4	**11**	1	4	
	T4	0	2	3	**1**	0	
	BP	0	0	0	0	**45**	
Order	T1	**1**	3	2	0	3	(61/95) 64.2%
	T2	0	**0**	10	0	3	
	T3	1	2	**14**	0	5	
	T4	2	0	2	**1**	1	
	BP	0	0	0	0	**45**	
Family	T1	**0**	1	2	4	2	(63/95) 66.3%
	T2	0	**2**	8	0	3	
	T3	1	2	**14**	0	5	
	T4	0	4	0	**2**	0	
	BP	0	0	0	0	**45**	
Genus	T1	**1**	0	2	5	1	(60/95) 70.6%
	T2	0	**0**	11	0	2	
	T3	1	3	**16**	0	2	
	T4	0	0	2	**0**	4	
	BP	0	0	2	0	**43**	
ASV	T1	**8**	0	1	0	0	(83/95) 87.4%
	T2	1	**8**	4	0	0	
	T3	0	2	**17**	3	0	
	T4	0	0	0	**6**	0	
	BP	0	0	1	0	**44**	

BP, Benign polyps, T1, T2, T3 and T4 were the four CRC stages.
For a given sample, the consistency between observed and diagnosed category was termed as a correct diagnosis, otherwise it was termed as a false diagnosis. Bold values represent the numbers of correct diagnoses.

and node size using NetShift. NESH is a neighbor shift score, which represents directional changes in individual node associations (Kuntal et al., 2019).

Results

General and clinical information

Ages of the 50 CRC patients ranged from 30 to 89 years, in which 7 patients (2 individuals were T2, and 5 patients were T3 stage) were younger than 40 years. This pattern reinforced the trend to younger CRC patients. The numbers of male patients were consistently higher (P = 0.0105, paired t test fixed CRC stage) than female patients over each CRC stage (Table S1). As expected, CEA values linearly increased along CRC severity (Figure S1A), while no significant differences in BMI among the four CRC stages (Figure S1B).

Differed microbiotas along CRC stages and BPs

Sequencing yielded a total of 12,128,782 (mean ± standard deviation, 81710 ± 47466) raw reads from the enrolled 95 samples. Rarefaction curves indicated sufficient sequencing depth was achieved (Figure S2), thus enabled us to compare diversity among groups. The Firmicutes, Bacteroidota, and Proteobacteria were the dominant bacterial phyla in BPs and tumor tissues, albeit difference in their relative abundances (Figure S3A). This composition was analogous to the composition of human gut microbiota. At the finer bacterial genus level, genera of *Collinsella*, *Parvimonas*, *Ruminococcus*, and *Bifidobacterium* significantly enriched in the tumors compared with BP, while the relative abundance of *Faecalibacterium* exhibited the opposing trend (Figure S3B).

There were no significant differences in the diversity of bacterial communities among BP and the tumors, as supported by both the Shannon diversity and Phylogenetic diversity (Figure S4). However, the CAP biplot demonstrated clear separation of bacterial communities between BP and along the four CRC stages (T1, T2, T3 and T4), in which CRC stage was imposed as a conditional factor. Overall, the bacterial communities were more dissimilar between BP and tumors than along the four CRC stages (Figure 1). These patterns were further corroborated by a comparison of the similarity between groups; the structures of bacterial community differed significantly (P < 0.05) between each paired groups, with the exception of T1 vs. T3 (Table S2). Furthermore, parametric permutational multivariate analysis of variance (perMANOVA) revealed that CRC stage and human age respectively constrained 8.4% (P < 0.001) and 2.1% (P = 0.002) variation in bacterial community, whereas the effect of host gender was insignificant (P = 0.103). The averaged AVD of bacterial communities in T1 (0.744 ± 0.016) was markedly increased (P < 0.05) compare with that in BP (0.752 ± 0.012). However, AVD values tended to be decreased over CRC progression (Figure 2).

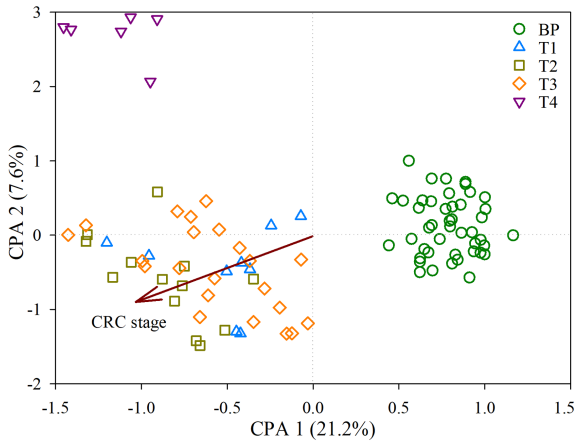

FIGURE 1
Constrained analysis of principal coordinates (CAP) depicting the effects of CRC stage on the bacterial communities derived from the distance matrix. Samples were coded by benign polyps (BP, here is controls) and along the four colorectal cancer (CRC) stages, T1, T2, T3 and T4.

Distribution of core taxa

In total, 1071 ASVs were uniquely detected in BP. Intriguingly, the numbers of unique ASVs linearly decreased along CRC progression, with 318, 243, 175 and 38 ASVs in T1, T2, T3 and T4 tumors, respectively. Similarly, there were gradual decreases in shared ASVs between adjacent stages. For example, the groups with the highest number of shared ASVs were BP and T1 (220 ASVs), followed by 90 shared ASVs between T1 and T2, 45 shared ASVs between T2 and T3, with the least shared ASVs between T3 and T4 (8 ASVs) (Figure 3A). In addition, only 153 ASVs (accounting for 4.25% of all ASVs) were shared across the five groups, while 19 ASVs were consistently detected among the four CRC stages (Figure 3A). In addition, there was no significant difference in diversity among

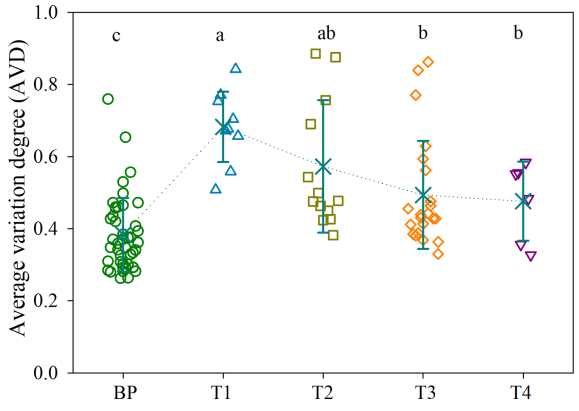

FIGURE 2
Average variation degree (AVD) for the bacterial communities in BP and along the four CRC stages. Different lowercase letters indicate significant differences among groups using one-way analysis of variance (ANOVA) with significant level of P < 0.05. Refer to Figure 1 for abbreviations.

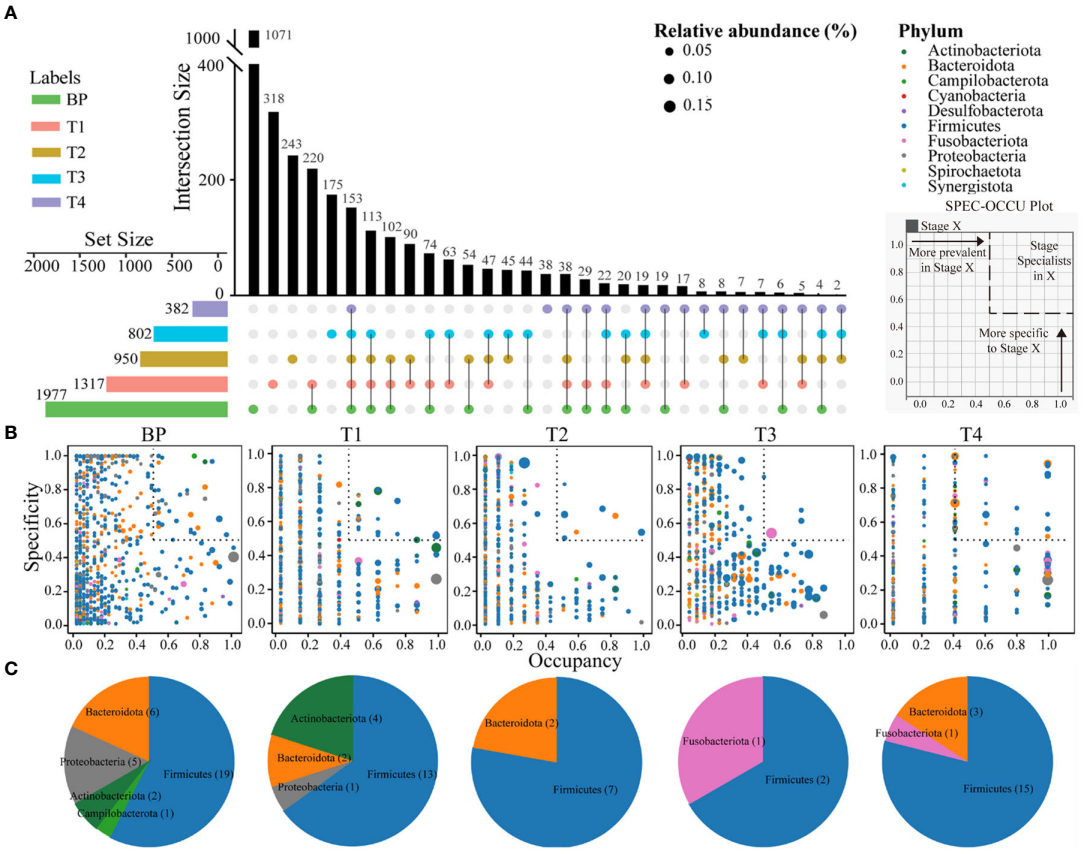

FIGURE 3
Upset plot displays the number of detected ASVs in each group (horizontal bars) and unique or shared ASVs (individual or connected points, respectively), in and among groups **(A)**. The SPEC-OCCU plots show 500 most abundant ASVs in each group; the x-axis represents occupancy, e.g., how well an ASV is distributed across biological replicates within each group; and the y-axis represents specificity, e.g., whether they are also found in other groups **(B)**. Pie charts showing the number of ASVs representing specialists in each group (See Table S3 for the list of these specialists) **(C)**. Refer to Figure 1 for abbreviations.

the five groups (Figure S4). These results indicated an increasing distinctness in bacterial communities over CRC progression.

To inspect how ASVs from each group are spread from BP to advanced CRC and also how specific they are to their stage, specificity and occupancy were calculated for each ASV, which were then projected onto a SPEC-OCCU biplot (Figure 3B). As indicated by the distribution of ASVs along the x-axis (occupancy), ASVs from BP displayed remarkably homogenous occupancy. To identify specialist taxa attributable to each group, we selected ASVs with specificity and occupancy higher or equal to 0.5 (dotted boxes in Figure 3B), that is, these ASVs are specific to a stage and common in their groups in most individuals. The number of these specialist ASVs substantially varied among groups. There was a decreasing trend in terms of observed richness from BP (33 ASVs represent), T1 (20 ASVs), T2 (9 ASVs) to T3 (3 ASVs) (Table S3), representing 1.7%, 1.5%, 0.95% and 0.37% of their total richness, respectively. Conversely, 19 specialist ASVs (representing 5.0%) were detected among the T4 tumors. Firmicutes species were the specialists across the all five groups (Figure 3C, Table S3).

Identification of CRC driver taxa

Comparison of the gut networks between BP and CRC stage 1 (T1), an important step to tumorigenesis, 13 taxa drove the network shift from BP to initial CRC (Figure 4A, Table S4). Specifically, ASV2473 *Phascolarctobacterium succinatutens*, ASV3703 *Muribaculum intestinale*, ASV853 *Neglectibacter timonensis*, ASV3538 *Porphyromonas endodontalis* among others were the driver nodes (ASVs) with higher NESH scores (red color and bigger nodes) (Figure 4A, Table S4). Of the 13 driver taxa, the relative abundances of six taxa significantly enriched, and only ASV1014 *Faecalibacterium prausnitzii* depressed in T1 compared with BP (Figure 4B). In particular, the six enriched driver taxa were also the most abundant over CRC progression (Figure 4C). However, abundances of the remaining seven driver taxa insignificantly changed between BP and T1, such as ASV3538 *Porphyromonas endodontalis*, ASV2131 *Ruminococcus torques* and ASV78 *Odoribacter splanchnicus* (Figure 4, Table S4), while case studies have reported their enrichments in CRC patients (Flemer

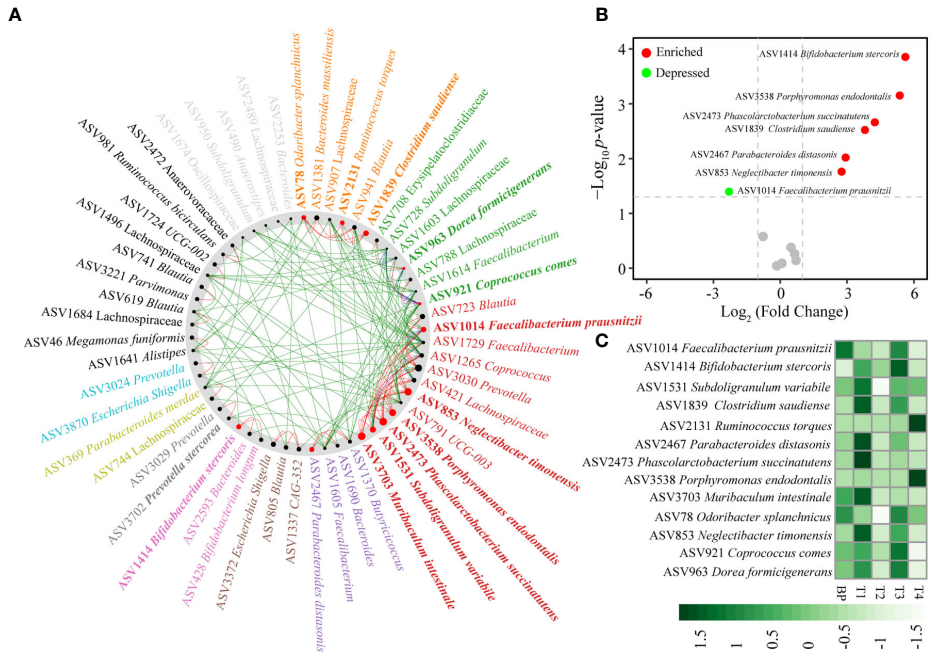

FIGURE 4
Identification of "driver" taxa from benign BP to CRC T1 tumor **(A)**, changes between T1 and BP **(B)**, distributions in BP and along the four CRC stages **(C)**. The nodes of the common sub-network were placed around a circle that was sorted by their identified community membership (in the CRC T1 network). All nodes belonging to the same community are assigned similar colors. Black nodes represent nodes that exist in both but interact directly with the common sub-network in either T1 tumor or BP. The size of the node is proportional to the NESH fraction of its scale, and the node is colored red if the intermediation of the node increases from BP to T1 tumor status. Thus, large red nodes are particularly important "driver" taxa. Refer to Figure 1 for abbreviations.

et al., 2017; Wolf et al., 2020). Based on the differential distribution across groups, 19 ASVs were spurted in CRC T1 tumors, which were substantially decreased in BP and along progressed CRC, including previously reported as CRC associated taxa, e.g., *Fusobacterium nucleatum* (Figure S5A). In addition, several potential pathogens, such as *Bacteroides fragilis*, *Clostridium perfringens* among others, were enriched and the most abundant in the advanced CRC stage 4 (T4) (Figure S5B).

Establishment of diagnosis model for diagnosing CRC stages

We randomly selected 67 samples (training data, 32 BPs and 35 CRC patients) for constructing the diagnosis model. The remained 28 samples (test data, 13 BPs and 15 patients) were used for validation. In order to distinguish the CRC stage effect from the confounded roles of host age in governing the bacterial community, we firstly identified the top age-discriminatory lineages (Figure S6). In addition, to evaluate whether the diagnosis accuracy was influenced by taxonomic level, we screened the discriminatory lineages at the bacterial phylum, class, order, family, genus, or ASV level, respectively. To this end, we found that the CRC stage-discriminatory ASVs contributed the highest accuracy of classification after excluding host age effect (Table 1). For this reason, CRC stage-discriminatory ASVs were applied as dependent variables for diagnosing the BP and CRC stages in the final diagnosis model.

We screened nine age-discriminatory ASVs from the BP that contributed an overall 92.4% diagnosis accuracy (Figure S6). After excluding the nine age-discriminatory taxa, we identified 15 common CRC stage-discriminatory ASVs. To visualize these biomarkers, we constructed a phylogenetic tree to identify their closest species (Figure S7). For example, the most predictive taxon belonged to the *Campylobacter* genus based on decrease in "Mean Decrease Accuracy" coefficient (Figure 5A), which was phylogenetically affiliated with *Campylobacter hominis* with 99% similarity (Figure S7). In general, the relative abundances of the CRC stage-discriminatory ASVs were varied significantly (11 out of 15 ASVs) among the five groups (Figure 5B). Importantly, using the profiles of the 15 CRC stage-discriminatory ASVs as dependent variable, the diagnosis model contributed an overall 87.4% accuracy (Table 1). In BP, 44 samples (accounting for 97.8% of the controls) were correctly diagnosed as BP individuals. Among the CRC patients, 44 individuals (80.0% of patients) were predicted accurately as corresponding CRC stage (Figure 5C). Of note, no CRC patients were falsely diagnosed as BPs by using the 15 CRC stage-discriminatory ASVs, namely, no false-negative (Table 1). It is worthy to emphasize that the diagnosis model could accurately diagnose the initiation of CRC (T1 stage, 8 out of 9 cases, 88.9%). In addition, the falsely diagnosed samples were not related to host gender (Figure S8A), though the numbers of male patients were consistently higher than female individuals along the fours CRC stages (Table S1). Also, the diagnosis model were unbiased by host age (Figure S8B), while ages were markedly varied among patients.

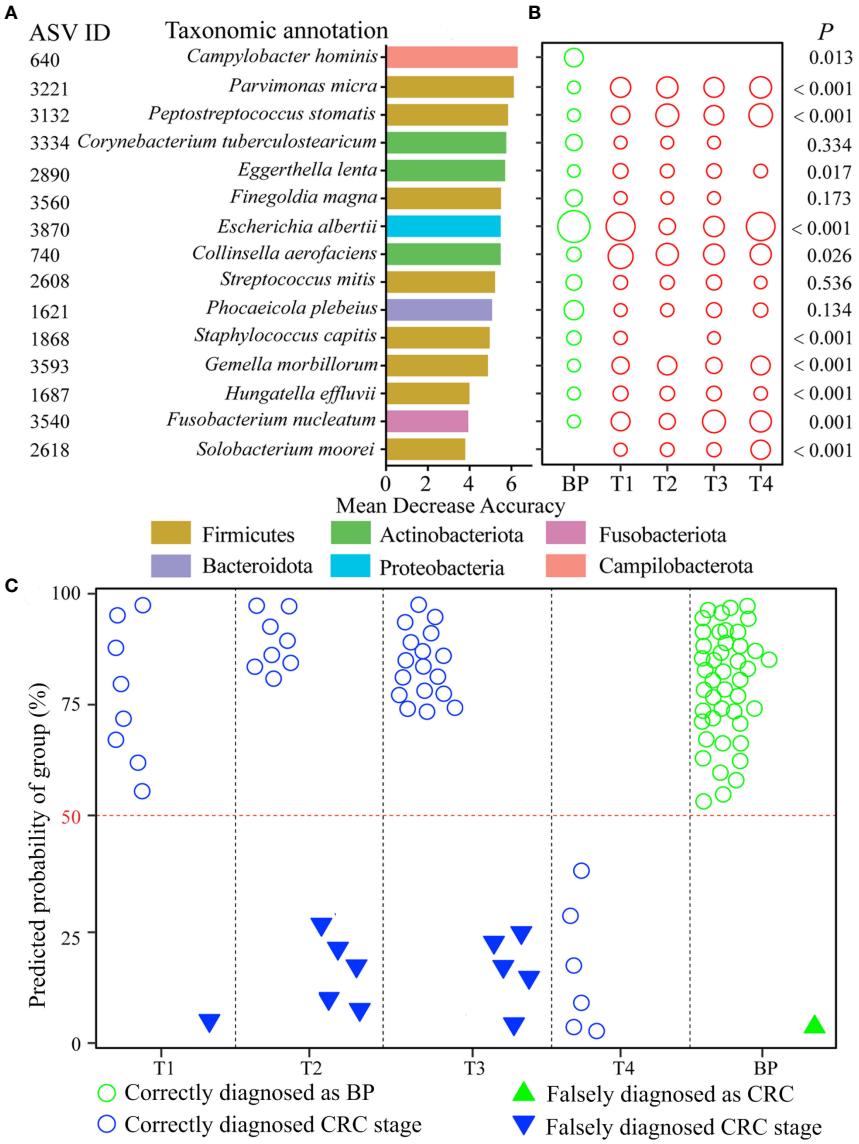

FIGURE 5
CRC diagnosis model using the CRC stage-discriminatory taxa. The top 15 CRC stage-discriminatory ASVs are ranked in descending order of importance to the accuracy of the diagnose model **(A)**. The diameters of the circles are proportional to the relative abundances of the 15 biomarkers **(B)**. The diagnosed CRC stages using profiles of the 15 CRC stage-discriminatory ASVs. The consistency between observed and diagnosed stage was termed a correct diagnosis with a cutoff of 50% **(C)**. Refer to Figure 1 for abbreviations.

We also screened the CRC stage-discriminatory taxa without exclusion of the age-discriminatory taxa. We found that the model performance was unsatisfactory, with a marked decrease in diagnosis accuracy (71.6% vs. 87.4%, Table S5). In this regard, our optimization procedure was imperative and valuable, which substantially improved the accuracy of diagnosis model.

Discussion

Currently there has been increased interest in the adenoma-specific markers that detect early CRC, partly due to the recognition that the bacterial communities are distinct along the control-adenoma-carcinoma sequence (Mizutani et al., 2020; Wu et al., 2021). By contrast, few studies have explored the bacterial communities over CRC progression, resulting in the uncertainty whether biomarkers could diagnose the four stages of CRC, rather than case-control. Additionally, as microbes are implicated in colorectal carcinogenesis, development and treatment outcome (Jin et al., 2019; Montalban-Arques and Scharl, 2019; Mizutani et al., 2020; Ting et al., 2022), understanding the dynamics in CRC development is a necessary initial step to developing a more complete understanding of both the ecology and etiology.

Increasingly evidence has shown that faecal bacterial communities are distinguishable from individuals with CRC or adenomas to controls (Wang et al., 2012; Flemer et al., 2018; Wu et al., 2021). In contrast, there is still lack of data about the bacterial profiles over CRC progression, especially these at cancerous tissue. Considering the

functional importance of microbes in promoting CRC tumorigenesis (Cheng et al., 2020; Mizutani et al., 2020), and given that faecal microbiota only partially reflects mucosal counterpart in CRC (Flemer et al., 2017), this ignorance could bias the identification of CRC associated taxa. Trying to comprehensively collect the tumors comprising the four CRC stages, our results depicted that there were distinct segregations in bacterial community structure between polyps and tumors, and along the four CRC stages (Figure 1, Table S2). These patterns suggest that, despite the extensive physical variances among individuals (e.g., age, gender, and CEA level, Table S1 and Figure S1), each stage exerts sufficiently unique conditions to assemble communities that are consistent in structure according to BP or CRC stage.

As the stability of the microbiota could be affected by host disease (Xiong, 2018; Aho et al., 2019), we compared with the stability of bacterial communities over CRC progression. CRC initiation (T1 stage) sharply disrupted the stability of bacterial community compared with BPs (Figure 2). There are several possible explanations for this pattern. First, the bloom of pathogenic taxa outcompete resident commensals, as supported by increased abundances of known pathogens in T1 tumors, such as *Fusobacterium nucleatum*, *Bacteroides fragilis*, and *Escherichia fergusonii* (Figure S6). Second, pathogen invasion could attenuate host filtering on the colonization of external taxa, leading to a dominance of stochastic processes (e.g., random birth and death) governing community assembly (Mallon et al., 2015; Adair and Douglas, 2017). Accordingly, there was a heterogeneous bacterial community (higher AVD value) in T1 tumors (Figure 2). However, as disease severity increased, inflammatory microenvironment could exert the dominant role of homogeneous selection, resulting in a convergent, disease-like microbial community (Subramanian et al., 2014; Xiong et al., 2017). Consistent with this assertion, the AVD values linearly decreased along CRC progression (Figure 2), whereas phylogenetic diversity exhibited an opposing trend (Figure S4B). Given that microbes are implicated in the outcome of CRC therapy (Montalban-Arques and Scharl, 2019; Mizutani et al., 2020; Ting et al., 2022), the convergence in tumor bacterial community may partially explain why advanced CRC is difficult to be curative.

It has proposed a stratification of individuals into three distinct enterotypes (Arumugam et al., 2011), while others supported a concept of stratification based on bacterial abundance gradients (Flemer et al., 2017). We detected a stratification of individuals between distinct enterotypes and abundance gradients, as supported by distinct structures of bacterial communities when integrated the abundance of ASVs, e.g., abundance gradients (Figure 1, Table S2). However, we also found rapid replacement of ASVs over CRC progression, because there were linearly deceased numbers and low proportions of overlapped ASVs between BP and advanced CRC, as well as shuffling between adjacent two stages, e.g., distinct enterotypes (Figure 3A). This is apparent in the SPEC-OCCU plots (Figure 3B), where the majority of the ASVs exhibited low occupancy, indicating that few of them are consistently detected among individuals. In accordance, the tumor communities harbored significantly lower homogeneity among individuals in CRC T1, T2 and T3 stages, compared with these in BP (Figure 2). These findings indicate that the tumor environments are less selective for a stable core community, resulting in heterogeneity in bacterial communities among patients. Considering that microbes determine the therapeutic efficiency of CRC, the heterogeneity in bacterial communities may guide the design of personalized medicine (Kong et al., 2019; Shi et al., 2020; Ting et al., 2022). Although this study was not designed to evaluate treatment outcome, clearly, there is a pressing need for longitudinal study exploring the associations between the tumor microbiota and treatment response in CRC patients.

Given that the gut microbiome is an important etiological element in the initiation and progression of CRC (Montalban-Arques and Scharl, 2019; Cheng et al., 2020; Mizutani et al., 2020; Guo et al., 2022), sufficient and accurate identification of CRC associated taxa could facilitate the targets for diagnosis and therapy. We identified the well known promoters in colorectal carcinogenesis, *Fusobacterium nucleatum* (Rubinstein et al., 2013), based on its sharply increased abundance in T1 stage tumors (Figure 5A). Similarly, three well known CRC-associated taxa, *F. nucleatum*, *Bacteroides fragilis*, and *Campylobacter concisus* (Guo et al., 2022), were enriched in the advanced T4 stage tumors (Figure 5B). Thus, there is a lack of consistency in the bacterial taxa associated with CRC progression, in accordance with studies conducted previously (Feng et al., 2015; Mizutani et al., 2020). This pattern supports the so-called "driver-passenger" model, which proposes that different bacteria sequentially implicate in CRC tumor initiation and progression. The "driver" bacteria are replaced by "passenger" bacteria that are better adapted to the conditions in and around cancerous cells (Tjalsma et al., 2012; Tilg et al., 2018). Of note, other bloomed ASVs have not been reported to be associated with CRC, instead, a few ASVs could be potential probiotics, such as *Corynebacterium vitaeruminis* (Colombo et al., 2017), and *Streptococcus alactolyticus* (Zhang et al., 2021). In this regard, it is cautious to identify CRC-associated taxa by increased abundance in the tumors. Going forward, we identified 13 "driver" bacterial ASVs by the NetShift model, moving beyond differential abundance, of which 6 ASVs were significantly enriched and the most abundant in T1 tumors (Figure 4). Among the 6 ASVs, ASV1839 *Clostridium saudiense* is recently identified as an opportunistic pathogen with the potential to cause hepatocellular carcinoma, which translocates from the gastrointestinal system to biliary system (Yoon et al., 2022). Unexpectedly, ASV1414 *Bifidobacterium stercoris* and ASV2467 *Parabacteroides distasonis* were significantly enriched in T1 tumors (Figure 4B). *B. stercoris* is able to produce acetic acid and lactic acid, and promote antitumor immunity (Sivan et al., 2015), while the later could attenuate toll-like receptor 4 signaling and thus blocks colon tumor formation (Koh et al., 2018). One possible explanation for this counterintuitive pattern is that the hosts release specific chemicals that favor the recruitment of beneficial microbes or of antagonists able to suppress the growth of pathogens, according to the "cry-for-help" hypothesis (Rolfe et al., 2019), although further study is needed to validate its applicability in human diseases. Conversely, the butyrate-producing AVS1014 *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium (Miquel et al., 2013), thus its reduction is expected to be associated with CRC. Consistent with the above, the ratio of *Fusobacterium nucleatum* to *Faecalibacterium prausnitzii* has been identified as a valuable biomarker for screening

early CRC (Guo et al., 2018). The beneficial ASVs decreased rapidly along advanced CRC (Figures 4, S5), which raises the intriguing possibility that tumors could recruit beneficial taxa to antagonize CRC-associated pathogens at the initial stage. Collectively, the taxa that trigger protumorigenic environments could be attributed to the enrichment of pathogenic strains, and also the depletion of probiotic species.

The establishment of gut microbiota—host health relationship and modeling algorithms facilitates the identification of bio-indicators diagnosing disease severity, which is a key goal of microbiome research. Multiple studies have extensively shown human age to be a strong covariate of the gut microbiota (Falony et al., 2016; Greenhalgh et al., 2016), thus host age may overshadow changes in commensals associated with CRC. For this reason, we teased apart the effect of ageing-related and CRC-related changes in the bacterial community. To achieve this, we identified age-discriminatory ASVs that featured the age of BP controls. The top nine age-discriminatory ASVs contributed a high consistency (r = 0.924, $P < 0.001$) between diagnosed and chronologic ages (Figure S6). After removal of the nine age-discriminatory ASVs, we further identified CRC stage-discriminatory taxa for diagnosing BPs and the four CRC stages, with an overall 87.4% accuracy. It is worthy to emphasize that this optimization substantially improves the performance of our diagnosis model compared with the model neglecting age effect (71.6% accuracy, Table S5). As a result, the diagnosis accuracy was not affected by host age (Figure S8). Our diagnosis model could add the designation of CRC stage-dependent clinical trials, especially the patients with poor pathological feature and un-reproducibility between two doctors (Quirke et al., 2007). Similarly and more importantly, no CRC patients were falsely diagnosed as BPs (Table 1), illustrating that there was no false-negative in diagnosing CRC when ignored the CRC stage. In this regard, our diagnosis model is reliable to warn the cancerization of CRC with 100% accuracy, which in turn alerts patients to further treatment. One might argue that the low sampling sizes at the T1 (N = 9) and T4 (N = 6) stages (Table S1), which could weaken statistics power. Here we collected unique and comprehensive tumors (four CRC stages), instead of fecal samples. We want to point out the CRC tend to be inspected at later stages, while T4 patients generally refuse surgery. Consequently, we collected limited tumors from T1 and T4 patients. However, the highly diagnosed accuracy of patients at the two stages indicated that our diagnosis model was not strongly affected by the sampling sizes (Table 1). Despite high heterogeneity of bacterial communities among patients at each CRC stage (Figure 1), we are surprised that, given the high number of ASVs (N = 3,601) identified across the 95 individuals, only 15 ASVs (accounting for 0.4%) accurately discriminated human BPs and CRC stages. This small subset affords unique experimental opportunities that could be prioritized based on their feature importances to the diagnosis model (Figure 5). For example, it has been shown that levels of *Parvimonas micra* in faecal samples from CRC patients are significantly higher compared to healthy individuals (Löwenmark et al., 2020), as also observed in the present study (Figures 5A, B). Thus, *P. micra* is proposed as non-invasive biomarkers for CRC (Löwenmark et al., 2020). Similarly, a metagenomic analysis of faecal microbiome reveals that *P. micra* and *Solobacterium moreii* are positively associated with CRC (Yu et al., 2017). Besides confirming known associations of ASV3221 *P. micra*, ASV2618 *S. moreii*, ASV3540 *Fusobacterium nucleatum* and ASV3132 *Peptostreptococcus stomatis* with CRC, we found significant associations with several species, including ASV640 *Campylobacter hominis*, ASV740 *Collinsella aerofaciens* among others (Figure 5). Clearly, the pressing steps would be to isolate representatives of these CRC stage-discriminatory ASVs and to explore their roles in CRC carcinogenesis.

Conclusion

This is the few attempts to explore CRC-associated taxa and underlying mechanisms in CRC progression from an ecological perspective. Tumor microenvironments are less selective for stable core community, leading to heterogeneity in bacterial communities among patients, as supported by higher AVD, lower occupancy and specificity. However, tumors could recruit beneficial taxa to antagonize CRC-associated pathogens at the initial stage, in accordance with the "cry-for-help" pattern that has not been recognized before. Based on the "driver-passenger" distribution of CRC-associated ASVs, CRC initiation could be attributed to the enrichment of pathogenic strains, and also the depletion of probiotic species. By distinguishing age- from CRC stage-associated taxa, the diagnosis model for accurately diagnoses (an overall 87.4% accuracy) BPs and the four CRC stages, especially without false-negative for CRC patients. The diagnosis model could add CRC stage stratification, especially the patients with poor pathological feature and un-reproducibility between doctors. Additional works will be imperative to eventually validate them in much larger cohorts before clinical deployment.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committees of Hwa Mei Hospital, University of Chinese Academy of Science (No. PJ-NBEY-KY-2020-042-01). The patients/participants provided their written informed consent to participate in this study.

Author contributions

XD, JL and JX conceived and designed the research. PC, JL, and HS conducted the experiments. JX contributed analytical tools. PC, JL, and HS analyzed the data. PC wrote the manuscript with help from JX, JL and XD. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author JL was employed by company Zhejiang KinGene Biotechnology Co., Ltd.

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.2023.1123544/full#supplementary-material

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Alteration of the gut microbiome and correlated metabolism in a rat model of long-term depression

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Objective: This study aims to investigate the composition and function of the gut microbiome in long-term depression using an 8-week chronic unpredictable mild stress (CUMS) rat model.

Materials and methods: Animals were sacrificed after either 4 weeks or 8 weeks under CUMS to mimic long-term depression in humans. The gut microbiome was analyzed to identify potential depression-related gut microbes, and the fecal metabolome was analyzed to detect their functional metabolites. The correlations between altered gut microbes and metabolites in the long-term depression rats were explored. The crucial metabolic pathways related to long-term depression were uncovered through enrichment analysis based on these gut microbes and metabolites.

Results: The microbial composition of long-term depression (8-week CUMS) showed decreased species richness indices and different profiles compared with the control group and the 4-week CUMS group, characterized by disturbance of Alistipes indistinctus, Bacteroides ovatus, and Alistipes senegalensis at the species level. Additionally, long-term depression was associated with disturbances in fecal metabolomics. D-pinitol was the only increased metabolite in the 8-week CUMS group among the top 10 differential metabolites, while the top 3 decreased metabolites in the long-term depression rats included indoxyl sulfate, trimethylaminen-oxide, and 3 alpha,7 alpha-dihydroxy-12-oxocholanoic acid. The disordered fecal metabolomics in the long-term depression rats mainly involved the biosynthesis of pantothenate, CoA, valine, leucine and isoleucine.

Conclusion: Our findings suggest that the gut microbiome may participate in the long-term development of depression, and the mechanism may be related to the regulation of gut metabolism.

KEYWORDS

long-term depression, CUMS, gut microbiome, metabolome, rat

Introduction

Depressive disorder, which has a detrimental impact on health systems and psychological well-being, remains among the leading causes of burden, with prevalence estimates and disability weights that are comparatively higher than those of many other diseases (Zhao et al., 2021; Ferrari et al., 2022). The gut microbiome is the major microbial community that settles in the human body and affects the nutrition, metabolism and immune function of the host (Hou et al., 2022). Disturbances in the gut microbial ecosystem have been linked with psychological conditions, including depression (Ortega et al., 2022).

Depression is inextricably linked to changes in gut flora richness and diversity. In healthy people, Bacteroidetes and Firmicutes are the most dominant bacteria, while the abundance of other phyla is relatively small, and the dynamic balance between different bacterial groups is critical to maintain normal intestinal function (Yu et al., 2017; Cheng et al., 2020). Compared with normal people, the richness and diversity of intestinal microorganisms in depressed patients decreases significantly, usually manifested as a decrease in Firmicutes, an increased proportion of Bacteroidetes, Proteobacteria and Actinobacteria at the phylum level, a decrease in Trichospiraceae and Ruminococcaceae at the family level, and a decrease in Faecium, Ruminococcus, Lactobacillus and Bifidobacterium at the genus level (Safadi et al., 2022). Although some studies may report contradictory flora changes due to differences in experimental conditions or subjects, the general consensus is that the flora composition of depressed patients is significantly different from that of healthy patients.

Similarly, changes in the gut microbiota have been reported in animal models of depression. The intestinal microbiota of depressed mice induced by chronic mild unforeseeable stimulation (CUMS) showed a reduced relative abundance of Corynebacterium, Colophilus, Lactobacillus and Faeccoccus (Sudo et al., 2004; Liang et al., 2015). Learned helplessness animal models of depression reduce the relative abundance of bacteria such as Lactobacillus and Clostridium spp. (Takajo et al., 2019). Li (Jianguo et al., 2019) investigated the potential correlation between depression-like symptoms and altered fecal metabolites in CUMS rats and found that altered intestinal microbiota may affect depression-like symptoms in CUMS rats through changes in intestinal metabolites. Transplantation of fecal microbiota from depressed patients into germ-free (GF) rodents resulted in altered depressive-like behavior compared with that in specific pathogen-free (SPF) rats (Yang et al., 2020). In addition, there were similar findings in the intestines of rodents subjected to the weaning isolation model, bilateral olfactory bulb resection model and social failure model. These mice showed typical depression-like behaviors, such as social avoidance and anhedonia, accompanied by intestinal flora changes [i.e., a decreased level of Firmicutes and an increased ratio of Bacteroidetes to Firmicutes (O'Mahony et al., 2009; Bharwani et al., 2017)]. These studies also provide evidence for the changes in the gut microbiota during the development of depression when they show the difference in the gut microbiota in depressed individuals and healthy individuals.

The gut microbiota can influence brain function and behavior through the microbiota-gut-brain axis (Zheng et al., 2021). However, depressive disorders are always long-lasting, and the gut microbial ecosystem is altered as the disease progresses. The specific role of microbiota metabolites in the long-term pathogenesis of depression has not been fully explored. Therefore, this study aims to explore the changes in intestinal microecology and microbial metabolites during the development of long-term depression.

In this study, we sought to investigate the composition and function of the gut microbiome in the long-term depression CUMS rat model. Animals were sacrificed after either 4 weeks or 8 weeks under CUMS to mimic long-term depression in humans. Based on 16S rRNA sequencing and metabolomics, we characterized the gut microbiota of depression and long-term depression, revealed the correlation between gut microbes and metabolites, and provided insights into the role of microbiota in the long-term development of depression.

Methods

Animal experiments

Animals

A total of 40 Sprague–Dawley (SD) male rats weighing 200 ± 10 g (6 weeks of age) were provided by Beijing Victory Lihua Experimental Animal Technology Co., Ltd. (experimental animal production license: SCXK (Beijing) 2016-0006). All animal procedures were approved by the Ethics Committee of the Institute of Basic Theory for Traditional Chinese Medicine at the China Academy of Chinese Medical Sciences (Permit No. IBTCMCACMS21-2110-05) and complied with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The rats were housed in the animal room of the Institute of Basic Theory of Traditional Chinese Medicine of the Chinese Academy of Chinese Medical Sciences under relatively steady conditions (12 h light-dark cycle at a humidity of 55 ± 5% and constant temperature of 20-25°C) and received food and water ad libitum. All rats were routinely reared for 2 weeks and were then randomly divided into 2 groups: the CUMS group (20) and the normal control group (20).

Chronic unpredictable mild stress

Three of the following stressors were arranged in a random order daily: food and water deprivation for 24 hrs, 30° cage tilt for 8 hrs or overnight, swimming in 4°C water for 5 min, 45°C heat for 5 min, restraint stress for 2 hrs, tail pinch for 1 min, and soiled cage for 8 hrs or overnight. The CUMS regimen started at week 2 (on 8-week-old rats) and lasted for 8 weeks. After 4 weeks (on 12-week-old rats) of CUMS exposure, 10 rats were sacrificed in each group.

Behavioral assays

Open field test: The test device consisted of two parts: the open field reaction chamber and the recording analysis system. The rat

open field reaction box was 35 cm in height, with a square bottom evenly divided into 25 small squares (4 cm * 4 cm). A digital camera was set up 2 m directly above the open field, which covered the entire open field. During the experiment, the number of rat traversals and the total distance of activity were recorded for 3 min and calculated by the Small Animal Behavior Recording Analysis System (Smart1.0, Panlab). Rats were given an open field test every four weeks.

Sucrose preference test: Rats were deprived of food and water for 24 hrs before the test. The rats were individually housed and habituated to a 1% sucrose solution and water for 24 h and were then exposed to two bottles for 1 h, one containing 1% sucrose solution and the other tap water. Total consumption of the sucrose solution and tap water was measured, and the sucrose preference ratio was then calculated accordingly.

16S rRNA gene sequencing and data processing

DNA extraction and PCR amplification of stool samples

The DNA Rapid Extraction Kit (Beijing Tiangen Biochemical Technology Co., Ltd.) was used to extract bacterial DNA after thawing stool samples. DNA concentration and quality were determined using an ultraviolet spectrophotometer and agarose gel electrophoresis.

The 16S rDNA V3-V4 variable region was amplified with primers synthesized by Beijing Ovichan Gene Technology Co., Ltd. (sequence: 5'-TCCTACGGGAGGCAGCAGT-3'; 5'-GGACTACCAGGGTATCTAATCCTGTT-3'). The PCR products were collected using the AxyPrep DNA gel extraction kit (AXYGEN).

16S rRNA gene sequencing

16S rRNA sequencing was completed by Beijing Ovison Gene Technology Co., Ltd. The SMRTbellTM Template Prep Kit (PacBio) was used for the generation of the sequencing libraries, with the library quality assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and FEMTO Pulse system. The PacBio Sequel platform was used for sequencing.

Data processing

Raw sequences were initially processed through the PacBio SMRT portal. The raw fastq files were quality-filtered using Trimmomatic and were then merged by FLASH. Sequence analysis was performed using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/). The SSUrRNA Database of the Silva Database (https://www.arb-silva.de/) was used based on the Mothur algorithm to annotate taxonomic information. Phylogenetic relationship construction was conducted using USCLE software (Version 3.8.31, http://www.drive5.com/muscle/).

Alpha diversity was applied in analyzing the complexity of species diversity for a sample through indices, including Observed-species, Chao1, Shannon, Simpson, ACE, and Good-coverage. All these indices in our samples were calculated with QIIME (Version 1.9.1) and were then displayed with R software (Version 2.15.3). Beta diversity was calculated by QIIME software (Version 1.9.1). Cluster analysis was preceded by principal component analysis (PCA) using the FactoMineR package and ggplot2 package in R software (Version 2.15.3).

Ultrahigh-performance liquid chromatography-mass spectrometry and data processing

UHPLC

Ultrahhigh-performance liquid chromatography (UHPLC) chromatographic separation was performed using an Acquity UHPLC system (Acquity LC, Waters) installed with a Waters UPLC column (ACQUITY UPLC BEH Amide 1.8 μm, 2.1×100 mm, Waters, Milford, MA). Mobile phase A included 25 mM ammonium acetate and 25 mM ammonium hydroxide in water, while mobile phase B was 100% ACN. The gradient program was as follows: 95% B, 0.5 min; 95%-65% B, 0.5-7 min; 65%-40% B, 7–8 min; 40% B, 9 min; 40% – 95% B, 9–9.1 min; and 95% B, 12 min. The injection volume was 2 μL, and the flow rate was 0.5 mL/min.

MS/MS spectra

MS/MS spectra were acquired using the QE HFX mass spectrometer in information-dependent acquisition (IDA) mode controlled by Xcalibur acquisition software (Thermo). The ESI source conditions were set as follows: 30 Arb for sheath gas flow rate, 10 Arb for Aux gas flow rate, 60000 for full MS resolution, 7500 for MS/MS resolution, capillary temperature at 350 °C, collision energy as 10/30/60 in NCE mode, and 3.6 kV or -3.2 kV for spray voltage.

Data processing

The raw data were converted to the mzXML format using ProteoWizard and were processed for peak detection, extraction, alignment, and then integration. Accurate mass matching (<25 ppm) and secondary spectrum matching were conducted for metabolite structure identification. An internal standard normalization method was employed. The R package MetaboAnalystR was used for principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) of the three-dimensional data (peak number, sample name, and normalized peak area). To check the robustness and predictive ability of the OPLS-DA model, 200 permutations were further conducted. The metabolites that met the following criteria were considered significantly altered metabolites: first principal component of variable importance in the projection (VIP) >1 and $p<0.05$ (Student's t-test) and fold change >1.5 or < 0.67. Online databases, including KEGG (http://www.kegg.jp) and MetaboAnalyst (http://www.metaboanalyst.ca/), were utilized for pathway enrichment of the metabolites.

Results

Behavioral characteristics of long-term depression rats

The experimental scheme is shown in Figure 1A. After 4 weeks of CUMS exposure, no significant difference in weight (CUMS: 486 ± 22.32 [mean ± SD]; control: 495 ± 10.97; p = 0.32) , significant decreases in sucrose consumption in the depressed rats (CUMS: 62.93% ± 14.29% [mean ± SEM%]; control: 93.62% ± 3.21%; p = 0.000), total distance traveled (CUMS: 47.50 ± 3.47[mean ± SD]; control: 168.75 ± 7.00; p =0.000) and number of grid crossings in the open field test(OFT)(CUMS: 15.45 ± 4.27 [mean ± SD]; control: 73.80 ± 7.69; p = 0.000) were observed in the CUMS group (Figures 1B–E). Compared with week 4, there was still no significant difference in weight (CUMS: 513 ± 30.24 [mean ± SD]; control: 540 ± 8.21; p = 0.13), but rats showed a further decrease in sucrose preference (CUMS: 31.82% ± 10.91% [mean ± SEM%]; control: 96.18 ± 2.75; p = 0.000), total distance traveled (CUMS: 23.80 ± 3.05 [mean ± SD]; control: 169.10 ± 6.17; p = 0.000), and number of grid crossings (CUMS: 7.40 ± 2.50 [mean ± SD]; control: 75.40 ± 5.48;p = 0.000) in the 8th week of CUMS (Figures 1B–E; n = 10 per group, P<0.01). These results suggested that our CUMS modeling was successful and that prolonged CUMS modeling led to more severe depression.

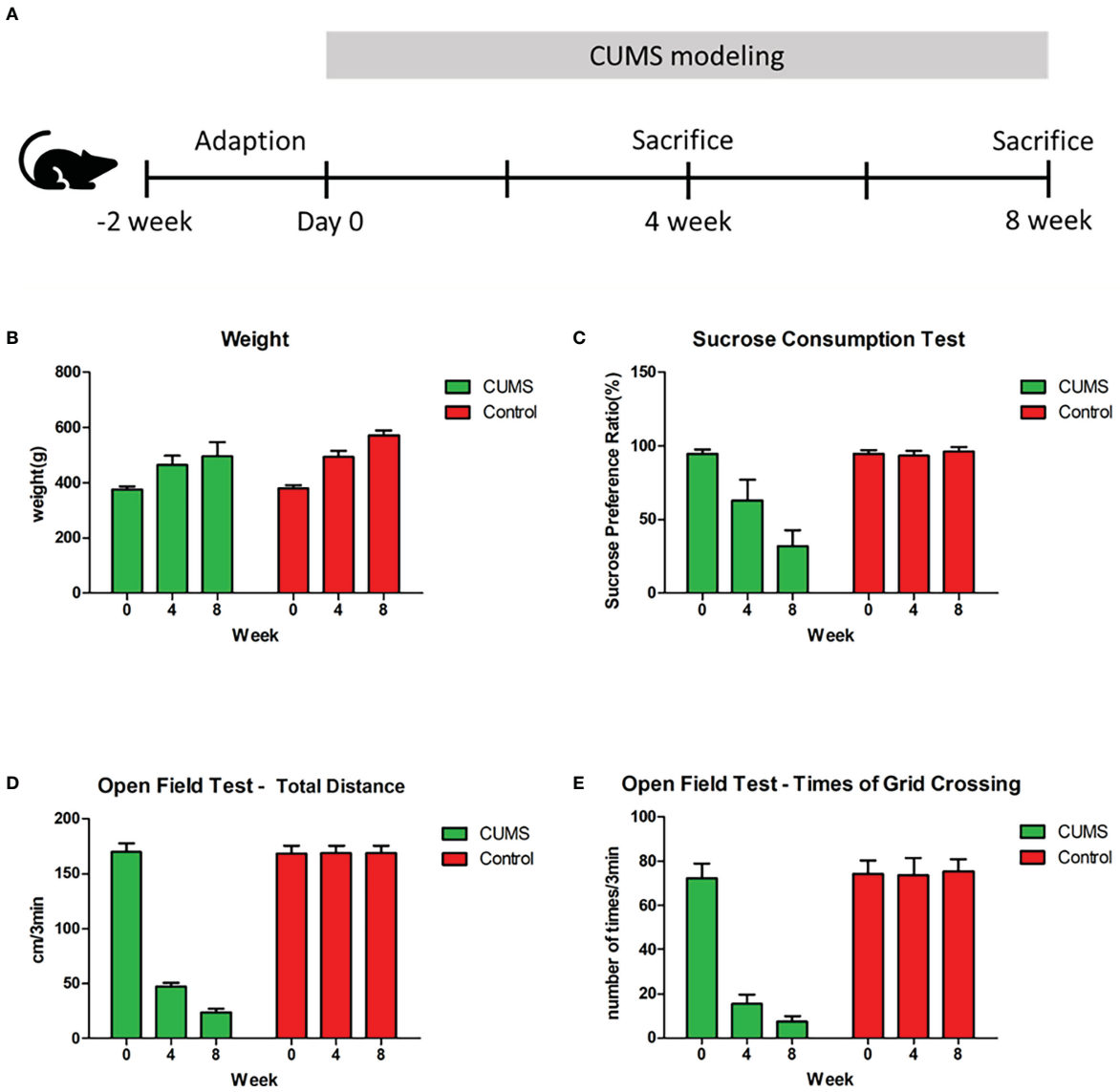

FIGURE 1
Experimental Schema and behavioral results. **(A)** Schedule of experimental procedures. **(B)** Changes in weight among control, rats under 4-week CUMS, and rats under 8-week CUMS. **(C)** Changes in sucrose preference among control, rats under 4-week CUMS, and rats under 8-week CUMS. **(D)** Changes in total distance in the open field test among control, rats under 4-week CUMS, and rats under 8-week CUMS. **(E)** Changes in times of grid crossing in the open field test among control, rats under 4-week CUMS, and rats under 8-week CUMS.

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Decreased species richness indices in the depression rats

The fecal microbial composition of control and depression rats was compared using 16S rRNA gene sequencing. Clean reads were clustered into 6,377 OTUs at 97% sequence similarity. The alpha-diversity values, including species diversity indices (Chao, observed species, PD whole tree, and Shannon), were lower in the CUMS groups than in the control group (Figure 2A). OPLS-DA revealed that the fecal microbial composition of the depression rats under

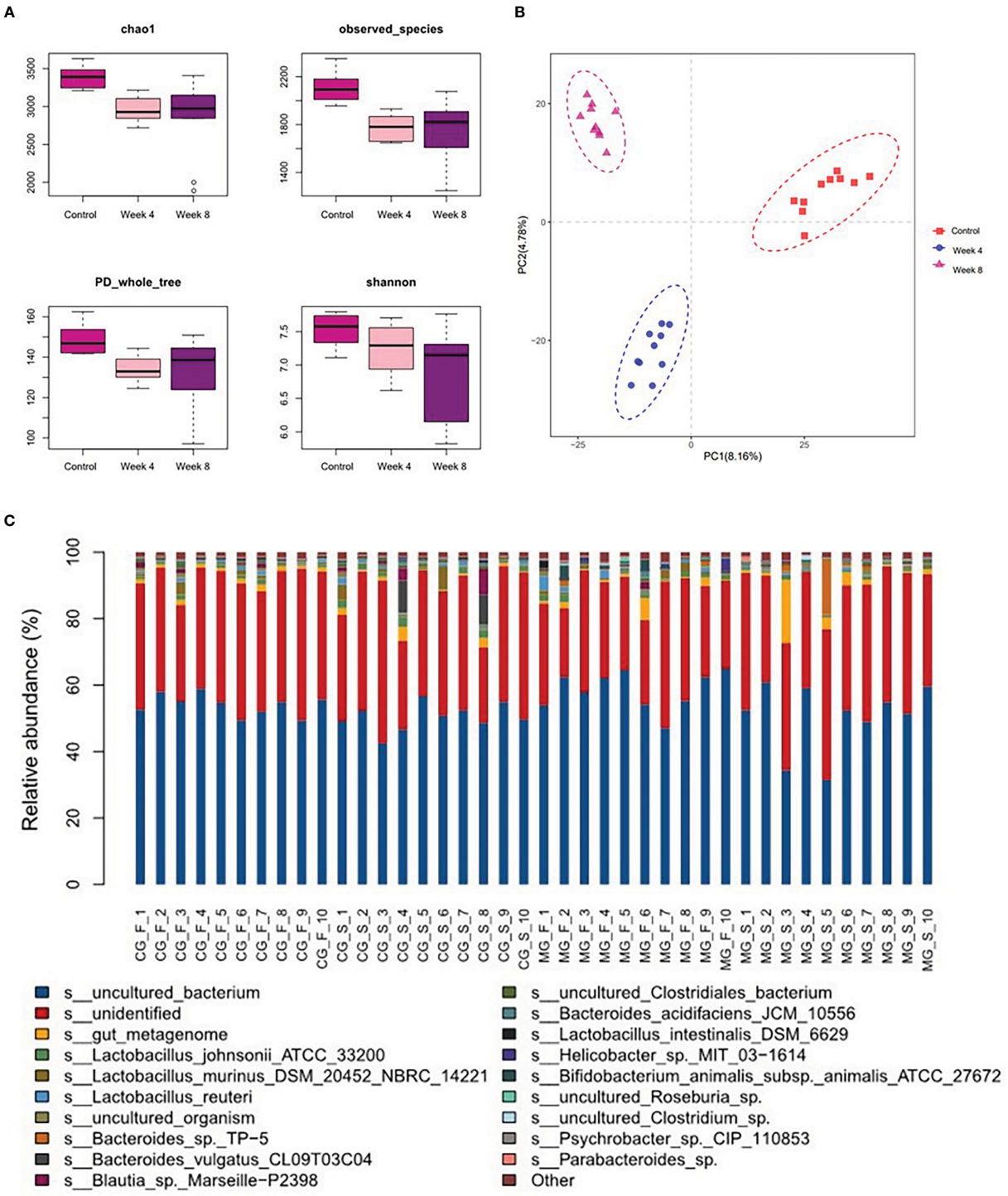

FIGURE 2
Characteristics of the gut microbiota in the depression rat model. **(A)** α-phylogenetic diversity analysis showing that CUMS rats were characterized by lower microbial richness relative to controls. **(B)** Orthogonal partial least squares discrimination analysis (OPLS-DA) showed that the gut microbial composition of CUMS rats was significantly different from that of the control group. **(C)** Relative abundance of gut microbes at the species level across samples (CG- normal control group; MG-CUMS group).

CUMS was significantly different from that in the control group (Figure 2B). The relative abundance of fecal microbes across samples at the species level is shown in Figure 2C.

Alterations in microbial composition in long-term depression rats

To further explore the characteristics of long-term depression, a comparison analysis between 4-week CUMS and 8-week CUMS was conducted. As shown in Figure 3A, rats in the 8-week CUMS group showed similar alpha-diversity indices. However, OPLS-DA showed that the gut microbial composition of 8-week CUMS rats was significantly different from that of 4-week CUMS rats in Figure 3B. The relative abundance of microbes at the species level is shown in Figure 3C. The top 20 differential microbes between rats under 4 weeks of CUMS and 8 weeks of CUMS included *Lactobacillus johnsonii*, *Lactobacillus murinus, Lactobacillus reuteri, Bifidobacterium animalis, Helicobacter, Bacteroides, Roseburia, Blautia Marseille, Psychrobacter, Lachnospiraceae bacterium, Lactobacillus intestinalis, Clostridiales bacterium, Clostridium, Allobaculum*, and *Rodentibacter ratti* (Figure 3D). The cooccurrence of the microbes altered in the depression groups is shown in Figure 3E at the genus and phylum levels. More co-occurrence relationships were detected in the 4-week CUMS group than in the 8-week CUMS group.

To identify the microbial characteristics distinguishing 4-week CUMS from 8-week CUMS, LEfSe analysis revealed the differential OTUs between the two groups (Figures 4A, B). The long-term depression group was characterized by disturbances in Alistipes indistinctus, Bacteroides ovatus, and Alistipes senegalensis at the species level (Figure 4B).

Characteristics of fecal metabolism in depression rats

The fecal metabolome is always considered the functional readout of the gut microbiome since it is known to be involved in metabolic regulation of the host. Thus, a UHPLC-MS-based metabolomic method was used to investigate the characteristics of fecal metabolism in depression rats. The differential metabolites were identified using "fold change > 2.00 and P value < 0.05" as a cutoff. The fecal metabolic phenotype of depression was distinguishable from that of the control group. Compared with the control group, there were 508 differential fecal features in the depression rats, of which 247 features increased and 261 features decreased. The fecal metabolic phenotype of depression was significantly different from that of the control (Figure 5A). The top 3 increased metabolites included 1,2-dimethylimidazole, D-pinitol, and dimethyl sulfone, while the top 3 decreased metabolites were resveratrol 4-o-glucuronide, succinate, and histamine (Figure 5B).

There were 545 differential fecal features in rats in the 8-week CUMS group compared with those in the 4-week CUMS group, of which 154 features increased and 391 features decreased (Figure 5C; n = 10 per group). D-pinitol was the only increased metabolite in the 8-week CUMS group among the top 10 differential metabolites, while the top 3 decreased metabolites in the long-term depression rats included indoxyl sulfate, trimethylamine n-oxide, and 3-alpha,7-alpha-dihydroxy-12-oxocholanoic acid (Figure 5D).

These differential metabolites were further used for KEGG pathway enrichment analysis. Among the top 20 pathways revealed, pantothenate and CoA biosynthesis was most significantly enriched (Figure 5E). Specifically, these differentially expressed metabolites were related to pantothenate and CoA biosynthesis; valine, leucine and isoleucine biosynthesis; glycine, serine and threonine metabolism; beta −alanine metabolism; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; riboflavin metabolism; thiamine metabolism; pyrimidine metabolism; the sulfur relay system; lysine degradation; biosynthesis of amino acids; glycerophospholipid metabolism; alanine, aspartate and glutamate metabolism; ubiquinone and other terpenoid−quinone biosynthesis; arginine and proline metabolism; alpha−linolenic acid metabolism; tyrosine metabolism; sulfur metabolism; and fatty acid degradation.

Correlations between gut microbes and fecal metabolites

Correlation analysis of the altered gut microbes and fecal metabolome was performed (Figure 6A). Nine of the gut microbes had a significant correlation with altered metabolites, including *Barnesiella intestinihominis, Parabacteroides, Bacteroides uniformis, Helicobacter, Ruminococcaceae bacteriumand, Adlercreutzia equolifaciens*, and *Bacteroides vulgatus*. Among the significantly correlated metabolites, we identified the three most representative metabolites that showed high diagnostic potential for depression with AUCs ≥ 0.8 in the diagnostic power test (Figure 6B). Our findings demonstrated that the depression rats were characterized by both an altered gut microbiome and a disturbed fecal metabolome, and potential regulatory relationships between the microbiota and metabolites may exist.

Discussion

In this study, we identified the different gut microbiome and fecal metabonomics among control, 4-week CUMS and 8-week CUMS rats. We found distinguishable microbial phenotypes in the depression rat groups. Compared with the control group, the CUMS rats showed decreased species richness indices in microbial composition (Figure 2A). The diversity of the human gut microbiota has been considered as one of the forms of evidence of health for decades (Eckburg et al., 2005). Normal intestinal flora diversity and flora metabolites are closely related to human health (Maier et al., 2018), and decreased species richness may indicate poor health conditions and disordered physiological processes. Bacteroides fragilis is a gram-negative brevibacterium with capsules in the intestine. It can activate and induce IL-10 to produce Tregs through its cell component capsular polysaccharide A and play an anti-inflammatory role. A decrease in the abundance of such bacteria reduces the immunity of mammals (Maynard et al., 2012). Normal

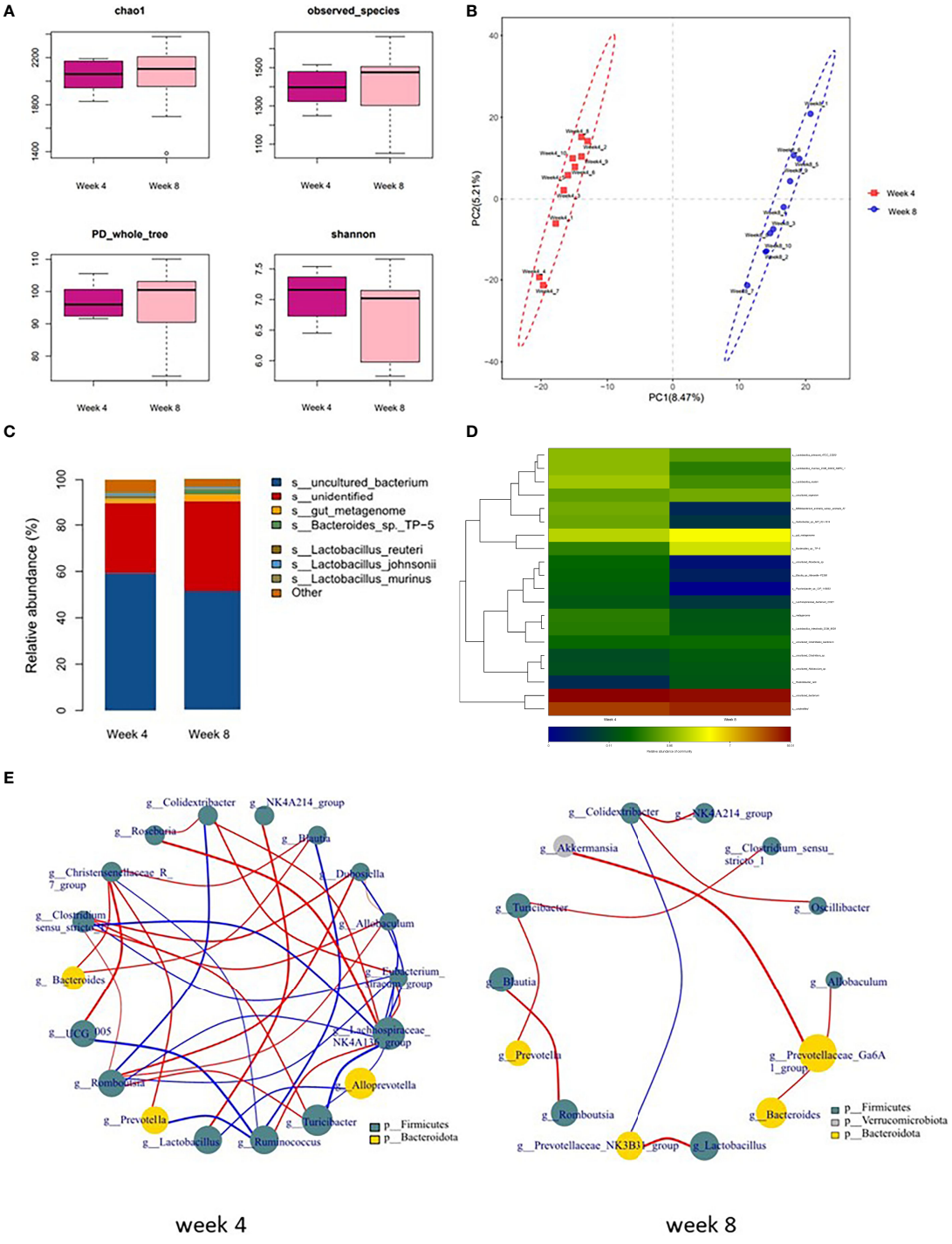

FIGURE 3
Microbial composition in long-term depression and co-occurrence of the species. **(A)** α-phylogenetic diversity analysis of 4-week CUMS and 8-week CUMS rats. **(B)** OPLS-DA showed that the gut microbial composition of 8-week CUMS rats was significantly different from that of 4-week CUMS rats. **(C)** Relative abundance of gut microbes at the species level. **(D)** Altered species (top 20) in the long-term depression group. **(E)** Co-occurrence of the species altered in the depression groups at the g and p levels.

coliform bacteria take Bacteroides, Bifidobacterium, Clostridium and Escherichia coli as the dominant bacteria, synthesize various complex digestive enzymes and metabolize sugars through glycolysis, pentose phosphate and anaerobic decomposition. When the abundance of these dominant bacteria decreases, it weakens the host's absorption of nutrients (Backhed et al., 2004). (Knuesel and Mohajeri, 2021) found that the diversity of intestinal flora in patients with depression decreased compared with that of healthy people. Other studies showed that the large reduction in Bacteroides in the intestine of patients with depression was closely related to depression symptoms

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FIGURE 4
Linear discriminant analysis effect size (LEfSe) analysis. Cladogram **(A)** and histogram **(B)** illustrating the species responsible for discriminating the rats under 4 weeks of CUMS and 8 weeks of CUMS (n = 10 per group).

and loss of pleasures (Mason et al., 2020; Wenzel et al., 2020). Therefore, the decreased species richness may indicate poor health conditions and disordered physiological processes.

Furthermore, the gut microbial composition of 8-week CUMS rats was significantly different from that of 4-week CUMS rats. More co-occurrence relationships were detected in the 4-week CUMS group than in the 8-week CUMS group. The top 20 differential microbes between rats under 4 weeks of CUMS and 8 weeks of CUMS included *Lactobacillus johnsonii, Lactobacillus murinus, Lactobacillus reuteri, Bifidobacterium animalis, Helicobacter, Bacteroides, Roseburia, Blautia Marseille, Psychrobacter, Lachnospiraceae bacterium, Lactobacillus intestinalis, Clostridiales bacterium, Clostridium, Allobaculum*, and *Rodentibacter ratti*. Among them, five species belong to Lactobacillus, while species of the same family have been recently reported to alleviate depression-related symptoms in a CUMS mouse model by regulating brain xanthine oxidase activity (Xu et al., 2022). To identify distinguishable microbial characteristics between 4-week CUMS and 8-week CUMS treatments, LEfSe analysis revealed the differential OTUs between the two groups (Figures 4A, B). The long-term depression group was characterized by disturbance of Alistipes indistinctus, Bacteroides ovatus, and Alistipes senegalensis at the species level (Figure 4B). Alistipes, primarily isolated from medical clinical samples, is a relatively new genus that is highly relevant in dysbiosis and disease, including mental signs of depression (Parker et al., 2020). A recent study revealed gut microbiota characteristics of ADHD and found that low levels of Bacteroides ovatus were associated with host cognitive impairment (Li et al., 2022). Bacteroides ovatus colonization has also been reported to influence the abundance of intestinal short-chain fatty acids and neurotransmitters (Horvath et al., 2022). Our study further detected its role in long-term depression development.

We also found that the development of depression was associated with disturbances in fecal metabolomics, which is always considered the functional readout of the gut microbiome since the gut microbiota is involved in the regulation of the metabolic pathways of the host. Compared with the control group, there were 508 differential fecal metabolites in the depression rats, of which 247 metabolites increased and 261 metabolites decreased. In addition, increased D-pinitol and decreased indoxyl sulfate, trimethylamine n-oxide, and 3alpha,7alpha-dihydroxy-12-oxocholanoic acid were observed. The active natural inositol D-pinitol reduces pancreas insulin secretion and increases circulating ghrelin levels in rats (Navarro et al., 2020); herein, the increase in D-pinitol in the long-term depression group may provide insight into the mechanism of depression-related weight gain. These differential metabolites were further used for KEGG pathway enrichment analysis. Among the top 20 pathways revealed, pantothenate and CoA biosynthesis was most significantly enriched (Figure 5E). Specifically, these differentially expressed metabolites were related to valine, leucine and isoleucine biosynthesis; glycine, serine and threonine metabolism; beta–alanine metabolism; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; riboflavin metabolism; thiamine metabolism; pyrimidine metabolism; the sulfur relay system; lysine degradation; biosynthesis of amino acids; glycerophospholipid metabolism; alanine, aspartate and glutamate metabolism; ubiquinone and other terpenoid–quinone biosynthesis; arginine and proline metabolism; alpha–linolenic acid metabolism; tyrosine metabolism; sulfur metabolism; and fatty acid degradation. Among them, the pathway with the highest enrichment factor was valine, leucine and isoleucine biosynthesis. A recent study showed that deficiency in the essential amino acids l-isoleucine, l-leucine and l-histidine can be used as predictors of moderate depression in elderly women (Solís-Ortiz et al., 2021). The enrichment of this KEGG pathway also reflected the important role of leucine and isoleucine in long-term depression development.

Increasing research evidence shows that the intestinal microbiota helps to maintain the metabolic homeostasis of the host, and in contrast, an imbalance in the microbiota affects the level of metabolites (such as branched-chain amino acids, hormones, vitamins, and short-chain fatty acids), leading to the development

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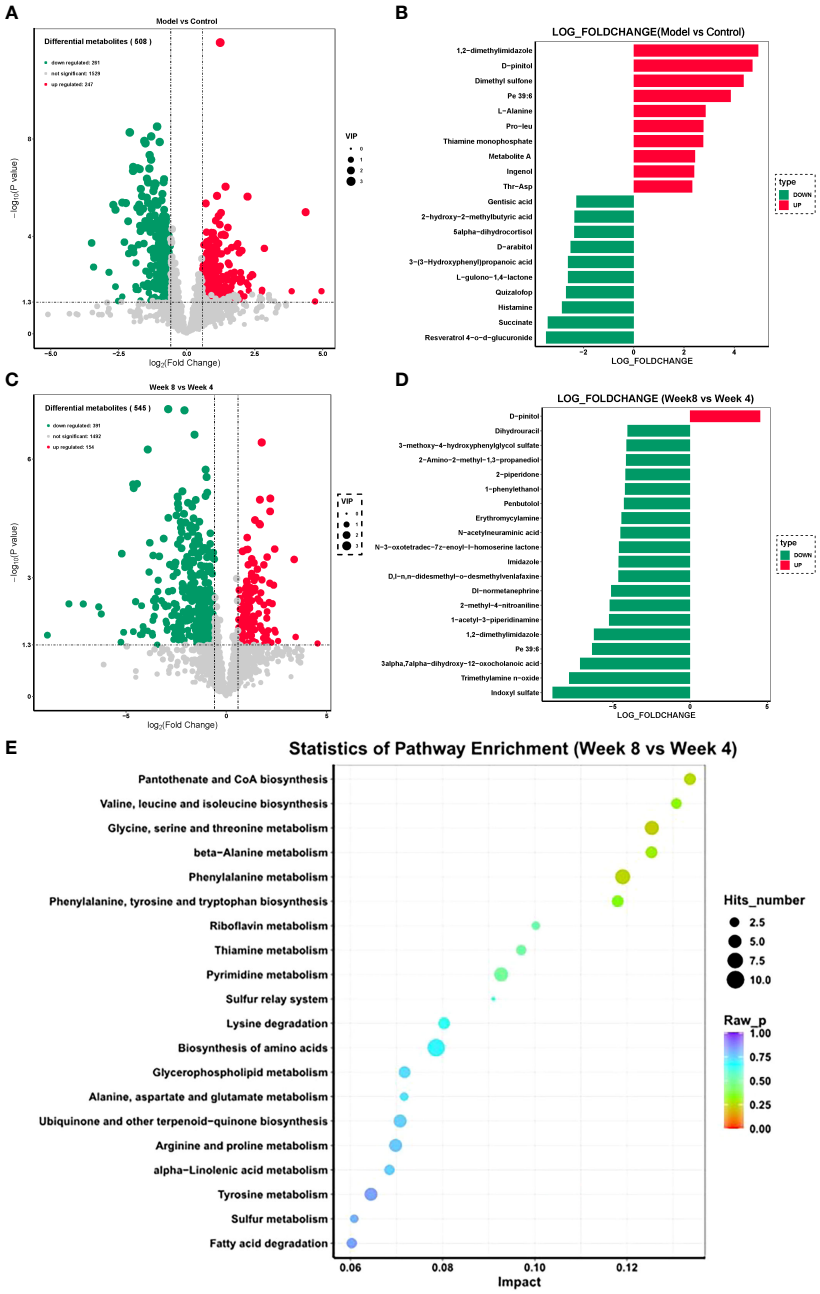

FIGURE 5
Fecal metabolism characteristics of CUMS rats. **(A)** Volcano plot of differential features between the CUMS group and the control group. **(B)** Top 10 differential metabolites between the CUMS group and the control group. **(C)** Volcano plot of differential features between the 8-week CUMS group and the 4-week CUMS group. **(D)** Top 10 differential metabolites between the 8-week CUMS group and the 4-week CUMS group. **(E)** KEGG pathways involved in the differential metabolites.

of obesity, insulin resistance, depression and other diseases (Caspani et al., 2019). It can be seen that the metabolic group and intestinal microbiota are closely related. At present, 16S sequencing technology is mostly used for the analysis of the intestinal microbiome, but this technology has certain limitations and cannot indicate the transcriptional activity of genes in each bacterial genome or distinguish between live bacteria and dead microbiota. Fecal metabolomics can better explain the metabolic interaction between the host, diet and intestinal microbiota and can provide functional data of microbiota to compensate for the lack of sequencing. Zierer J et al. found that the fecal metabolome reflected the composition of the intestinal flora to a large extent. The composition of the intestinal flora explained 67.7% of the changes in the level of 710 metabolites on average. There was a high correlation between the intestinal microbiome and fecal metabolome (Zierer et al., 2018). It has been reported in the literature that depression is related to main microbial metabolites (short-chain fatty acids - SCFAs -, bill acids, amino acids, tryptophan - trp - derivatives, and more) (Ortega et al., 2022). We

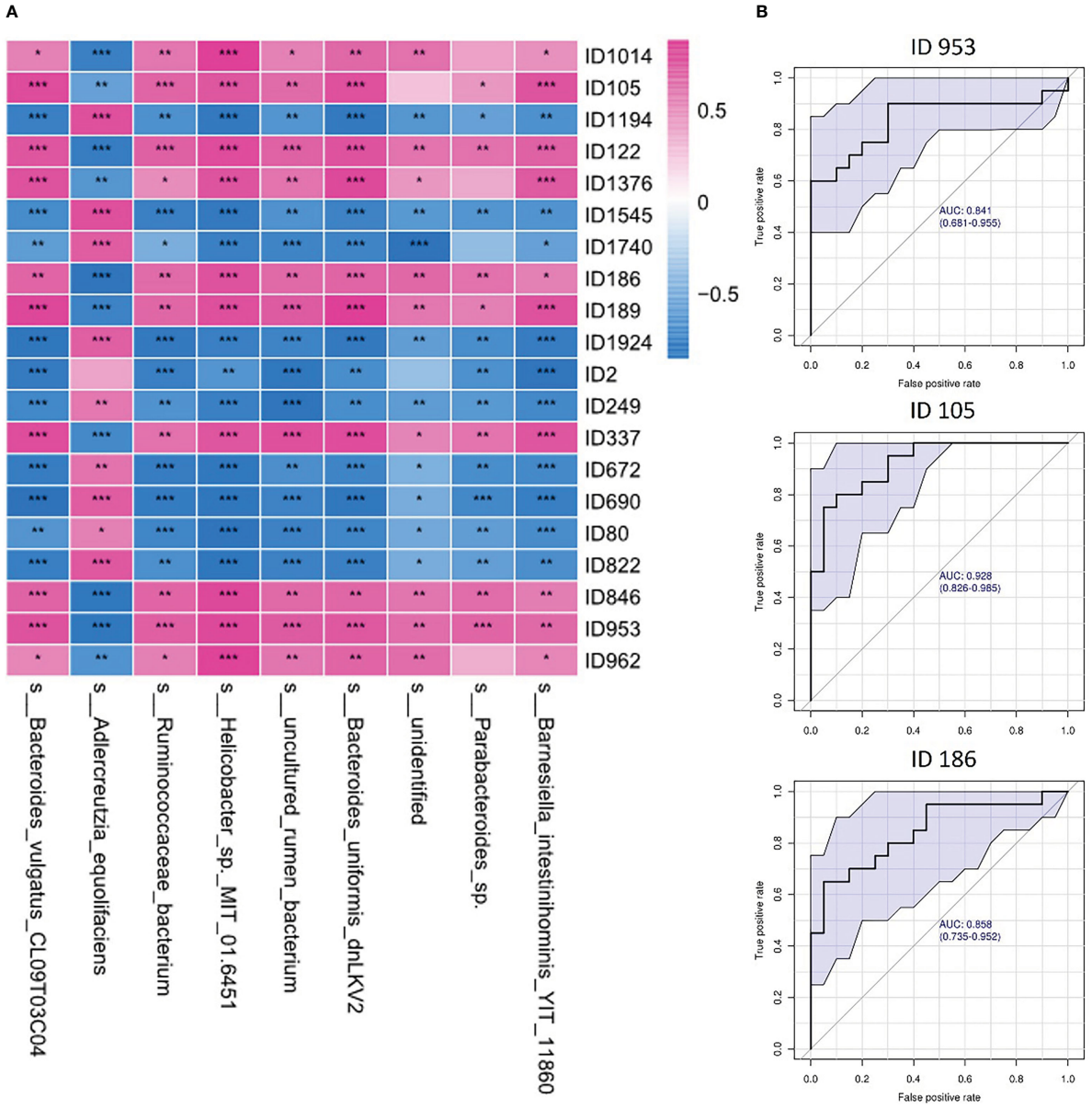

FIGURE 6
Correlations between gut microbes and fecal metabolites. **(A)** Heatmap of correlations between gut microbes and fecal metabolites in depression rats; **(B)** ROC curve of the top 3 representative metabolites related to gut microbes (ID953: N-acetyl-d-glucosamine; ID105: 3-(3-Hydroxyphenyl) propanoic acid; ID186: N-acetyl-d-mannosamine). One asterisk (*) represents a p-value less than 0.05, two asterisks (**) represent a p-value less than 0.01, and three asterisks (***) represent a p-value less than 0.001.

conducted a study on the correlation between gut microbes and fecal metabolites and found that at least nine gut microbes were significantly correlated with at least twenty fecal metabolites, such as Bacteroides uniformis with trimethoprim (ID1376) (Silva et al., 2000), Bacteroides vulgatus with N-acetyl-d-glucosamine (ID953) (Nihira et al., 2013; You et al., 2023), Helicobacter with trigonelline (ID1194) (Kyung et al., 2019), and Helicobacter with N-acetyl-d-glucosamine (ID953) (Dai et al., 2021). Some correlations between intestinal microorganisms and metabolites found in our study are consistent with those reported in the literature, but the names of metabolites are more specific, and the correlation between them has also been reported in other diseases, such as metabolic disorders and tumors. In long-term depression, the experimental results can provide ideas for further study.

Conclusion

In summary, we outlined the landscapes of gut microbiota and fecal metabolites in rats under 4-week and 8-week CUMS using multiomics data analysis. We found that the gut microbiome was distinguishable between depression and long-term depression rat models, and the altered microbiota may participate in the long-term development of depression through metabolism regulation. Our

findings provide a new perspective for understanding the pathogenesis of long-term depression.

Data availability statement

The data are divided into two parts, which have been uploaded. SubmissionID: SUB12502472, BioProject ID: PRJNA917078, https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB12502472/overview. SubmissionID: SUB12518854, BioProject ID: PRJNA919495, https://submit.ncbi.nlm.nih.gov/subs/bioproject/SUB12518854/overview.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of Institute of Basic Theory for Traditional Chinese Medicine, China Academy of Chinese Medical Sciences.

Author contributions

Conception and design: YXS and ZZhan. Performed the experiments: YBL, JLL, HXL, YKZ, YJL, ZBS, ZZhai and YPY. Performed the microbiome and metabolomic analysis: YXS, JLL, RC, MW and YJC. Drafted the manuscript: YXS and YBL. Final approval of the completed manuscript: YBL and ZZhan. 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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Oral microbiome changes associated with the menstrual cycle in healthy young adult females

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The relationship between the menstrual cycle and the oral microbiome has not been clarified. The purpose of this study was to assess potential changes in the oral microbiome of healthy young adults using 16S rRNA-based sequencing. Eleven females (aged 23–36 years) with stable menstrual cycles and without any oral problems were recruited. Saliva samples were collected before brushing every morning during the menstrual period. Based on basal body temperatures, menstrual cycles were divided into four phases, namely the menstrual, follicular, early luteal, and late luteal phases. Our results showed that the follicular phase had a significantly higher abundance ratio of the *Streptococcus* genus than the early and late luteal phases, whereas the abundance ratios of the *Prevotella* 7 and *Prevotella* 6 genera were significantly lower in the follicular phase than those in the early and late luteal phases and that in the early luteal phase, respectively. Alpha diversity by the Simpson index was significantly lower in the follicular phase than that in the early luteal phase, and beta diversity showed significant differences among the four phases. Using the relative abundance data and copy numbers of the 16S rRNA genes in the samples, the bacterial amounts in the four phases were compared, and we observed that the follicular phase had significantly lower amounts of the *Prevotella 7* and *Prevotella* 6 genera than the menstrual and early luteal phase, respectively. These results indicate reciprocal changes with the *Streptococcus* genus and *Prevotella* genera, particularly in the follicular phase. In the present study, we showed that the oral microbiome profiles are affected by the menstrual cycles of healthy young adult females.

KEYWORDS

menstrual cycle, oral microbiome, saliva, *Prevotella*, *Streptococcus*

1 Introduction

A menstrual cycle begins with the onset of menstrual flow. The menstrual phase generally lasts for 4–6 days with the shedding of the thickened endometrium, which is also known as menstrual bleeding. The follicular or proliferative phase continues until ovulation. And the luteal, or secretory phase begins at ovulation and continues until the onset of the next menstrual flow (Muizzuddin et al., 2006).

What has recently come to light is that the fluctuation of two hormones, estradiol and progesterone, plays a crucial role in neurological and psychological development and function, which impacts brain function, cognition, emotional status, sensory processing, and appetite (Farage et al., 2008). The menstrual cycle comes periodically with these physiological changes from menarche until menopause, except during pregnancy and childbearing. Because changes in these female hormones induce a variety of effects on the female's physical and mental health, it is important to clarify the relationship between physical and psychological changes associated with the menstrual cycle and the mechanisms of their effects in order to maintain and promote individual health and wellbeing.

The oral cavity, which is the gateway to the gastrointestinal tract, communicates with the outside environment and contains as many as 5–10 billion bacteria, comprising a unique commensal microbiome (Dewhirst et al., 2010). In fact, the oral cavity harbors the second most abundant microbiota next to the gastrointestinal tract. According to the expanded Human Oral Microbiome Database (eHOMD) (updated in November 2017) (Chen et al., 2010), approximately 770 bacterial genera have been identified as oral commensals. It is well known that altered oral microflora has been observed in several diseases such as diabetes, bacteremia, endocarditis, cancer, autoimmune disease, and preterm births. Therefore, it becomes crucial to understand the fluctuation of the oral microbial diversity under diseased/perturbed conditions (Verma et al., 2018).

The relationship between pregnancy and oral microbiome has been previously summarized in literature; oral microbiome composition can contribute to pregnancy complications (Saadaoui et al., 2021). The pregnant body is affected by a series of hormonal, metabolic, and immunological changes, resulting in the divergence of the oral microbiome composition (Lain and Catalano, 2007; Wang et al., 2016). Multiple studies have examined microbiome differences between pregnant and nonpregnant females (Basavaraju et al., 2012; Borgo et al., 2014; Fujiwara et al., 2017). One of these studies showed that the total number of viable oral microbes was significantly increased in all stages of pregnancy compared to nonpregnant females (Fujiwara et al., 2017). Moreover, it has been found that the growth and proliferation of multiple bacterial taxa, such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Escherichia* genera, were altered during pregnancy (Pelzer et al., 2012).

While there are many studies on pregnancy and oral contraceptives, few studies have examined the effects of menstruation on the oral microbiome. Balan et al. reported that as many as 43% of females experience some oral symptoms during their menstrual cycle (Balan et al., 2012). Other groups showed an increase in inflammatory cytokines, such as IL-1β and TNF-α, in the saliva before menstruation (Baser et al., 2009; Khosravisamani et al., 2014). Furthermore, Machtei et al. found that the gingival index was significantly higher during ovulation and premenstruation than during menstruation, even though the mean plaque indices were similar at all time points (Machtei et al., 2004). These reports indicate that the oral environment changes in accordance with the menstrual cycle (Gao et al., 2018). To date, the relationship between oral microbiome and menstrual cycles remains uncertain. Fischer et al. concluded that there was no specific pattern in bacterial changes during the menstrual cycle (Fischer et al., 2008), while Calil et al. reported that anaerobic bacteria counts remained unchanged (Calil et al., 2008). On the contrary, Kawamoto et al. showed that *Prevotella intermedia* (*P. intermedia*) and *Porphyromonas gingivalis* (*P. gingivalis*) were significantly higher during the ovulatory phase compared to the follicular phase (Kawamoto et al., 2010; Kawamoto et al., 2012).

While these reports on menstrual cycles used culture-based methods, Bostanci et al. have recently conducted a metagenomics-based study (Bostanci et al., 2021). Saliva samples were collected from 103 females of reproductive age during the menstrual, follicular, and luteal phases of the cycle (n = 309), and the abundances of oral bacteria were analyzed by 16S rRNA V3–V4 sequencing. No significant differences in α-diversity or clustering were observed among the three phases. Nonetheless, the authors found that the richness of *Campylobacter*, *Hemophilus*, *Prevotella*, and *Oribacterium* genera varied throughout the cycle, especially during the luteal phases.

The relationship between menstrual cycle and oral microbiome has not been clarified. Therefore, the purpose of this study was to clarify the changes in the oral microbiome during the menstrual cycle in 11 healthy young adult females. One of the pitfalls of oral microbe studies involves the precise timing of sample collection because oral samples are affected by brushing and ingesting drinks/foods. In order to unify collecting conditions, saliva samples were collected daily for approximately one month from the start of menstruation, after waking up and before brushing.

2 Materials and methods

2.1 Criteria of subject selection

Female researchers recruited 11 (Subjects #F1-F11) females (aged 23–36 years) with stable menstrual cycles, who had not taken any oral contraceptives or antibiotics within one month prior to the beginning of this study (Table S1). Five (Subjects #M1–M5) males (aged 22–32 years), who had not taken any antibiotics within one month prior to the beginning of this study, were recruited as controls. The participants were staffs, undergraduate students, and graduate students.

2.2 Saliva collection

Every morning, two milliliters of saliva were collected from each participant into a drool collection kit before brushing teeth. The saliva samples were transferred into storage tubes and stored in a freezer. For DNA extraction, 100 μL of whole saliva was diluted with 400 μL of phosphate buffered saline to reduce viscosity, and the

diluted saliva was transferred to a Pathogen Lysis Tube S (QIAGEN N.V.) and mechanically disrupted for 10 min using a Disruptor Genie (Scientific Industries). The resultant solution was subsequently processed using the QIAamp UCP Pathogen Mini Kit (QIAGEN N.V.) according to the manufacturer's instruction. Double stranded DNA concentrations were measured using the Qubit HS Assay Kit (ThermoFisher SCIENTIFIC). Additionally, the collected saliva was thawed and centrifuged at 1500 g for 15 min, and the supernatants were utilized in an ELISA testing below.

2.3 Phase classification

Basal body temperature was measured every morning upon waking up with a gynecological thermometer for approximately one month from the first day of menstruation to the beginning of the following month. An obstetrician and several midwives estimated ovulation periods based on the subjects' temperatures (Figure S1). For some of participants, we also measured salivary progesterone concentrations using an ELISA kit (SALIMETRICS) to estimate periods as complementary data (Figure S2). Because the periods of Subject #F2 and #F6 were not estimated, their data were excluded. The luteal phase was divided into two subphases because the first and second halves of a luteal phase can be affected by the follicular cycle and the upcoming menstruation, respectively. In addition, the menstrual cycle was divided into four phases: menstrual, follicular, early luteal, and late luteal phase (Figure S1). Male subjects used samples from the periods corresponding to the number of days (first duration, Days 1–2; second duration, Day 14; third duration, Day 21–22; fourth duration, Day 28).

2.4 Next generation sequencing and microbiome analysis

We prepared libraries of the V3–V4 region of the 16S rRNA gene with KAPA HiFi HS ReadyMix (KAPA BIOSYSTEMS) using the salivary DNAs as templates according to the 16S rRNA metagenomics protocol for MiSeq system (Illumina). The PCR samples were cleaned up by AMPure XP beads (Beckman Coulter) and the read data were obtained by a MiSeq sequencer using Miseq Reagent Kit v3 and PhiX Control v3 (Illumina). The raw reads data have been registered with DNA Data Bank of Japan under the accession number DRA015154 (Table S1). The obtained read data were analyzed by the QIIME2 program (Caporaso et al., 2010) (https://qiime2.org/). After merging the pair end reads, quality check and chimera checks were conducted with the DADA2 plugin (Callahan et al., 2016) . To analyze taxonomy, a classifier was trained using SILVA 16S rRNA database (Ver. 123) (Quast et al., 2013). The samples were rarefied at 10,000 depths of sequences.

2.5 Quantitative PCR

Quantitative PCR targeting part of the 16S rRNA gene (regions 321–524) was performed using Quantifast SYBR kit (QIAGEN) with a pair of primers: Forward 5'-(ACTGAGAYACGGYCCA)-3' and Reverse 5'-(CTGCTGGCACGDAGTTAGCC)-3' (Wang and Qian, 2009). The copy numbers of the gene in saliva were calculated using the indicated copy numbers of the *Staphylococcus caprae* strain JMUB145, which contains six copies of 16S rRNA genes in a chromosome (Watanabe et al., 2018). The bacterial amount was calculated by multiplying the relative abundance ratio by the copy number.

2.6 Statistical analysis

Data were expressed as box plots (the middle horizontal line refers to the median value and the width of the box represents the interquartile range), and all statistical analyses were performed in SPSS version 24 (IBM) or R version 4.1.1. Alpha diversity was calculated as the observed number of species as well as Simpson, Shannon, and Chao1. Differences among menstrual cycles or trimesters were calculated with the Friedman test, followed by *post hoc* pairwise comparisons using the Bonferroni test. Correlations were calculated with Spearman's rank correlation coefficient test. Beta diversity was calculated on Bray-Curtis distances and clustered on complete linkage. The factors that affect bacteria compositions on beta diversity assessed through a permutational multivariate analysis (PERMANOVA). A p-value < 0.05 was considered significant.

3 Results

3.1 Demographic data of subjects

The saliva samples were taken from healthy young adult females (aged: 23–36 years, body mass index: 18.0–30.6, and menstrual duration: 25–43 days), and males (aged: 22–32 years and body mass index: 18.7–34.5). All subjects had no oral problems, and no habit of using dental floss (Table S1).

3.2 Genera abundances in the oral microbiome

The oral microbiome in both females and males mainly consists of *Streptococcus*, *Prevotella*, *Veillonella*, *Porphorymonas*, *Nesseria*, and *Gemella* genera (Figure 1). With respect to the *Prevotellaceae* family, the *Prevotella* 7, *Prevotella* 6, and *Prevotella* (unnumbered) genera were detected. Species in the *Prevotella* 7, *Prevotella* 6, and *Prevotella* (unnumbered) genera in the SILIVA database (Ver. 123) are listed in Table S2. Among the abundance ratios of the top 20 oral microbiomes, Friedman tests revealed significant differences in five genera among females. The abundance ratio of the *Streptococcus* genus in the follicular phase was significantly higher than that in the early and late luteal phases ($P < 0.05$, Figure 2A), while the abundance ratios of the *Prevotella* 7 and *Prevotella* 6 genera were significantly lower in the follicular phase than in the early and late luteal phases ($P < 0.05$, Figure 2B) and in the early luteal phase ($P < 0.05$, Figure 2C), respectively. The abundance ratio of the *Saccharibacteria* genus was significantly lower in the follicular phase than in the late luteal phase ($P < 0.05$, Figure 2D). While Friedman test detected significant difference in the abundance

ratio of Atopobium genus, there was no significant difference by post hoc pariwise comparisons (Figure 2E). Furthermore, the abundance ratio of the Oribacteirum genera tended to be lower in the follicular phase than in the early lutealphase ($P = 0.064$, Figure 2F). In contrast, there were no significant differences in the abundance ratios of genera among males.

3.3 Alpha diversity and beta diversity

In females, the Simpson index was significantly lower in the follicular phase than in the early luteal phase ($P < 0.05$, Figure 3A) and tended to be lower than in the late luteal phase ($P = 0.064$, Figure 3A), while neither the Shannon nor the Chao1 index showed significant differences among the four phases. In contrast, beta diversity revealed significant differences among the four phases ($P < 0.01$, Figure 3B). There were no significant differences among the periods in males.

3.4 Copy numbers of the 16S rRNA genes

In order to examine changes in bacterial amounts during the menstrual cycles, the copy numbers of the 16S rRNA genes were calculated by quantitative PCR (Figure 4). In females, Friedman test revealed that the 16S rRNA gene copy number absolute value tended to be difference ($P = 0.072$, Figure 4). In contrast, there were no significant differences in the copy numbers of the 16 S rRNA gens among males.

3.5 Bacterial amounts

To estimate changes in the numbers of each bacterial genus during the menstrual cycles, we multiplied the copy numbers by the abundance ratio. Our results demonstrate there were no significant differences in the amount of the *Streptococcus* genus among the four phases (Figure 5A). In contrast, the amount of the *Prevotella 7* genus was significantly lower in the follicular phase than in the menstrual phase ($P < 0.01$, Figure 5B) while that of the *Prevotella 6* genus was significantly lower in the follicular phase than in the early luteal phase ($P < 0.05$, Figure 5C) but not in the late luteal phase. Finally, the total amount of the *Saccharibacteria* genus was significantly lower in the follicular phase than in the late luteal phase ($P < 0.05$, Figure 5D).

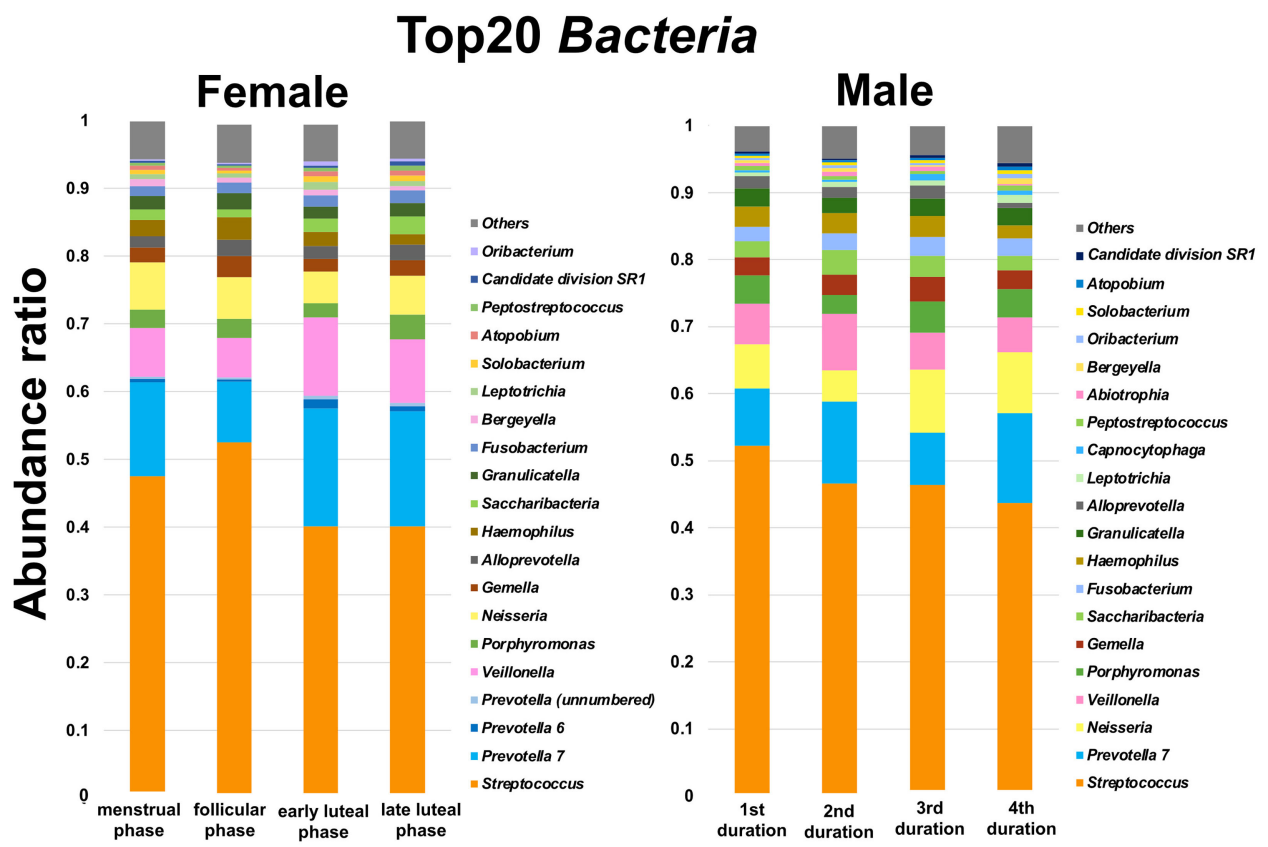

FIGURE 1
Abundance ratios of the top 20 bacteria genera in the oral microbiome. Stacked bar plots showing the composition of the most dominant genera color-coded and ranked according to abundance, while *Prevotella 6* and Prevotella unnumbered genera were arranged next to *Prevotella 7* genus. The abundance ratios in females are shown in four phases: menstrual, follicular, early luteal, and late luteal phases. The abundance ratios in males are shown with four durations corresponding to the number of days: days 1–2, 1st duration; day 14, 2nd duration; days 21–22, 3rd duration; day 28, 4th duration. Females, N = 9; Males, N = 5.

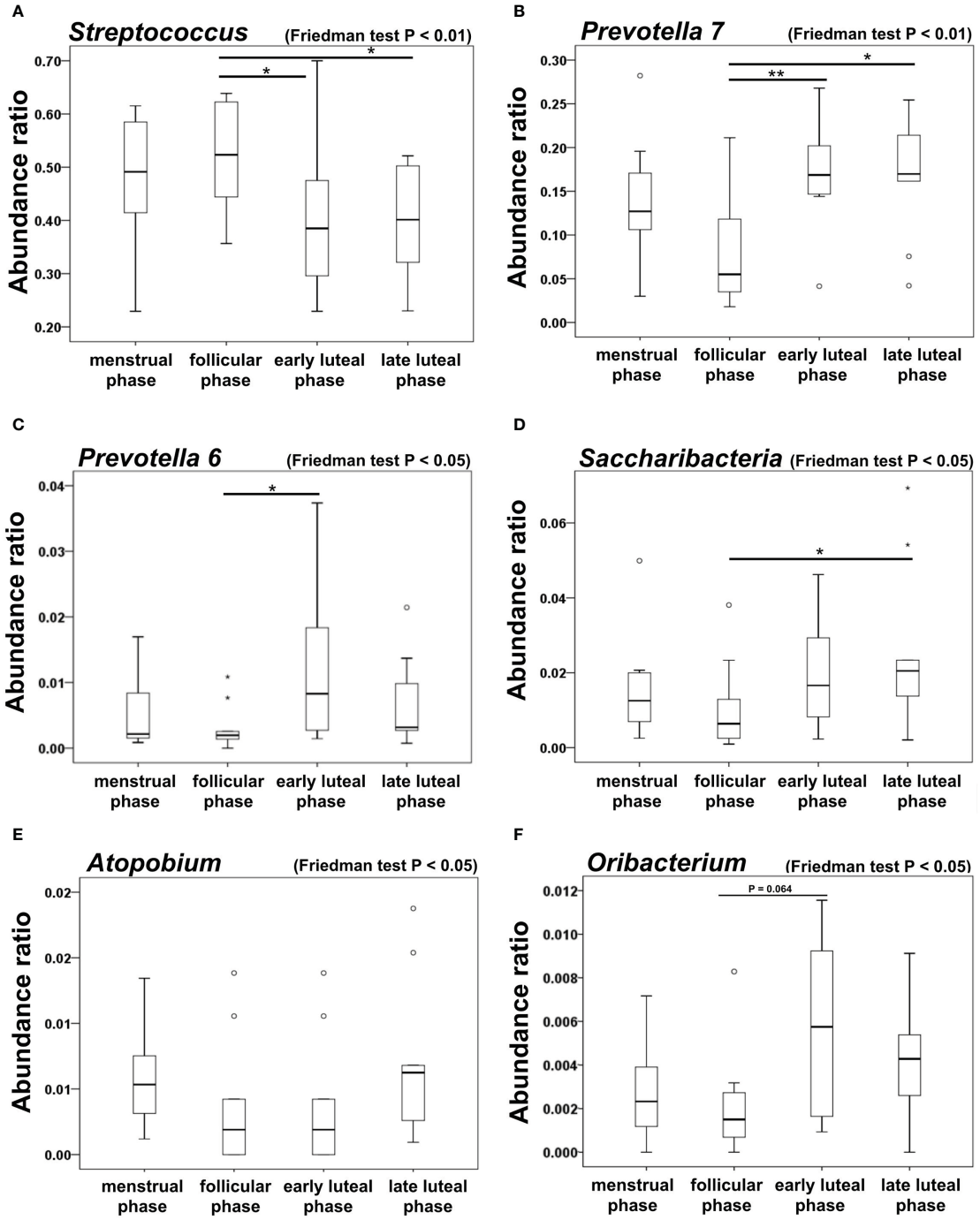

FIGURE 2
Abundance ratios of each bacterial genus in the oral microbiome of female subjects. Boxplots showing the abundance ratios of each bacterial genus during menstrual cycles: **(A)** *Streptococcus* genera. **(B)** *Prevotella 7* genera. **(C)** *Prevotella 6* genera, **(D)** *Saccharibacteria* genera, **(E)** *Atopobium* genera, and **(F)** *Oribacterium* genera (menstrual, follicular, early luteal, and late luteal phases). Friedman test and Bonferroni test, *P <0.05 and **P < 0.01. N = 9.

3.6 Correlation of bacteria genera in the oral microbiome

The Spearman's rank correlation coefficients were r = −0.850, −0.783, and −0.850 in the menstrual, follicular, and early luteal phase, respectively, indicating a negative correlation between the *Streptococcus* and *Prevotella 7* genera ($P < 0.05$), while there was no significant correlation in the late luteal phase (Table 1). Correlation between the *Streptococcus* genus and *Prevotella 6* genera was observed only in the late luteal phase (r = −0.717, $P < 0.05$). There was no correlation between the *Streptococcus* genus and *Prevotella* (unnumbered) genera in the four phases. Although *Prevotella (unnumbered)* and *Porphyromonas* genera tended to be positively correlated in the early luteal phase (r = 0.6, $P = 0.088$), the abundance ratios were correlated negatively in the follicular phase (r = −0.817, $P < 0.05$) (Table S3).

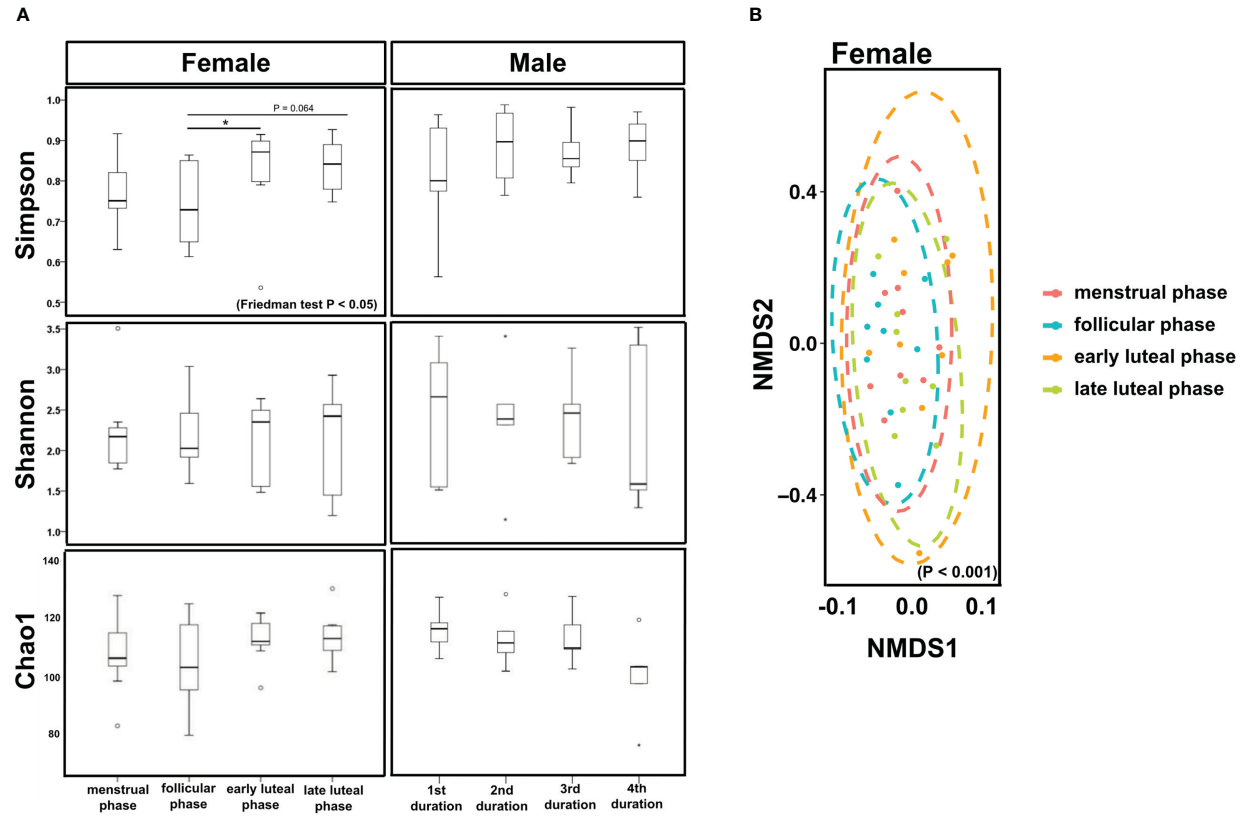

FIGURE 3
Bacterial diversity. **(A)** Box plots showing the alpha diversity indices, Simpson, Shannon, and Chao1, based on the phases of the menstrual cycles (menstrual, follicular, early luteal, and late luteal phases) or durations (days 1–2, 1st duration; day 14, 2nd duration; days 21–22, 3rd duration; day 28, 4th duration). Friedman test and Bonferroni test, *P <0.05. Females, N = 9; Males, N = 5. **(B)** Nonmetric multidimensional scaling plot showing the dissimilarities of all samples collected in this study according to menstrual cycles (menstrual, follicular, early luteal, and late luteal phases). Ellipsoids represent a 95% confidence interval used to group each sample.

4 Discussion

Several studies have addressed the changes in the oral environment during the menstrual cycle (Baser et al., 2009; Balan et al., 2012; Khosravisamani et al., 2014). However, the relationship between the menstrual cycle and the oral microbiome has not been clarified. Therefore, in this study, we used 16S rRNA-based sequencing to examine the cyclical changes

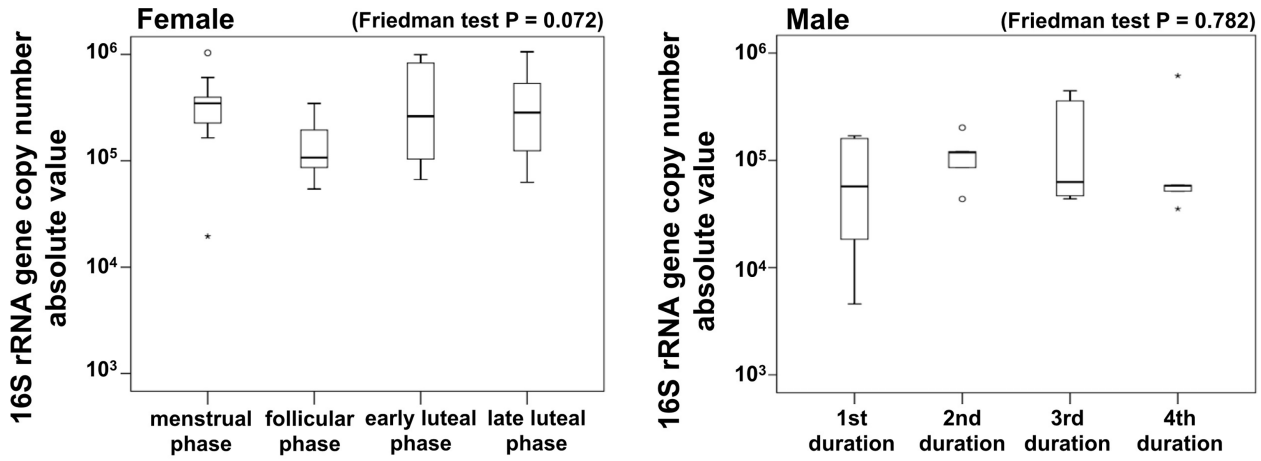

FIGURE 4
Copy numbers of the 16S rRNA gene collected by saliva samples. Data is shown in box plots. Friedman test and Bonferroni test. Females, N = 9; Males, N = 5. The vertical axis was a base-10 log scale. rRNA, ribosomal RNA.

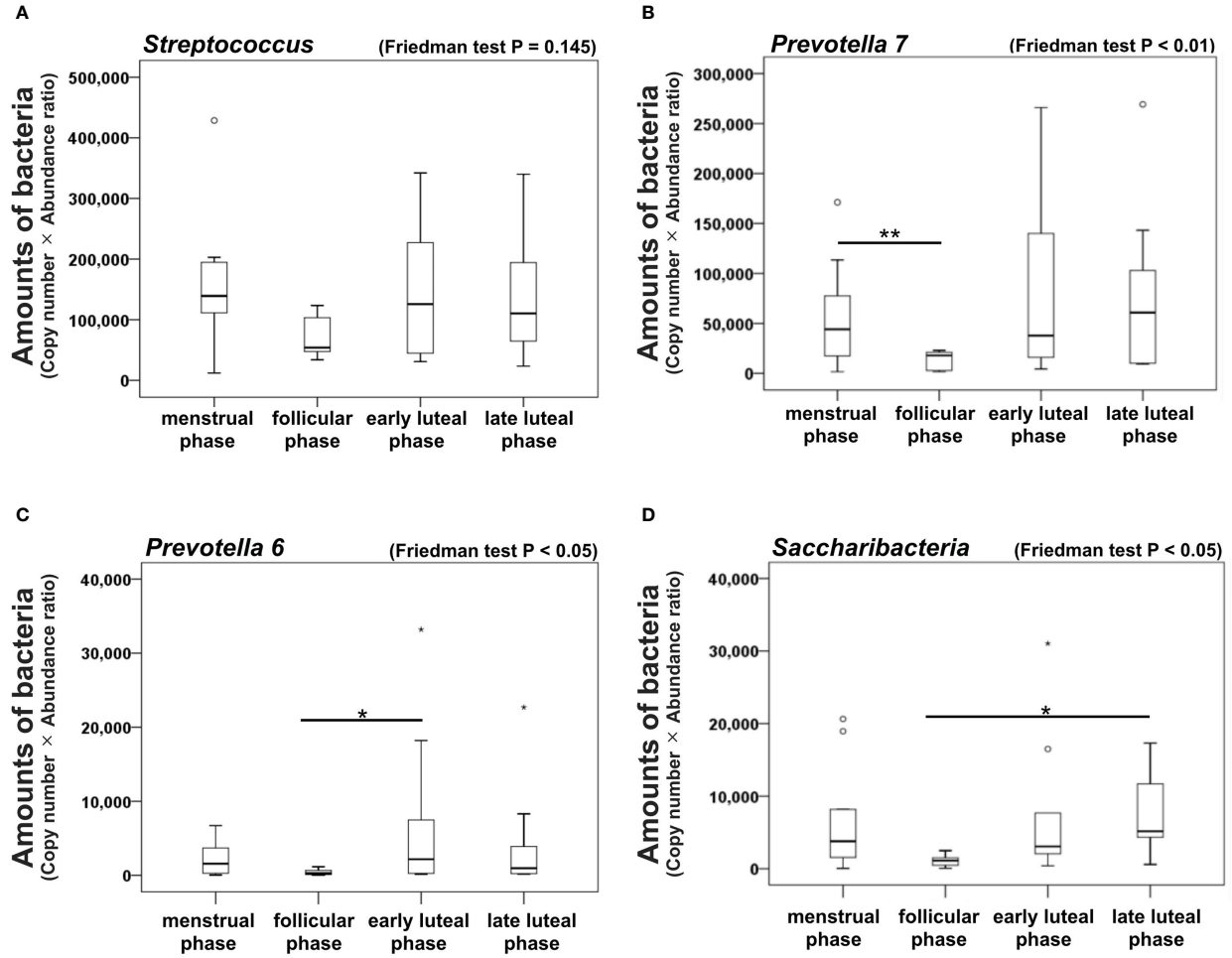

FIGURE 5
Amounts of dominant bacteria genera in the oral microbiome of female subjects. Box plots showing the amounts of **(A)** *Streptococcus*, **(B)** *Prevotella 7* genera, **(C)** *Prevotella 6*, and **(D)** *Saccharibacteria* genera during the menstrual cycles. Friedman test and Bonferroni test, *$P < 0.05$ and **$P < 0.01$. N = 9.

in oral microbiome and assess their impact on females' health and wellbeing. Our study revealed the presence of various microorganisms during the menstrual cycles of healthy young adult females, indicating reciprocal changes with the *Streptococcus* genus and *Prevotella* genera, especially in the follicular phase. To the best of our knowledge, this is the first study to prove the cyclical changes with specific bacteria genus during the menstrual cycle by 16S rRNA-based sequencing. The results of this study may provide a basis for etiological studies of female hormone-influenced oral disorders.

The main finding of this study is that changes in the *Streptococcus* and *Prevotella* genera abundances in healthy young adult females in Japan occur in accordance with their menstrual cycles. Previously, Bostanci et al. reported that the abundances of these bacteria were not altered during the menstrual cycles in adult females in Denmark (Bostanci et al., 2021). This discrepancy might be attributed to the methods used for the collection of samples. In this study, we collected saliva samples immediately after waking, while Bostanci et al. collected their samples after 30 minutes of fasting in the hospital. In addition, dietary and racial differences

TABLE 1 Correlation of the abundance ratios of bacterial genera in menstrual cycles.

	Menstrual	Follicular	Early Luteal	Late Luteal
Streptococcus/Prevotella 7	−0.850*	−0.783*	−0.850**	−0.433
Streptococcus/Prevotella 6	−0.433	−0.583	−0.100	−0.717*
Streptococcus/Prevotella (unnumbered)	−0.533	−0.150	−0.483	−0.417

Spearman's rank correlation coefficient test, *P < 0.05 and **P < 0.01. N = 9.

could also be associated with this discrepancy. In our study, bacteria of the *Streptococcus* genus were the most predominant followed by bacteria of the *Prevotella 7* genus. In a previous report, bacteria of the *Prevotella* genus (including all *Prevotella 1—9* and unnumbered genera) were the most abundant followed by bacteria of the *Streptococcus* genus, indicating that the effect of menstrual cycle correlates with the composition of the oral microbiome.

Alpha diversity represents microbiome richness and community diversity in a sample or site, and it is calculated by different formulas based on the Simpson, Shannon, and Chao1 indices. Generally, the Simpson index gives more weight to higher abundant genera. In our study, compared with the Shannon or Chao1 index, the Simpson index revealed was significantly decreased in the follicular phase, suggesting notable changes in the predominant *Streptococcus* and *Prevotella* genera. To our knowledge, there are no variations in the alpha diversity of the oral microbiome between healthy young people and depression. However, it has been previously reported that the Shannon index is different in the depressed group than in healthy adults, although there was no significant difference in the mean values of the diversity (Wingfield et al., 2021). Several reports concluded that there was no significant difference in the alpha diversity of oral microbiome between pregnant and nonpregnant groups (Sparvoli et al., 2020), between patients with anxiety and depression and control subjects (Simpson et al., 2020), or between postpartum and nonpregnant females (Khadija et al., 2021). Further analysis is required to clarify whether changes in the alpha diversity of oral microbiome depend on the oral environment, disease, and/or mouth conditions.

Furthermore, the present study found that the amount of the *Prevotella 7* genus significantly decreased in the follicular phase. During that phase, estradiol reaches its highest levels, as opposed to progesterone, which reaches its highest levels during the early luteal phase. Previous studies have investigated the relationships between female hormones (estradiol and progesterone) and bacterial amounts. For example, ovariectomy in mice showed that oral bacterial amounts increased as a result of estradiol deficiency (Lucisano et al., 2021), although this report did not focus on *Prevotella* species. In addition, *Prevotella* species have been found to change their characteristics according to estradiol levels (Könönen et al., 2022), of which *Prevotella intermedia*, *Prevotella nigrescens*, and *Prevotella pallens* increased biofilm formation in the presence of estradiol (Fteita et al., 2015; Fteita et al., 2017). The decrease observed in the *Prevotella 7* genus in saliva might be due to the fact that estradiol-induced biofilm prevents *Prevotella* from flowing into the saliva. Regarding progesterone, *Prevotella* species as well as *Porphyromonas gingivalis* utilize progesterone as a source of nutrition (Basavaraju et al., 2012; Butera et al., 2021), indicating that the increase in the amount of *Prevotella 7* genus in the late luteal phase is due to a progesterone-dependent growth. Further studies are required to identify the mechanisms with which female hormones influence the amount of *Prevotella* species.

Interestingly, the *Prevotella 7* genus was negatively correlated with the *Streptococcus* genus in the menstrual, follicular, and early luteal phases, and the *Prevotella* 6 genus was negatively correlated with the *Streptococcus* genus in the late luteal phase. These results demonstrate that the *Prevotella* genera are associated with the menstrual cycle and/or other bacterial genera under different regulations. According to the SILIVA database (Ver. 123), *Prevotella 7* contains *P. buccae*, *P. dentasini*, *P. denticola*, *P. enoeca*, and *P. melaninogenica* (Table S2). Among them, *P. melaninogenica* was found in high proportions in the saliva of 225 systemically healthy individuals (Mager et al., 2003), although this species is recognized as pathogenic. However, it remains unknown whether the increase and decrease in *P. melaninogenica* and other *Prevotella 7* genera in this study affected the health status of the females. *P. intermedia* and *P. nigrescens*, members of the *Prevotella (unnumbered)* (Table S2), are associated with inflammatory periodontal diseases together with *Porphyromonas gingivalis* (Lafaurie et al., 2017; Iuşan et al., 2022). Although the abundance ratio of *Prevotella (unnumbered)* and *Porphyromonas* tended to be positively correlated in the early luteal phase, the abundance ratios were significantly negative in the follicular phase (Table S3). These results indicate that the menstrual cycle is associated with periodontal diseases through affecting interaction between *Prevotella (unnumbered)* and *Porphyromonas*.

The SILVA database is a comprehensive web resource for up-to-date, quality-controlled databases of aligned rRNA gene sequences from the Bacteria, Archaea and Eukaryota domains (Quast et al., 2013). Several previous studies have also used the SILVA database for oral microbiome analysis (Kennedy et al., 2016; Zhao et al., 2021; Zhao et al., 2022). Furthermore, a recent systematic review of oral peri-implant and periodontal microbiota (Gazil et al., 2022) showed a specific database for oral microbiomes, such as the HOMD (Chen et al., 2010) (https://www.homd.org/). Further analysis using oral-specific databases, such as the HOMD would be interesting.

Limitations

Our study has several limitations. First, only saliva, but not intragingival, samples were collected. Consequently, it is unclear whether our results do reflect the bacterial flora in the gingiva. Second, the subjects included in this study had no oral issues; therefore, it is unclear how the affected oral microbiome in subjects with oral problems may influence our results. Third, our sample size was small and limited (only included Japanese subjects), restricting the generalization of our present findings. Finally, our participants included one female with overweight and one male with obesity, thereby restricting the generalization of our present findings.

5 Conclusion

Oral microbiome profiling in menstrual cycles using 16S rRNA sequencing revealed the presence of a community of microorganisms in healthy young adult females, indicating reciprocal changes with the *Streptococcus* and *Prevotella* genera, especially in the follicular phase. Further studies are required to identify the mechanism with which these cyclical changes affect oral microbiome.

Data availability statement

The data presented in the study are deposited in the repository of DNA Data Bank of Japan (DDBJ), accession number DRA015154.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Kanazawa University (Approval No. 953-1). The patients/participants provided their written informed consent to participate in this study. This study was carried out in accordance with the Ethical Principles for Medical Research Involving Human Subjects (Declaration of Helsinki), the Ethical Guidelines for Medical Research Involving Human Subjects (Ministry of Health, Labor and Welfare), the Regulations on Medical Research Involving Human Subjects at Kanazawa University, and the Kanazawa University Regulations for Prevention of Misconduct in Research Activities.

Author contributions

AY conducted the ELISA experiments and protein concentration calculation, analyzed all data, and wrote this manuscript. KOgu analyzed the sequence data and revised the draft of this manuscript. KMi analyzed body temperatures and the ELISA data to determine the menstrual cycles. KOga performed DNA extraction and sequencing processes. TH collected the saliva samples and clinical data. SO supervised AY and contributed to the discussion part. KMu conceived the study, collected the saliva samples and clinical data, conducted part of the experiments, and revised the manuscript critically for content. All authors reviewed and accepted the 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.

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Supplementary material

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Use of probiotic lactobacilli in the treatment of vaginal infections: *In vitro* and *in vivo* investigations

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The vaginal microbiome is a distinct component of the human microbiome that is colonized by a wide variety of microorganisms. Lactobacilli are the most frequently identified microorganisms in the healthy human vagina. These Gram-positive bacilli can acidify the vaginal microenvironment, inhibit the proliferation of other pathogenic microorganisms, and promote the maintenance of a eubiotic vaginal microbiome. However, a vaginal flora with a reduced proportion or abundance of lactobacilli is associated with various vaginal infections that have been linked to serious health consequences such as infertility, preterm birth, pelvic inflammatory disease, premature rupture of membranes, and miscarriage. Due to their "Generally Recognized as Safe" classification and critical role in vaginal health, probiotic lactobacilli have been widely used as an alternative or adjunct to traditional antibiotic therapy for the treatment of vaginal infections and restoration of the vaginal microbiome. This review focuses on the significant role of probiotic lactobacilli in the vaginal microenvironment and discusses the use of probiotic lactobacilli in the treatment of female vaginal infections *in vitro* and *in vivo*.

KEYWORDS

lactobacilli, vaginal microbiome, probiotic, vaginal infections, treatment

1 Introduction

The vaginal microbiome is now largely recognized as a balanced ecosystem dominated by *Lactobacillus* species, with notable fluctuation over time and across individuals (Armstrong and Kaul, 2021; Lehtoranta et al., 2022). Under physiological conditions, this *Lactobacillus*-dominant arrangement has long been regarded as an indicator of vaginal health (Cappello et al., 2023; Shen et al., 2023). The production of lactic acid, biosurfactants, bacteriocin-like chemicals, and hydrogen peroxide (H_2O_2) by these Gram-positive bacilli, which varies from strain to strain, can acidify the vaginal microenvironment, inhibit the proliferation of other pathogenic microorganisms, and thereby promote the maintenance of a eubiotic vaginal microbiome (Chee et al., 2020; Das

et al., 2023). However, a vaginal flora with a reduced proportion or abundance of lactobacilli can lead to vaginal dysbiosis, which is related to vaginal infections such as bacterial vaginosis (BV), vulvovaginal candidiasis (VVC), aerobic vaginitis (AV), human papillomavirus (HPV) infection, human immunodeficiency virus (HIV) infection, herpes simplex virus 2 (HSV-2) infection, and other sexually transmitted infections (STIs) including gonorrhea, trichomoniasis, *Chlamydia* infection, *Mycoplasma* infection, and *Ureaplasma* infection, as well as mixed infections of these diseases (Han et al., 2021).

Owing to their "Generally Recognized as Safe" status, probiotic lactobacilli have been used as alternatives and adjuncts to antibiotics, reducing antimicrobial resistance caused by the overuse of antibiotics in the treatment of illnesses (Aslam et al., 2021; Magalhães et al., 2021; Mei and Li, 2022). In addition, the efficacy of lactobacilli as a prophylactic agent has been demonstrated in long-term administration, although most probiotics are recommended for gastrointestinal use, several are marketed for vaginal health (Lagenaur et al., 2021; Nader-Macías et al., 2021). In recent years, clinical research and development of lactobacilli for the prevention and treatment of vaginal infections in women has advanced rapidly (Lagenaur et al., 2021; Sadahira et al., 2021; Schenk et al., 2021). This paper focuses on the importance of lactobacilli in the vaginal microenvironment and summarizes the use of probiotic lactobacilli in the treatment of several different types of vaginal infections through *in vitro* and *in vivo* investigations.

2 Lactobacilli in the vagina and their crucial function

A landmark study conducted by Ravel et al. revealed that the vaginal microbiome of most reproductive-age women is clustered into five microbial community state types (CSTs), four of which are dominated by *Lactobacillus* (CST I: *L.crispatus*-dominated CST, CST II: *L. gasseri*-dominated CST, CST III: *L. iners*-dominated CST, and CST V: *L. jensenii*-dominated CST), where CST IV is characterized by a lower level of *Lactobacillus* and a higher level of anaerobic bacteria (Ravel et al., 2011). Subsequently, Gajer et al. further divided CST IV into two subgroups, CST IV-A and CST IV-B. CST IV-A contains a modest fraction of *L. iners* as well as anaerobic bacteria, where CST IV-B contains a significant amount of BVAB (Gajer et al., 2012). It is worth noting that vaginal CSTs are changeable owing to menstruation, pregnancy, and sexual activity (Amin et al., 2023). For example, *Gardnerella* and *Ureaplasma* are more abundant during the third trimester of a normal pregnancy than during the first and second trimesters, but this change corresponds with a high *Lactobacillus* abundance (Park et al., 2022; Amin et al., 2023).

Lactobacilli can restore the vaginal microbiome to homeostasis *via* several mechanisms. Co-aggregation with pathogens may occur when the cell surface of the lactobacilli contains various mucin-binding proteins, fbronectin-binding proteins, and collagen-binding proteins, and these surface proteins can enhance the ability of lactobacilli to adhere to pathogens (Spacova et al., 2020; Dell'Anno et al., 2021; Zawistowska-Rojek et al., 2022). Production of defense metabolites including lactic acid, H_2O_2, bacteriocin, and biosurfactants may also occur. As fundamental components of vaginal defense, D- and L-lactic acid isomers can maintain the pH of the vaginal environment between 3.5 and 4.5, thereby making it inappropriate for the growth of pathogenic bacteria species (Kerry-Barnard et al., 2022). Most lactobacilli produce both the D- and L-chiral isomers of lactic acid. However, *L. iners* can only produce L-isomers. It should be noted that D-lactic acid directly affects host tissues by modulating the immune system and gene expression (France et al., 2022). H_2O_2 is an oxidizing chemical that is hazardous to catalase-negative bacteria, including most anaerobes. It exhibits significant antimicrobial action *in vitro*, and colonization with lactobacilli that generate H_2O_2 has been linked to a decreased incidence of BV, preterm delivery, and HIV infection (Bonneville et al., 2021). Bacteriocins are a category of potent, ribosomally produced peptides that are active against Gram-positive and Gram-negative bacteria, as well as some fungi (Mokoena et al., 2021; Darbandi et al., 2022). Biosurfactants are a structurally diverse group of surface-active molecules. They can be extremely important in lowering the ability of pathogens to attach to host cells, which is a prerequisite for the growth of biofilms (Nelson et al., 2020; Jeyanathan et al., 2021; Patel et al., 2021). Another mechanism involves competitive adhesion. Lactobacilli adhesins can cause significant adhesion to the vaginal wall. The strong adhesion of lactobacilli to the vaginal epithelium results in exclusion and rejection of harmful pathogens (Lee et al., 2021; Kawahara et al., 2022). Lactobacilli also exhibit immunomodulatory effects by increasing monocytes and macrophages which play a crucial role in the innate immune response *via* activation of Toll-like receptors (TLR) and the generation of cytokines (Mitchell et al., 2021). Specifically, lactobacilli and their derivatives can inhibit the expression of various pro-inflammatory cytokines, such as interleukin (IL)-6, IL-1β, IL-2, and tumor necrosis factor (TNF)-α, and promote the production of IL-10, which can prevent systemic and local acute inflammation (Hao et al., 2021; Hu et al., 2022a). Lactobacilli can also affect the maintenance of epithelial cell tight connections. Lactobacilli can accelerate the re-epithelialization of vaginal epithelial cells and increase the production of vascular endothelial growth factor, an essential factor in tissue healing which is recognized as an epithelial cell migration inducer (Albuquerque-Souza et al., 2021).

As mentioned above, lactobacilli in the vagina are thought to be a key defensive mechanism against infection. Consequently, lactobacilli replenishment of the vaginal microbiome is an intriguing strategy for vaginal infection prevention (van de Wijgert and Verwijs, 2020; Mei and Li, 2022). Furthermore, probiotic lactobacilli have been found to be effective in changing the vaginal microbiota and improving individual health (López-Moreno and Aguilera, 2021). There are approximately 170 species of lactobacilli, but only a few such as *L. acidophilus*, *L. brevis*, *L. crispatus*, *L. delbrueckii*, *L. fermentum*, *L. gasseri*, *L. helveticus*, *L. jensenii*, *L. johnsonii*, *L. plantarum*, *L. paracasei*, *L. reuteri*, *L. rhamnosus*, and *L. salivarius* have been used to treat vaginal infections (Joseph et al., 2021; Piccioni et al., 2021). *L. crispatus* and *L. rhamnosus* are the most often employed species,

although *L. rhamnosus* is less prevalent in the natural vaginal microbiome (Veščičík et al., 2020; Petrova et al., 2021). Furthermore, despite the fact that *L. iners* is common in the vaginal environment, it has not been used as a probiotic in the vagina. This may be due to its cultural complexity and uncertain importance (Zheng et al., 2021; Novak et al., 2022).

3 Probiotic lactobacilli in the treatment of vaginal infections

Vaginal infections have been linked to serious health consequences such as infertility, preterm birth, pelvic inflammatory disease (PID), premature rupture of membranes, and miscarriage (Huang et al., 2021; Chen et al., 2021a; Dong et al., 2022). As shown in Figure 1, vaginal infections such as BV, VVC, AV, viral infections, and other STIs are frequently linked to a vaginal flora containing a lower proportion of lactobacilli and a larger number of pathogens, leading to epithelial cell and mucosal damage in the vagina. The emergence and rapid spread of antibiotic-resistant diseases, particularly multidrug-resistant bacteria, has restricted the use of antibiotics throughout the past century (Larsson and Flach, 2022). Hence, the use of probiotic lactobacilli against pathogens has evolved as an alternative or supplementary vaginal infection therapy. In this chapter, we will outline results from *in vitro* experiments, *in vivo* animal model investigations, and clinical trials of probiotic lactobacilli in the treatment of various vaginal infections.

3.1 Probiotic lactobacilli use in BV

BV is one of the most prevalent vaginal diseases in women of reproductive age, and it is characterized by the replacement of beneficial lactobacilli by an anaerobic bacterial overgrowth of *Gardnerella vaginalis*, *Prevotella*, *Atopobium*, and *Mobiluncus* (Chen et al., 2021b; Abou Chacra et al., 2022). Even though multiple studies have demonstrated a link between BV and the presence of a variety of pathogen genera and species, the significance of these organisms in the etiology and pathophysiology of the illness remains unclear (Hu et al., 2022b). In BV therapy, probiotic lactobacilli have been provided either orally or intravaginally for the maintenance and restoration of a healthy vaginal microbiome (Basavaprabhu et al., 2020).

A considerable number of recent *in vitro* investigations have shown that lactobacilli exhibit antibacterial activity against BV pathogens. Several *Lactobacillus* strains isolated from dairy products and the vaginal ecology of healthy women from different countries have antagonistic activity against BV pathogens such as *P. bivia*, *A. vaginae*, and *G. vaginalis* (Happel et al., 2020; Kumherová et al., 2021). More specifically, the acetic acid and lactic acid generated by lactobacilli can alter the morphology of *G. vaginalis*

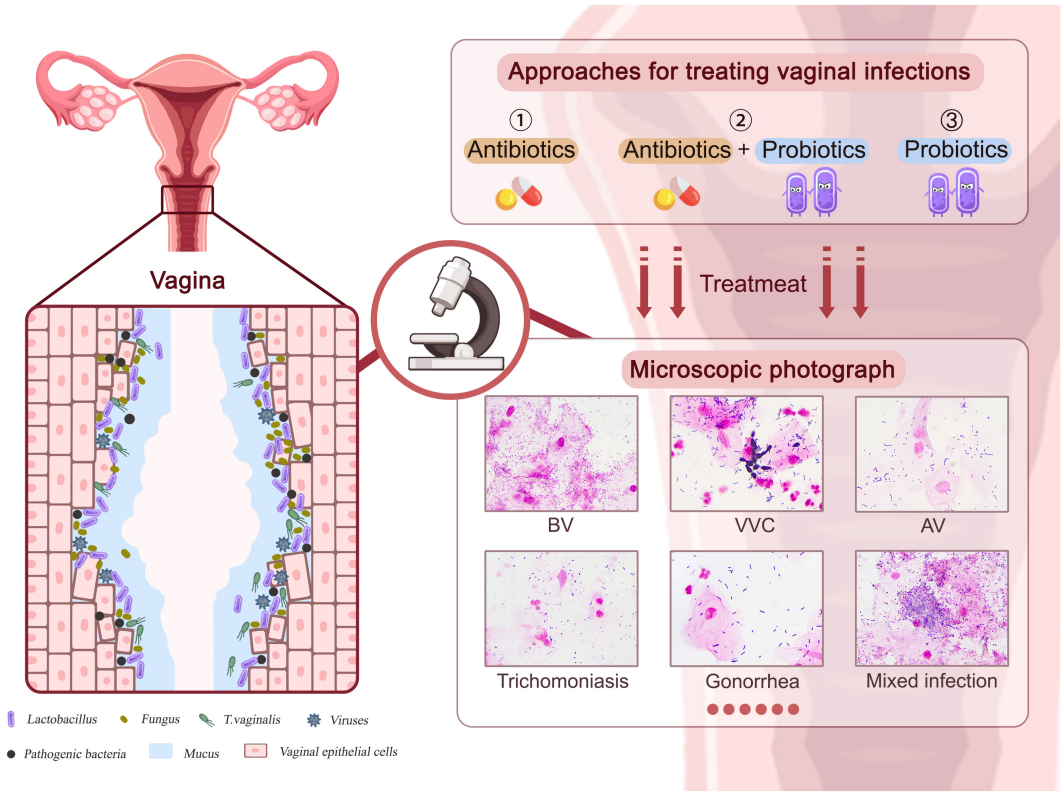

FIGURE 1
The approaches to treating various vaginal infections. Pathogens associated with BV, VVC, AV, viral infections, and other STIs can cause damage to vaginal epithelial cells and mucosa. Approaches including antibiotics, probiotics, and antibiotics plus probiotics could affect therapeutic efficacy.

cells, ultimately causing the cells to shrink or burst (Huang et al., 2022). These two chemicals can also impair the activity of the Na^+/K^+-ATPase of *G. vaginalis*, resulting in aberrant ATP metabolism and, eventually, inhibiting *G. vaginalis* growth and reproduction (Huang et al., 2022). The cell-free supernatants of lactobacilli have also been shown to dramatically reduce *G. vaginalis* biofilm formation (Qian et al., 2021). Specifically, these probiotics can influence different phases of *Gardnerella* biofilm development and exhibit the highest inhibitory impact when introduced at the early stage of *Gardnerella* biofilm formation (He et al., 2021). Lactobacilli can also prevent the adherence of vaginal infection-causing pathogens such as *Gardnerella* and *Mobiluncus* to vaginal epithelial cells by increasing the production of the proinflammatory cytokines IL-8 and IL-1β, as well as that of human β-defensin 2, and by decreasing the concentration of secretory leukocyte protease inhibitor (He et al., 2020). Quantitative PCR analysis has shown that *L. plantarum* can reduce the pathogenicity of *G.vaginalis* by repressing the expression of the genes related to virulence factors, adhesion, biofilm formation, metabolism, and antimicrobial resistance (Qian et al., 2021).

In vivo studies performed on animal models also confirm the benefits of probiotic lactobacilli on BV infection. *L. gasseri* can reduce viable *G. vaginalis* numbers, inhibit sialidase activity, regulate TNF-α and IL-1β expression, and decrease myeloperoxidase activity in experimental mouse models (Zhang et al., 2022). Similarly, in mouse vaginal tissue lysates, a combination of five lactobacilli strains can greatly inhibit *G. vaginalis* proliferation and considerably lower myeloperoxidase activity, pro-inflammatory cytokine levels (TNF-α, IL-1β, and IL-6), and nitric oxide levels (Choi et al., 2022). In experiments using a *Caenorhabditis elegans* model, *L. plantarum* strain P1 has shown potent antibacterial action against *G. vaginalis* and considerably prolonged the lifespan of *C. elegans* after exposure to infection agents (Li et al., 2022).

Based on the positive efficacy of lactobacilli in treating BV infection in animal models, clinical trials on the effectiveness of a single strain or combination of probiotics given orally or intravaginally in the treatment of BV infection have been conducted. Previously, *L. rhamnosus* GR-1 and *L. reuteri* RC-14, the most thoroughly studied vaginal probiotics, have been administered orally to BV patients and have resulted in a notable improvement in vaginal flora (Martinez et al., 2009; Vujic et al., 2013). Recent clinical trials using these two strains, however, have not achieved ideal outcomes. A randomized controlled trial conducted in Shenzhen, China, was performed to evaluate the effectiveness of metronidazole alone and oral probiotics in addition to antibiotics in the treatment of BV. When *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were administered orally for 30 days as an adjunct to metronidazole, no significant difference was seen in the total cure rate (Zhang et al., 2021). One possible cause may be that the *L. rhamnosus* GR-1 and *L. reuteri* RC-14 bacteria isolated from the vaginas of Caucasian and African American women might not work in Chinese people. Therefore, ethnicity might be a key factor. Aside from these two well-known *Lactobacillus* strains, other strains such as *L. acidophilus* GLA-14, *L. crispatus* LMG S-29995, *L. crispatus* DSM32717, *L. crispatus* DSM32720, *L. paracasei* LPC-S01, and *L. rhamnosus* HN001 have also been used orally in clinical investigations (Russo et al., 2019a; Koirala et al., 2020; Reznichenko et al., 2020; Mändar et al., 2023). These trials revealed that oral probiotics can enhance the recovery rate and relieve the symptoms of BV, as well as improve the vaginal microbial profile. As shown in Figure 2, in addition to oral delivery, intravaginal administration is becoming increasingly popular among clinical studies (Marcotte et al., 2019; Baldacci et al., 2020; Cohen et al., 2020; Armstrong et al., 2022; Mändar et al., 2023), possibly because intravaginal administration avoids the passage of lactobacilli into the highly acidic stomach, preventing loss of probiotics and accelerating the onset of effect. Compared to orally administered probiotics, which reach the vagina in approximately seven days, *Lactobacillus* strains administered intravaginally show effects in two to three days (Mogha and Prajapati, 2016). Most importantly, fueled by the draft guidance of the Food and Drug

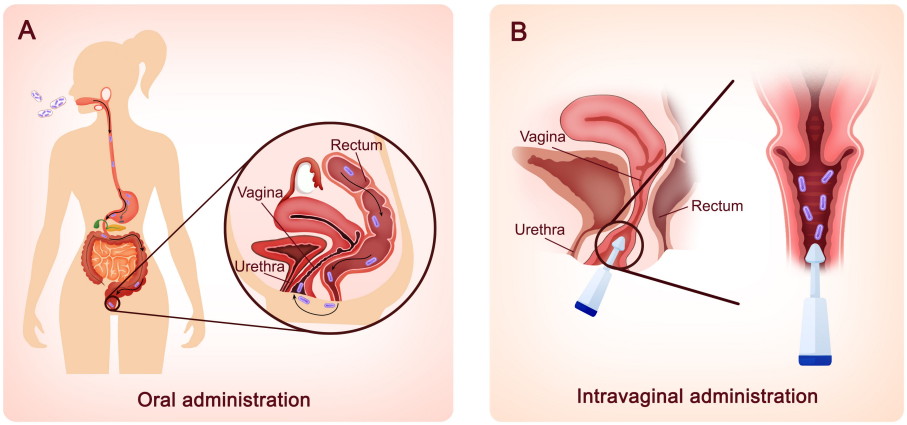

FIGURE 2
Two different routes for probiotic lactobacilli administration. **(A)** Oral administration. Probiotic lactobacilli taken orally must travel *via* the mouth, stomach, intestines, and colon before reaching the vagina *via* skin contact in the perineum. The probiotics are delivered to the vagina in approximately seven days. **(B)** Intravaginal administration. Probiotic lactobacilli can also be administered intravaginally using external devices. Within 2–3 days, lactobacillus strains manifest their effects.

Administration (FDA) to develop live biotherapeutic products (LBPs), LACTIN-V (including *L. crispatus* CTV-05) has been developed as an adjuvant therapy and might be the first microbiome-based LBP to prevent the recurrence of BV, a breakthrough in BV treatment (Cohen et al., 2020; Lagenaur et al., 2021; Armstrong et al., 2022).

3.2 Probiotic lactobacilli use in VVC

VVC is considered the second most common vaginal infection after BV, and it is mainly caused by the opportunist fungus *Candida* (Willems et al., 2020). According to published research, fluconazole is widely used in the treatment of VVC; however, its function is to inhibit the growth of *Candida* but not eradicate it (Dunaiski et al., 2022). This can lead to the emergence of fluconazole resistance. As an alternative or adjuvant therapy, probiotic lactobacilli have shown considerable promise in the treatment of VVC (Mohankumar et al., 2022; Sun et al., 2023).

Candida may undergo a morphological change from round-ovoid yeast cells to hyphal mycelial growing organisms, allowing it to thrive as both a commensal colonizer and a pathogen (McKloud et al., 2021). Studies conducted *in vitro* using vaginal yeast and lactobacilli co-cultures suggest that lactobacilli inhibit the *Candida* yeast-to-hypha switch, reducing the level in the vagina and competing with *Candida* for adhesion sites on epithelial receptors, owning to their higher affinity (McKloud et al., 2021; Spaggiari et al., 2022). For example, lactic acid secreted by lactobacilli can inhibite *Candida* overgrowth and its transition from the commensal yeast form to the pathological hyphal form by modulating *Candida* protein expression, including agglutinin-like sequence protein (AlS3) and the hypha-associated proteins (HGC1, Ece1, Hwp1and Hyr1) (Chow et al., 2021). Furthermore, inhibiting the development of pathogenic hyphal forms of *Candida* species by lactobacilli can restrict the capacity of these yeasts to create biofilms (Nelson et al., 2020; McKloud et al., 2021; Vazquez-Munoz et al., 2022). The biosurfactants extracted from lactobacilli such as iturin, lichenysin, and surfactin have the capacity to limit *Candida* biofilm formation and prevent biomass expansion (Nelson et al., 2020). Lactobacilli can also reduce *C. albicans* pathogenicity by boosting the local immune system response of human cells by modifying immune cytokines (Andrade et al., 2021; Charlet et al., 2022). In particular, the oleic acid and palmitic acid generated by *L. johnsonii* can boost the expression of IL-10 and reduce the production of TNF-α, IL-6, and IL-12 in lipopolysaccharide-stimulated macrophages (Charlet et al., 2022). It should be noted that not all *Lactobacillus* species are advantageous and protective in VVC patients (Kalia et al., 2020). As previously documented, VVC-positive women exhibited an increase in the relative abundance of *L. iners*. *L. iners* can induce a marked increase in biofilm formation by *C. albicans*, enhance hyphal growth, and upregulate expression of the typical hyphae-associated genes *hwp1* and *ece1* (Sabbatini et al., 2021). This might limit *L. iners* use in treating vaginal infections.

Recent investigations in animal models have demonstrated the efficacy of oral and intravaginal probiotic therapy in treating VVC. In a mouse experimental model, biosurfactants derived from a vaginal *L. crispatus* strain can lower viable cell counts of *C. albicans* and leukocyte influx (De Gregorio et al., 2020). Similarly, in the murine VVC model, prophylactic *L. casei*/pPG612.1-BLF treatment increased vaginal mucosa IL-17 production, decreased IL-23 levels, and led to reduced *Candida* burden after 5 days of therapy (Liao et al., 2019).

Notably, several clinical investigations have demonstrated an improvement in the treatment of VVC with or without antibiotic therapy, plus oral or intravaginal probiotic lactobacilli administration. In a randomized, double-blind, placebo-controlled study, eight weeks of probiotic lactobacilli treatment was found to be helpful for pregnant women, particularly in its ability to relieve vulvovaginal symptoms and VVC recurrences, along with decreased emotional and social discomfort (Ang et al., 2022). Similarly, oral or vaginal administration of three *L. crispatus* strains can lower the combined scores of two of the most important symptoms in VVC patients, the amount of discharge and the level of itching/irritation (Mändar et al., 2023). In addition to lactobacilli alone, the combination of lactobacilli with antibiotics is also an effective therapy for vaginal *Candida* infection. By improving the composition of vaginal flora and reestablishing vaginal microecology, probiotic lactobacilli vaginal capsules combined with clotrimazole vaginal tablets can enhance the effectiveness of treatments for simple VVC (Zeng et al., 2023). The combination of *L. acidophilus* GLA-14, *L. rhamnosus* HN001, and bovine lactoferrin dramatically improved itching and discharge in VVC patients at 3 and 6 months, and throughout the six-month follow-up, the intervention group had considerably fewer recurrences than the placebo group (Russo et al., 2019b).

3.3 Probiotic lactobacilli use in AV

AV is characterized by a sharply reduced lactobacilli level, high levels of aerobic bacteria, including *Streptococcus agalactiae*, *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis*, and vaginal inflammation (Prasad et al., 2021; Wójkowska-Mach et al., 2021). Antibiotics that target aerobic bacteria such as clindamycin, meclocycline, and kanamycin have been used to treat AV (Ma et al., 2022). However, it is highly improbable that oral use of any of the antibiotics listed above have a long-term beneficial effect on the vaginal environment. Regular and sustained use of probiotic lactobacilli is a safer method in women with AV-associated vaginal flora (Das et al., 2023).

The potential benefits of probiotic lactobacilli have been Investigated *in vitro* with AV pathogens. Extracellular vesicles, phenyl-lactic acid, bacteriocins, and exopolysaccharides, isolated from lactobacilli display remarkable antioxidant and antiproliferative activity and inhibit *S. aureus, E. faecalis, S. agalactiae*, and *E. coli* with noticeable antibiofilm activity (Xiu et al., 2020; Abdul-Rahim et al., 2021; Croatti et al., 2022; Jiang et al., 2022). Studies have shown that lactobacilli can inhibit *S. aureus* through interfering with the staphylococcal quorum-sensing system *agr*, a key regulator of virulence genes, and by suppressing the generation of toxic shock syndrome toxin-1 in *S. aureus* (Li et al., 2011; Singh et al., 2022). Most importantly, as the most

prevalent risk pathogen for both early-onset and late-onset newborn sepsis, *S. agalactiae* viability can also be inhibited by lactobacilli *via* multiple strategies. Recent studies have shown that supernatants from lactobacilli can inhibit *S. agalactiae* biofilm formation on endometrial cells (Shiroda et al., 2020; Cajulao and Chen, 2021). Lactobacilli can also lessen the pathogenicity of *S. agalactiae* by enhancing the local immunological response of human cells. For example, in a model of *S. agalactiae*-infected primary endometrial epithelial cells, the colonization of lactobacilli can decrease the expression of IL-6, which is elevated in the presence of pathogens, and increase the production of IL-1 (Chenoll et al., 2019).

The potential effectiveness of lactobacilli against AV-associated bacteria has been proven in several studies using animal models. Intravaginally administered *L. reuteri* have reduced physiological index, vaginal bacterial burden, and histopathological changes in vaginal tissues in *E. faecalis* infected mice. The probiotic *L. reuteri* can increase the expression of the anti-inflammatory cytokines Foxp3, interferon-gamma (IFN-γ) and reduce overexpression of the pro-inflammatory cytokines IL-6 and IL-1β caused by *E. faecalis* infection (Shazadi et al., 2021; Shazadi et al., 2022). Similarly, lactobacilli also have a protective effect against *E. coli* infection. By the induction of a host IFN-1 response, which in turn increases the production of cathepsin D within lysosomes to kill the pathogen, *L. crispatus* can alleviate bladder uropathogenic *E. coli* infection in a mouse model (Song et al., 2022). In mice, serial vaginal inoculation with probiotic *L. reuteri* gives partial protection against *S. agalactiae* infections, and this effect is mediated in part by mucosal immunity (Brokaw et al., 2021).

Clinical trials performed to determine whether probiotic lactobacilli can be used to treat AV have demonstrated considerable promise. In a pilot study, pregnant women ingested *L. salivarius* CECT 9145 daily from week 26 to week 38, and the treatment can reduce the number of *S. agalactiae*-positive women throughout pregnancy and the number of women with intrapartum antibiotic prophylaxis during birth (Martín et al., 2019). Similarly, in China, vaginal and rectal *S. agalactiae*-positive pregnant women who consumed two probiotic capsules containing *L. rhamnosus* and *L. reuteri* also significantly decreased the species abundance of *Enterococcus*, *Staphylococcus*, and *Streptococcus* in the vaginal flora and improved pregnancy outcomes (Liu et al., 2020). A meta-analysis also found that taking probiotics throughout pregnancy was related to lower GBS recto-vaginal colonization at 35-37 weeks and a healthy perinatal profile (Menichini et al., 2022).

3.4 Probiotic lactobacilli use in vaginal virus infections

HIV, HPV, and HSV-2 are the three most prevalent sexually transmitted viruses that have a substantial impact on female health. According to the statistics provided by UNAIDS for 2018, there were 36.9 million individuals living with HIV and 1.8 million newly diagnosed cases in 2018 (Baxi et al., 2020). More importantly, young women of reproductive age continue to be one of the most vulnerable and impacted populations, with much higher rates of HIV infection than men of the same age (De Oliveira et al., 2017). HPV can infect the stratified squamous epithelium and stimulate cellular proliferation, resulting in benign hyperplasia or in some cases, cervical cancer following prolonged, unresolved infection (Szymonowicz and Chen, 2020; Alimena et al., 2022). HSV-2 can produce genital herpes in the form of painful sores at the site of viral replication and shedding and has the capacity to penetrate the central nervous system and induce latent dorsal root ganglia infection when in touch with the genital mucosal surface (Chentoufi et al., 2022). Because each of these illnesses raises the chance of developing another STI, the epidemiology of these infections is exceedingly convoluted (Torcia, 2019).

Based on its essential role in the female vagina, lactobacilli have shown potential to relieve various viral infections *in vitro*. By blocking HIV-1-cell receptor connections, extracellular vesicles generated by lactobacilli can protect human cervico-vaginal cells. This inhibition was related to a decrease in viral envelope protein exposure due to the steric hindrance of gp120 (Costantini et al., 2022). In addition to wild-type strains, bioengineered lactobacilli that express anti-HIV molecules, such as human CD4, bn nanobodies, have also demonstrated potent anti-HIV activity and have the potential to be used as a live virucide in the vaginal mucosa of women at high risk for HIV infection (Wei et al., 2019; Kalusche et al., 2020). Lactobacilli also possess antiviral activity against HSV-2, and a vaginal microbiome that is dominated by lactobacilli can produce a considerable decrease in the load of HSV-2 in vaginal epithelial cells (Amerson-Brown et al., 2019). HPV is the most common virus among women, and it was discovered that supernatants of lysates and heat-inactivated lactobacilli can suppress the production of the human papillomavirus genes e6/e7, which are the leading cause of cervical cancer (Hu et al., 2021). Additionally, immune-related pathways in cell models indicate that the *L. casei* strain can cause the production of large quantities of IL-2, a cytokine with well-established anti-cervical cancer effects (Jacouton et al., 2019a).

In vivo studies in animal models indicate that lactobacilli also possess anti-viral properties. In a humanized mouse model, scientists have engineered *Lactobacillus acidophilus* to express the HIV-1 receptor human CD4 on the cell surface. The modified strains can block HIV-1 infection locally at the intrarectal site of infection but not systemically, reducing the efficiency of HIV-1 sexual transmission (Wei et al., 2019). Similarly, in animal models, lactobacilli have also been modified to express the granulocyte-macrophage colony-stimulating factor and IL-17A cytokine which can cause cytolytic and proliferative responses in splenocytes that are specific to cervical cancer (Jacouton et al., 2019b; Abdolalipour et al., 2022). Therefore, this may be used as a candidate HPV mucosal vaccine with cross-neutralizing activity against diverse HPV types.

Clinical investigations have been conducted to validate the effects of lactobacilli for the treatment of people afflicted with viruses; nevertheless, the findings are not always positive. In a double-blind placebo-controlled clinical trial, intervention with *L. plantarum* and *Pediococcus acidilactici* was found to be safe and to lead to increases in the CD4/CD8 ratio and reductions in sCD14 in HIV-1 infected patients (Blázquez-Bondia et al., 2022). However,

using the probiotic *Lactobacillus casei* Shirota had no discernible impact on immunological activation indicators, NK cells, CD4+ and CD8+ subpopulations, or sCD14 levels in HIV-infected patients receiving suppressive antiretroviral treatment with poor CD4+ recovery over the course of a 12-week period (Tenore et al., 2020). Recent preventive HPV vaccinations have been demonstrated to prevent genital infection with multiple HPV types; however, individuals who were infected with HPV prior to vaccination will likely see little benefit. In patients with cervical intraepithelial neoplasia (CIN) 3, an oral agent that expresses the HPV 16 E7 antigen on the surface of *L. casei*, is a breakthrough. Studies showed that 70% of patients receiving the optimized dose encountered a pathological down-grade to CIN 2 after 9 weeks of therapy, and 75% of CIN 3 patients were cured in a subsequent phase 2a clinical trial (Ikeda et al., 2019; Park et al., 2019). Comparable to engineered bacteria, long-term treatment with wild *Lactobacillus* species also demonstrates promising benefits in clinical studies against HPV infection. Long-term use of natural *Lactobacillus* species can change the CST status and increase HPV clearance in women with dysbiosis and concurrent HPV-infections, and thus may have a beneficial influence on HPV infection management (DI Pierro et al., 2021). Acyclovir is the most common first-line therapy in HSV-2 infected individuals with recurrent genital infections (Sharma et al., 2023). A randomized double-blind controlled trial comparing the effectiveness and safety of the multi-strain probiotic and acyclovir found no significant difference in resolution of episode, viral shedding, lesion healing time, or proportion of discomfort, demonstrating the great promise of probiotic lactobacilli against HSV-2 infection (Mohseni et al., 2018).

3.5 Probiotic lactobacilli use in other STIs

Apart from the above-mentioned infections, STIs such as gonorrhea, trichomoniasis, *Chlamydia* infection, *Mycoplasma* infection, and *Ureaplasma* infection can invariably result in a major burden for female patients, even though they are not often fatal (Van Gerwen et al., 2022). These STIs are associated with consequences such as PID, ectopic pregnancy, infertility, seronegative arthropathy, and neurological and cardiovascular disorders (Van Gerwen et al., 2022). STIs during pregnancy can also result in fetal or neonatal mortality, early birth, newborn encephalitis, ocular infections, and pneumonia (Glassford et al., 2020). There is an increasing concern for the level of antibiotic resistance in STIs, which is frequently underestimated by clinicians (Tien et al., 2020; Williams et al., 2021). Urgent demand exists for "antibiotic-free" techniques for the control of STIs. Acting as a significant barrier to pathogens in the promotion of female vaginal health, lactobacilli have a great potential for use against vaginal pathogens (Loeper et al., 2018).

Experiments conducted *in vitro* established that lactobacilli can protect the lower female genital tract against infection caused by *N. gonorrheae*. *L. crispatus* can decrease the adhesion and invasiveness of *N. gonorrhoeae* through reducing the expression of genes responsible for pro-inflammatory cytokines like TNF-α and CCL20 in *N. gonorrhoeae*-infected epithelial cells (Płaczkiewicz et al., 2020). Similarly, the cell surface aggregation-promoting factor from *L. gasseri* can block *Trichomonas vaginalis* adhesion to human vaginal ectocervical cells in a dose-dependent manner (Phukan et al., 2018; Malfa et al., 2023). The three main intracellular parasites in the female vagina, *Mycoplasma*, *Chlamydia*, and *Ureaplasma*, have also been shown to be inhibited from infecting the vagina by lactate, bacteriocins, and the acidic environment induced by lactobacilli (Parolin et al., 2018; García-Galán et al., 2020; Garza et al., 2021; Chen et al., 2022). Lactobacilli cells can also directly inactivate the extracellular infectious elementary body of *C. trachomatis* in a concentration-dependent manner, although to a lower extent than their supernatants, by inducing quick and dynamic membrane changes (Parolin et al., 2018).

Several studies indicate that lactobacilli can suppress *Chlamydia* infectivity in animal models, which provides valuable information for the development of lactobacilli as an additional therapy or vaccine for *Chlamydia* infection. Female BALB/c mice intravaginally administered with lactobacilli mixtures, but not a single *Lactobacillus* strain, following genital *Chlamydia* infection showed dramatically reduced levels of *Chlamydia* shedding in both the lower vaginal tract and the intestinal tract, decreased production of cytokines such as TNF-α, IFN-γ, and IL-1β in the vagina, and attenuated upper genital tract pathogenicity and inflammation (Chen et al., 2022). An engineered strain of *L. plantarum* containing the *C. trachomatis* antigen, also known as heterologous immuno-repeat 2, has the potential to be used as a mucosal booster vaccine to elicit H2-specific IgA responses in the vaginal mucosa, which is relevant to the prevention and treatment of *C. trachomatis* genital infections in female B6C3F1 mice (Kuczkowska et al., 2017).

To the best of our knowledge, just one clinical experiment has been conducted evaluating the effectiveness of probiotic lactobacilli against vaginal *Ureaplasma parvum*. Participants in the treatment group of a prospective, monocentric, randomized controlled trial took one sachet a day of a defined probiotic supplement containing four different *Lactobacillus* strains for a period of four weeks. After the intervention period, there was a significant difference in the relative abundance of *U. parvum* between the control group (3.52%) and the intervention group (0.77%) (Schenk et al., 2021).

3.6 Probiotic lactobacilli use in mixed vaginal infections

Mixed vaginal infections are characterized by the presence of at least two separate vaginal pathogens at the same time, both of which contribute to an aberrant vaginal environment and result in signs and symptoms (Qi et al., 2021). Even when two different bacteria have been detected in a co-infection, there is still a possibility that one of the infections may not be the source of the vaginal symptoms that are already present (Tumietto et al., 2019). Indeed, it has been documented that the interaction between different pathogens might affect the antibiotic sensitivity of both organisms (Gilbert et al., 2021).

Based on the essential role of lactobacilli in the vaginal microbiome, nearly all probiotic *Lactobacillus* species can combat distinct vaginal pathogens according to *in vitro* studies. Two commercially available *Lactobacillus* strains, *L. acidophilus* GLA-14 and *Lactobacillus rhamnosus* HN001, were tested in a co-culture assays against four distinct pathogens that cause both BV and AV (Bertuccini et al., 2017). *L. rhamnosus* CA15 generated H_2O_2, organic acid, and lactic acid, and showed a broad spectrum of antagonistic action and enhanced colonization resistance to urinary tract pathogens, such as *E. faecalis*, *E. coli*, *C. albicans*, *G. vaginalis*, and *S. agalactiae* (Pino et al., 2022a). Similarly, several *Lactobacillus* strains isolated from the vaginal ecology of healthy women also showed antagonistic activity against the pathogens that potentially cause BV, VVC, and AV (Kumherová et al.,2021).

Despite few animal studies have examined the effects of probiotic lactobacilli on mixed infections in the vagina, several clinical trials have examined whether probiotics can enhance the efficacy of antimicrobial treatment for co-infections. The oral probiotic product prOVag®, comprised of three *Lactobacillus* strains, decreased the recurrence rate of BV/AV mixed infections by 51% compared with placebo (Heczko et al., 2015). Adding probiotic lactobacilli to the conventional treatment of TV in patients who also had BV can also significantly raise the cure rates of both TV and BV (Sgibnev and Kremleva, 2020). Participants taking daily oral probiotic lactobacilli capsules can lead to a substantial improvement in the vaginal flora in terms of an increase in lactobacilli and a decrease in enterococci, staphylococci, *Gardnerella* spp., and *Candida* spp. in mixed vaginal infected patients (Pino et al., 2022b; Rapisarda et al., 2023).

4 Conclusion

Over the past decades, a significant amount of knowledge has been gained regarding the vaginal microbiome and how it is related to host health. A considerable amount of development and advancement have been achieved in the use of probiotic lactobacilli in the prevention and treatment of vaginal infections. The primary benefit of probiotic lactobacilli is the recovery of a healthy, natural microbiome in in the vagina, transforming it from a disease-causing, dysbiotic ecosystem to a healthy, symbiotic microbiome. However, despite the well-established safety of probiotics, not all clinical studies have met their effectiveness objectives. There is a great deal of heterogeneity across clinical trials in terms of probiotic strains/combinations used and the target population by ethnicity, age, life stage, and research methodologies. The FDA has not yet authorized any probiotics for the prevention and treatment of female vaginal infections, thus, these probiotics can only be categorized as safe and beneficial dietary supplements (Stavropoulou and Bezirtzoglou, 2020).

In recent years, studies demonstrating the efficiency of fecal microbiota transplants (FMTs) for the treatment of recurrent *Clostridioides difficile* infections have prompted research into vaginal microbiota transplants as a possible treatment for female vaginal infections (Lev-Sagie et al., 2019). Like FMTs, vaginal samples from healthy donors must be thoroughly tested to rule out infectious microorganisms to prevent severe adverse effects, particularly in immunocompromised patients (Zhang et al., 2018). As opposed to stools, which may be easily obtained from healthy donors, it is difficult to acquire enough vaginal samples from donors due to the small amount of vaginal discharge and the unwillingness of some women to donate. To overcome these restrictions, a platform including transplants of large synthetic microbial consortia, as opposed to individual probiotics or undefined community consortia that may contain infectious pathogens, has been developed. While startup companies like Vedanta Biosciences and the Federation Bio have created artificial microbiome products that are in the commercial development stage, these products are focused more on recurrent *Clostridioides difficile* infection, cancers, inflammatory bowel disease, and allergies (Seydel, 2021; Dsouza et al., 2022). For the treatment of female vaginal infections, it is possible that the synthesis of diverse microbiomes like the CSTs of healthy women will be achieved. Based on the application of emerging sequencing technologies, a synthetic microbial microbiome may have the potential to function as personalized medicine for people who possess complex and distinctive characteristics.

Author contributions

PL drafted the manuscript for publication. PL and XC prepared the draft of Figures. YL provided the microscope images of Figure 1. XC and RL reviewed and 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.

Publisher's note

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Characterization of the gut microbiome of patients with *Clostridioides difficile* infection, patients with non–*C. difficile* diarrhea, and *C. difficile*–colonized patients

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Introduction: *Clostridioides difficile* infection (CDI) is the main cause of nosocomial diarrhea in developed countries. A key challenge in CDI is the lack of objective methods to ensure more accurate diagnosis, especially when differentiating between true infection and colonization/diarrhea of other causes. The main objective of this study was to explore the role of the microbiome as a predictive biomarker of CDI.

Methods: Between 2018 and 2021, we prospectively included patients with CDI, recurrent CDI (R-CDI), non-CDI diarrhea (NO-CDI), colonization by *C. difficile*, and healthy individuals. Clinical data and fecal samples were collected. The microbiome was analyzed by sequencing the hypervariable V4 region of the 16S rRNA gene on an Illumina Miseq platform. The mothur bioinformatic pipeline was followed for pre-processing of raw data, and mothur and R were used for data analysis.

Results: During the study period, 753 samples from 657 patients were analyzed. Of these, 247 were from patients with CDI, 43 were from patients colonized with *C. difficile*, 63 were from healthy individuals, 324 were from NOCDI, and 76 were from R-CDI. We found significant differences across the groups in alpha and beta diversity and in taxonomic abundance. We identified various genera as the most significant biomarkers for CDI (*Bacteroides, Proteus, Paraprevotella, Robinsoniella*), R-CDI (*Veillonella, Fusobacterium, Lactobacillus, Clostridium*

sensu stricto I), and colonization by *C. difficile* (*Parabacteroides, Faecalicoccus, Flavonifractor, Clostridium XVIII*).

Discussion: We observed differences in microbiome patterns between healthy individuals, colonized patients, CDI, R-CDI, and NOCDI diarrhea. We identified possible microbiome biomarkers that could prove useful in the diagnosis of true CDI infections. Further studies are warranted.

KEYWORDS

CDI, *C. difficile*, microbiome, R-CDI, 16S rRNA

1 Introduction

Clostridioides difficile infection (CDI) is the main cause of nosocomial diarrhea in developed countries (Dubberke and Olsen, 2012). The clinical severity of CDI can be classified as follows: absence of symptoms; mild, moderate, and severe disease; pseudomembranous colitis; and toxic megacolon, sepsis, and death (Rupnik et al., 2009).

Risk factors associated with CDI include advanced age, hospital stay, treatment with proton pump inhibitors, and prolonged antibiotic treatment or treatment with multiple antibiotics (Bignardi, 1998). The risk factor most closely associated with disease is use of antibiotics, as it is directly related to dysbiosis of the gut microbiota, which enables germination of and colonization by *C. difficile* (Theriot and Young, 2015; Theriot et al., 2016; Thanissery et al., 2017). Dysbiosis caused by antibiotic treatment is reflected in decreased diversity and loss of specific taxa, both of which alter the variety and quantity of metabolites present in the gut (Jernberg et al., 2007; Dethlefsen et al., 2008; Jernberg et al., 2010; Britton and Young, 2014).

Age also plays an important role in the risk of CDI, as the gut microbiota becomes less diverse with age. In addition, the presence of chronic diseases and the use of multiple drugs, including antibiotics, considerably affects the microbiota, thus increasing the risk of colonization by *C. difficile* in elderly patients (Odamaki et al., 2016; Gao et al., 2018).

Because of the close relationship between gut microbiota and *C. difficile*, guidelines recommend fecal microbiota transplantation for recurrent episodes of CDI in order to restore patients' gut microbiota and prevent further recurrences (McDonald et al., 2018). However, more preventive strategies are needed.

The considerable associated health and economic burden of CDI calls for novel strategies by which CDI can be prevented in susceptible patients (Heimann et al., 2018; Reigadas Ramirez and Bouza, 2018). Profiling differences between the gut microbiota of patients with CDI, that of healthy individuals, and that of individuals with diarrhea due to other causes could help us to predict which patients are at immediate risk for CDI, which will progress better or worse, and which will experience recurrence of CDI.

One of the challenges in diagnosing CDI is that of being able to distinguish between patients with true CDI and those who are colonized (Planche and Wilcox, 2015). The search for new methods for the detection of *C. difficile* and for distinguishing true infection from colonization in diarrhea of other causes is ongoing (Kelly et al., 2020; Sandlund et al., 2020; Ng et al., 2021). Profiling the microbiome and identifying possible microbiome biomarkers could help us differentiate between infection and colonization by *C. difficile* and diarrhea of other causes (NOCDI) (Planche and Wilcox, 2015).

The main objective of this study was to explore the role of the microbiome as a predictive biomarker of true CDI.

2 Methods

2.1 Setting, design, and study population

The study was carried out at Hospital General Universitario Gregorio Marañón in Madrid (Spain), a 1,350-bed tertiary university hospital. Determination of toxigenic *C. difficile* is routinely performed on all loose stool samples from patients older than 2 years. The microbiology laboratory receives samples both from the hospital itself and from 13 outpatient centers in the same area.

We conducted a prospective study from 2018 to 2021. We enrolled patients whose stool samples were sent to the microbiology laboratory and healthy individuals. We collected clinical data and diagnostic fecal samples from all patients. The participants were classified into the following groups: healthy individuals, patients with primary CDI (CDI), patients with recurrent CDI (R-CDI), patients colonized by toxigenic *C. difficile* (colonized) and patients with diarrhea who tested negative for *C. difficile* (NOCDI).

2.2 Definitions

An episode of CDI was defined as the presence of a positive toxigenic CDI test, together with diarrhea (≥3 loose stools in 24 hours) or findings of pseudomembranous colitis by colonoscopy,

following the definitions set out in the guidelines of the Society for Healthcare Epidemiology of America (SHEA) and Infectious Diseases Society of America (IDSA) (McDonald et al., 2018).

We considered a patient to be colonized with toxigenic *C. difficile* when he/she tested positive for toxigenic *C. difficile* but did not meet clinical criteria for CDI, as defined above.

The severity of the CDI episode was defined according to the SHEA and IDSA guidelines (McDonald et al., 2018).

R-CDI was defined as CDI recurring within 8 weeks of a previous episode, provided the symptoms of the previous episode resolved after completion of initial treatment (van Prehn et al., 2021). Having new symptoms and a positive sample after 60 days was considered a new episode.

Death was considered CDI-related when there were no other attributable causes and/or it occurred within 10 days after the diagnosis of CDI and/or was due to known complications of CDI.

Healthy individuals were defined as those who did not meet any of the following criteria: body mass index lower than 17 or higher than 30, any type of disease including microbiota-related disease (cholelithiasis, colorectal cancer, hepatic encephalopathy, idiopathic constipation, inflammatory bowel disease, irritable bowel syndrome, familial Mediterranean fever, gastric lymphoma or carcinoma, arthritis, asthma, atopy, dermatitis, psoriasis, autoimmune disease, fatigue syndrome, diabetes mellitus, hypercholesterolemia, idiopathic thrombocytopenic purpura, myocardial ischemia, metabolic syndrome, behavioral disorders, multiple sclerosis, myoclonus dystonia, non-alcoholic fatty liver disease, oxalate kidney stones, Parkinson's disease), gastrointestinal disorders, immunologic disease, immunocompromise, alcohol intake higher than 50 g/day, and use of antibiotics, probiotics, immunosuppressants, proton pump inhibitors, or vaccines in the previous three months.

As for microbiome-related definitions, we considered richness as the number of different species found in a sample (Whittaker et al., 2001). Evenness was defined as the degree to which different species are similar or uniform in abundance. Diversity indicated the degree of species richness and abundance, where alpha diversity referred to the diversity within an individual and beta diversity referred to the difference in diversity between individuals (Whittaker et al., 2001).

2.3 Detection of *C. difficile*

Samples were processed using a rapid detection kit for toxigenic *C. difficile*. This rapid test involves detection of the antigen by immunochromatography (C Diff Quik-Chek Complete assay, TechLab, Blacksburg, VA, USA) and a real-time polymerase chain reaction (PCR) of the toxin B gene (XpertTM*C. difficile* Assay, GeneXpert, Cepheid, Sunnyvale, CA, USA).

In addition, all samples were cultured on *C. difficile* selective agar (bioMeriéux, Marcy l'Etoile, France). Suspected toxigenic *C. difficile* colonies were confirmed using immunochromatography (C Diff Quik-Chek Complete assay, TechLab, Blacksburg, VA, USA).

2.4 Clinical data

The demographic data collected included age and sex. Regarding clinical data, underlying conditions were recorded using the McCabe and Jackson score for prognosis of underlying diseases (McCabe and Jackson, 1962); comorbidity was graded according to the Charlson comorbidity index (Charlson et al., 1987). Other clinical data collected included antibiotic treatment, proton pump inhibitor treatment, nasogastric tube use, mechanical ventilation, surgery, and chemotherapy or radiotherapy in the month prior to CDI diagnosis. For the CDI episode, data on severity, treatment received, treatment failure, recurrence, mortality, and CDI-related mortality were recorded.

2.5 Sample processing

Immediately upon receipt, the fecal samples were homogenized, aliquoted, and stored at −80°C until the day of analysis. Total DNA was extracted from fecal samples using the Qiagen Fast QiaAmp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol with the inclusion of a physical lysis step. The sample was lysed in FastPrep-24 (MPBio, Derby, UK) with lysis matrix E tubes (MPBio, Derby, UK) twice at 6.5 m/s for 45 seconds. The hypervariable V4 region of the 16s rRNA gene was amplified by polymerase chain reaction with 515-806 primers tailed with sequences to incorporate Illumina flow cell adapters and indexing barcodes (Illumina, San Diego, USA).

Primer dimers and low-molecular-weight products were removed using Agencourt Ampure Beads (Beckman Coulter, Spain) and samples were quantified and quality checked for amplicon size using the 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Amplicons were pooled in equimolar amounts and sequenced (2 × 250) on an Illumina Miseq system (Illumina, San Diego, USA) according to standard protocols.

2.6 Data analysis

The raw data were pre-processed and grouped by operational taxonomic units (OTUs) with 97% similarity and classified taxonomically using mothur software (Patrick D. Schloss, PhD, © 2019, Michigan, USA) and SILVA and RDP Ribosomal Database Project databases. Species richness (OTUs observed), evenness (Pielou index), alpha diversity (Shannon index, inverse Simpson index), and beta diversity (Bray-Curtis distance, unweighted unifrac distance) were analyzed using mothur and R software (R Project for Statistical Computing) (R Core Team, 2021, Vienna, Austria).

Statistical analyses were performed with R (R Core Team, 2021, Vienna, Austria). Frequencies were calculated for qualitative variables, and proportions were calculated with their 95% confidence interval following a binomial distribution. For quantitative variables, the median and interquartile range (IQR) or mean and standard deviation (SD) were calculated. Microbiota

analyses were performed with R using the Bioconductor packages phyloseq, microbiome, microbiomeStat, vegan, DESeq2, and microeco.

Biomarkers were found using linear discriminant analysis effect size (LEfSe) and random forest (RF). For the LEfSe analysis, the Kruskal-Wallis non-parametric factorial rank sum test was first performed, then the pairwise groups were analyzed using the unpaired Wilcoxon rank sum test. Following these steps, linear discrimination analysis (LDA) was used. LDA score higher than 3 was used. For random forest analysis bootstrapt test number selected was 30 and 1000 trees to grow. Method for adjusted p-values was false discovery rate and Mean Decrease Gini was selected as the indicator value in the analysis.

Differences between groups were determined using the $\chi 2$ test; differences for continuous variables were assessed using the *t* test. The Mann-Whitney test was used for non-normal distributions. The normality of the distribution of continuous variables was tested using the Kolmogorov-Smirnov test with the Lilliefors correction.

3 Results

During the study period, a total of 753 samples from 657 patients were analyzed. Of these, 247 samples were obtained from 233 patients with CDI, 43 samples from 40 patients colonized with *C. difficile*, 63 samples from 63 healthy individuals, 324 samples from 264 patients with diarrhoea without *C. difficile*; and 76 samples from 57 R-CDI.

3.1 Demographic and clinical characteristics

The median age of the patients was around 70 years, with more females in all groups except NOCDI, as follows: CDI, 56.2% (131/233); colonized, 52.5% (21/40); NOCDI, 49.2% (130/264); and R-CDI, 68.4% (39/57) (p=0.053). Most healthy individuals were female (52.4%), and the median age was 32 years (range, 0-76). The most common underlying conditions were cardiovascular, metabolic, endocrine, nephro-urological, and gastrointestinal diseases (Table 1). The median Charlson comorbidity index was 4 (IQR: 2-6) in the CDI, R-CDI, and NOCDI groups and 4 (IQR: 2.8-5.2) in the colonized group. The percentage of patients with underlying diseases related to microbiota abnormalities was high in all groups, although it was higher in R-CDI: colonized, 60.0% (24/40); NOCDI, 63.6% (168/264); CDI, 67.4% (157/233); and R-CDI, 80.7% (46/57). The most common underlying diseases were diabetes mellitus and myocardial ischemia (Table 1). The lowest number of immunosuppressed patients was found among colonized patients: 32.5% (13/40) vs CDI with 36.9% (86/233), NOCDI with 49.6% (131/264), and R-CDI with 43.9% (25/57) (p=0.018) (Table 1).

The main risk factor for developing CDI was antibiotics in the month prior to sampling, with greater percentages in CDI and R-CDI: CDI, 91.5% (226/247); colonized, 86.0% (37/43); NOCDI, 83.5% (269/324); R-CDI, 93.4% (71/76) (p=0.007). Overall, the most used antibiotic groups in all patients were penicillins, third generation cephalosporins, quinolones and carbapenems (Table 2, Supplementary Table 1). With regard to the other CDI risk factors assessed, treatment with proton pump inhibitors and hospitalization were common in most patients. However, no significant differences were found between the groups for proton pump inhibitors, nasogastric tube use, mechanical ventilation, chemotherapy, radiotherapy, or dialysis (Table 2).

As for severity, most episodes were mild (CDI, 63.6%; R-CDI, 65.8%). There were three cases of toxic megacolon and two cases of pseudomembranous colitis in the CDI group. Most CDI cases were hospital-onset, healthcare facility–associated (53.0% [131/247], while in the R-CDI patients, they were community-onset, healthcare facility–associated (50.0% [38/76]; $p < 0.001$). All groups contained patients who received treatment for CDI, as follows: CDI patients, 96.7% (238/246); colonized patients, 37.2% (16/43); NOCDI patients, 4.6% (15/324); and R-CDI patients 97.4% (74/76) ($p<0.001$). Most patients were treated with vancomycin. The recurrence rate was 25.7% in the primary CDI patients (53/206), and 30.0% in R-CDI (15/50). Five CDI patients (5/233; 2.1%) had a probable CDI-related death and four (4/233; 1.7%) had a clearly CDI-related death. In the R-CDI group, 3 (3/57; 5.3%) patients had a probable CDI-related death and 1 (1/57; 1.8%) a clearly CDI-related death (Table 3).

3.2 Community structure (diversity)

Comparison of the 5 groups revealed significant differences in richness, alpha diversity, and evenness (all $p < 0.001$); these differences were maintained when the healthy individuals were removed from the comparison (all $p < 0.05$). In all cases, the R-CDI patients had the lowest richness, the lowest alpha diversity, and the lowest evenness (Figure 1).

When we stratified groups by age, all differences were maintained for those aged 16-69 years and for those over 69 years. When healthy individuals were removed from the comparison, there were significant differences in richness, alpha diversity, and evenness for those aged >69 years and in evenness for those aged 19-69 years. We found a series of significant differences, as follows: between CDI and NOCDI in richness, alpha diversity, and evenness (all $p < 0.05$); between CDI and R-CDI in richness and alpha diversity (all $p < 0.05$); between NOCDI and colonized patients in richness and evenness ($p<0.05$); and between R-CDI and colonized patients in richness and alpha diversity ($p<0.05$). No significant differences were found between CDI and colonized patients.

In all cases, we found significant differences between healthy individuals and each of the other groups, with higher richness, diversity, and evenness ($p < 0.001$) (Figure 1). Examination of each group individually revealed significant differences in alpha diversity and evenness among CDI with inflammatory bowel disease (IBD), with lower Shannon and Pielou indices. Differences in alpha diversity and evenness were also seen in patients who had taken a probiotic in the month prior to the episode, with lower Shannon, inverse Simpson, and Pielou indices than patients who had not

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TABLE 1 Clinical characteristics of patients excluded healthy subjects.

	CDI N 229	COLONIZED N 42	NOCDI N 264	RCDI N 53	p.value
INSTITUTIONALISED PATIENT	17 (7.4%)	4 (9.5%)	17 (6.5%)	4 (7.5%)	0.901
N-Miss	0	0	1	0	
HIV	10 (4.4%)	1 (2.4%)	8 (3.0%)	1 (1.9%)	0.738
SOLID ORGAN TRANSPLANT	30 (13.1%)	6 (14.3%)	54 (20.5%)	6 (11.3%)	0.105
MALIGNANCY	50 (21.8%)	6 (14.3%)	80 (30.3%)	14 (26.4%)	0.054
CARDIOLOGICAL DISEASE	171 (74.7%)	29 (69.0%)	173 (65.5%)	45 (84.9%)	0.015
PULMONARY DISEASE	56 (24.5%)	12 (28.6%)	48 (18.2%)	13 (24.5%)	0.23
GASTROINTESTINAL DISEASE	79 (34.5%)	16 (38.1%)	101 (38.3%)	26 (49.1%)	0.267
LIVER DISEASE	52 (22.7%)	7 (16.7%)	62 (23.5%)	15 (28.3%)	0.609
HEMATOLOGIC MALIGNANCIES	40 (17.5%)	10 (23.8%)	58 (22.0%)	12 (22.6%)	0.556
ENDOCRINE DISEASE	98 (42.8%)	20 (47.6%)	108 (40.9%)	24 (45.3%)	0.825
METABOLIC DISEASE	111 (48.5%)	18 (42.9%)	105 (39.8%)	28 (52.8%)	0.146
INFECTIOUS DISEASE	29 (12.7%)	3 (7.1%)	26 (9.8%)	3 (5.7%)	0.375
ALERGIC DISEASE	4 (1.7%)	1 (2.4%)	5 (1.9%)	1 (1.9%)	0.994
RHEUMATIC DISEASE	58 (25.3%)	11 (26.2%)	60 (22.7%)	16 (30.2%)	0.68
NEUROLOGICAL DISEASE	68 (29.7%)	15 (35.7%)	67 (25.4%)	17 (32.1%)	0.418
NEPHROUROLOGICAL DISEASE	89 (38.9%)	23 (54.8%)	91 (34.5%)	26 (49.1%)	0.03
IMMUNE-MEDIATED DISEASE	10 (4.4%)	3 (7.1%)	18 (6.8%)	5 (9.4%)	0.469
NUMBER OF DISEASE Median (Q1, Q3)	5.0 (3.0, 6.0)	4.5 (3.0, 6.0)	4.0 (3.0, 6.0)	5.0 (4.0, 7.0)	0.008
CHARLSON COMORBIDITY INDEX Median (Q1, Q3)	4.0 (2.0, 6.0)	3.0 (2.0, 5.0)	4.0 (2.0, 6.0)	4.0 (2.0, 6.0)	0.479
MICROBIOTA DYSBIOSIS RELATED DISEASE	153 (66.8%)	25 (59.5%)	168 (63.6%)	44 (83.0%)	0.039
DIABETES MELLITUS	73 (47.7%)	13 (52.0%)	82 (48.8%)	16 (36.4%)	0.476
COLELITHIASIS	21 (13.7%)	0 (0.0%)	17 (10.1%)	9 (20.5%)	0.061
MYOCARDIAL ISCHEMIA	37 (24.2%)	11 (44.0%)	34 (20.2%)	12 (27.3%)	0.071
AUTOIMMUNE DISEASE	5 (3.3%)	3 (12.0%)	12 (7.1%)	1 (2.3%)	0.148
ASTMA	15 (9.8%)	3 (12.0%)	10 (6.0%)	6 (13.6%)	0.319
ATOPIA	0 (0.0%)	1 (4.0%)	1 (0.6%)	0 (0.0%)	0.072
PSORIASIS	3 (2.0%)	0 (0.0%)	2 (1.2%)	0 (0.0%)	0.691
GASTRIC LYMPHOMA OR CARCINOMA	1 (0.7%)	0 (0.0%)	3 (1.8%)	0 (0.0%)	0.598
COLORRECTAL CARCINOMA	12 (7.8%)	0 (0.0%)	16 (9.5%)	5 (11.4%)	0.375
IRRITABLE BOWEL	5 (3.3%)	1 (4.0%)	7 (4.2%)	3 (6.8%)	0.778
INFLAMMATORY BOWEL DISEASE	14 (9.2%)	1 (4.0%)	20 (11.9%)	0 (0.0%)	0.076
IDIOPATHIC THROMBOCYTOPENIC PURPURA	2 (1.3%)	0 (0.0%)	1 (0.6%)	1 (2.3%)	0.715
SKIN DISEASE	2 (1.3%)	0 (0.0%)	1 (0.6%)	1 (2.3%)	0.715
PARKINSON DISEASE	10 (6.5%)	2 (8.0%)	8 (4.8%)	4 (9.1%)	0.704
HEPATIC ENCEPHALOPATHY	5 (3.3%)	0 (0.0%)	4 (2.4%)	1 (2.3%)	0.802
BEHAVIOURAL DISORDERS	2 (1.3%)	1 (4.0%)	1 (0.6%)	1 (2.3%)	0.489
CELIAC DISEASE	1 (0.7%)	0 (0.0%)	1 (0.6%)	1 (2.3%)	0.662

(Continued)

TABLE 1 Continued

	CDI N 229	COLONIZED N 42	NOCDI N 264	RCDI N 53	p.value
ARTHRITIS	9 (5.9%)	0 (0.0%)	10 (6.0%)	5 (11.4%)	0.291
METABOLIC SYNDROME	1 (0.7%)	0 (0.0%)	1 (0.6%)	1 (2.3%)	0.662
IDIOPATHIC CONSTIPATION	2 (1.3%)	2 (8.0%)	2 (1.2%)	2 (4.5%)	0.078
NON-ALCOHOLIC FATTY LIVER	4 (2.6%)	1 (4.0%)	7 (4.2%)	3 (6.8%)	0.631
NUMBER OF MICROBIOTA DYSBIOSIS RELATED DISEASE Median (Q1, Q3)	1.0 (1.0, 2.0)	2.0 (1.0, 2.0)	1.0 (1.0, 2.0)	1.0 (1.0, 2.0)	0.417
ALCOHOL INTAKE > 50GR/DAY	11 (4.8%)	1 (2.4%)	8 (3.0%)	3 (5.7%)	0.634
N-Miss	1	1	0	0	
COLECTOMY OR ILEOSTOMY	26 (11.4%)	6 (14.3%)	44 (16.7%)	5 (9.4%)	0.279
CHOLECYSTECTOMY	27 (11.8%)	5 (11.9%)	37 (14.0%)	7 (13.2%)	0.9
IMMUNOCOMPROMISED	87 (38.0%)	11 (26.2%)	131 (49.6%)	25 (47.2%)	0.007

CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile diarrhoea*; RCDI, recurrent CDI.
N-Miss, Number of cases with no information.
Underlined values are significant p values (lower than 0.05).

taken probiotics (Supplementary Table 2). No significant differences in alpha diversity were seen in CDI, colonized patients, or R-CDI related to the presence or absence of toxin detected by Quick Check.

As for the NOCDI group, we found significant differences due to various factors, as follows: higher diversity and evenness values in patients over 69 years old; less diversity in patients who received antibiotics; higher diversity values in patients with underlying

TABLE 2 CDI development risk factors.

	CDI N 255	COLONIZED N 44	NOCDI N 324	RCDI N 67	p.value
ADMITTED PATIENT	174 (68.2%)	24 (54.5%)	260 (80.2%)	25 (37.3%)	< 0.001
ANTIBIOTIC TREATMENT	233 (91.4%)	38 (86.4%)	269 (83.5%)	63 (94.0%)	0.012
N-Miss	0	0	2	0	
NUMBER OF ANTIBIOTIC Median (Q1, Q3)	2.0 (1.0, 4.0)	2.0 (1.0, 3.0)	3.0 (1.0, 4.0)	2.0 (1.0, 3.8)	0.251
PROTON PUMP INHIBITOR TREATMENT	220 (86.3%)	36 (83.7%)	269 (83.0%)	56 (84.8%)	0.76
N-Miss	0	1	0	1	
NASOGASTRIC TUBE	34 (13.3%)	7 (15.9%)	51 (15.7%)	6 (9.1%)	0.512
N-Miss	0	0	0	1	
MECANIC VENTILATION	37 (14.5%)	9 (20.5%)	53 (16.4%)	8 (12.1%)	0.622
N-Miss	0	0	0	1	
SURGERY	44 (17.3%)	11 (25.0%)	66 (20.4%)	12 (18.2%)	0.589
N-Miss	0	0	1	1	
CHEMOTHERAPY OR RADIOTHERAPY	42 (16.6%)	6 (13.6%)	78 (24.1%)	10 (14.9%)	0.06
N-Miss	2	0	0	0	
DIALYSIS	10 (3.9%)	4 (9.1%)	8 (2.5%)	5 (7.5%)	0.066
IMMUNOSUPPRESS TREATMENT	87 (34.3%)	15 (34.1%)	158 (48.8%)	22 (32.8%)	0.001
N-Miss	1	0	0	0	
ANTIFUNGIC TREATMENT	32 (12.6%)	5 (11.6%)	54 (16.7%)	8 (11.9%)	0.445
N-Miss	1	1	1	0	

CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile diarrhoea*; RCDI, recurrent CDI. N-Miss, Number of cases with no information. CDI development risk factor in the month prior to sample collection.
Underlined values are significant p values (lower than 0.05).

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TABLE 3 Patients'evolution after sample collection.

	CDI N 255	COLONIZED N 44	NOCDI N 324	RCDI N 67	p.value
CDI-RELATED DEATH					<0.001
No_related	5 (2.0%)	3 (6.8%)	0	3 (4.5%)	
Probably_related	5 (2.0%)	0 (0.0%)	0	3 (4.5%)	
Clearly_related	4 (1.6%)	0 (0.0%)		1 (1.5%)	
TREATMENT FAILURE	5 (2.2%)	0 (0.0%)	0	0 (0.0%)	
N-Miss	26	32	324	10	
30 DAYS MORTALITY	19 (7.5%)	5 (11.4%)	11 (3.4%)	3 (4.5%)	0.053
90 DAYS MORTALITY	35 (13.7%)	7 (15.9%)	35 (10.8%)	7 (10.4%)	0.590
RECURRENCE	51 (25.8%)	0	0	20 (48.8%)	0.004
N-Miss	31	32	263	12	

CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile diarrhoea*; RCDI, recurrent CDI.N-Miss, Number of cases with no information. CDI probably related death, Death was considered CDI-related when there were no other attributable causes and/or it occurred within 10 days after the diagnosis of CDI and/or was due to known complications of CDI.
Underlined values are significant p values (lower than 0.05).

disease related to gut microbiota abnormalities and metabolic or cardiac disease; and lower diversity in patients with hematological disease, colectomy or ileostomy, colorectal cancer, or being immunosuppressed (Supplementary Table 2). Finally, in the R-CDI group, the group which presented the lowest microbiome diversity compared to the other CDI, colonized and healthy groups, we also found some differences within the R-CDI group. Patients presenting with colorectal cancer and colectomy or ileostomy had significantly higher diversity and evenness values than R-CDI patients without this condition, except for evenness in colorectal cancer (Supplementary Table 2).

We found significant differences in beta diversity between all the groups in general and in paired terms between all the groups except colonized patients and R-CDI (Figure 2). Regarding the homogeneity of the variance of the intra-patient samples, we found that the most homogenous patients were the healthy individuals, followed in decreasing order by CDI, R-CDI, colonized patients, and NOCDI patients, who were the most heterogeneous (Figure 2).

3.3 Community composition (relative abundance of taxa)

Regarding the relative abundance of the different taxonomic groups, we found that the major phylum, in all groups except CDI, was Firmicutes (healthy, 51.13%; NOCDI, 41.42%; colonized, 49.06%; and R-CDI, 39.86%), followed by Bacteroidetes (healthy, 31.23%; NOCDI, 36.37%; colonized, 25.09%; and R-CDI, 26.03%); in the CDI patients, the most common phylum was Bacteroidetes (39.59%), followed by Firmicutes (37.53%) (p<0.001). Differences in relative abundance between the groups were significant for Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and

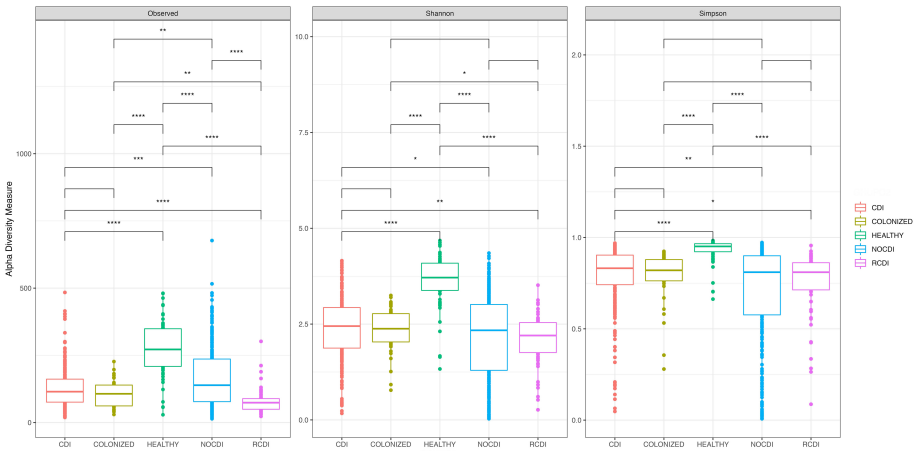

FIGURE 1
Box plot of Alpha diversity index (Shannon and Simpson) and Richness index (Observed) in all groups of patients. CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile* diarrhoea; RCDI, recurrent CDI; Colonized, patients colonized with *C. difficile*; Healthy, Healthy subjects. *, p <= 0.05; **, p <= 0.01; ***, p <= 0.001; ****, p <= 0.0001.

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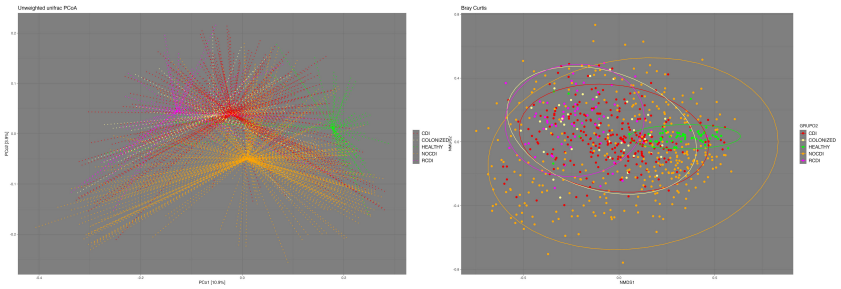

FIGURE 2
Beta- diversity. Left: Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances. Right: Non-metric multidimensional scaling (NMDS) plot based on the Bray-Curtis dissimilarity index. CDI, *Clostridioides difficile* infection; NOCDI, No *C. difficile* diarrhoea; RCDI, recurrent CDI; Colonized, patients colonized with *C. difficile*; Healthy, Healthy subjects.

Proteobacteria. In the healthy individuals, we found a higher proportion of Actinobacteria and a lower proportion of Proteobacteria than in the other groups (p<0.001). When we compared the CDI, colonized, NOCDI, and R-CDI groups, we still found significant differences in Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Table 4).

Within the taxonomic family level, we found that Bacteroidaceae was the most abundant in the NOCDI, colonized, and CDI groups (NOCDI, 27.11%; colonized, 18.56%; and CDI, 29.99%), followed by Enterobacteriaceae (NOCDI, 15.29%; colonized, 15.26%; and CDI, 14.79%). However, in the healthy patients, the most abundant family was Lachnospiraceae (25.06%), followed by Bacteroidaceae (19.86%), and in the R-CDI patients, the most abundant was Enterobacteriaceae (23.06%), followed by Bacteroidaceae (20.14%). (Table 5) (Figure 3)

When we reach the taxonomic level of genus, we find the following differences in abundance between the different groups (Figure 4).

3.3.1 CDI vs healthy

For CDI patients vs. healthy, we found a significantly lower abundance in *Bifidobacterium, Collinsella, Slackia, Blautia, Butyrivibrio, Clostridium_XlVb, Coprococcus, Dorea, Fusicatenibacter, Ruminococcus, and Faecalibacterium, and a greater abundance of Staphylococcus, Enterococcus, Lactobacillus, Streptococcus, Parvimonas, Clostridium_XlVa, Robinsoniella, Peptostreptococcus, Clostridium_XVIII, Coprobacillus, Veillonella, Fusobacterium, Campylobacter, Proteus, Pseudomonas*, and *Akkermansia* (all p–0.05).

3.3.2 CDI vs NOCDI

For CDI vs. NOCDI, we found genera with significant differences between the two groups. Decreases were recorded for *Dysgonomonas, Streptococcus, Clostridium_IV, Ruminococcus, Clostridium_XIVb, Megamonas, Acinetobacter*, and *Enterococcus*. Increases were recorded for *Blautia, Anaerostipes, Butyrivibrio, Clostridium_XlVa, Coprococcus, Robinsoniella, Butyricicoccus, Coprobacillus, Fusobacterium, Campylobacter*, and *Akkermansia* (all p<0.05).

3.3.3 CDI vs colonized

For CDI vs. colonized, we recorded a reduction in *Faecalicoccus, Proteus, Weissella* and an increase in *Staphylococcus, Clostridium_XlVb, Coprococcus, Peptostreptococcus, Olsenella, Butyricicoccus, Robinsoniella, Megasphaera*, and *Alloprevotella* (all p<0.05).

3.3.4 Colonized vs NOCDI

For colonized patients vs. NOCDI, we found significant differences, such as the decrease in the genera *Clostridium XIVb, Coprococcus, Staphylococcus*, and *Megasphaera* and a significant increase in *Coprobacillus, Faecalicoccus, Weisella*, and *Fusobacterium* (all p<0.05).

TABLE 4 Mean relative abundance (%) in each group at phylum level.

Phylum	CDI	COLONIZED	HEALTHY	NOCDI	RCDI	p.adj	p.adj without healthy
Actinobacteria	2.70	1.54	12.75	2.92	1.08	< 0.001	0.015
Bacteroidetes	**39.59**	25.09	31.23	36.37	26.03	< 0.001	0.003
Firmicutes	37.53	**49.06**	**51.13**	**41.42**	**39.86**	< 0.001	0.006
Fusobacteria	0.83	1.76	0.00	0.31	2.66	< 0.001	
Proteobacteria	16.20	17.11	2.96	16.90	24.77	< 0.001	0.001
Verrucomicrobia	3.15	5.43	1.93	2.09	5.59		

CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile diarrhoea*; RCDI, recurrent CDI.
Bold values are the most abundant phyla in that group of patients.

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TABLE 5 Mean relative abundance (%) in each group at family level.

Phylum	Family	CDI	COLONIZED	HEALTHY	NOCDI	RCDI	p.adj	p.adj without healthy
Actinobacteria	Actinomycetaceae	0.10	0.05	0.04	0.34	0.03	0.010	< 0.001
	Bifidobacteriaceae	1.73	1.07	8.01	1.52	0.92	< 0.001	
	Coriobacteriaceae	0.85	0.41	4.70	1.02	0.11	< 0.001	< 0.001
Bacteroidetes	Bacteroidaceae	**29.99**	**18.56**	19.86	**27.11**	20.14	0.018	
	Porphyromonadaceae	4.86	3.46	2.96	4.72	2.76	< 0.001	< 0.001
	Rikenellaceae	2.63	2.05	2.93	3.12	0.99	< 0.001	< 0.001
Firmicutes	Bacillales_Incertae_Sedis_XI	0.02	0.01	0.01	0.02	0.01		< 0.001
	Enterococcaceae	7.25	6.97	0.26	14.75	4.20	< 0.001	
	Clostridiaceae_1	0.25	0.49	0.35	0.40	1.00	< 0.001	< 0.001
	Lachnospiraceae	10.31	10.97	**25.06**	8.07	11.96	< 0.001	< 0.001
	Peptostreptococcaceae	2.43	2.49	0.95	0.32	3.65	< 0.001	< 0.001
	Ruminococcaceae	5.70	6.98	14.99	5.24	3.17	< 0.001	0.009
	Erysipelotrichaceae	0.66	2.02	0.05	0.59	0.63	< 0.001	< 0.001
	Acidaminococcaceae	2.41	3.06	3.14	2.03	2.24	0.007	0.003
	Veillonellaceae	5.50	10.40	2.28	3.68	8.95	< 0.001	< 0.001
Fusobacteria	Fusobacteriaceae	0.83	1.76	0.00	0.31	2.66	0.003	
Proteobacteria	Sutterellaceae	0.37	0.22	0.47	0.38	0.45	< 0.001	
	Desulfovibrionaceae	0.51	0.68	0.27	0.45	0.44	0.034	
	Enterobacteriaceae	14.79	15.26	2.12	15.29	**23.06**	< 0.001	0.005

CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile diarrhoea*; RCDI, recurrent CDI.
Bold values are the most abundant family in that group of patients.
Underlined values are p values lower than 0.001.

Of note, genera, such as *Enterococcus, Fusobacterium, Proteus, Faecalicoccus, Veillonella*, and *Akkermansia* and some *Clostridium* increased in all groups with respect to healthy individuals, and some of these genera maintained the same differences between the CDI and colonized groups vs. NOCDI. Other groups maintained the opposite pattern, and in the groups with *C. difficile*, several genera were less abundant, for example, *Bifidobacterium, Collinsella, Olsenella, Blautia, Butyrivibrio, Slackia, Coprococcus, Fusicatenibacter*, and *Megamonas*.

As for similarity between the OTUs of the different groups, we found that CDI and NOCDI shared the highest number of OTUs (4,523), followed by NOCDI and healthy individuals (3,296); the lowest values were found for R-CDI and healthy (1,172), colonized and healthy (1,128), and R-CDI and colonized (1,007).

3.4 Biomarker analysis: linear discrimination analysis and random forest analysis

The biomarker search was based on a linear discrimination analysis, which revealed various genera to be possible discriminating biomarkers for patients with high scores (> 3).

The potential biomarkers identified for colonization were *Parabacteroides, Faecalicoccus, Flavonifractor*, and *Clostridium_XVIII*. For CDI group, the biomarkers were *Bacteroides, Proteus, Paraprevot ella, Butyrivibrio, Senegalimassilia, Holdemanela, Robinsoniella*, and *Eggerthellas*. In R-CDI patients, they were *Veillonella, Fusobacterium, Lactobacillus, Enterococcus, Clostridium_XIVa*, and *Clostridium_sensu_stricto*. Finally, in the healthy patients, we identified, among others, *Bifidobacterium, Blautia, Faecalibacterium*, and *Roseburia* (Figures 5, 6).

We also performed a random forest analysis, which identified the same discriminating genera for each patient (Figure 5).

4 Discussion

In the present study, we outlined the differences in the microbiota of five groups of patients (healthy, colonized, CDI, NOCDI, and R-CDI) from a large cohort, finding marked differences that could be useful as diagnostic markers of true CDI infection.

Several studies have characterized microbiota in patients with CDI, although the groups compared were very heterogeneous. Some

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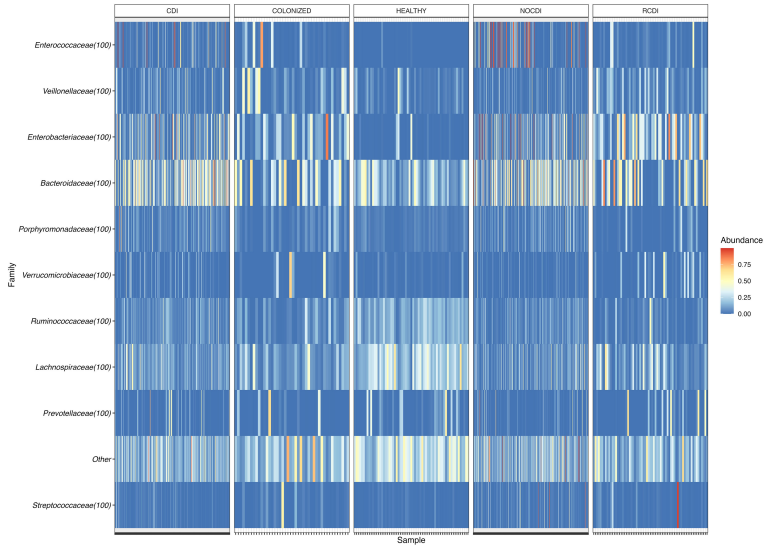

FIGURE 3
Heatmap of family relative abundance in each sample. CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile* diarrhoea; RCDI, recurrent CDI; Colonized, patients colonized with *C. difficile*; Healthy, Healthy subjects.

studies based their control group on "healthy" individuals, which is a confusing term, since "healthy" can include patients with other diseases but not CDI or diarrhea, patients receiving different treatments for their underlying disease, persons without diarrhea who have not taken antibiotics, and even stool donors for fecal transplantation (Schubert et al., 2014; Milani et al., 2016; Amrane et al., 2019; Sánchez-Pellicer et al., 2021). We compared various groups, including both healthy individuals and patients with diarrhea in whom *C. difficile* has been ruled out but who share diseases, treatments, and other characteristics that may more closely resemble those of CDI patients.

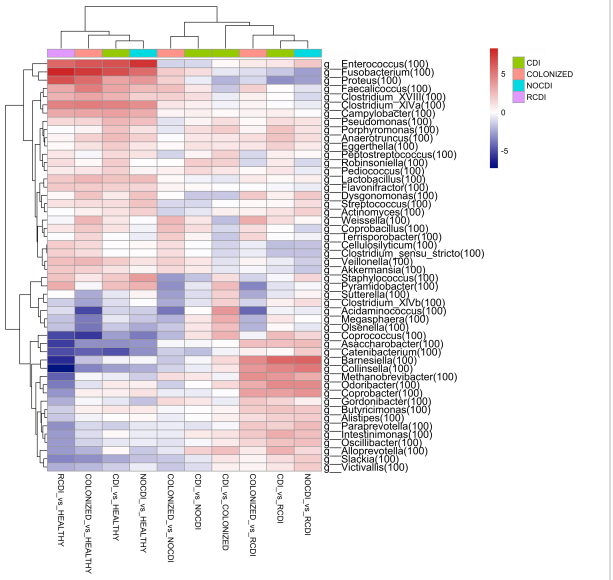

FIGURE 4
Genera heatmap of Log2 Fold Change represent differentially abundance genus between groups. CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile* diarrhoea; RCDI, recurrent CDI; Colonized, patients colonized with *C. difficile*; Healthy, Healthy subjects.

The comparison with healthy individuals revealed a considerable difference in the state of the intestinal microbiota compared with patients with gastrointestinal disorders, whether due to *C. difficile* or not, namely, greater richness, alpha diversity, and evenness. These differences are also evident in beta diversity, where the healthy individuals group differs significantly from all the others.

It should also be noted that we did find differences in alpha diversity and richness between the CDI and NOCDI groups, in contrast with observations reported elsewhere, perhaps because our sample was larger than those of other studies (Sangster et al., 2016; Jeon et al., 2019; Herrera et al., 2021). Most studies comparing the microbiota of patients with CDI with that of patients with diarrhea due to other causes and with the microbiota of persons colonized by *C. difficile* include a low number of individuals for each study group (Zhang et al., 2015; Allegretti et al., 2016; Milani et al., 2016; Sangster et al., 2016; Jeon et al., 2019; Herrera et al., 2021). This observation is very important, since the intestinal microbiome is constantly changing due to factors such as diet, environmental factors, sports, and medication (Dominianni et al., 2015; Rashid et al., 2015; Mohr et al., 2020). Therefore, in order to obtain more reliable results, it is necessary to include a considerable number of persons in each group, as in our study.

Consistent with the literature, the lowest diversity and richness values were recorded for patients with R-CDI (Chang et al., 2008; Allegretti et al., 2016; Gazzola et al., 2020). Both our data and that of other authors show the existing damage to the microbiota or dysbiosis in patients with diarrhea due to *C. difficile* or other causes and that of patients colonized by *C. difficile* (Sangster

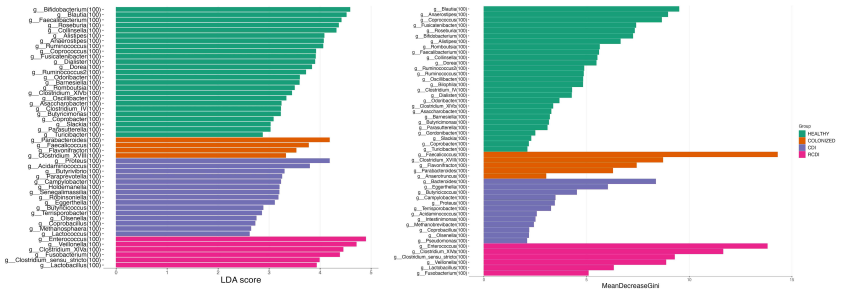

FIGURE 5
List of possible genera biomarkers that enable discrimination between groups. Left (Linear discriminant analysis (LDA). Right Random Forest (RF)). CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile* diarrhoea; RCDI, recurrent CDI; Colonized, patients colonized with *C. difficile*; Healthy, Healthy subjects.

et al., 2016; Sokol et al., 2018; Crobach et al., 2020; Sánchez-Pellicer et al., 2021; Wan et al., 2022). In addition, the greater damage to the microbiota in patients with repeated episodes of CDI is highlighted. In this study, we observed that patients with CDI and IBD presented less alpha diversity and evenness than CDI patients without IBD.

Interestingly, we found that among patients with diarrhea, there were markedly significant differences in beta diversity between those with diarrhea due to CDI and those with diarrhea due to other causes (NOCDI). Some authors have reported similar findings (Schubert et al., 2014; Sangster et al., 2016; Han et al., 2020), whereas others have not (Antharam et al., 2013; Jeon et al., 2019; Gazzola et al., 2020; Herrera et al., 2021). Moreover, we observed that beta diversity differed significantly in patients with CDI and patients colonized by *C. difficile*. This finding has not been reported in the few studies carried out that include these groups, probably owing to the limited number of samples analyzed (Han et al., 2019; Crobach et al., 2020; Sánchez-Pellicer et al., 2021).

In agreement with the literature (Crobach et al., 2020), we observed that alpha and beta diversity were significantly altered by specific CDI risk factors (antibiotic treatment in the previous month, previous episodes of CDI, treatment with proton pump inhibitors or probiotics in the previous month, nasogastric tube in the previous month), colorectal cancer, solid organ transplant, neoplasia, and hematological disease.

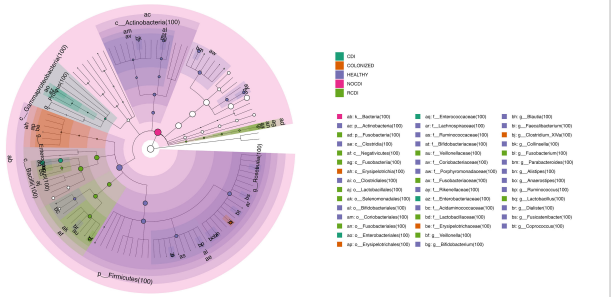

FIGURE 6
Cladogram with taxa different abundance between groups CDI, *Clostridioides difficile* infection; NOCDI, Non *C. difficile* diarrhoea; RCDI, recurrent CDI; Colonized, patients colonized with *C. difficile*; Healthy, Healthy subjects.

As for differences in abundance, we found that, except in patients with CDI, the most abundant phylum in all five groups was Firmicutes followed by Bacteroidetes. In the CDI group, this order was inverted, with Bacteroidetes being the most abundant phylum, as reported elsewhere (Antharam et al., 2013; Sánchez-Pellicer et al., 2021), although the opposite has also been observed (Zhang et al., 2015; Han et al., 2019; Han et al., 2020). These contradictions in the literature may be due to the different methodologies used for sample extraction, amplification, and analysis, together with the limited number of samples processed. Consistent with other observations in the literature, we also found a decrease in Actinobacteria and an increase in Proteobacteria in patients with CDI compared to healthy individuals (Amrane et al., 2019; Han et al., 2019; Han et al., 2020; Sánchez-Pellicer et al., 2021). When we assessed the genera by groups, we found that *Ruminococcus*, *Clostridium*_IV, *Streptococcus*, *Acinetobacter*, and *Megamonas* decreased in the CDI group compared to CDI. The opposite occurred *Blautia* and *Coprococcus*, which increased compared to NOCDI. Other genera that were more abundant in CDI compared to both healthy individuals and NOCDI were *Lactobacillus, Clostridium_XIVa, Robinsoniella, Coprobacillus, Fusobacterium, Campylobacter*, and *Akkermansia*. The decrease in butyrate-producing bacteria, such as *Ruminococcus*, and the increase in genera of lactic acid–producing bacteria, such as *Blautia*, *Lactobacillus*, and *Fusobacterium*, has been associated with an increased risk of CDI (Vakili et al., 2020).

Significant differences were found for the presence of genera observed in CDI patients versus NOCDI or those for CDI patients versus colonized patients. This enabled us to carry out an exhaustive search for microbial biomarkers that could help respond to the as yet unresolved need to identify diarrhea truly caused by *C. difficile*. Few studies have attempted to outline these differences.

Among the few studies comparing the intestinal microbiota of patients with CDI and *C. difficile* colonization, the number of patients included is lower than in ours, with very few significant differences between the two groups (Zhang et al., 2015; Han et al., 2019; Crobach et al., 2020; Sánchez-Pellicer et al., 2021). Furthermore, the study conducted by Han et al. did not discriminate between toxigenic and non-toxigenic *C. difficile* strains, meaning that the *C. difficile* study groups included

patients with toxigenic and non-toxigenic *C. difficile*, thus making it difficult to interpret and extrapolate their results (Han et al., 2019). We identified a series of changes in the microbiota that enabled us to outline the differences between colonized patients and those with true CDI infection. In CDI patients, we observed two genera that decreased significantly, namely, *Faecalicoccus* and *Proteus*, both of which are increased in colonized patients. We also found a significant increase in *Clostridium_XIVb, Coprococcus, Staphylococcus*, and *Robinsoniella* among patients with true CDI. These differences could facilitate appropriate categorization of these groups of patients, which, as mentioned above, remains unresolved.

To the best of our knowledge, this is the first study to have carried out such a broad comparison to identify biomarkers that differentiate between so many subgroups of patients, especially between CDI patients and colonized patients. The few previous studies on this area focused on finding biomarkers to be able to distinguish between CDI and healthy individuals (Antharam et al., 2013), CDI and NOCDI (Schubert et al., 2014; Herrera et al., 2021), and CDI and R-CDI (Allegretti et al., 2016).

Our results were also consistent with those of previous studies. We observed a decrease in certain families or genera involved in the production of butyric acid, such as Ruminococcaceae (*Faecalibacterium* and *Ruminococcus*) and Lachnospiraceae (*Blautia* and *Coprococcus*), both in CDI patients and in colonized patients (Antharam et al., 2013; Zhang et al., 2015; Milani et al., 2016). For CDI and colonized patients, we also observed a decrease in *Bifidobacterium*, a genus with anti-inflammatory and antimicrobial properties. (Imaoka et al., 2008) We identified a series of genera that could be of use as biomarkers. These included *Parabacteroides*, *Faecalicoccus*, *Flavonifractor*, and *Clostridium_XVIII* for the colonized group, and *Veillonella*, *Fusobacterium*, *Lactobacillus, Enterococcus, Clostridium_XIVa*, and *Clostridium_sensu_sricto* for the R-CDI group. We also identified genera that better define true CDI infection, such as *Bacteroides*, *Proteus*, *Paraprevotella*, *Robinsoniella*, and *Eggerthella*.

Our study is limited by its single-center design, which requires our results to be validated in multicenter studies. However, as our sample is the largest microbiome analysis of CDI to date, our results can be considered robust. Basing our study on 5 different groups of patients enabled us to analyze the different microbiota profiles and thus better characterize those microbiota abnormalities caused solely by *C. difficile* infection. We also separated primary CDI from R-CDI, where the progressive damage that *C. difficile* produces in the intestinal microbiota is evident.

In conclusion, we found several genera that could be used as biomarkers to differentiate between *C. difficile*–colonized patients, true CDI episodes, and diarrhea due to other causes. Our approach will enable us to improve the diagnosis, management, and treatment of CDI. Further research is warranted.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB57947.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Hospital General Universitario Gregorio Marañón in Madrid (number MICRO.HGUGM.2016-029). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ER and EB designed the study. SV-C performed the collection of data and samples. SV-C and NG performed the data analysis. SV-C, ER, EB, MO, LA, MM, PM, contributed to data interpretation. SV-C wrote the first draft of the manuscript. SV-C, ER, EB, MO, LA, MM, PM, AF and LV, contributed with the intellectual content and approved the final draft. SV-C, ER, and EB had access to and verified the underlying study data. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Conflict of interest

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

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Supplementary material

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Mapping the human oral and gut fungal microbiota in patients with metabolic dysfunction-associated fatty liver disease

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Metabolic dysfunction-associated fatty liver disease (MAFLD) is a phenotype of liver diseases associated with metabolic syndrome. The pathogenesis MAFLD remains unclear. The liver maintains is located near the intestine and is physiologically interdependent with the intestine *via* metabolic exchange and microbial transmission, underpinning the recently proposed "oral-gut-liver axis" concept. However, little is known about the roles of commensal fungi in the disease development. This study aimed to characterize the alterations of oral and gut mycobiota and their roles in MAFLD. Twenty-one MAFLD participants and 20 healthy controls were enrolled. Metagenomics analyses of saliva, supragingival plaques, and feces revealed significant alterations in the gut fungal composition of MAFLD patients. Although no statistical difference was evident in the oral mycobiome diversity within MAFLD and healthy group, significantly decreased diversities were observed in fecal samples of MAFLD patients. The relative abundance of one salivary species, five supragingival species, and seven fecal species was significantly altered in MAFLD patients. Twenty-two salivary, 23 supragingival, and 22 fecal species were associated with clinical parameters. Concerning the different functions of fungal species, pathways involved in metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, and carbon metabolism were abundant both in the oral and gut mycobiomes. Moreover, different fungal contributions in core functions were observed between MAFLD patients and the healthy controls, especially in the supragingival plaque and fecal samples. Finally, correlation analysis between oral/gut mycobiome and clinical parameters identified correlations of certain fungal species in both oral and gut niches. Particularly, *Mucor ambiguus*, which was abundant both in saliva and feces, was positively

correlated with body mass index, total cholesterol, low-density lipoprotein, alanine aminotransferase, and aspartate aminotransferase, providing evidence of a possible "oral-gut-liver" axis. The findings illustrate the potential correlation between core mycobiome and the development of MAFLD and could propose potential therapeutic strategies.

KEYWORDS

metabolic dysfunction-associated fatty liver disease, oral mycobiome, gut mycobiome, oral-gut axis, metagenomics

1 Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a condition of fat accumulation in the liver in combination with obesity, type 2 diabetes, or metabolic dysfunction (Demir et al., 2022). Formerly known as nonalcoholic fatty liver disease, MAFLD has a global prevalence of 25%, and has surged to 29.2% in China's mainland (Fan et al., 2021; Lu et al., 2022; Zhuge et al., 2022). The prevalence will continue to increase with the growing numbers of obese individuals with metabolic syndrome. The pathophysiology of MAFLD is complex and includes several mechanisms, with metabolic syndrome and insulin resistance playing major roles (Kuchay et al., 2020; Sakurai et al., 2021).

Accumulating evidence has indicated the role of gut microbiota in the development and progression of MAFLD, since the "gut-liver axis" was first proposed by Marshall in 1987(Volta et al., 1987). Given the direct connection between the intestine and the liver *via* the portal vein, the liver is more susceptible to gut microbiota, bacterial products, endotoxins, and microbiome inflammatory molecules(Jiang et al., 2020). Intestinal bacteria could migrate through the portal vein into the liver and lead to the abnormal activation of the immune system(Milosevic et al., 2019; Albillos et al., 2020). In turn, this would lead to an inflammatory response in patients with MAFLD. Most studies have focused on gut bacteria, with little attention given to fungal alterations(Heng et al., 2022).

The human gastrointestinal tract harboring a complex diversity of microorganisms, including bacteria, fungi, viruses, protozoa, and archaea. Fungi constitute only approximately 0.1% of the human gut microbiome(Huffnagle and Noverr, 2013). Yet, they are an indispensable part of this microbiome and have vital roles in multiple physiological processes. The oral cavity is the second-largest microbial community in the human body(Pathak et al., 2021). It is among the most diverse microbial ecosystems in the human body, together with the gut, harboring over 600 bacterial species and 100 fungal species(Fidel et al., 2021). Recent studies have supported a possible pathogenic impact of the oral microbiome and oral disease on gastrointestinal and liver diseases. For example, preclinical and clinical research have shown periodontitis, a common periodontal disease initiated by oral microbial dysbiosis, can exacerbate MAFLD(Albuquerque Souza and Sahingur, 2022; Wang et al., 2022).

The oral microorganisms vary across niches, such as dental plaque, tongue, saliva, and gingival sulcus. Oral microorganisms could translocate to the gut and further disseminate from the gut lumen to the liver *via* the portal vein and cause liver disease(Park et al., 2021; Zhang et al., 2022). Since the gastrointestinal tract receives a constant flow from mouth to anus, it is possible that the microbiota or their by-products in the oral cavity disseminate to the liver after the oral-gut microbial translocation, and thus contribute to the pathogenesis of liver diseases. For example, *Porphyromonas gingivalis*, which causes periodontitis, is associated with liver inflammation(Albuquerque Souza and Sahingur, 2022; Wang et al., 2022). However, the oral fungal microbiome has received much less attention, albeit its ecological and clinical significance.

This study is the first characterization of the oral and gut fungal microbiomes using metagenomics sequencing in patients diagnosed with MAFLD. The fungal composition of oral and gut niches are revealed. Alterations of oral and gut mycobiota between MAFLD and healthy controls were investigated. Correlations between oral/gut mycobiota and clinical parameters were assessed to characterize MAFLD-related fungi. Functional analyses were performed. The possible link between oral-gut *Mucor ambiguus* that was evident suggests the microbial transmission between oral and gut niches in the pathogenesis of MAFLD.

2 Methods

2.1 Study participants

This study followed the Declaration of Helsinki on medical protocols and ethics, and was approved by the Ethics Committee of School and Stomatology Wenzhou Medical University (approval no. WYKQ2021006). Twenty-one MAFLD patients who met the inclusion and exclusion criteria were enrolled in the study from July 2021 to June 2022. Twenty healthy controls were enrolled by matching age. Inclusion criteria were age over 18 years and clinically proven MAFLD according to the Asian Pacific Association for the Study of the Liver guidelines. The exclusion criteria were as follows: (1) presence of other liver diseases, including viral hepatitis, autoimmune hepatitis, and hepatolenticular degeneration; (2) hepatic steatosis induced by

drugs, such as tamoxifen, amiodarone, valproate, methotrexate, and glucocorticoids; (3) other factors that may cause hepatic steatosis, including long-term total parenteral nutrition, inflammatory bowel disease, celiac disease, hypothyroidism, Cushing's syndrome, lipoprotein deficiency, lipid-atrophic diabetes, and others; (4) use of lipid-lowering drugs in the 6 months preceding enrollment; (5) type 1 or type 2 diabetes; (6) current oral diseases, including untreated oral abscess, precancerous lesions, oral cancer, oral fungal infection, missing more than eight teeth, periodontitis and others; (7) use of probiotics, antifungal drugs; and (8) other conditions, including pregnant or lactating women, long-term heavy smoking, use of antibiotics for more than 5 days within the preceding 6 months, severe acute episode of a systematic disease, abnormal thyroid function, familial hyperlipidemia, and others. Written informed consent was obtained from all participants prior to enrollment. Patient characteristics are summarized in Table 1.

2.2 Acquisition of clinical variables

Demographic information of all patients, including weight, height and dietary habits, were obtained by trained staff in Wenzhou Medical University. Clinical parameters were collected with hematologic examination after 12 h of fasting. These parameters included total cholesterol (TC), total triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT), fasting blood glucose (FBG), fasting serum insulin (FSI), glycated hemoglobin A1c (HbA1c), white blood cell (WBC) and plasma high-sensitivity C-reactive protein (CRP). As an approximation of insulin resistance (IR), the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) score was calculated as [FPG (mmol/L) × FSI (mU/L)]/22.5.

2.3 Sample collection

A total of 123 samples (41 saliva, 41 supragingival plaque, and 41 feces samples) were collected from the 41 participants as previously described(Zhao et al., 2020; Chen et al., 2022). Briefly, each participant were required to rinse their mouth and avoid eating and drinking for at least 1 h before collection of the oral sample. Saliva was collected and preserved in 50 mL sterile tubes (Corning, New York, NY, USA) containing saliva DNA preservation solution (Huayueyang Biotech, Beijing, China). Supragingival plaque was collected before eating in the morning in accordance with the methods described in the Manual of Procedures for the Human Microbiome Project. The fecal sample from each participant was freshly collected in specially provided collection containers. Each fecal sample was transported to the laboratory in a box containing an ice pack within 2 h of collection and stored at -80°C. Whole-blood samples were collected in tubes with anticoagulants (Improve Medical, Guangzhou, China) after at least 8 h of fasting.

TABLE 1 Clinical characteristics of the study cohort.

Characteristics	Control (n=20)	MAFLD (n=21)	*P* value
Sex (M/F)	16-Apr	16-May	1
Age (years)	29.00 ± 8.89	32.43 ± 6.59	0.1778
Height (cm)	172.10 ± 7.59	169.33 ± 6.71	0.2344
Weight (kg)	65.68 ± 8.55	74.81 ± 13.08	0.0142*
BMI (kg/m2)	22.09 ± 1.92	25.96 ± 3.27	0.0001*
TC (mmol/L)	4.30 ± 0.64	4.87 ± 1.02	0.0429*
TG (mmol/L)	1.07 ± 0.44	1.82 ± 0.82	0.0010*
LDL-C (mmol/L)	2.59 ± 0.50	3.13 ± 0.91	0.0291*
HDL-C (mmol/L)	1.25 ± 0.24	1.09 ± 0.23	0.0395*
GGT (U/L)	16.40 ± 4.98	35.10 ± 18.93	0.0002*
AST (U/L)	18.10 ± 5.13	28.33 ± 20.88	0.0443*
ALT (U/L)	17.05 ± 9.13	39.33 ± 23.32	0.0004*
FBG (mmol/L)	4.71 ± 0.33	4.85 ± 0.44	0.2652
FSI (mU/L)	5.90 ± 2.18	11.30 ± 5.20	0.0002*
HOMA-IR	1.28 ± 0.48	2.47 ± 1.27	0.0003*
HbA1c	5.18 ± 0.21	5.33 ± 0.28	0.0664
CRP (mg/L)	1.15 ± 1.28	2.11 ± 2.38	0.1296
WBC (′ 109/L)	5.90 ± 1.12	6.28 ± 1.25	0.3282

BMI, body mass index; TC, total cholesterol; TG, total triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; FBG, fasting blood glucose; FSI, fasting serum insulin; HbA1c, glycated hemoglobin A1c; WBC, white blood cell; CRP, plasma high-sensitivity C-reactive protein; IR, insulin resistance; HOMA-IR, the Homeostatic Model Assessment for Insulin Resistance. Values are presented as mean and standard deviation. Student's t-test or the Kruskal-Wallis test was applied for analysis of all clinical variables. *P < 0.05.

2.4 DNA extraction and metagenomic sequencing

Total bacterial genomic DNA was extracted from the collected supragingival samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's protocols. The concentration and purification of the extracted DNA were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA quality was checked by 1% agarose gel electrophoresis.

The extracted DNA was fragmented to an average size of approximately 400 bp using a Covaris M220 ultrasonicator (Gene Company Limited, Huzhou, China) for paired-end library construction. The paired-end library was constructed using NEXTFLEX® Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt-end of fragments. Paired-end sequencing was performed using a Novaseq 6000 device (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using NovaSeq Reagent Kits according to the manufacturer's instructions. The data were analyzed on the free online Majorbio Cloud Platform. Briefly, the paired-end Illumina reads were trimmed of adaptors, and low-quality reads (length<50 bp or with a quality value <20 or having N bases) were removed by fastp (https://github.com/OpenGene/fastp, version 0.20.0).

2.5 Gene prediction, taxonomy, and functional annotation

Open reading frames (ORFs) from each assembled contig were predicted using Prodigal(Hyatt et al., 2010) /MetaGene(Noguchi et al., 2006). The predicted ORFs with a length ≥ 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table.

A non-redundant gene catalog was constructed using CD-HIT (Fu et al., 2012) with 90% sequence identity and 90% coverage. High-quality reads were aligned to the non-redundant gene catalogs to calculate gene abundance with 95% identity using SOAPaligner.

Representative sequences of non-redundant gene catalog were aligned to NR database with an e-value cutoff of 1e-5 using Diamond for taxonomic annotations. Cluster of orthologous groups of proteins (COG) annotation for the representative sequences was performed using Diamond against eggNOG database with an e-value cutoff of $1e^{-5}$. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was performed using Diamond against the KEGG database with an e-value cutoff of $1e^{-5}$.

2.6 Statistical analyses

The clinical variables are demonstrated by counts. Chi-squared test was used for differential analyses. Continuous variables are presented as mean ± standard deviation. Student's *t*-test or the Kruskal-Wallis test was applied for analysis of all clinical variables. A *P*-value < 0.05 was considered significant. Alpha diversity was calculated using the Shannon, Simpson, and Chao-1 indices. The equality of variance was confirmed with Levene's test. Normality of the data was evaluated using the Shapiro–Wilk test. The significance of genotype was assessed with the Student's *t* test. Beta diversity was assessed by the principal coordinate analysis (PCoA) with Bray–Curtis distance used to calculate the distance metric. Analysis of similarities (ANOSIM) test was used for the statistical analysis. The Kruskal–Wallis test was used to detect significant differences in abundance, and the Wilcoxon rank-sum test was used for *post hoc* comparison. A *P*-value < 0.05 was considered significant. Linear discriminant analysis effect size (LEfSe) was used to identify the significant differential fungal biomarkers with a linear discriminant analysis (LDA) score > 2.0. Correlation analysis was constructed to investigate the interaction between fungal species and clinical parameters, and Spearman coefficient $|r| > 0.5$ and $P < 0.05$ are shown. Spearman's correlations of fungal abundance among different sample sites were tested with a coefficient $|r| > 0.5$ and $P < 0.05$. P < 0.05 is marked with *, $P < 0.01$ with ** and $P < 0.001$ with ***.

3 Results

3.1 Study population and clinical characteristics

A total number of 41 participants were recruited for the study. Twenty-one had been diagnosed with MAFLD and 20 were healthy controls (Table 1). The two confounding factors of age and gender were matched at the time of inclusion of the study subjects. Overall, there were no significant differences between the two groups concerning age and gender. In the absence of significant differences in height between the two groups, the MAFLD group had significantly higher body weight and correspondingly higher body mass index (BMI) than the control group. Compared to the control group, the MAFLD group had lower HDL-C levels and higher levels of TC, TG, LDL-C, GGT, AST, and ALT. There was no difference in FBG between the two groups, but FSI was relatively higher in the metabolic group, resulting in a higher HOMA-IR in the MAFLD group than in the control group.

3.2 Fungal diversity of oral and gut mycobiome in MAFLD patients

To investigate the mycobiome profile of two groups, metagenomic sequencing was performed on the oral (saliva and supragingival plaques) and gut (feces) samples from the 41 participants. Alpha diversity analysis demonstrated no significant difference in the fungal diversity (Shannon and Simpson indices) and fungal richness (Chao index) of the oral mycobiome between the MAFLD patients and healthy controls. Lower alpha diversity was observed with the fecal mycobiome in the MAFLD patients, as indicated by the significantly decreased Shannon and Simpson

indices (P = 0.0052 and 0.0085, respectively; (Figure 1A). PCoA plots were measured by Bray-Curtis distance of the three sample types Figure 1B). ANOSIM revealed the significant difference of the gut fungal structure between MAFLD patients and the healthy controls (P = 0.002). Little difference was observed with regard to the oral mycobiome, although a statistical tendency (P = 0.056) was detected in the suragingival plaques (Figure 1C).

3.3 Composition of oral and gut mycobiome in MAFLD patients

The oral and gut fungal community of the current cohort mainly consisted of six phyla, including *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Microsporidia*, *Mucoromycota*, and *Zoopagomycota* (Figure 2A). The top 50 most abundant fungal species of three loci were then identified. Seven species in feces, five in supragingival plaques, and one in saliva presented significantly different abundance between the MAFLD and control groups (P < 0.05, Figure 2B). To further identify the significantly enriched fungal species in MAFLD patients, LEfSe was used to identify fungi that differed significantly between the MAFLD and control groups. Species with LDA scores ≥ 2.0 were confirmed and are shown in Figure 2C. Focusing on the top 50 species, *Mucor ambiguous* (*M. ambiguous*), a member of the *Mucoromycota* phylum, was enriched in both saliva and feces in MAFLD patients. *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*S. pombe*), were more abundant in supragingival plaques of MAFLD groups. Conversely, these two species were more abundant in feces of the healthy group.

3.4 Correlation analysis of oral/gut mycobiome and clinical indicators

To evaluate the clinical significance of the prevailing fungal species, Spearman correlation analyses were performed to assess the associations between the clinical indicators and the top 50 species,

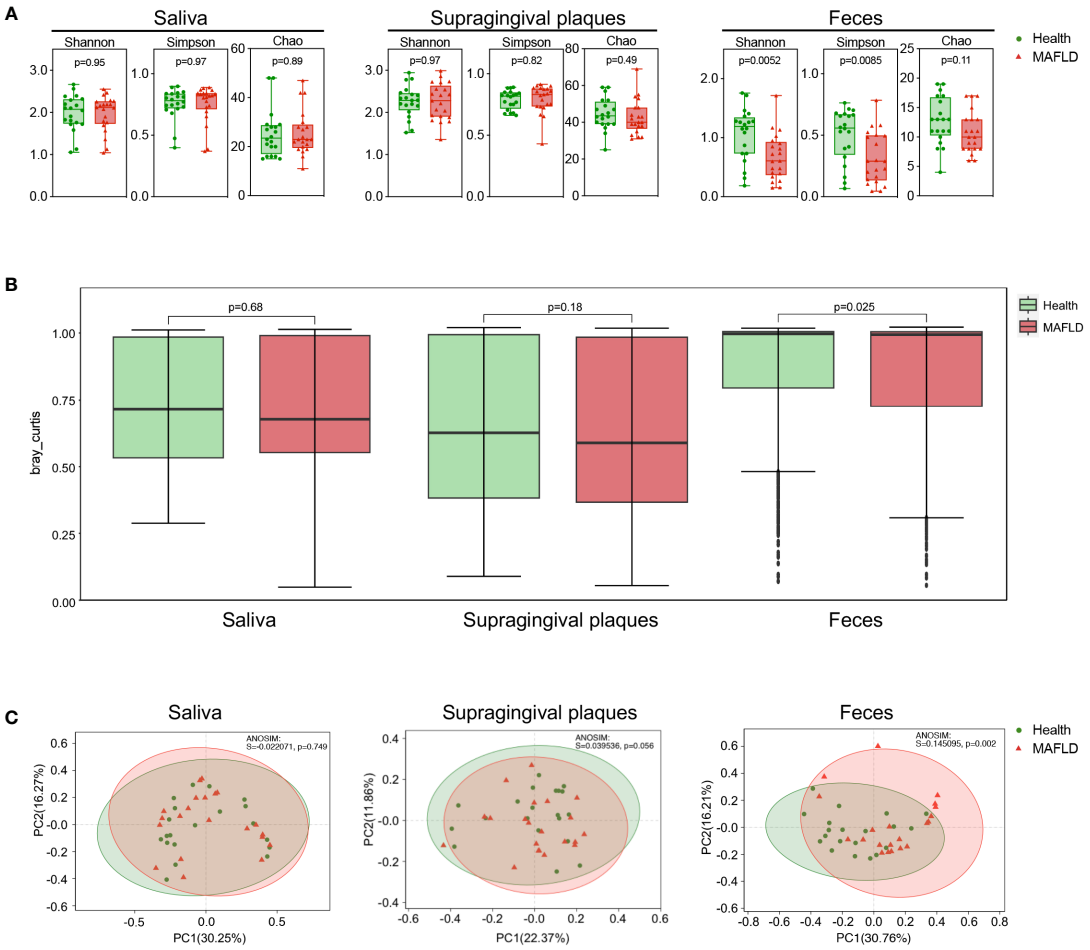

FIGURE 1
Fungal diversity of oral and gut mycobiome in MAFLD patients. **(A)** Shannon, Simpson, and Chao1 indices of the oral (saliva and supragingival plaques) mycobiome and gut (feces) mycobiome between MAFLD patients and healthy controls (*t* test). **(B)** Bray–Curtis distance of the beta-diversity in the oral and gut mycobiome in MAFLD patients and healthy controls. **(C)** Principal coordinate analysis (PCoA) plots and analysis of similarities (ANOSIM) statistical data of the oral and gut mycobiome between MAFLD patients and healthy controls. P < 0.05 with Wilcoxon rank-sum test followed by Kruskal-Wallis test.

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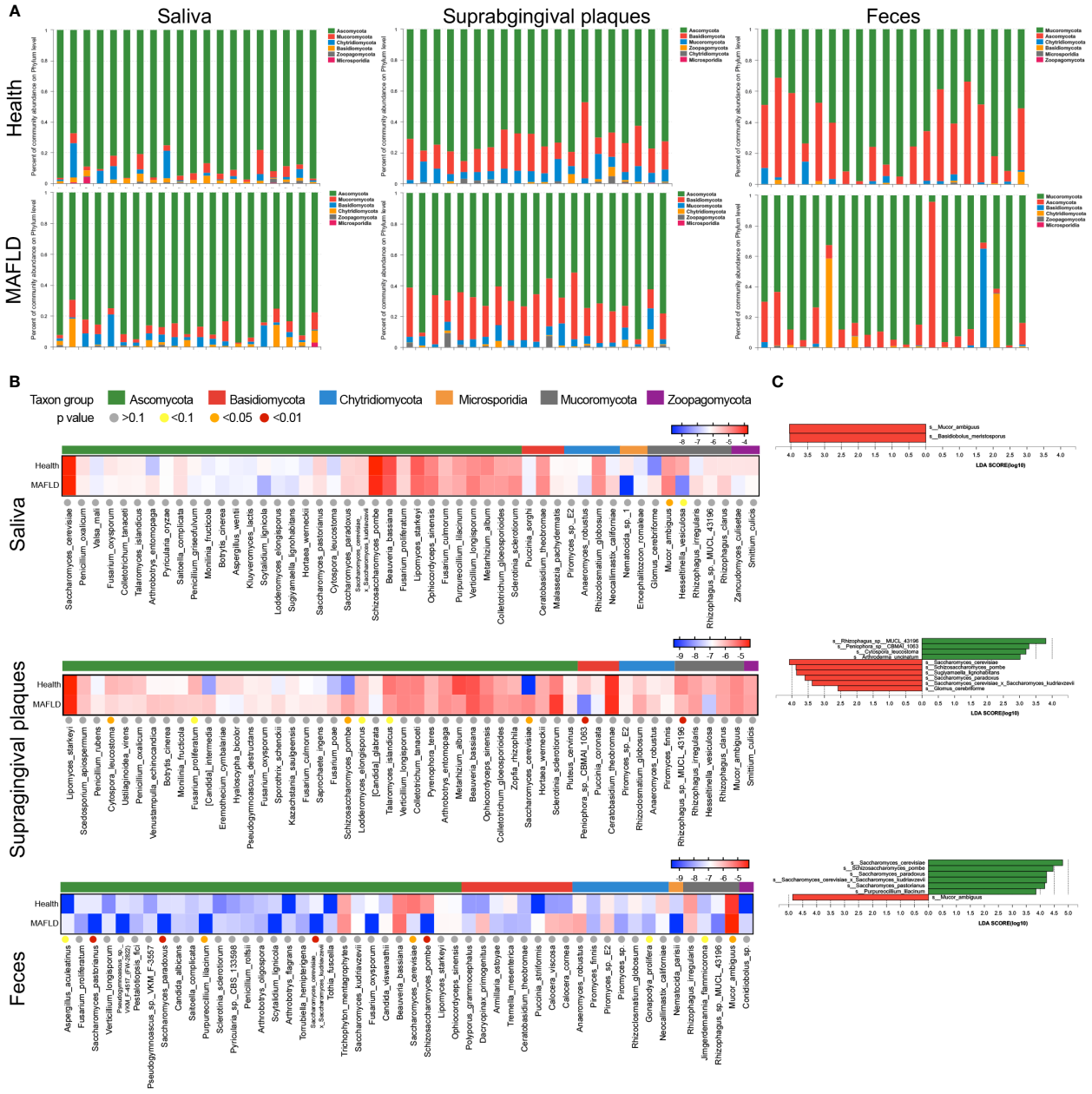

FIGURE 2
Compositional alterations of oral and gut mycobiome between MAFLD patients and healthy controls. **(A)** Relative abundance of oral (oral and supragingival plaques) and gut (feces) mycobiota at the phylum level. **(B)** Clustering heatmaps of the common logarithm of abundance of the top 50 species. The color of dots reflects the *P*-value of the differential analysis. **(C)** Differential fungal species in the saliva, supragingival mycobiome and gut mycobiome between MAFLD patients and healthy controls.

focusing on distinct species among three loci (Figure 3). Among the top 50 species in oral and gut samples, significant associations with various clinical parameters were evident for 22 species in saliva, 23 species in supragingival plaques, and 22 species in feces (Figures 3A, B, C). Significantly enriched fungal species within the top 50 species in the MAFLD and healthy groups were separately highlighted in red and green in the figure. Significantly enriched in the saliva of MAFLD patients, *M. ambiguus* was positively associated with BMI and HbA1c ($P < 0.05$, Figure 3A). Simultaneously presenting higher abundance in the feces of MAFLD patients, *M. ambiguus* was positively correlated with TC ($P < 0.05$), LDL-C ($P < 0.05$), ALT ($P < 0.01$), and AST ($P < 0.01$) (Figure 3C). Being enriched in the feces of healthy subjects, *S. cerevisiae* was negatively correlated with GGT ($P < 0.05$) and FSI ($P < 0.01$, Figure 3C). *S. pombe* in

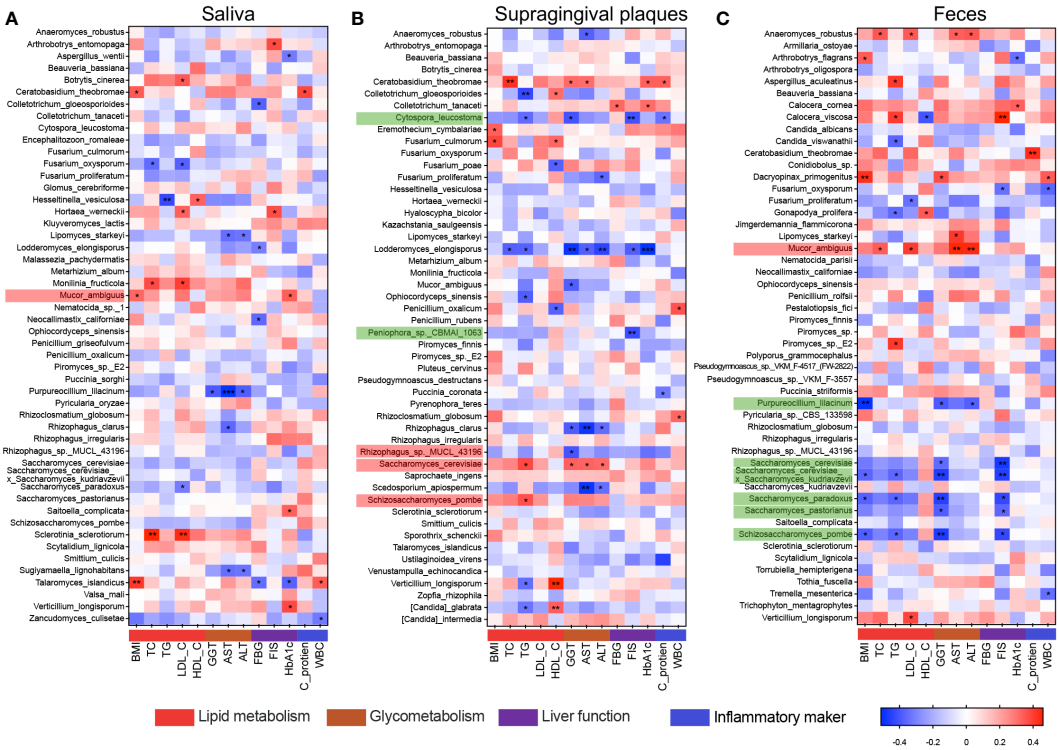

FIGURE 3
Correlation analysis of oral/gut mycobiome and clinical indicators. Heatmaps of Spearman correlation coefficients between clinical indicators and the top 50 species in saliva **(A)**, supragingival plaques **(B)**, and feces **(C)**. Species highlighted in red and green are those significantly enriched in MAFLD patients and healthy controls, respectively. BMI, body mass index; TC, serum total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; GGT, gamma-glutamyl transferase; AST, aspartate transaminase; ALT, alanine transaminase; FBG, fasting blood glucose; FSI, fasting serum insulin; HbA1C, glycosylated hemoglobin type A1C; CRP, C-reactive protein; WBC, white blood cells. *P (FDR) < 0.05.

supragingival plaques was positively associated with TG ($P < 0.05$), whereas *S. pombe* was negatively correlated with TG ($P < 0.05$), BMI ($P < 0.05$), FSI ($P < 0.05$), and GGT ($P < 0.01$) in feces.

3.5 Functional differences of oral/gut mycobiome in MAFLD patients

To elucidate functional profiles of mycobiome in saliva, supragingival plaques, and feces, gene were annotated using the KEGG database. Redundancy analysis (RDA) revealed the top 10 functional pathways at KEGG level 3 related to clinical indicators. Pathways involved in metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments and carbon metabolism were of high abundant in all loci, suggesting that these are core functions (Figures 4A, C, E). The contributions of prevalent fungi on the top 10 functional pathways were further analyzed. In saliva, *S. cerevisiae* and *S. pombe* were the main contributors of the top 10 pathways, and slight contribution difference of each pathway was observed between MAFLD patients and healthy controls (Figure 4B). In supragingival plaques, different taxa contribution was observed in several disease-related pathways, including glycolysis/gluconeogenesis, carbon metabolism, microbial metabolism, biosynthesis of secondary metabolites, and metabolic pathways, and contributions of *S. cerevisiae* to these pathways were significantly higher in the MAFLD group than the healthy group (Figure 4D). In feces however, less contributions of *S. cerevisiae* to these pathways were identified in MAFLD patients (Figure 4F). Among the top 3 abundant functions, the taxa contribution was also significantly different between the MAFLD patients and healthy controls, particularly for supragingival plaques and feces. Network analysis indicated an increased number of taxa that involved in core functions from healthy group to MAFLD group (Figure S1).

3.6 Correlation analysis of oral and gut mycobiome in MAFLD patients

The gut-transiting oral microorganism has been linked to human health. Whether this is also the case for MAFLD was investigated here. Among the top 50 species in healthy individuals, 49 fungal species in saliva, 43 in supragingival plaques, and 28 in feces were identified, with 22 shared species in all three loci (Figure S2). In MAFLD patients, 48 species in saliva, 50 in supragingival plaques, and 18 in feces were identified, with 18 shared species (Figure S2). The distribution of the top 50 fungal species at the three loci differed significantly between MAFLD patients and healthy subjects (Figure 5A). To understand the potential oral-gut connection, Spearman's correlation analysis

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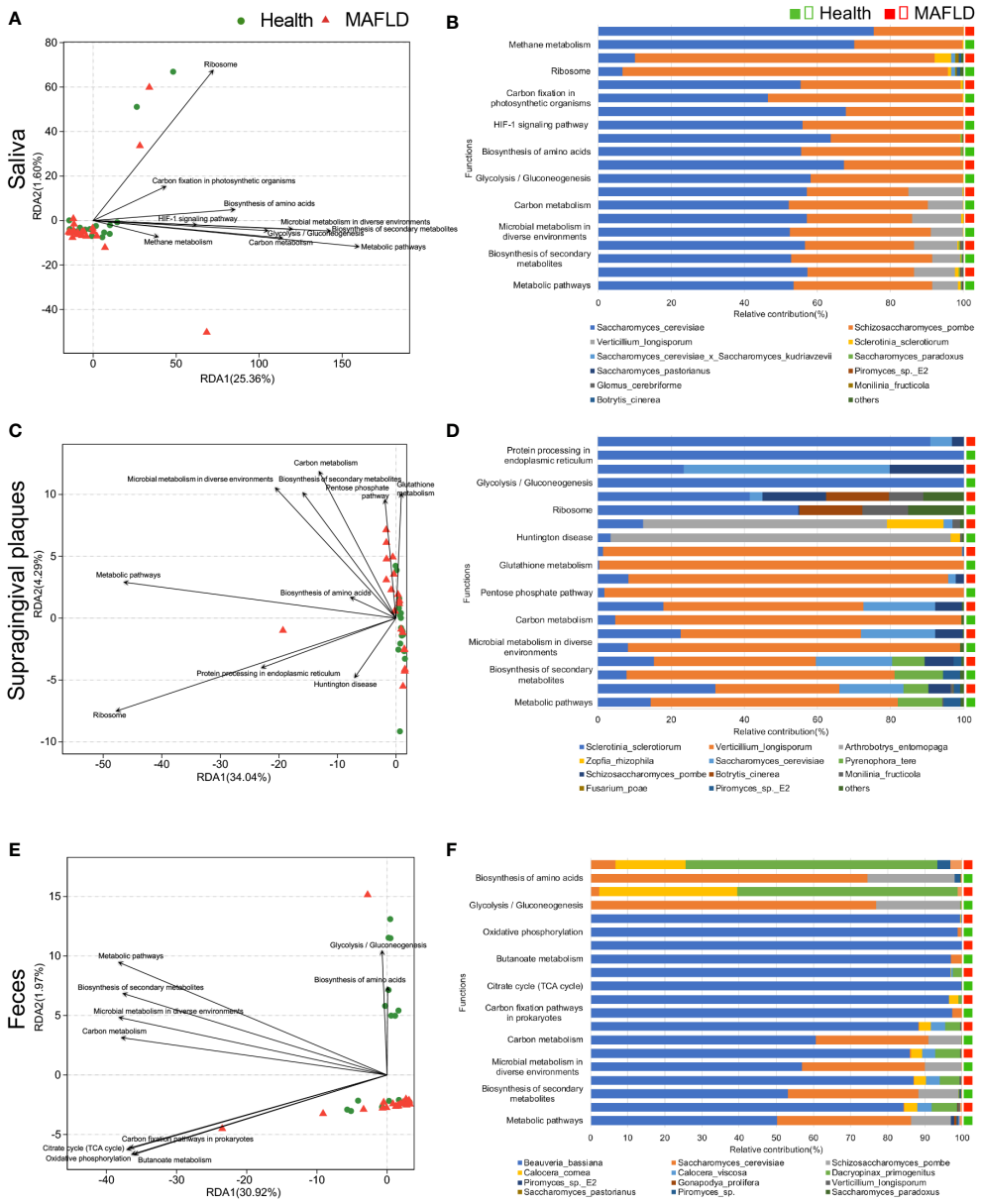

FIGURE 4
Functional differences of the oral/gut mycobiome in MAFLD patients. The relationship between clinical indicators and KEGG pathway in saliva **(A)**, supragingival plaques **(C)**, and feces **(E)**. The different colors and shapes of the points represent different groups, and the distance between the points represents the similarity and difference of the functional composition between the samples. The length of the arrow line represents the degree of correlation between clinical indicators and the KEGG pathway distribution (the longer the line, the greater the correlation). Species contribution analysis for the above 10 KEGG pathways in saliva **(B)**, supragingival plaques **(D)**, and feces **(F)**. The vertical axis represents the KEGG pathways, and the horizontal axis represents the proportion of different fungal species.

based on the taxa relative abundance was performed. Among the top 50 species, there were 100 significant correlations between the feces and saliva and 95 significant correlations between the feces and supragingival plaques. *M. ambiguus* was found in all three loci in MAFLD patients (Figure 5A). In addition, the abundance of *M. ambiguous* in feces was positively correlated with its abundance in saliva (Figure 5B). The findings suggest that the potential ectopic colonization of *M. ambiguus* was more likely involved in the pathogenesis and progression of MAFLD. The decrease of *Cytospora leucostoma* in supragingival plaques was also positively correlated with the decrease of *S. paradoxus* and *Saccharomyces_cerevisiae_x_Saccharomyces_kudriavzevii* in feces (Figure 5C). These three species were both enriched in heathy controls and decreased in MAFLD patients, suggesting the beneficial roles in health.

To further identify the import role of *M. ambiguus*, receiver operating characteristic (ROC) analysis was performed to explore whether *M. ambiguus* can be used as a microbial marker of

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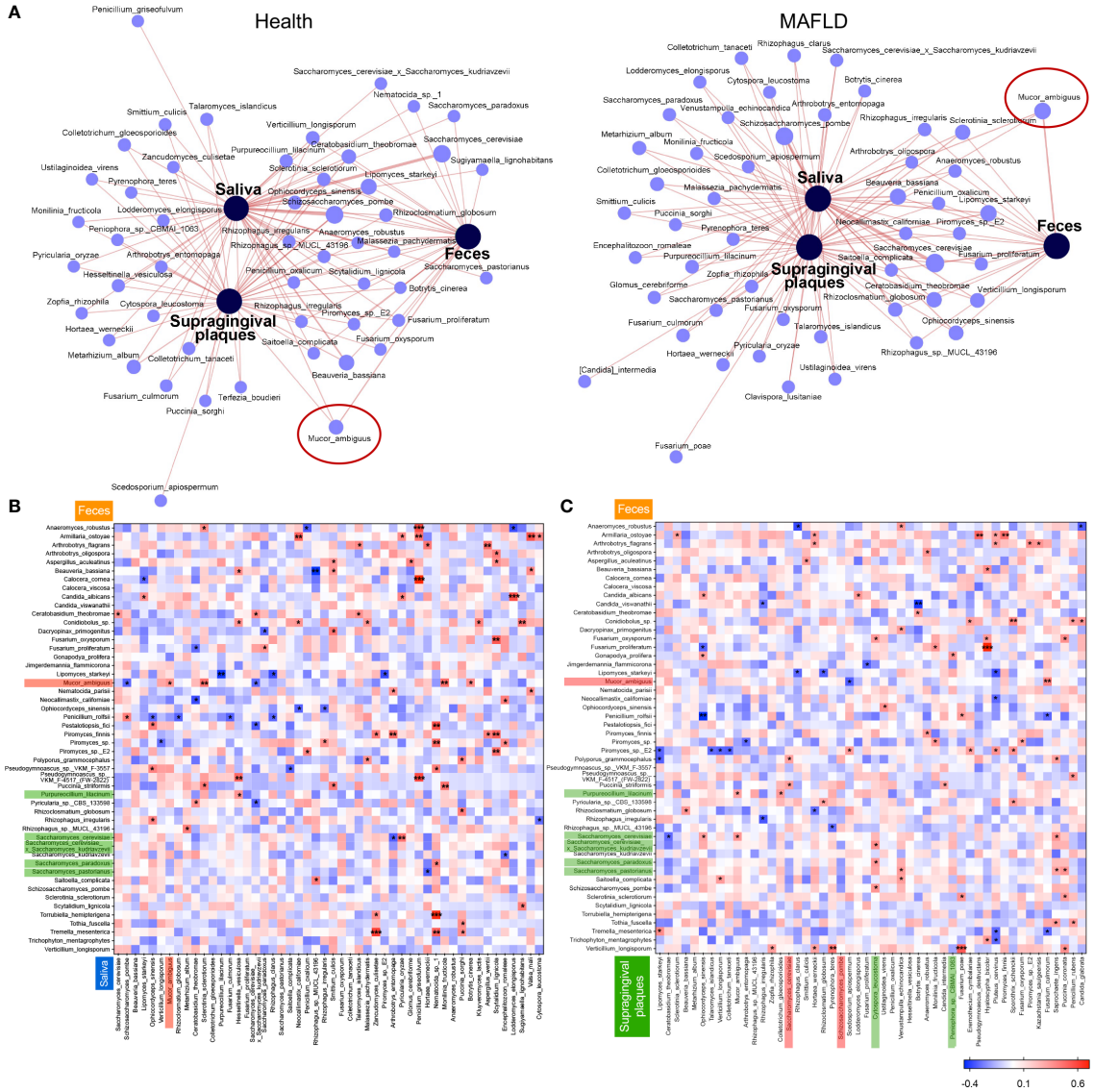

FIGURE 5
Communications between oral and gut microbiota in participants with or without MAFLD. Co-occurrence network analysis of top 50 fungal species among the three loci in healthy controls and MAFLD patients **(A)**. Heatmaps of Spearman's correlation coefficients between relative abundances of shared genera in salivary **(B)** or supragingival **(C)** microbiota and those in feces. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

MAFLD. The area under the ROC curve were 0.76 and 0.73 in saliva and feces, respectively (Figures 6A, B), implicating salivary *M. ambiguus* as being potential for the diagnosis of MAFLD.

4 Discussion

Numerous studies have addressed the involvement of the bacterial microbiome in the development of MAFLD(Oh et al., 2020; Drozdz et al., 2021; Rao et al., 2021). However, knowledge of the fungal microbiome and MAFLD remains limited. The fungal community is a prominent component of the oral microbiome (Baker et al., 2017), yet the mycobiome has been much less studied compared to the bacterial community at any anatomical site, including the oral cavity. In this study, the fungal microbiome from two niches (oral and gut) with three different types of samples (saliva, supragingival plaques, and feces) in a cohort of MAFLD participants and healthy controls was investigated, and the functions in different niches was further illustrated. Considering that the patients' gender is one of the main factors affecting the composition of gut microbiota(Fransen et al., 2017; Pepoyan et al., 2018), a non-parametric analysis of the gender of the recruited patients was performed. There was no difference in gender between the two groups in the present study, which also removed the confounding factors introduced by gender. The redesignation of MAFLD from NAFLD reflects the demonstrated involvements of

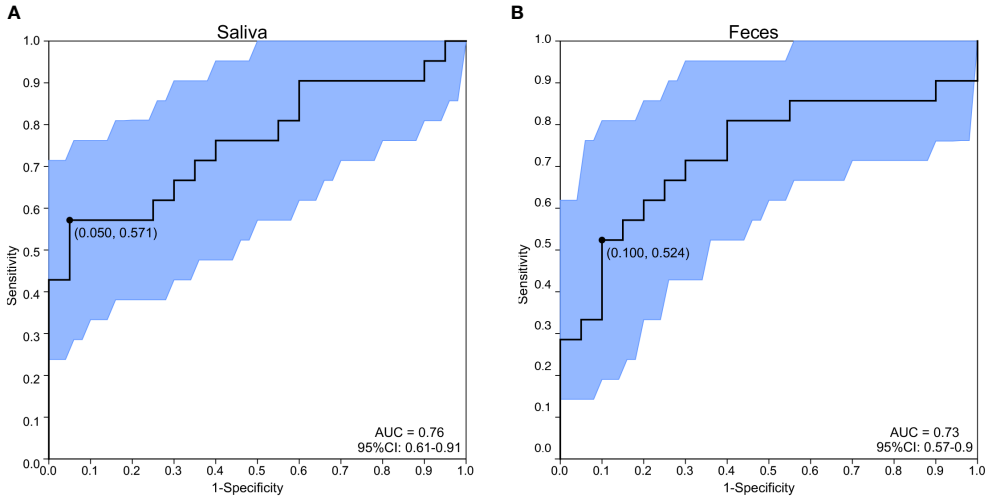

FIGURE 6
ROC analysis of *M. ambiguus* as a marker for MAFLD. ROC analysis of *M. ambiguus* in saliva **(A)** and feces **(B)**. The area under the ROC curve (AUC) of *M. ambiguus* classification. The black bars denote the 95% confidence interval (CI) and the area between the two outside curves represents the 95% CI shape.

metabolic alterations and metabolism-related pathways, such as biosynthesis of amino acids, carbon metabolism, and biosynthesis of secondary metabolites.

The richness of the gut mycobiome showed a similar tendency in MAFLD patients, and the community diversity was significantly decreased in MAFLD patients compared to healthy individuals. Both alpha and beta diversities were barely changed in the oral mycobiome, although reduced diversity was evident in the supragingival plaques of MAFLD patients compared with the healthy controls. *Ascomycota* and *Basidiomycota* constituted the two main predominant phyla in the oral mycobiome in both MAFLD patients and healthy controls. This finding is consistent with previous descriptions of proportions of *Ascomycota* and *Basidiomycota* of 75.5% and 24.5%, respectively, from 304 healthy individuals. The fungal composition of the plaques differed from the saliva samples, with a lower proportion of *Ascomycota*, and a higher proportion of *Basidiomycota*. As the initial site of the gastrointestinal tract, the oral niche features some special variables not present in feces, such as oral hygiene, oral health (bleeding gums, mouth ulcer, and false teeth), and others. The human mouth harbors a range of substrates, including teeth, tongue, cheeks and gums. Each habitat supports a complex, distinctive community(Mark et al., 2020). *S. cerevisiae* is the most abundant fungal species in the saliva. It was observed no significant difference in the prevalence of *S. cerevisiae* between MAFLD patients the healthy controls. Moreover, a higher proportion of *S. cerevisiae* was observed in the supragingival plaques of MAFLD patients and the gut mycobiome of the healthy controls. Though the tendency was independent in the two different niches, *S. cerevisiae* remained the biomarker species according to the LEfSe results. *S. cerevisiae* is one of the most concerned microbial species used for industrial production and is an important model organism to understand the biology of the eukaryotic cells and humans(Liu et al., 2021). A study followed 298 pairs of healthy mothers and offspring from 36 weeks of gestation until 2 years of age to explore the gut fungi in maternal and offspring samples(Schei et al., 2017). The authors detected very few *S. cerevisiae* in offspring at 10 days and 3 months after birth. However, once solid food was introduced to the diet, *S. cerevisiae* became the dominant species(Schei et al., 2017). Another study investigating the gut mycobiome variations across geography, ethnicity and urbanization, demonstrated a high enrichment of *S. cerevisiae* in urban populations compared with rural populations, with a significant inverse correlation with liver pathology-associated blood parameters, including AST, ALT, GGT, and direct bilirubin, indicating that *S. cerevisiae* may protect against liver-injury associated diseases(Sun et al., 2021). Concordant findings were observed in the present study, presenting inverse correlations between fecal *S. cerevisiae* and GGT and FIS

As for the significant biomarker, *M. ambiguus* was enriched in saliva and fecal samples of MAFLD group. *Mucor* sp. are common soil fungi but also known as agents of human infections (mucormycosis) and used in food production and biotechnology (Petrikkos et al., 2012; Walther et al., 2013; Wagner et al., 2020). These organisms can increase the intestinal permeability in epithelial cell monolayers(Mueller et al., 2019). It was observed a positive association between *M. ambiguus* and some clinical parameters, including BMI, HbA1c, TC, LDL-C, ALT and AST. Moreover, the log-ratios were of interest, given the findings of previous studies indicating that the log-ratios of *Mucor* sp./*S. cerevisiae* and *Candida albicans*/*S. cerevisiae* were independently associated with higher inflammatory activity. Munevver et al. reported a significant higher log-ratio of *Mucor* sp./*S. cerevisiae* in patients with non-alcoholic steatohepatitis, which also associated with serum glucose levels, AST levels, stage of fibrosis, and grade of liver inflammation(Demir et al., 2022). A random forest model indicated a predictive effect of *M. ambiguus* in saliva and feces for MAFLD, which might suggest an oral-gut axis in the development of MAFLD. Preclinical and clinical studies have shown the

relationship between oral diseases and systematic diseases(Farrell et al., 2012; Ramos-Garcia et al., 2021). Although defined as two different niches in healthy state, some oral microbiota can translocated to the gut, where they cause microbial dysbiosis and disrupt intestinal permeability, further aggravating systematic inflammation. Oral fungi are expected to be biomarkers for the early diagnosis of many diseases and have great clinical applications. Compared to the intestinal ROC, greater ROC of oral *Mucor* was observed in the current project, suggesting that oral *Mucor* can served as a potential biomarker for the MAFLD diagnosis.

Limitations of this study include the lack of data on oral hygiene and dental status of the participants, which restricted the correlation between the oral health and clinical parameters of MAFLD. Moreover, since *M. ambiguus* was a significant biomarker in both the oral and gut mycobiomes, its role and effects should be further investigated with *in vitro* and *in vivo*.

In conclusion, the present study provides the first data of the composition and function of oral mycobiome and gut mycobiome in MAFLD patients. A strong correlation between *S. cerevisiae* and MAFLD-associated clinical parameters was found. The findings indicate a potential oral-gut communication of *M. ambiguus*. These data reinforce the role of fungal microbiota in the development of MAFLD, and strengthen the correlation of the oral-gut axis in metabolic diseases.

Data availability statement

The data presented in the study are deposited in the NCBI sequencing read archive repository, accession number SRP429021.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of School and Stomatology Wenzhou Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZH, RM and YP designed the study. CN, MW, PZ, XL, XH and YY collected clinical samples and conducted experiments. YT, ZC, WT, TD, MM, QJ and KY analysed the data. CN and YT prepared manuscript. ZH, HL, LH and JL 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.

The reviewer NX declared a shared affiliation with the authors ZH, CN, YT, QJ, ZC, PZ, JL, HL, KY, XH, XL, YY, MM, TD, WT, LH and RM to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1157368/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Correlation analysis between function and fungal species of healthy controls and MAFLD patients. Correlation network analysis between functional alterations and differential fungal species. Spearman correlation > 0.5 or ≤ 0.5 is represented.

SUPPLEMENTARY FIGURE 2
Shared species in participants with or without MAFLD. Venn diagrams showing the unique and shared species among saliva, supragingival plaques, and feces. The 50 most abundant species were used for the Venn diagrams.

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Gut microbiota in children with split-dose bowel preparations revealed by metagenomics

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Objective: Split-dose polyethylene glycol (PEG) is routinely used for bowel preparation before colonoscopy. This study aimed to investigate the composition of gut microbiota and its functions in pediatric patients undergoing split-dose PEG bowel preparation for colonoscopy to understand the stability and resilience of gut microbiota.

Material and methods: From September to December 2021, 19 pediatric patients were enrolled at Shenzhen Children's Hospital and 76 samples (4 time points) were analyzed using metagenomics. Time points included Time_1 (one day before bowel preparation), Time_2 (one day after colonoscopy), Time_3 (two weeks after bowel preparation), and Time_4 (four weeks after bowel preparation).

Result: Alpha diversity comparison at both the species and gene levels showed a decrease in community richness after colonoscopy, with little statistical significance. However, the Shannon diversity index significantly decreased ($P<0.05$) and gradually returned to pre-preparation levels at two weeks after bowel preparation. The genus level analysis showed six genera (*Eubacterium*, *Escherichia*, *Intertinibacter*, *Veillonella*, *Ruminococcaceae unclassified*, and *Coprobacillus*) significantly different across the four time periods. Additionally, at the species level, the abundance of *Escherichia coli*, *Bacteroides fragilis*, and *Veillonella parvula* significantly increased at one day after colonoscopy before gradually decreasing at two weeks after bowel preparation. In contrast, the abundance of *Intertinibacter bartlettii* decreased at one day after colonoscopy but then recovered at two weeks after bowel preparation, reaching the preoperative level at four weeks after bowel preparation. Furthermore, five functional pathways (base excision repair, biosynthesis of ansamycins, biosynthesis of siderophore group nonribosomal peptide, flavonoid biosynthesis, and biosynthesis of type II polyketide products) were significantly different across the four time periods, with recovery at two weeks after bowel preparation and reaching preoperative levels at four weeks after bowel preparation.

Conclusions: Gut microbiota at the genus level, species level, and functional pathways are impacted in pediatric patients undergoing split-dose PEG bowel preparation and colonoscopy, with recovery two weeks following bowel

preparation. However, the phylum level was not impacted. Modifications in gut microbiota composition and function may be investigated in future studies of bowel preparation. This study highlights the stability and resilience of gut microbiota among pediatric patients during bowel preparation.

KEYWORDS

bowel preparation, children, colonoscopy, gut microbiota, metagenomics, resilience, split dose, stability

1 Introduction

Colonoscopy is a commonly used method for screening and treating intestinal diseases in children. Achieving good preparation of the bowel is crucial for improving diagnostic and therapeutic outcomes. The drug of choice for bowel preparation is polyethylene glycol (PEG) due to its high efficacy, safety, and ease of use, as confirmed by several studies. Both adult and child guidelines recommend PEG as the preferred laxative for bowel preparation (Pashankar et al., 2004; Safder et al., 2008; Pall et al., 2014; ASGE Standards of Practice Committee et al., 2015; Tsunoda et al., 2017; Mytyk et al., 2018). Split-dose PEG is commonly used for bowel preparation in adults before colonoscopy (Samarasena et al., 2012; Zawaly et al., 2019). Research has shown that split-dose PEG is more effective than a single dose for bowel preparation in children (Tripathi et al., 2020).

The human microbiome is composed of various microorganisms, including bacteria, archaea, fungi, protozoa, and viruses, with more than 100 times the number of genes as the human body (Qin et al., 2010; Ursell et al., 2012). These genes encode pathways that produce biologically active molecules from diet and metabolism (Lynch and Pedersen, 2016). The gut microbiota plays a vital role in obtaining energy from dietary fiber and linking its metabolites to the occurrence of various diseases, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Shreiner et al., 2015). Although previous studies have shown that colonoscopy and bowel preparation affect gut microbiota, there is still limited research in children (Drago et al., 2019). Furthermore, the impact of split-dose PEG bowel preparation on changing gut microbiota in children has not been well studied, and there are no metagenomic studies in this area. To obtain a more comprehensive view of the microbial community, metagenomics was employed for investigating gut microbiota of children in this study. Metagenomics can detect both bacterial and non-bacterial microorganisms, providing information about the functional potential of the microbial community. In contrast, 16S rRNA sequencing only detects bacterial microorganisms and does not provide functional potential information. Thus, our study aimed to characterize the potential differences in gut microbiota composition and functional capacity in children undergoing colonoscopy with split-dose PEG bowel preparation.

2 Results

2.1 Alpha diversity analysis between the 4 time points at the species and gene levels

We compared alpha diversity at the species and gene levels between the 4 time points, as detailed in Table S2. Our analysis showed that although abundance decreased after bowel preparation, it was not significant (Figures 1A, B). However, the Shannon diversity index significantly decreased ($P<0.05$) at both the species and gene levels, which gradually returned to baseline levels by two weeks after bowel preparation (Figures 1A, B).

2.2 Beta diversity analysis between the 4 time points

There was no significant difference in beta diversity between the 4 time points at the phylum level ($P=0.564$), genus level ($P=0.545$) and species level ($P=0.624$) (Figure 2). Moreover, PCoA based on Bray-Curtis distance was used to compare the changes in microbiota composition at different time points. The dots did not overlap, suggesting that the composition of the microbiota was altered after bowel preparation.

2.3 Analysis of differential microbiota at the phylum level

A comparison of gut microbial compositional features at the phylum level between the 4 time points (detailed data in Table S3). No significant difference was observed at the phylum level among the four time points ($P>0.05$) (Figure 3).

2.4 Analysis of differential microbiota at the genus level

A comparison of gut microbial compositional features at the genus level between the 4 time points (detailed data in Table S4). At the genus level, six genera (*Eubacterium*, *Escherichia*, *Intertinibacter*, *Veillonella*, *Ruminococcaceae_unclassified* and *Coprobacillus*) were

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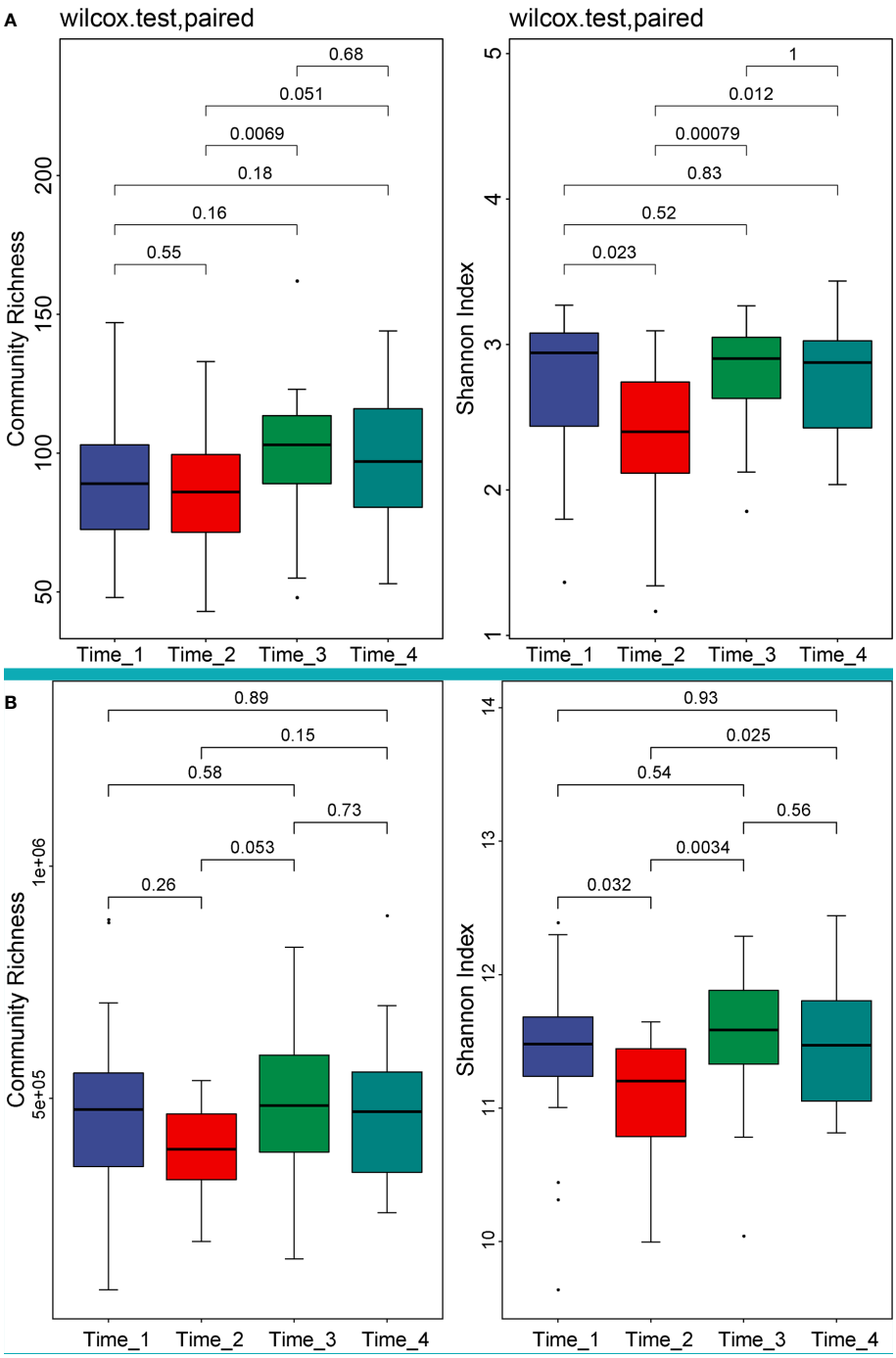

FIGURE 1
Alpha diversity analysis of 4 time points at the species and gene levels. (Left of **A**) Community richness at the species level showed that the abundance declined after bowel preparation, but not significantly. (Right of **A**) The Shannon diversity index decreased significantly at the species level (*P*=0.023) and gradually returned to the level before bowel preparation at Time_3. (Left of **B**) Community richness at the gene level showed that the abundance declined after bowel preparation, but not significantly. (Right of **B**) The Shannon diversity index decreased significantly at the gene level (*P*=0.032) and gradually returned to the level before bowel preparation at Time_3. Note, The Wilcoxon rank sum test was used to test the difference between groups, and the value above the horizontal line was the P-value of the difference test. Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation.

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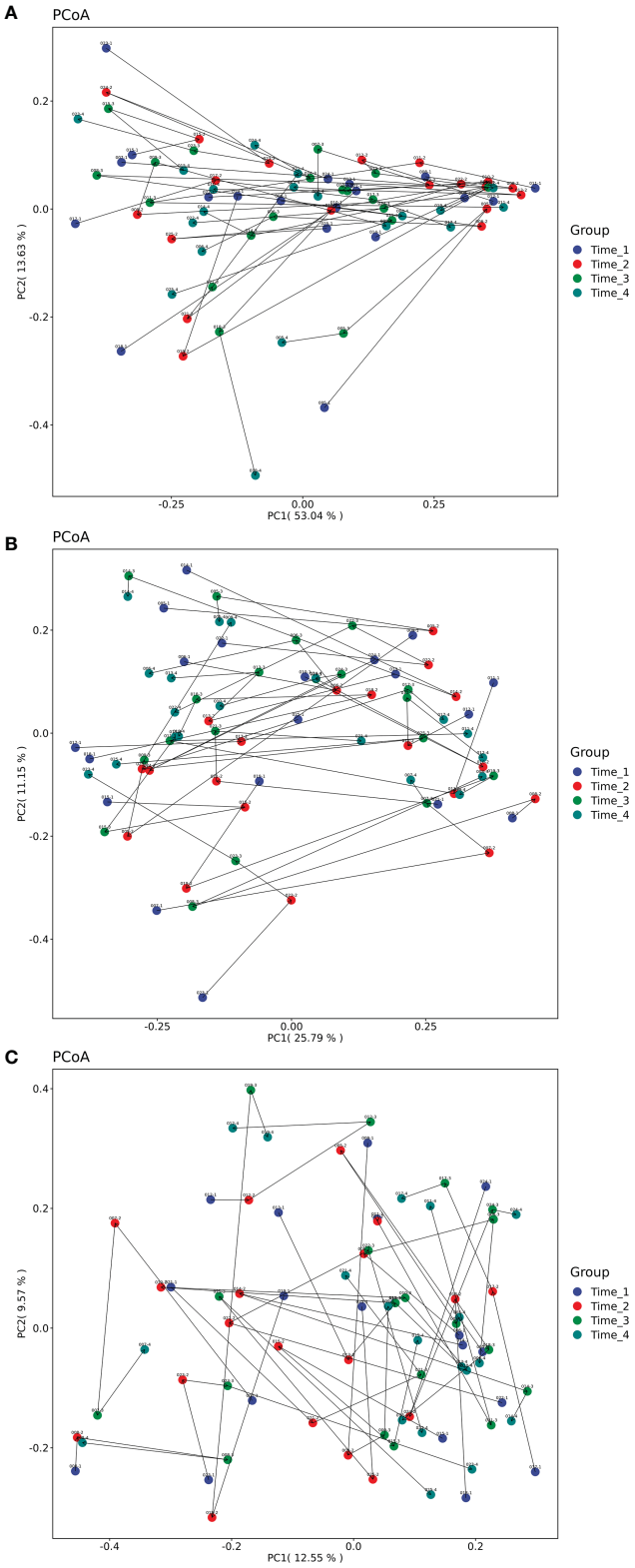

FIGURE 2
PCoA analysis based on Bray-Curtis distance of microbiota composition at 4 time points. **(A)** Beta diversity analysis of 4 time points at the phylum level. There was no significant difference in beta diversity between the 4 time points at the phylum level (P=0.564). **(B)** Beta diversity analysis of 4 time points at the genus level. There was no significant difference in beta diversity between the 4 time points at the genus level (P=0.545). **(C)** Beta diversity analysis of 4 time points at the species level. There was no significant difference in beta diversity between the 4 time points at the species level (P=0.624). None of the dots overlapped. Note, Different colored dots indicate different times. The line and arrow are connected for the same patient with sample ID (detailed in Table S1). Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation.

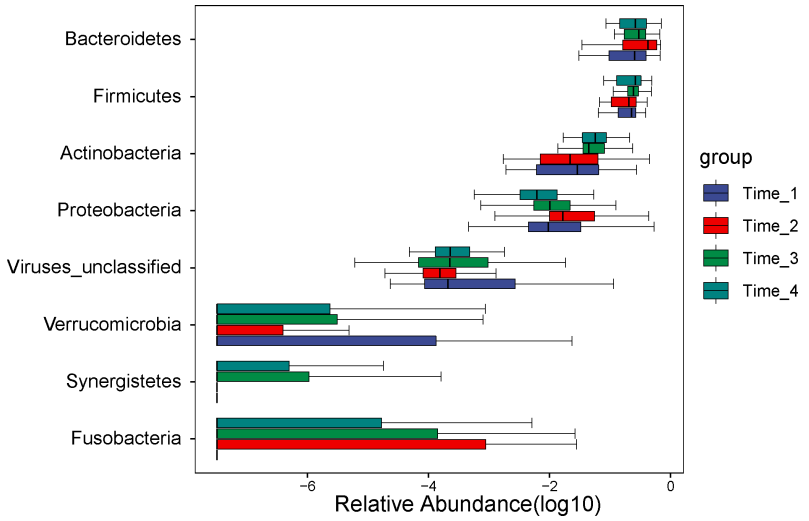

FIGURE 3
Analysis of differential microbiota at the phylum level. No differential microbiota was found at the phylum level (P>0.05). Note, Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation.

significantly different between the 4 time points (Figure 4). The abundance of *Eubacterium* increased at two weeks after bowel preparation. While the abundance of *Escherichia* and *Veillonella* increased at two days after bowel preparation and then gradually returned to the baseline level at two weeks after bowel preparation, the abundance of *Intertinibacter* decreased at two days after bowel preparation and was restored to the baseline level at two weeks and four weeks after bowel preparation.

2.5 Analysis of differential microbiota at the species level

A comparison of gut microbial compositional features at the species level between the 4 time points (detailed data in Table S5). At the species level, we discovered that the abundance of *Escherichia coli* (P=0.028), *Bacteroides fragilis* (P=0.039) and *Veillonella parvula* (P=0.002) increased significantly at two days after bowel preparation and gradually decreased at two weeks after bowel preparation (Figure 5). The abundance of *Intertinibacter bartlettii* (P=0.003) decreased obviously at two days after bowel preparation, recovered at two weeks after bowel preparation, and approached the baseline level at four weeks after bowel preparation.

2.6 KEGG functional pathway analysis

The Kruskal-Wallis test was used, and P<0.05 was used as the screening condition for significant differences in functional pathways (detailed data in Table S6). Five functional pathways (base excision repair, biosynthesis of ansamycins, biosynthesis of siderophore group nonribosomal peptide, flavonoid biosynthesis and biosynthesis of type II polyketide products) were significantly different in the four time periods (P<0.05) (Figure 6). While the pathway of biosynthesis of siderophore group nonribosomal peptide was decreased obviously at two days after bowel preparation, other pathways were increased significantly at two days after bowel preparation, but all of them recovered at two weeks after bowel preparation and approached the baseline level at four weeks after bowel preparation.

3 Discussion

The impact of bowel preparation on the composition of gut microbiota was first reported by Mai's team in 2006 (Mai et al., 2006). Since then, there have been a total of studies on this subject, with only one study reported in children (Table 1) (Mai et al., 2006; Harrell et al., 2012; Gorkiewicz et al., 2013; O'Brien et al., 2013; Jalanka et al., 2015; Drago et al., 2016; Shobar et al., 2016; Shaw et al., 2017; Powles et al., 2022). These studies comprised a sample size ranging from 4 to 23 and included both healthy and those with diseases such as IBD. Most of the studies utilized 16S rRNA, with no reported use of metagenomics. Thus, our study was the first to utilize metagenomics in investigating the effects of split-dose PEG bowel preparation and colonoscopy on gut microbiota in children.

Our study revealed a decrease in alpha diversity on the second day after bowel preparation, which gradually returned to the level before bowel preparation over the following two weeks. This finding is consistent with another study that reported a decrease in alpha diversity three days after bowel preparation that returned to pre-preparation levels at six weeks (Powles et al., 2022). Additionally, another study reported a reduction in the Shannon index only in biopsy samples from IBD patients (Shobar et al., 2016). These results suggest that bowel preparation does indeed affect alpha diversity, but its levels return to normal over time. However, beta diversity was not influenced by bowel preparation at the phylum, genus, and species levels. We observed distinct dots, which indicates

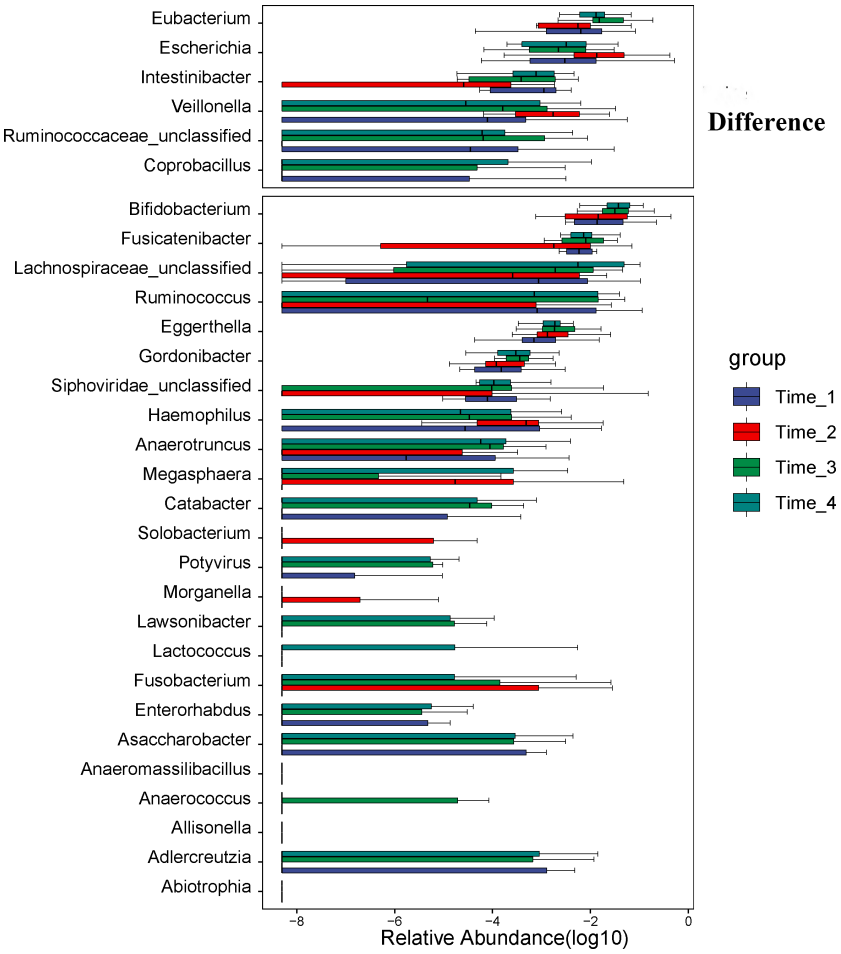

FIGURE 4
Analysis of differential microbiota at the genus level. At the genus level, six genera (*Eubacterium*, *Escherichia*, *Intertinibacter*, *Veillonella*, *Ruminococcaceae_unclassified* and *Coprobacillus*) were significantly different between the 4 time points. The abundance of *Eubacterium* increased at Time_3. While the abundance of *Escherichia* and *Veillonella* increased at Time_2 and then gradually returned to the level before bowel preparation at Time_3, the abundance of *Intertinibacter* decreased at Time_2 and was restored to the level before bowel preparation at Time_3 and Time_4. Note: Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation.

that the composition of gut microbiota was altered after bowel preparation. To date, no studies have reported on the effect of bowel preparation and colonoscopy on beta diversity of gut microbiota.

Our study did not reveal any significant difference at the phylum level, which was consistent with previous research (Harrell et al., 2012). However, some studies have reported changes at the phylum level following bowel preparation (Gorkiewicz et al., 2013; Jalanka et al., 2015; Drago et al., 2016; Shaw et al., 2017). Notably, in these studies, the PEG dose was relatively high or a single dose was used. It should be noted that one of these studies reported that bowel preparation using a single dose led to more alterations in gut microbiota than two separate doses (Jalanka et al., 2015). This suggests that split-dose PEG bowel preparation only has a minor effect on gut microbiota at the phylum level.

At the genus level, we found that six genera, including *Eubacterium*, *Escherichia*, *Intertinibacter*, *Veillonella*, *Ruminococcaceae_unclassified* and *Coprobacillus*, were significantly different. Some increased after bowel preparation, while some decreased, but most of them returned to normal after 2 weeks. Many studies have reported changes at the genus level after bowel preparation (Harrell et al., 2012; Jalanka et al., 2015; Shobar et al., 2016; Shaw et al., 2017). Each study reported that different genera are changed after bowel preparation. A pediatric study showed a significant increase in *Faecalibacterium* and significant decreases in *Ruminococcus*, *Escherichia*, *Pseudobutyrivibrio* and *Subdoligranulum* (Shaw et al., 2017). This was different from what we reported. This may be related to the origin of the patients. Although our two subjects were about the same age, we took more stool samples than that study. We collected 19 stool samples at each time period, but that study collected 11 pre-bowel preparation stool samples, 7 post-bowel preparation stool samples, seventeen biopsy samples, sixteen luminal content samples and eighteen swabs taken at colonoscopy.

At the species level, we discovered that the abundance of *Escherichia coli*, *Bacteroides fragilis* and *Veillonella parvula* increased significantly at two days after bowel preparation, gradually decreased two weeks later and gradually recovered. The abundance of *Intertinibacter bartlettii* decreased obviously at two

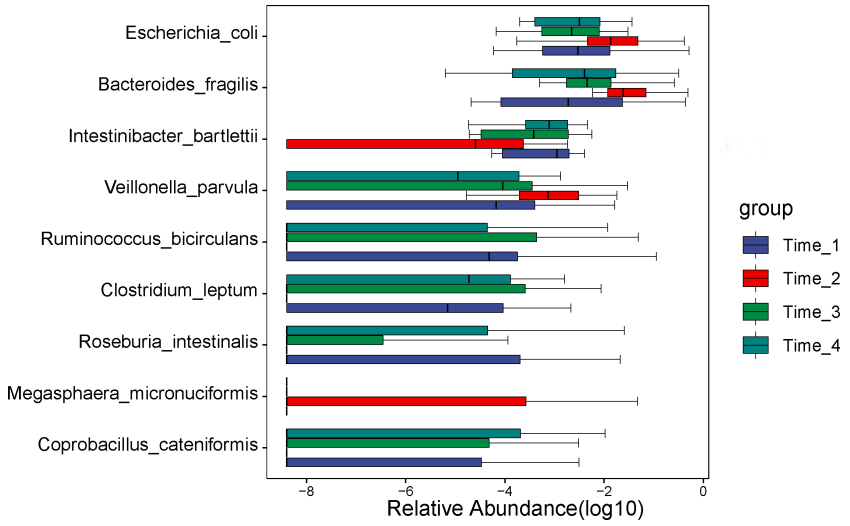

FIGURE 5
Analysis of differential microbiota at the species level. At the species level, we discovered that the abundance of *Escherichia coli, Bacteroides fragilis* and *Veillonella parvula* increased significantly at Time_2 and gradually decreased at Time_3. The abundance of *Intertinibacter bartlettii* decreased obviously at Time_2, recovered at Time_3, and approached the level before bowel preparation at Time_4. Note: Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation.

days after bowel preparation, recovered at two weeks later, and approached the preoperative level at four weeks. To date, no reports have been published on the effects of bowel preparation and colonoscopy on gut microbiota at the species level.

In our final analysis, we evaluated the functional diversity of gut microbiota at time points during bowel preparation. Our findings revealed a distinct decrease in the pathway of biosynthesis of siderophore group nonribosomal peptide two days after bowel preparation. Additionally, four pathways (baseision repair, biosynthesis of ansamycins, flavonoid biosynthesis, and biosynthesis of type II polyketide products) increased two days after bowel preparation, but all pathways recovered their pre-preparation levels within two weeks and gradually continued to recover. Our findings indicate that bowel preparation can decrease the biosynthesis of siderophore group nonribosomal peptide, while enhancing base excision repair, biosynthesis of ansamycins, flavonoid biosynthesis, and biosynthesis of type II polyketide products. Such effects of bowel preparation on gut microbiota have not been reported previously.

The results of our study show that after bowel preparation, the composition and functional pathways of gut microbiota undergo a change, which then gradually returns to baseline, demonstrating the stability and resilience of gut microbiota. Such qualities are considered basic ecological characteristics of gut microbiota (Lozupone et al., 2012). The ability of gut microbiota to restore its balance after infection with a pathogen or antibiotic treatment is referred to as resilience (Sommer et al., 2017; Palleja et al., 2018). The gut microbiota's stability and resilience are affected by factors such as unhealthy status, antibiotic use, and dietary factors (Fassarella et al., 2021). Our study represents the first utilization of metagenomics in children with split-dose PEG bowel preparation to understand the stability and resilience of gut microbiota. We also discovered that the primary outcome across studies in this field both in children and adults is the compositional alteration of gut microbiota after bowel preparation and colonoscopy, after which it recovers with time. Our findings are generally consistent with previous studies, indicating that split-dose PEG can be widely utilized for bowel preparation in children.

Our study had two limitations. Firstly, no detailed food frequency questionnaires were included which could account for the potential influence of diet on the observed differences in gut microbiota. Secondly the disease spectrum of the subjects was not consistent, which may have influenced the results.

In conclusion, our research indicates that split-dose PEG bowel preparation and colonoscopy induce changes in gut microbiota of children at the genus and species levels, as well as in functional pathways. However, the phylum level remains unaffected. It is possible that future bowel preparation research may target these compositional and functional changes in gut microbiota. Our study also exhibits the stability and resilience of gut microbiota, laying the foundation for future research.

4 Materials and methods

4.1 Study subjects

This study was carried out with the approval of the Human Ethics Committee of Shenzhen Children's Hospital. Participants aged from 2.5 to 16.8 years old (average age of 10.01 ± 3.47 years) were enrolled, including 11 boys and 8 girls, from September to December 2021 at Shenzhen Children's Hospital (Table 2). Before colonoscopy, these

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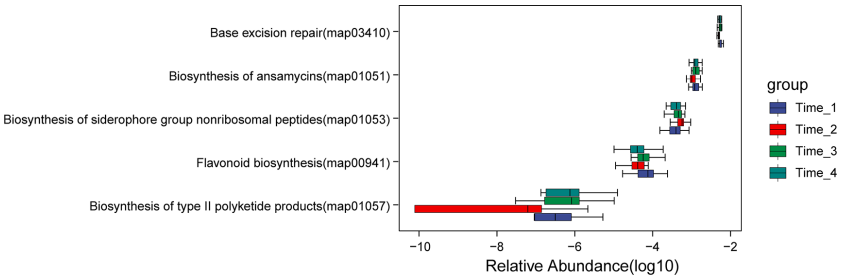

FIGURE 6
Analysis of differential functional pathways. Five functional pathways (base excision repair, biosynthesis of ansamycins, biosynthesis of siderophore group nonribosomal peptide, flavonoid biosynthesis and biosynthesis of type II polyketide products) were significantly different in the four time periods ($P<0.05$). While the pathway of biosynthesis of siderophore group nonribosomal peptide was decreased obviously at Time_2, other pathways were increased significantly at Time_2, but all of them recovered at Time_3 and approached the level before bowel preparation at Time_4. Note: Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation.

patients received a split dose of PEG electrolyte solution (total 80 mL/kg, ≤ 3000 mL, divided into two parts, two-thirds and one-third); the two-thirds in the evening and the one-third on the following morning. The mean BMI was 17.36 kg/m2. Patients who recently (past 90 days) used probiotics or antibiotics were excluded.

4.2 Sample collection and metagenomic sequencing

Fecal samples were obtained from all recruited subjects for metagenomic sequencing. Seventy-six samples of 19 cases were

TABLE 1 Research associated with the effects of bowel preparation on gut microbiota.

Research (ref)	Age (years)	Mean BMI (kg/cm^2)	No. patients	Bowel cleansing	Sample	Sampling time	Detection method	Result
Present research	10.01 ± 3.47	17.36	19 children	Split-dose PEG	Stool	Before bowel preparation after two days after two weeks after four weeks	Metagenomics	The genus level, species level and functional pathways were affected, but they recovered two weeks later. The microbiota did not change at the phylum level.
Mai et al. (21)	/	/	5 adults	/	Stool	Before colonoscopy during Colonoscopy after 6-8 weeks	DGGE	The composition of microbiota was disturbed in patients undergoing colonoscopy.
Harrell et al. (22)	25-48	/	12 healthy adults	PEG 4 L	Mucosa	Before colonoscopy after 1 week	16S rRNA sequencing	The phylum level was not significantly changed, but the genus level was differences observed.
O'Brien et al. (23)	46-69	/	20 adults	PEG 2 L + bisacodyl 10 mg	Stool	Before colonoscopy after 1 week after 4 weeks after 12-24 weeks	DGGE and 16S rRNA sequencing	Bowel preparation did not have a lasting influence on the composition of the microbiota.
Gorkiewicz et al. (24)	36-47	24-26.6	4 healthy adults	PEG 150 g, for 3 days	Stool and mucosa	Before colonoscopy after 4 days	16S rRNA sequencing	The phylum level was significantly changed both in the stool and mucosa.
Jalanka et al. (25)	25-27	23-23.3	23 healthy adults	PEG 1 L × 2 vs 2 L	Stool	Before colonoscopy during	16S rRNA sequencing	The split-dose bowel preparation introduced fewer effects to the gut microbiota than a single dose. The composition of the microbiota was

(Continued)

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TABLE 1 Continued

Research (ref)	Age (years)	Mean BMI (kg/cm^2)	No. patients	Bowel cleansing	Sample	Sampling time	Detection method	Result
						colonoscopy after 2 weeks after 4 weeks		decreased, and they would restored within 14 days, the rate of recovery was dose dependent.
Drago et al. (26)	40-68	24.6	10 adults	PEG 4 L	Stool	Before colonoscopy during colonoscopy after 4 weeks	16S rRNA sequencing	The gut microbiota at the phylum, class, and family level were changed.
Shobar et al. (27)	49-55.4	22.15-32.2	8 IBD and 10 healthy adults	/	Stool and mucosa	Before colonoscopy during colonoscopy	16S rRNA sequencing	The composition and diversity of the fecal and luminal microbiota were affected.
Shaw et al. (28)	4-17	/	16 children	Sodium picosulfate with magnesium citrate and senna	Stool, mucosa, swab	Before colonoscopy during colonoscopy after 54 days	16S rRNA sequencing	Bowel preparation had a clear transient effect on the microbiota during colonoscopy, but no significant long-term effect.
Powles et al. (29)	41	23.4	11 adults	MoviPrep	Stool and urine	Before colonoscopy after 3 days after 6 weeks	16S rRNA sequencing	Bowel preparation temporarily reduced the alpha diversity of gut microbiota without significant changes in fecal and urine metabolites.

PEG, polyethylene glycol; DGGE, denaturing gradient gel electrophoresis;/, not mention; IBD, inflammatory bowel disease; MoviPrep, included Macrogol 3350, sodium sulfate anhydrous, sodium chloride, potassium chloride, ascorbic acid and sodium ascorbate; our study is highlighted in red.

TABLE 2 Characteristics of the study cohort.

Case	Age (years)	Gender (Male : Female 11:8)	Height (cm)	Weight (kg)	BMI (kg/cm^2)
Case1	12.1	Male	156	49.5	20.34
Case2	10.1	Male	135	27.0	14.81
Case3	12.9	Male	161	59.8	23.07
Case4	12.9	Male	154	43.4	18.29
Case5	13.6	Female	153	45.1	19.26
Case6	2.5	Male	97	15.2	16.15
Case7	13.4	Female	164	46.2	17.17
Case8	9.4	Female	128	21.4	13.06
Case9	6.2	Female	123	19.4	12.82
Case10	10	Female	149	36.5	16.44
Case11	16.8	Male	175	83.6	27.29
Case12	9.0	Male	139	33.2	17.18
Case13	7.0	Female	128	23.4	14.28
Case14	7.0	Female	126	23.6	14.86
Case15	9.0	Male	142	29.2	14.48
Case16	9.0	Female	140	39.6	20.20
Case17	12.9	Male	157	57.0	23.12
Case18	5.2	Male	112	16.4	13.07
Case19	11.1	Male	131	24.0	13.98

collected in four time periods (Sample ID in Table S1). Time_1: one day before bowel preparation; Time_2: two days after bowel preparation (one day after colonoscopy); Time_3: two weeks after bowel preparation; Time_4: four weeks after bowel preparation (Figure 7). Each sample was frozen immediately at −80°C before transport to the laboratory within 24 h. Stool sample DNA was extracted using the CTAB method.

A sequencing library was generated using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to each sample. The DNA libraries were sequenced on an Illumina platform, and 150 bp paired-end reads were generated.

4.3 Metagenomic analysis

The adapter was trimmed, and low-quality reads were filtered using trimmomatic (version 0.39). Then, host sequences were removed by aligning sequencing reads back to the host genome reference (hg38) using soap2 (version 2.20) when sequence identity exceeded 90% (Qin et al., 2012).

Taxonomic profiling of the metagenomic samples was performed using MetaPhlAn (version 3.0.7), which uses clade-specific markers to provide panmicrobial (bacterial, archaeal, viral and eukaryotic) quantification at the species level (Beghini et al., 2021). MetaPhlAn was run with the parameters '–read_min_len 50 –add_viruses –unknown_estimation'.

At the same time, the high-quality reads were aligned to the updated gut microbiome gene catalog using SOAP2 (version 2.20) with a threshold of more than 90% identity and 95% read length (Li et al., 2014). The gene abundance profile was calculated as previously described (Li et al., 2014). Next, the relative abundances of KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologous (KO) groups were summed from the relative abundances of their respective genes to obtain a functional profile.

4.4 Bioinformatic and statistical analysis

4.4.1 Alpha and beta diversity

Alpha diversity was measured by observed counts and the Shannon index at the gene and species levels with an in-house Perl script. We performed the Wilcoxon rank-sum test for the difference in α diversity. Unless otherwise stated, all statistical analyses were performed in R software, and P values <0.05 were considered statistically significant.

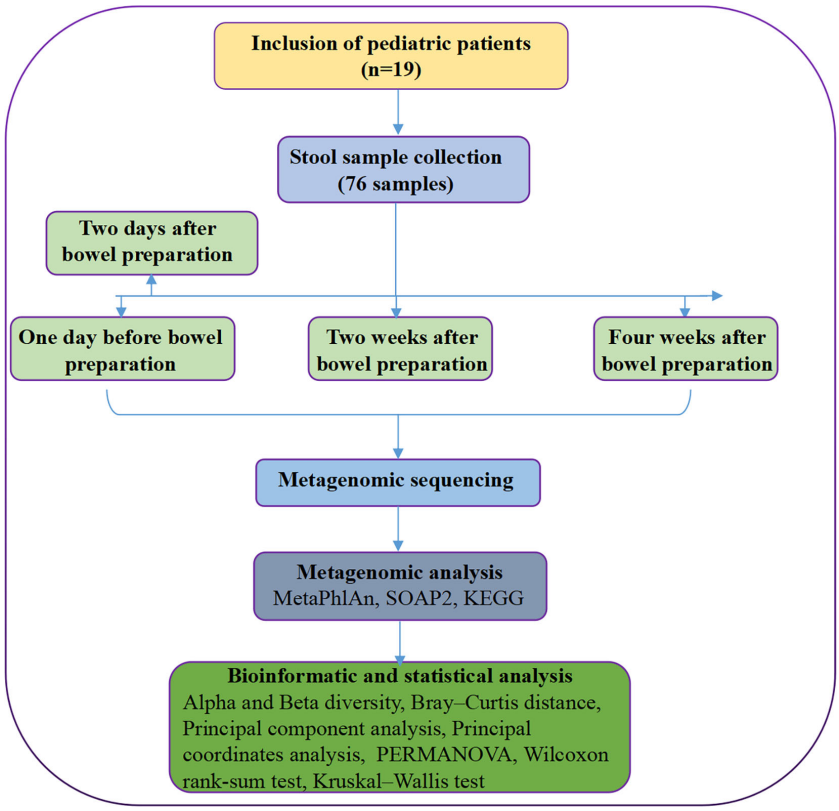

FIGURE 7
Flowchart of the study design. The samples were collected at 4 time points, including one day before bowel preparation, two days after bowel preparation, two weeks after bowel preparation and four weeks after bowel preparation. Note, KEGG, Kyoto Encyclopedia of Genes and Genomes; PERMANOVA, permutation multivariate analysis of variance.

The Bray-Curtis distance was calculated using the Python module scipy (version 1.5.1). Principal component analysis (PCA) was performed using the R package FactoMineR and factoextra. Principal coordinates analysis (PCoA) was used to visualize beta diversity using the Bray-Curtis distance matrix data in R with ggplot2. The R packages vegan and ggplot2 were used to analyse and visualize NMDS (nonmetric multidimensional scaling) using Bray-Curtis distance.

4.4.2 PERMANOVA

PERMANOVA (permutation multivariate analysis of variance) was used to assess the effects of different phenotypes on metagenomic profiles. We used Bray distance and 999 permutations in R (version 3.6.3, vegan package).

4.4.3 Analysis of differential microbiota

We performed a Kruskal-Wallis test for the difference in microbiota. All statistical analyses were performed in R software, and P values < 0.05 were considered statistically significant.

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: European Bioinformatic Institute (EBI, https://www.ebi.ac.uk/) database under accession code PRJEB56144.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shenzhen Children's Hospital. 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

Conceptualization: YZ, SHZ, SMZ, JZ. Data collection: MC. Formal analysis: SL, QF. Funding acquisition: SMZ, JZ. Project administration: YZ, SHZ, SMZ, JZ. Writing original draft: YZ, SHZ, SMZ, JZ. Writing-review & editing: YZ, SHZ, SMZ, JZ. All authors have read and approved 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1202007/full#supplementary-material

SUPPLEMENTARY TABLE 1
Sample ID, group and pair of 19 cases.

SUPPLEMENTARY TABLE 2
Alpha diversity data at the species level.

SUPPLEMENTARY TABLE 3
The data of differential microbiota at the phylum level.

SUPPLEMENTARY TABLE 4
The data of differential microbiota at the genus level.

SUPPLEMENTARY TABLE 5
The data of differential microbiota at the species level.

SUPPLEMENTARY TABLE 6
KEGG functional pathway data.

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Revealing a causal relationship between gut microbiota and lung cancer: a Mendelian randomization study

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Background: The gut microbiota has been found to be associated with the risk of lung cancer. However, its causal relationship with various types of lung cancer remains unclear.

Methods: We conducted a Mendelian randomization (MR) study using the largest genome-wide association analysis of gut microbiota data to date from the MiBioGen consortium, with pooled statistics for various types of lung cancer from the Transdisciplinary Research in Cancer of the Lung, the International Lung Cancer Consortium, and FinnGen Consortium R7 release data. Inverse variance weighted, weighted model, MR-Egger regression, and weighted median were adapted to assess the causal relationship between gut microbiota and various types of lung cancer. Sensitivity analysis was used to test for the presence of pleiotropy and heterogeneity in instrumental variables. A reverse MR analysis was performed on these bacteria to determine their potential role in causing lung cancer. A reverse MR analysis was performed on these bacteria to determine their potential role in causing lung cancer. Multivariable Mendelian randomization (MVMR) was conducted to assess the direct causal impact of gut microbiota on the risk of various types of lung cancer.

Results: Using IVW as the primary analytical method, we identified a total of 40 groups of gut microbiota with potential causal associations with various subtypes of lung cancer, of which 10 were associated with lung cancer, 10 with lung adenocarcinoma, 9 with squamous cell lung cancer, and 11 groups of bacteria associated with small cell lung cancer. After performing FDR correction, we further found that there was still a significant causal relationship between Peptococcaceae and lung adenocarcinoma. Sensitivity analyses demonstrated the robustness of these results, with no heterogeneity or pleiotropy found.

Conclusions: Our results confirm a causal relationship between specific gut microbiota and lung cancer, providing new insights into the role of gut microbiota in mediating the development of lung cancer.

KEYWORDS

lung cancer, gut microbiota, Mendelian randomization, causal inference, genome-wide association study

1 Introduction

According to statistics, lung cancer accounts for 2.2 million new cases and 1.79 million fatalities each year (Thai et al., 2021), making it the second most commonly occurring cancer type globally and the primary cause of cancer-related deaths (Sung et al., 2021; Huang et al., 2022). Several well-known risk factors contribute to the development of lung cancer, including smoking, previous lung diseases, air pollutants, and occupational carcinogens (Samet et al., 2009; Wang et al., 2020). Identifying and discovering potentially modifiable risk factors is crucial to reducing the incidence of lung cancer, which is conducive to early diagnosis and treatment of lung cancer.

Recently, it has been suggested that the gut microbiota (GM) has an impact on the progression of lung cancer. GM refers to the highly complex community of microorganisms that reside within the gastrointestinal tract, including archaea, bacteria, eukaryotes, parasites, and viruses (Ge et al., 2021). Dysbiosis of microbial ecology may lead to changes in metabolism, immunosuppression, and the recruitment of inflammatory factors that can drive lung carcinogenesis. In the study by JinC and LagoudasGK et al., mouse models showed that the local microbiota associated with tumor development promotes inflammatory progression through lung-resident γδ T cells (Jin et al., 2019) and is thought to be associated with lung cancer. The most recent theory holds that even though the gastrointestinal tract and respiratory tract are physically separate, they share an embryonic origin and a high degree of structural similarity (Georgiou et al., 2021). There is a distinct crosstalk between the respiratory tract and the gastrointestinal tract known as the gut–lung axis (Dang and Marsland, 2019; Enaud et al., 2020; Zhang et al., 2020). The gut-lung axis is the pathway through which the GM interacts with the lung (Young et al., 2016; Liu et al., 2021), Microbiota-accessible carbohydrates can alter the GM and improve short-chain fatty acid (SCFA) levels, hence shaping lung immunity (Arrieta et al., 2015; Budden et al., 2017). Through T cell receptor signaling, SCFAs may activate innate lymphoid cells that produce IL-22 type 3, regulatory T cells, and Th2 cells in the lung to reduce inflammation and thus reduce the incidence of lung cancer (Corrêa et al., 2022). SCFAs can also exert effective anti-inflammatory and immunomodulatory effects by activating G protein-coupled cell surface receptors and inhibiting histone deacetylases, as well as exert immunomodulatory effects by activating G protein-coupled cell surface receptors and inhibiting histone deacetylases (Ubachs et al., 2021). Nonetheless, whether there is a clear causal relationship between lung cancer and GM still needs further proof.

In order to investigate the potential causal link between lung cancer and GM, Mendelian randomization (MR) analysis was used. MR analysis is a statistical strategy based on genome-wide association analysis, following the Mendelian rule of "parental alleles randomly assigned to offspring". It is therefore a natural randomized controlled trial (RCT). MR analysis has been extensively utilized to investigate the connection between GM and diseases such as pre-eclampsia and eclampsia (Li et al., 2022), autoimmune diseases (Xu et al., 2021), and psychiatric disorders (Zhuang et al., 2020). In this research, we employed the two-sample MR analysis method to examine the potential impact of GM in various types of lung cancer. The findings of this research can serve as a foundation for understanding the etiology and diagnosis of lung cancer.

2 Materials and methods

2.1 Study design

A bidirectional two-sample MR analysis was conducted to investigate the causal relationship between GM and various subtypes of lung cancer. The flow chart of the study is shown in Figure 1. The aim of reverse MR analysis is to mitigate the potential effect of various subtypes of lung cancer on the GM and improve the confidence of the results.

2.2 Data sources

The MiBioGen study provides genome-wide association summary data for the GM (Kurilshikov et al., 2023) and is the largest meta-analysis of its kind to date. The study includes 24 cohorts comprising 18,340 participants of various races and ages. To ensure data quality, the majority of cohorts employed comparable methods for interpolation and subsequent filtering. Direct taxonomic binning was used to classify the taxonomy, resulting in 211 taxa being included in the analysis, representing 131 genera, 35 families, 20 orders, 16 classes, and 9 phyla.

To bolster statistical power, validate research findings, and foster the exploration of potential new associations, we leveraged a comprehensive set of three lung cancer datasets. The Transdisciplinary Research in Cancer of the Lung (TRICL) is a member of an organization focused on Genetic Associations and Mechanisms in Oncology (GAME-ON consortium). The International Lung Cancer Consortium (ILCCO) is an international organization of lung cancer researchers that was founded in 2004 to share data from ongoing lung cancer epidemiological studies with the overall goal of maximizing statistical power. We obtained summary data from the MR-Base database for both TRICL and ILCCO. The FinnGen consortium R7 (http://r7.finngen.fi/) results provided us with summary data on small-cell lung cancer. All the data is listed in Table 1. We used all summary data from published studies and publicly available GWAS abstracts, and therefore did not require additional ethical approval or consent.

2.3 Instrumental variable selection

For the MR analysis, we examined 211 taxa of gut microbes, but excluded 15 taxa from unclassified groups, leaving 196 bacterial taxa (9 phyla, 16 classes, 20 orders, 32 families, and 119 genera) to be included in the analysis. To ensure data robustness and reliability of results, quality control of SNPs was performed to obtain compliant instrumental variables:(1) After consulting the relevant research (Lv

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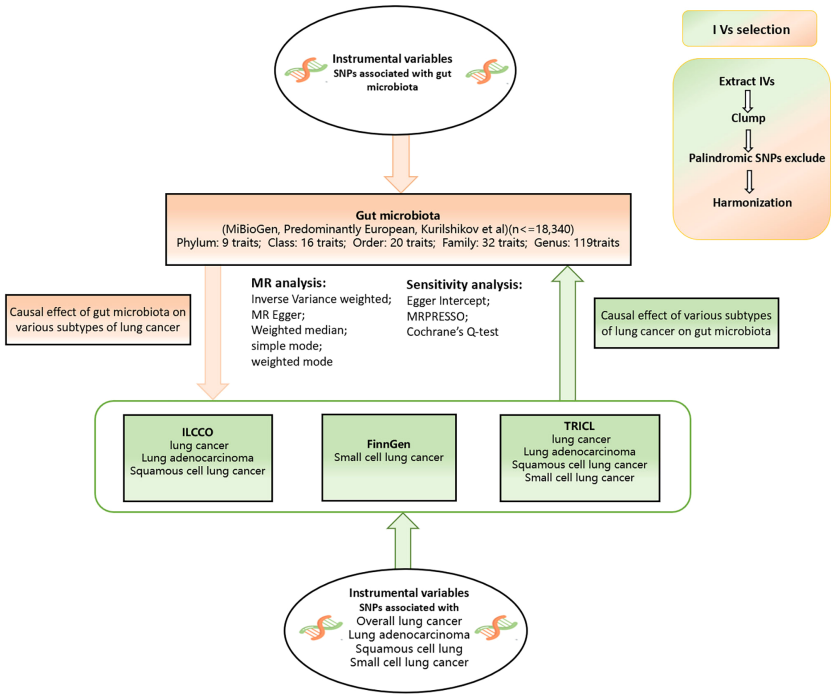

FIGURE 1
Flow chart of our study design. This study was a bidirectional Mendelian randomization analysis testing the causal effects between the gut microbiota and different types of lung cancer. Inverse variance weighted (IVW) was adopted as the primary method for univariable MR. To provide robust evidence of MR estimates, sensitivity analyses were used.

TABLE 1 The data source of exposure, outcome, and multivariate Mendelian randomization.

Overview of the source of gut microbiota.							
Traits	Sample size	Consortium	Population	download URL			
Gut microbiota	18340	MiBioGen	mixed	https://mibiogen.gcc.rug.nl/			
Overview of the source of various subtypes of lung cancer.							
GWAS ID	Trait	Consortium	Sample size	Case	Control	Number of SNPs	Population
ieu-a-987	Lung cancer	TRICL	85,449	29,863	55,586	10,439,018	European
ieu-a-984	Lung adenocarcinoma	TRICL	65,864	11,245	54,619	10,345,176	European
ieu-a-989	Squamous cell lung cancer	TRICL	62,467	7,704	54,763	10,341,529	European
ieu-a-988	Small cell lung carcinoma	TRICL	23,371	2,791	20,580	7,438,318	European
ieu-a-966	Lung cancer	ILCCO	27,209	11,348	15,861	8,945,893	European
ieu-a-965	Lung adenocarcinoma	ILCCO	18,336	3,442	14,894	8,881,354	European
ieu-a-967	Squamous cell lung cancer	ILCCO	18,313	3,275	15,038	8,893,750	European
finn-b-C3_SCLC	Small cell lung carcinoma	NA	218,792	179	218,613	16,380,466	European
Multivariate Mendelian randomization							
GWAS ID	Trait	Consortium	Sample size	Case	Control	Number of SNPs	Population
finn-b-J10_COPD	COPD	NA	447,485	6915	186723	16,380,382	European
ieu-a-1283	Alcohol consumption	UK Biobank	112,117	NA	NA	12,935,395	European
ieu-b-142	Cigarettes smoked per day	GSCAN	249,752	NA	NA	12,003,613	European

NA, Not Applicable.

et al., 2021; Liu et al., 2022; Ren et al., 2023; Su et al., 2023), we adjusted the threshold to the locus-wide significance level ($p < 1 \times 10-5$); (2) As strong linkage disequilibrium may cause biased results, we performed linkage disequilibrium (LD) analysis with a threshold of r2< 0.001 and an aggregation window of 10,000kb, using a reference panel of 1000 Genome Project European samples; (3) Exclusion of palindromic sequences and SNPs with allelic inconsistencies between the two samples; and (4) To evaluate the strength of the SNP, we calculated the F-statistic. An F-statistic value of ≥10 indicates a lack of strong evidence for weak instrument bias. We excluded weak instruments with F-statistics less than <10.

2.4 Statistical analysis

2.4.1 MR analysis

To identify the connection between the GM and various types of lung cancer, we utilized four approaches in this study: inverse variance weighted (IVW), MR-Egger, weighted model, and weighted median. IVW is a method for MR to meta-summarize wald estimates for each locus when analyzing multiple SNPs. The IVW results would be objective if horizontal pleiotropy did not exist (Pierce and Burgess, 2013). The MR-Egger method employs the InSIDE assumption to perform a weighted linear regression of exposure outcomes, while the weighted median method assigns greater weight to SNPs with larger beta values during estimation. Weighted median is the median of the distribution function obtained by sorting all individual SNP effect values by weight. To obtain robust estimates in our research, we require at least 50% of the information to be derived from valid instrumental variables. We performed validation using the Benjamini-Hochberg correction. Only significant data with a corrected p-value of less than 0.05 were considered to have a strong causal relationship. However, we suggest that bacteria with a p-value of less than 0.05 but not significant after correction may still have a potential causal relationship with lung cancer. Furthermore, we conducted additional reverse MR analyses to investigate whether different types of lung cancer have a causal impact on the significant GM identified in our study. In order to investigate the influence of the GM on different subtypes of lung cancer, we conducted a multivariate Mendelian randomization analysis. This analytical approach allowed us to explore the causal relationship between the GM and lung cancer subtypes while controlling for potential confounding factors such as smoking, alcohol consumption, and chronic obstructive pulmonary disease (COPD).

2.4.2 Sensitivity analysis

We utilized MR-PRESSO to evaluate horizontal pleiotropy, while MR-Egger avoided enforcing the regression line through zero to account for the existence of directional genetic pleiotropy. In cases where the regression intercepts were non-zero and $p<0.05$, we regarded them as statistically significant indicators of genetic pleiotropy. Furthermore, we conducted Cochran's Q test, and we observed heterogeneity if $p<0.05$. Finally, we carried out a leave-one-out sensitivity test to determine if a single instrumental variable had a significant impact on the causal effect.

The R software was used to perform all statistical analyses (Version 4.2.2). "TwoSampleMR" package (version 0.5.6) (Hemani et al., 2017) and "MRPRESSO" (version 1.0) package (Verbanck et al., 2018) were used to perform MR analysis. The code used for our analysis is available on GitHub [GitHub].

3 Results

The results of MR estimates for the relationship between the GM and different types of lung cancer are presented in Supplementary Table 1 and Figure 2. After correction, one microbial taxon was found to be significant at a significance level of 0.05.

3.1 Causal effect of GM on various subtypes of lung cancer

For overall lung cancer, as shown in Supplementary Figure 1, we identified a total of 11 causal relationships between the GM and lung cancer in the ILCCO and TRICL datasets. Among these 11 microbial communities, Prevotella9 ($P = 0.004$), Coprococcus3 ($P = 0.016$), Holdemanella ($P = 0.005$), and Peptococcus ($P = 0.017$) were considered positively correlated with the risk of lung cancer, while Bifidobacteriaceae ($P = 0.025$), Collinsella ($P = 0.018$), and Ruminiclostridium6 were ($P =0.029$) deemed negatively correlated with lung cancer risk. Clostridiumsensustricto1 ($P_1 = 0.022$, $P_2 = 0.028$) and "Bifidobacteriales" ($P_1 = 0.02$, $P_2 = 0.025$) both exhibited positive results in two lung cancer datasets. Notably, "family Bifidobacteriaceae" and "order Bifidobacteriales" demonstrated a consistent effect in the same lung cancer dataset ($P = 0.025$).

For lung adenocarcinoma, as shown in Supplementary Figure 2, we identified a total of 11 causal relationships between the GM and lung adenocarcinoma in two datasets, among which 7 demonstrated an inversely proportional relationship with the risk of lung adenocarcinoma. To be specific, these encompass Mollicutes ($p = 0.048$), Defluviitaleaceae ($p = 0.009$), Peptococcaceae ($p =0.001$), Eubacteriumhalliigroup ($p = 0.004$), Collinsella ($p = 0.04$), Gordonibacter ($p=0.06$), and Tenericutes ($p= 0.048$), possibly because Mollicutes represent the class level, while Tenericutes represent the phylum level, with the latter encompassing the former. This is why these two microbial taxa exhibited similar effects on lung adenocarcinoma. Conversely, Prevotella9 ($p = 0.003$) and Anaerotruncus ($p= 0.009$) displayed a direct correlation with the risk of lung adenocarcinoma. It is worth noting that the Holdemanella has exhibited remarkable efficacy in both datasets. The Peptococcaceae was also found to be significant after P-value adjustment ($p\_adj = 0.04$).

For squamous cell lung cancer, as demonstrated in Supplementary Figure 3, nine bacteria were identified by MR analysis as being associated with squamous cell lung cancer. Genetically predicted risk of squamous cell lung cancer was correlated with Ruminococcustorquesgroup ($P = 0.022$), Actinomyces ($p = 0.004$), Eggerthella ($P = 0.041$), Flavonifractor ($P = 0.025$), and Peptococcus ($P = 0.014$). The genetically predicted

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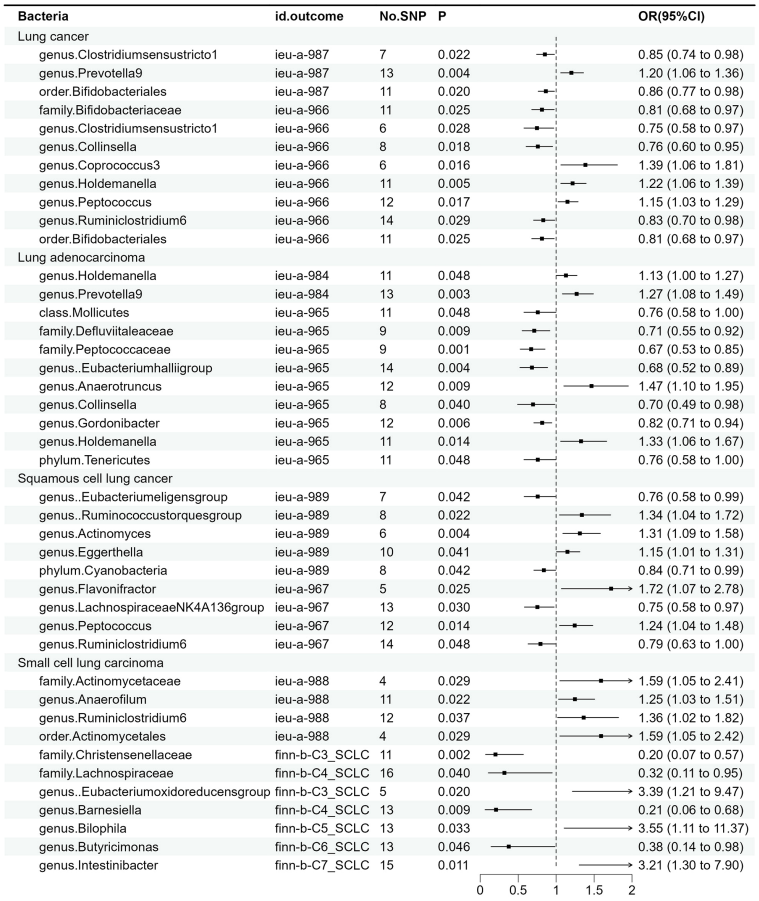

FIGURE 2
Associations between gut microbiota and various subtypes of lung cancer. The odds ratios are scaled per 1 standard deviation increase in gut microbiota.CI, confidence interval; OR, odds ratio.

protective roles of microbiota are Eubacterium eligens (P = 0.042), Collinsella (p = 0.04), Lachnospiraceae (p = 0.03), and Ruminiclostridium6 (p = 0.048).

Furthermore, we also identified 11 bacteria associated with small cell lung cancer, as illustrated in Supplementary Figure 4. The results of IVW showed a causal correlation of Actinomycetaceae (P = 0.029), Actinomycetales (P = 0.029), Anaerofilum (P = 0.022), Ruminiclostridium6 (P = 0.037) Eubacterium oxidoreducens (P = 0.02), Bilophila (p = 0.033), and Intestinibacter (P = 0.011) on the risk of small cell lung cancer. Furthermore, the microbial communities Christensenellaceae (P = 0.002), Lachnospiraceae (P = 0.04), Barnesiella (P = 0.009), and Butyricimonas (P = 0.046) conferred a protective effect against small cell lung cancer.

3.2 Sensitivity analysis

Sensitivity analyses were performed to ensure the results' robustness. The findings of Cochran's Q showed that the instrumental variables were homogeneous. According to the results of the MR-Egger regression, no evidence of pleiotropic effect was observed (all P intercept >0.05), as well as MR-PRESSO global test (all P global test >0.05); this finding shows that IVs seem to be unlikely to influence lung cancer risk through pathways other than the GM with significant results. All results can be found in the Supplementary File. Furthermore, Supplementary Figures 5-8 summarize the results of the causal effect of significant taxa on various subtypes of lung cancer in the leave-one-out analysis.

3.3 Reverse MR analysis

A reverse MR analysis was carried out to investigate whether different types of lung cancer have any causal impact on the observed significant bacteria. The reverse MR analysis process was identical to the former MR analysis. The majority of results indicated that there is no reverse causality (Supplementary Tables 2-4). Only the effect of small cell lung cancer on Holdemania exhibited a reverse causal relationship, and to ensure the rigor of our results, we excluded this finding.

3.4 MVMR analysis

To ascertain whether the significantly positive microbial community observed after calibration directly or indirectly affects

cancer risk through common cancer risk factors, we performed additional MVMR analysis. Although multivariable Mendelian randomization (MR) has been used in some previous studies to consider the joint effects of multiple variables, we did not find that this approach was suitable for our current research. After considering important confounding factors such as smoking, alcohol consumption, and chronic obstructive pulmonary disease in our analysis, we were unable to identify any common SNPs across the majority of the GM, which limits the ability to establish a robust causal relationship using multivariable MR; the results are shown in Supplementary Table 5. Previous studies have shown that conducting multivariable MR analyses in the presence of insufficient common SNPs may lead to the accumulation of bias and errors in conclusions, lacking scientific justification (Sanderson, 2021). Only Peptococcus yielded common SNPs during the multivariable Mendelian randomization analysis; however, after adjustment, the results were no longer significant.

4 Discussion

In this study, we performed a two-sample MR analysis using the summary statistics of the GM from the MiBioGen consortium's largest GWAS data and the summary statistics of various subtypes of lung cancer from the TRICL, ILCCO, and FinnGen consortium R7 release data to evaluate the causal association between GM and lung cancer. We found 10 GM to be causally associated with overall lung cancer, 10 with lung adenocarcinoma, 9 with squamous cell carcinoma, and 11 with small cell lung cancer. After FDR correction, we found Peptococcaceae to be causally associated with lung adenocarcinoma.

The GM primarily influences the occurrence of lung cancer *via* the gut-lung axis, exerting its effects by regulating the lung immune system, influencing lung inflammation response, and producing metabolites (Zhao et al., 2021). Alterations in the GM may lead to disruption of the intestinal mucosal barrier, resulting in the occurrence of inflammation. These inflammatory factors may enter the lungs through the gut-lung axis (Enaud et al., 2020; Zhao et al., 2021), and long-term inflammatory responses can promote the occurrence of lung cancer by activating signal transduction pathways. In addition, intestinal immune cells such as ILC2s, ILC3, and TH17 can migrate directly from the intestine to the respiratory tract *via* the bloodstream to influence the immune activity of the respiratory system (Ma et al., 2022). The GM can also produce various metabolites, such as SCFAs, carotenoids, and bile acids (Du et al., 2022; Soriano-Lerma et al., 2022). These metabolites may directly or indirectly affect the occurrence of lung cancer by entering the lungs through the gut-lung axis.

The family of Bifidobacteriaceae, as a probiotic, has been reported in a study to have a protective effect against oxidative stress-induced DNA damage *in vitro*. This study suggests that Bifidobacterium bifidum, a member of the Bifidobacteriaceae family, possesses antioxidant properties that may prevent diseases such as lung cancer by reducing DNA damage (Bhatt et al., 2017). Our research findings are consistent with the notion that Bifidobacteriaceae has a strong causal relationship with lung cancer and serves as a protective factor against it. With regard to cyanobacteria, phycocyanin can be produced, which has a variety of biological functions such as anti-tumor, anti-oxidant, immunomodulatory, and anti-inflammatory activities. Several studies have demonstrated that phycocyanin exerts a dual role in NSCLC cells by not only reducing the activity and proliferation of A549 cells (Hao et al., 2018a), but also inhibiting the proliferation of various NSCLC cell lines (such as H1299, H460, and LTEP-a2) while inducing apoptosis (Hao et al., 2018b). These findings provide a basis for future applications of cyanobacteria in the treatment of NSCLC. It is worth mentioning that our findings align with those of Nam et al., who found that patients with the inflammatory disease "rosacea" had lower levels of Peptococcaceae in the gut (Nam et al., 2018). Furthermore, chronic inflammation is known to be linked to lung cancer. Based on this, we propose that Peptococcaceae may play a role in mitigating lung inflammation damage and could potentially impact the development of lung cancer. However, there is a lack of relevant research evidence to confirm the specific mechanism of the potential causal link between these specific microbial taxa and the incidence of lung cancer.

The study by J et al. suggests that the dysbiosis of lung microbiota in lung cancer patients is associated with upregulation of the PI3K signaling pathway and cancer progression. Specific lung cancer-associated bacteria, such as Prevotella, may impact the tumor microenvironment and initiate a cascade reaction in cancer cells, leading to the upregulation of the PI3K/AKT signaling pathway and cancer development. Overall, this basic research reveals the potential role of Prevotella and other specific lung cancer-associated bacteria in lung cancer development, providing a possible avenue for researching new preventive and therapeutic strategies (He et al., 2022). There is also a study that shows a potential association between Actinomyces and lung cancer development. The relative abundance of Actinomyces is increased in both COPD and NSCLC patients, and its abundance is positively correlated with the risk of developing lung cancer. This study reveals an association between the dysbiosis of lung microbiota and the occurrence of lung cancer. However, further research is necessary to determine the precise impact of Actinomyces on lung cancer development (Peters et al., 2022). It is worth noting that there is currently no research directly determining how the genus Ruminiclostridium 6 and Actinomycetales influence lung cancer. However, a study has shown that patients with a higher relative abundance of Actinomycetales have a lower risk of lung cancer recurrence after surgery (Wei et al., 2023). Further research is needed to determine the exact mechanism of action of Actinomycetales and other bacterial genera in lung cancer development.

It is worth noting that our research shows significant differences in results when compared to two other studies, those by Long et al. and Wei et al (Long et al., 2023; Wei et al., 2023), with the primary difference being that we used a larger and more comprehensive database. This makes our results more reliable, confirming previous research and providing a novel perspective on the potential causal relationship between GM and lung cancer. Additionally, we had stringent requirements for maintaining consistency between beta values and the IVW method in our analysis, which led to the exclusion of certain results due to slight differences. Additionally,

unlike studies with limited sample sizes or specific populations, the use of MR studies has the advantage of overcoming confounding factors. MR uses natural genetic variation as instrumental variables to explore causality between the exposure and outcome. This approach can eliminate reverse causality and collinearity and exclude most confounding factors during the estimation process, leading to results that are more likely to reflect the true cause-effect relationship. However, there may still be some confounding factors present in the study, such as smoking, diet, medications, and COPD, which could interfere with the composition of the GM and increase the risk of developing lung cancer. As such, future research should continue to explore and implement advanced methods to reduce the influence of confounding factors.

This research has several advantages. First, we utilized MR analysis to minimize the effects of reverse causation and confounding, thus enhancing the causal inference of lung cancer and the GM. Second, we chose a more comprehensive and larger database, which yielded more results. Additionally, to confirm the robustness of our results, we also employed the MR-Egger regression intercept term test and MR-PRESSO. Nevertheless, this study still has some limitations. One limitation is that the molecular biology of the GM is not yet fully understood, which limits our ability to deduce the molecular mechanisms that link GM and lung cancer. Additionally, the lung cancer cases are primarily from cross-sectional studies, which may introduce a survival bias effect. At the same time, potential influential factors such as smoking, diet, or treatment effects may also affect the results of genome-wide association studies (GWAS). Finally, the majority of data in this study comes from European populations, and while we have included some other ethnic groups, our findings may be somewhat biased.

5 Conclusions

By conducting an MR analysis on the causal relationship between the GM and various subtypes of lung cancer, we identified potential causal associations between 10 microbial communities and lung cancer, 10 with lung adenocarcinoma, 9 with lung squamous cell carcinoma, and 11 with small cell lung cancer. Following correction, Peptococcaceae still displayed a strong causal relationship with lung adenocarcinoma. However, definitive experimental research is required to further explore the precise mechanism underlying this relationship.

Data availability statement

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

Author contributions

MZ and YW contributed to the concept and design of this study. YL and KW were responsible for the statistical analysis and writing of the report. YZ and JY reviewed the article and provided critical feedback to improve and structure the report. YL and KW are regarded as co-first authors with the same degree of contribution. 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.2023.1200299/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Scatter plots for the causal association between gut microbiota and lung cancer. MR, Mendelian randomization; SNP, single-nucleotide polymorphism.

SUPPLEMENTARY FIGURE 2
Scatter plots for the causal association between gut microbiota and lung adenocarcinoma. MR, Mendelian randomization; SNP, single-nucleotide polymorphism.

SUPPLEMENTARY FIGURE 3
Scatter plots for the causal association between gut microbiota and squamous cell lung cancer. MR, Mendelian randomization; SNP, single-nucleotide polymorphism.

SUPPLEMENTARY FIGURE 4
Scatter plots for the causal association between gut microbiota and small cell lung cancer. MR, Mendelian randomization; SNP, single-nucleotide polymorphism.

SUPPLEMENTARY FIGURE 5
Leave-one-out plots for the causal association between gut microbiota and lung cancer. MR, Mendelian randomization.

SUPPLEMENTARY FIGURE 6
Leave-one-out plots for the causal association between gut microbiota and lung adenocarcinoma. MR, Mendelian randomization.

SUPPLEMENTARY FIGURE 7
Leave-one-out plots for the causal association between gut microbiota and squamous cell lung cancer. MR, Mendelian randomization.

SUPPLEMENTARY FIGURE 8
Leave-one-out plots for the causal association between gut microbiota and small cell lung cancer. MR, Mendelian randomization.

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Single-cell analysis and spatial resolution of the gut microbiome

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Over the past decade it has become clear that various aspects of host physiology, metabolism, and immunity are intimately associated with the microbiome and its interactions with the host. Specifically, the gut microbiome composition and function has been shown to play a critical role in the etiology of different intestinal and extra-intestinal diseases. While attempts to identify a common pattern of microbial dysbiosis linked with these diseases have failed, multiple studies show that bacterial communities in the gut are spatially organized and that disrupted spatial organization of the gut microbiome is often a common underlying feature of disease pathogenesis. As a result, focus over the last few years has shifted from analyzing the diversity of gut microbiome by sequencing of the entire microbial community, towards understanding the gut microbiome in spatial context. Defining the composition and spatial heterogeneity of the microbiome is critical to facilitate further understanding of the gut microbiome ecology. Development in single cell genomics approach has advanced our understanding of microbial community structure, however, limitations in approaches exist. Single cell genomics is a very powerful and rapidly growing field, primarily used to identify the genetic composition of microbes. A major challenge is to isolate single cells for genomic analyses. This review summarizes the different approaches to study microbial genomes at single-cell resolution. We will review new techniques for microbial single cell sequencing and summarize how these techniques can be applied broadly to answer many questions related to the microbiome composition and spatial heterogeneity. These methods can be used to fill the gaps in our understanding of microbial communities.

KEYWORDS

microbiome, single cell, genomics, sequencing, spatial resolution

Introduction

The human organism is colonized by a vast community of microorganisms, which support and maintain many aspects of our health. The intestinal microbiota contributes to multiple physiological functions of the host, including metabolic homeostasis, immunity, and neuronal activity. In turn, the host provides a stable colonization niche for commensal

microorganisms and ensures continuous influx of dietary nutrients. Despite advances in sequencing and culturing techniques, a large amount of undiscovered and otherwise undescribed microbial taxa remains unknown, including in the human gut (Almeida et al., 2019). The intestinal microbiota has been implicated in the etiology of a variety of human diseases, including those localized to the gastrointestinal tract such as inflammatory bowel disease (IBD) (Glassner et al., 2020), Crohn's Disease (Pascal et al., 2017), susceptibility to pathogenic bacterial infection (Ivanov et al., 2009; Theriot et al., 2014; Velazquez et al., 2019), as well as a number of other extraintestinal diseases like cardiovascular diseases (Jie et al., 2017; Yoo et al., 2021), depression (Limbana et al., 2020) and obesity (Liu et al., 2021). Research efforts are focusing on exploring causality between changes in the gut microbiota and disease, with the aim to improve understanding to lead to therapeutics as well as more robust prevention strategies. Sequencing techniques are being continually refined, with a current goal being the focus on sequencing at the single cell level, rather than bulk sequencing, in order to gain finer resolution and understanding of microbial communities.

Advancements in technologies and techniques in the field of DNA sequencing, particularly with regards to the gut microbiota, have added to our understanding of the role of the microbiota in human health and disease. However, until recently, the study of the gut microbiota was limited to the study of those microbes which could be isolated and cultured. The usage of ribosomal RNA genes as a classification system for microbes, along with Sanger sequencing which allowed for the automated sequencing of DNA in the late 1970s, set the stage for the study and classification of a number of microbes, culturable or not (Escobar-Zepeda et al., 2015). Improvements made to Sanger sequencing method, such as the replacement of radio isotopes with the use of fluorometric based detection methods, as well as detection via capillary-based electrophoresis lead to the development of the first DNA sequencing machines in the late 1980s and early 1990s, allowing for the sequencing of bacterial and other more complex genomes (Heather & Chain, 2016). Sequencing based on the Sanger method, or dideoxy method, was prevalent for a number of years, until second-generation DNA sequencing technology was developed. Pyrosequencing, unlike Sanger sequencing, does not require the use of labeled dideoxy nucleotides and subsequent visualization, rather, it takes advantage of an enzymatic reaction by which light is produced proportional to the amount of nucleotide binding (Heather & Chain, 2016). The advent of pyrosequencing, like Sanger sequencing, revolutionized the field, as now sequencing reactions could be run in parallel with near instant results. Later, Ion Torrent sequencing was developed, which does not require the use of fluorescence or luminescence, but rather measures nucleotide incorporation by the change in pH cause by the proton release during polymerization, allowing for very rapid sequencing (Heather & Chain, 2016). However, one of the most commonly used second-generation DNA sequencing technologies is the Illumina next generation sequencing system. The Illumina sequencing platform uses sequencing by synthesis methodology where sequencing takes place in multiple cycles that capture the fluorescence signal emitted when a correct base is added to the growing DNA strand (Gloor et al., 2010). Presently, third-generation technologies are being developed with the aim of having longer read length capabilities with lower cost, however, for many researchers, second-generation sequencing techniques are most often used (Escobar-Zepeda et al., 2015). One such third-generation sequencing technology is Pacific Biosciences' single molecule, real-time sequencing technology (SMRT) that generates raw reads longer than 10kb in length and is popular for sequencing complex microbial communities (Tsai et al., 2016; Sadowsky et al., 2017; Li et al., 2021).

Investigation of the microbiome primarily relies on meta-omics, or the analyses of microbial DNA, RNA, or metabolites recovered from samples. The most common method used by researchers today is the 16S rRNA gene-amplicon sequencing (Tolonen and Xavier, 2017). However, this method and other commonly used sequencing techniques have drawbacks, including taxonomic blind spots, as well as the loss of information of low abundance members of the microbiota (Hatzenpichler et al., 2020; Bowers et al., 2022). The advent of single cell isolation and sequencing have been crucial to addressing this issue, however, there still remain further hurdles to the application of single cell technology for the analysis of gut microbiota. Many technical issues are being addressed by advances in technologies which will be described here. Here, we will discuss new technologies and techniques for the sequencing of the human microbiome, including the spatial aspect of the microbial community.

Single cell isolation methodologies

Sequencing the microbiome at a resolution of individual microbes has been recently gaining popularity. There has been substantial development for single cell genomics approaches for prokaryotes and eukaryotes, enhancing the feasibility of such experiments for researchers. However, single cell sequencing from microbial communities poses challenges unique to microbes that are not considered for mammalian single cell sequencing. A few limitations with isolating single microbial cells include aggregation of bacteria which makes it difficult to isolate single cells efficiently (Trunk et al., 2018); bacterial cell walls pose a challenge for many single cell sequencing approaches and therefore require to be permeabilized (Blattman et al., 2020; KuChina et al., 2021); low biomass and low abundance of mRNA (Blattman et al., 2020; KuChina et al., 2021). For these techniques, the first step involves isolation of single cells (Figure 1). Despite the progress in the field of genomics, limitations exist for the first step of isolating single cells, an important part of the workflow to perform high-throughput single cell genomics. Several widely used methods of isolating single cells include fluorescence-activated cell sorting (FACS), micromanipulation, and microfluidics (Blainey, 2013; Tolonen and Xavier, 2017).

FACS is one of the most commonly used high-throughput methods for isolation of individual cells (Stepanauskas and Sieracki, 2007). Microbial cells can be individually sorted on the basis of their size and fluorescence by FACS (Figure 1). Rinke and colleagues described a protocol to isolate single cells from environmental microorganisms using FACS followed by extraction and amplification of their genomes (Rinke et al., 2014). A key

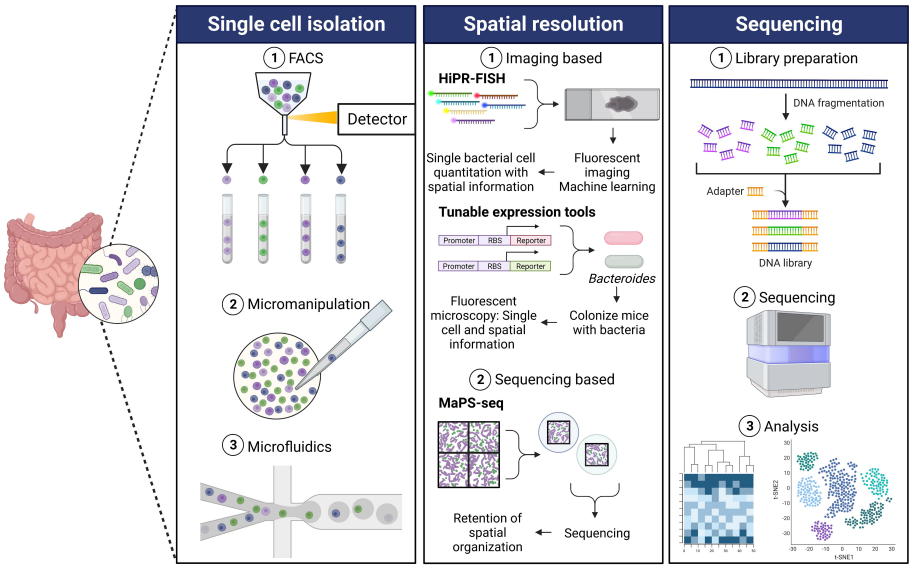

FIGURE 1
Single cell sequencing workflow. Single cell isolation: The first step for performing single cell microbial genomics is isolating single cells. Some commonly used single cell isolation techniques include fluorescence-activated cell sorting (FACS), micromanipulation, and microfluidics. (1) FACS involves size- and fluorescence-based separation that separates single cells from a complex microbial community (Rinke et al., 2014). (2) The traditional micromanipulation method includes micro-pipetting in combination with an inverted microscope for the isolation of single cells from a mix of microbial cells (Ishøy et al., 2006). (3) The microfluidics approach combined with droplet encapsulation involves encapsulating individual cells in hydrogel microspheres resulting in isolated single cells in each droplet (Marcy et al., 2007). There have been different modifications of the microfluidics method, based on the core concept of encapsulating single cells in droplets, to yield single cells. Spatial resolution: Various new techniques have been developed in order to obtain spatial genomics information of the gut microbiota, generally either imaging based or sequencing based. (1) High phylogenetic resolution fluorescence *in-situ* hybridization (HiPR-FISH) employs a binary barcode system based on hybridization of distinct fluorophores (Shi et al., 2020). Spectra are measured using fluorescence microscopy, and spectral barcodes are decoded using machine learning. Identification and spatial visualization of taxa are possible. Tunable expression tools (Whitaker et al., 2017) is a platform for engineering Bacteroides using a novel phage promoter and translation tuning strategy to enable imaging of fluorescent bacteria. Unique fluorescent signals can be used to allow differentiation of species within the gut. (2) Metagenomic plot sampling by sequencing (MaPS-seq) combines genomic and spatial resolution (Sheth et al., 2019). Intact microbiota samples are fractured into particles and are encapsulated in droplets before deep sequencing. This results in the retention of spatial information and can identify species that tend to co-localize in complex samples such as the gut microbiota. Sequencing: (1) The sequencing step of the single cell genomics workflow involves using DNA from the isolated cells to prepare a library. (2) This is followed by high-throughput sequencing which yields (3) critical information identifying the genetic composition microbes and associated gene expression changes in a complex microbial community.

advantage of using FACS to isolate single cells is minimized risk of contamination by extracellular DNA because of the low volume requirement. Other advantages of using FACS as the method to separate individual cells for genomic sequencing are that it has high throughput, can be automated, and is compatible with downstream applications. On the other hand, some limitations of using FACS for single cell genomics are the inability to reduce reaction volumes to nanogram range, increased caution necessary to avoid external contamination during open-plate workflows, and lack of ability to inspect cells visually (Stepanauskas, 2012; Rinke et al., 2014; Hu et al., 2016; Woyke et al., 2017).

In addition to the use of FACS, traditional methods of micromanipulation that include using micro pipetting combined with an inverted microscope as a visual aid have been used to isolate single bacterial cells (Figure 1) (Stepanauskas, 2012; Hu et al., 2016; Woyke et al., 2017). Using this approach, individual cells are selected and physically delivered to be processed for downstream applications. Micromanipulation has been used to capture single microbial cells from different bacterial habitats including a low pH and high temperature hot spring to study the microbial ecology in their natural environments (Ishøy et al., 2006). Hohnadel and colleagues developed an improved micromanipulation method to isolate and detect single microbial cells in food samples (Hohnadel et al., 2018). Automated versions of this method to select single cells using capillary micropipettes and associated robotics have been developed and used for bacterial single cell gene expression analysis (Anis et al., 2008; Gao et al., 2011). Major drawbacks of this approach are that it is extremely time-consuming, labor-intensive, and low-throughput (Blainey, 2013; Chen et al., 2017). Another limitation associated with this method is the risk of contamination from the laboratory environment, equipment, and RNA contamination (Blainey, 2013; Hodne and Weltzien, 2015; Chen et al., 2017).

Another widely growing technique for isolating single cells for the downstream application of genomics is the microfluidics method (Figure 1). Microfluidics was one of the first methods used for cell isolation for microbial single cell studies (Marcy et al., 2007; Leung et al., 2012). Key advantages of microfluidics include the ability to visualize target cells and reduce reaction volumes. The microfluidics approach provides the benefits of high-throughput isolation and barcoding individual genomes (Leung et al., 2012; Chen et al., 2017; Woyke et al., 2017). Newer techniques involving a

combination of microfluidics and single cell encapsulation in droplets are rapidly growing. The droplet microfluidics method involves encapsulating single cells in hydrogel microspheres or generating water-in-oil droplets. This is followed demulsifying the droplets followed by sequencing (Tauzin et al., 2020; Pryszlak et al., 2022) or by fragmenting and barcoding individual genomes from the cells in each droplet, enabling pooled sequencing of many tagged genomes simultaneously (Zengler et al., 2002; Hosokawa et al., 2017; Woyke et al., 2017). Lan and colleagues successfully used gel microdroplets combined with microfluidics to perform single cell genomics of a synthetic community of Gram-negative and Gram-positive bacteria (Lan et al., 2017). Lim and colleagues developed PCR-Activated Cell Sorting (PACS) that utilizes the microfluidic droplet method to encapsulate individual bacteria in picoliter volume droplets which are then subjected to TaqMan PCR to identify bacteria of interest (Lim et al., 2015). These can then be used for downstream applications including genome sequencing. This method offers the advantage of performing single cell genomics in complex ecosystems.

Bacterial single cell whole genome sequencing

Bacterial single cell whole genome sequencing field is advancing rapidly. There are many technologies that have contributed to the efficient characterization of microbes at a single cell resolution. Chijiiwa et al. reported identification of gut bacteria that responded to dietary fiber by using a novel single cell genome sequencing method (Chijiiwa et al., 2020). This method includes single cell isolation of gut microbes by capturing them in agarose gel beads by a microfluidic droplet generator. This was followed by amplification of single cell amplified DNA, captured into the gel beads, as a single cell amplified genome (SAG) library. The SAG-gel platform allows for the specific sequencing of researcher-selected samples out of the large numbers of SAGs and is also cost-efficient. Lan et al., described a high-throughput single-cell genomic sequencing (SiC-seq). SiC-seq utilizes droplet microfluidics to capture individual microbial cells in microgels. This is followed by cell lysis, DNA fragmentation and barcoding, pooling tagged DNA fragments, and sequencing. The workflow of SiC-seq was validated using an artificial microbial community comprising of yeast, Gram-negative bacteria, and Gram-positive bacteria (Lan et al., 2017). A newer high-throughput single cell sequencing method called Microbe-seq was developed by Zheng and colleagues. The Microbe-seq methodology yielded a large number of individual microbial genomes-without culturing-from longitudinally collected human gut microbial samples. This technique involves encapsulating individual microbes in droplets using a microfluidics platform followed by performing whole-genome amplification and barcoding DNA within the droplets, generating multiple SAGs per sample. The tagged DNA is then pooled and sequenced. The SAGs obtained from the Microbe-seq were then co-assembled to yield strain-level resolution of the gut microbiota samples (Zheng et al., 2022). However, techniques such as SiC-seq and Microbe-seq offer low recovery of genomes. Hosokawa and colleagues used the SAG-gel technology to recover high-quality, near complete bacterial genomes from propidium monoazide-treated human gut microbiome samples (Hosokawa et al., 2022). Arikawa et al., integrated single cell genomics and metagenomics to create a single-cell metagenomics workflow to improve the recovery of strain-resolved genomes from human microbiota samples which yielded high-quality recovery of nearly complete microbial genomes (Arikawa et al., 2021). Jin and colleagues developed a high-throughput method called Barcoding Bacteria for Identification and Quantification (BarBIQ) that provides abundance and identification at an individual microbe resolution. BarBIQ involves encapsulation of barcoded-single cells in droplets and uses 16s rRNA sequences to classify and quantify individual microbes into cell-based operational taxonomy units. This study validated the workflow of BarBIQ by comparing the effect of vitamin A deficiency on proximal and distal cecal microbiota abundance and composition in mice (Jin et al., 2022). One limitation of SAGs is that they have incomplete sequences because of the introduction of biased sequencing during amplification cycles. To address this, Kogawa and colleagues developed a single-cell amplified genome long-read assembly workflow that enables construction of complete SAGs using long reads (Kogawa et al., 2023).

Another technique for single cell genomic sequencing used by investigators is the single droplet multiple displacement alignment (sd-MDA) method. This technique also involves the encapsulation of single cells in droplets followed by whole genome alignment (WGA). Single cells are passed through a droplet generator. Hosokawa and colleagues successfully used this method for single cell genome sequencing involving both bacterial cells and human cancer cells. Some advantages of this method include increased efficiency of sample preparation and reduced cost and labor investment (Hosokawa et al., 2017). Despite being indispensable for whole-genome sequencing, there are some limitations of this method associated with MDA. The limitations include low coverage due to genome coverage bias and potential DNA contamination (Džunková et al., 2014; Hosokawa et al., 2017). Advances in the isolation of single cells have made downstream single cell sequencing possible.

Bacterial single cell transcriptomics

Single cell sequencing has tremendously helped in studying phenotypic heterogeneity of microbes. It has also enabled exploring rare organisms using high throughput single cell analysis (KuChina et al., 2021). A number of new technologies have emerged which have refined single-cell transcriptional analysis of microbes, especially in mixed and complex communities like the human gut microbiome.

One of the recently developed single cell transcriptomics that can be applied to microbes is split-pool ligation-based transcriptome sequencing (SPLiT-Seq). SPLiT-seq overcomes the need for microfluidics as it utilizes a combinatorial barcoding approach. In this method, cells are distributed in individual wells and barcoded primers are used to synthesize cDNA through

intracellular reverse transcription. This step is followed by multiple rounds of pooling and splitting accompanied by barcoding in every round. Finally, the reads are combined by referring to the barcode combination to assemble the transcriptome. An advantage of this technique is that it circumvents the need of isolating single cells, and it enables scalable multiplexing (Rosenberg et al., 2018). Kuchina and colleagues developed a modified version of SPLiT-seq tailored to identify microbial subpopulations and associated gene expression changes, called microSPLiT (microbial split-pool ligation-based transcriptomics). Transcriptional responses to heat shock exposure to *Escherichia coli* and *Bacillus subtilis* were reliably detected using microSPLiT. Furthermore, this technique also captured signature transcriptional changes through the growth cycle of *B. subtilis*. Transcriptional changes in stress responses, regulation of carbon utilization, developmental decisions, and metal uptake were revealed through microSPLiT, indicating its capabilities of identifying heterogeneity in cellular and regulatory pathways. Together, these analyses demonstrate the potential of microSPLiT for detecting differential gene expression associated with heterogeneous cell populations in varied environments (KuChina et al., 2021).

Another recently developed high throughput prokaryotic single cell RNA-seq (scRNA-seq) technique is prokaryotic expression profiling by tagging RNA *in situ* and sequencing (PETRI-Seq) (Blattman et al., 2020). PETRI-seq uses an *in situ* combinatorial indexing approach to barcode bacterial transcripts. The methodology of PETRI-seq involves fixation and permeabilization of cells, split-pool barcoding, and library preparation for sequencing. Blattman and colleagues developed PETRI-seq and successfully used it for high-throughput sequencing *E. coli* and *Staphylococcus aureus* with high single cell purity. The authors demonstrated that PETRI-seq enabled successful distinction between *E. coli* populations in different growth phases. This was achieved by using complementary approaches of comparing operon expression patterns and Gene Ontology terms associated with exponential and stationary phases. PETRI-seq, owing to its high throughput capacity, detected a rare subpopulation of *S. aureus* undergoing prophage induction by applying principal component analysis to 6,663 single cell transcriptomes of *S. aureus* (Blattman et al., 2020).

A FACS-based bacterial scRNA-seq workflow was developed by Imdahl et al. which utilizes poly(A)-independent multiple annealing and dC-tailing-based quantitative scRNA-seq (MATQ-seq) protocol. The bacteria are sorted into single cells using FACS and are then enzymatically lysed. The MATQ-seq protocol is used to obtain cDNA from individual bacterial cells followed by tagmentation and library preparation to generate libraries for sequencing. The study used this scRNA-seq workflow to characterize global transcriptomes of *Salmonella* populations under different growth conditions resolved at a single cellular level (Imdahl et al., 2020). The same group recently developed an improved version of this workflow that resulted in improved gene detection limit and coverage at a single bacterial cell resolution. This version of scRNA-seq involves the use of a more efficient reverse transcriptase and has a Cas9-based rRNA depletion step integrated in the workflow (Homberger et al., 2023).

Another FACS-based scRNA-seq was developed by Nishimura and colleagues which incorporates RamDA-seq in its workflow. The methodology involves FACS-based isolation of single bacterial cells followed by cell lysis. The workflow includes library construction using the RamDA-seq technique which is a full-length RNA sequencing method. Before sequencing, the amplified cDNA libraries are then depleted of rRNA using Cas9. The study used this hybrid scRNA-seq approach to reveal heterogeneity in different growth stages of live *E. coli* and in heat-shocked *E. coli* populations in about a quarter of *E. coli* genes at a single cell resolution (Nishimura et al., 2023).

An additional high-throughput scRNA-seq technique that has been successfully used for bacterial populations is the BacDrop that is droplet-based. The workflow of BacDrop involves fixation and permeabilization of cells followed by rRNA and gDNA depletion. The next step is generation of barcoded cDNA by reverse transcription which is then followed by capturing single cells in droplets and droplet barcoding. The last step is library preparation for sequencing. The group used BacDrop to characterize previously unknown heterogeneity based on mobile genetic elements in *Klebsiella pneumoniae* at a single cell resolution. However, the working efficiency of BacDrop remains to be validated on more complex microbial communities involving unknown genomes (Ma et al., 2023).

Together, these methodologies of single cell isolation and single cell sequencing offer the flexibility to choose the best suited approach of isolating and sequencing single cell genomes of bacteria, depending on the need of experiments.

Gut biogeography through the lens of community analysis

Single cell sequencing at the base level does not maintain information about where in space the cells were located in the original sample, but newer methods have been applied to tackle this problem.

The spatial localization of pathogens within the host has been understood as a key factor to their pathogenesis, however, less is understood about the localization of commensals, and how the change in localization affects health and disease (Lopez et al., 2016; Spiga and Winter, 2019; Rogers et al., 2021). Localization of gut microbiota members along the gastrointestinal tract differs in both cross-sectional (from epithelium and mucus-associated to the lumen) and longitudinal (from stomach to distal colon) heterogeneity, the latter of which does not require novel technologies to study, as researchers can sample from different locations longitudinally along the GI tract (Donaldson et al., 2016; Tropini et al., 2017). However, identification of localization of gut microbiota members in the cross-sectional aspect is more difficult to address. There are many factors known to influence not only the gut microbiota, but also the spatial distribution of the members of the microbiota, such as oxygen and ROS species (Albenberg et al., 2014; Lopez et al., 2016; Miller et al., 2020), the physical barrier mucus (Van der Sluis et al., 2006; Johansson and Hansson, 2016), pH (O'May et al., 2005; Ilhan et al., 2017), various immune effectors

produced by the host, such as antimicrobial peptides (Bevins and Salzman, 2011), and availability of nutrients (Koropatkin et al., 2012). Additional habitat filters varying both longitudinally and cross-sectionally include electron acceptor availability (Miller et al., 2021; Liou et al., 2022).

Research on changes in the microbiota has often been focused on large changes in community composition associated with disease, especially with the growth of certain pathogenic species. It is now becoming better appreciated that changes in the spatial distribution of species in the gut is also associated with disease and infection, as well as several chronic conditions, such as IBD (Swidsinski et al., 2005), and colorectal cancer (Dejea et al., 2014; Saffarian et al., 2019), and various other perturbations such as starvation, antibiotics, and surgery (Zaborin et al., 2020). It is therefore necessary for analysis of gut microbial communities to move beyond defining the composition of the microbiota to a spatial understanding, to better probe questions about microbiota function and interaction between other species and the host. Answering questions about how certain members of the microbiota are interacting with both the host as well as other microbiota members will shed light on a variety of human diseases, as well as help define homeostasis and dysbiosis of the gut, and moving towards causation of the later (Tiffany and Bäumler, 2019).

High-throughput -omics techniques have revolutionized the field of gut microbiota research. Much work has relied on the use of fecal samples, whose use is pervasive due to the ease of sample collection, however, fecal samples usage faces drawbacks such as decay of microbes, and the loss of information about spatial differences in community members, both along the GI tract and in the cross-sectional plane. Many genomic techniques rely on a homogenized sample, which therefore results in the loss of important spatial information in the larger picture of the host-microbiota environment. Laser Capture Microdissection (LCM) has provided a method of high-resolution site-specific sampling of microbial communities (Espina et al., 2006). Using LCM, researchers can select a sample region with use of a microscope, capturing the specific area of interest, and the sample can be used downstream for metagenomic or other analyses. Several studies have delved into newer technologies providing single-cell spatial genomic information. Generally, these technologies can be classified as image-based or sequencing-based.

Spatial resolution of DNA sequencing

The use of *in situ* hybridization (ISH), an image-based approach, has been available to researchers for a number of years (Rudkin and Stollar, 1977). ISH depends on labeled probes hybridizing specific DNA or RNA targets, often employing use of a fluorophore, as in fluorescent *in situ* hybridization (FISH), which allows for the location of the target of interest under the microscope. A limitation of FISH in microbiota samples is the limited number of fluorophores available for visualization and discrimination of different targets. Advances in the field of RNA-FISH have alleviated issues with multiplexity and other problems with FISH, such as the analysis of targets with low copy numbers and could be applied to DNA targets in the microbiota in the future (Eng et al., 2019).

A recently developed method known as High Phylogenetic Resolution FISH (HiPR-FISH) combines FISH, a visual assay targeting ribosomal RNA for visualization and identification, with a binary barcoding scheme and machine learning of fluorophore combinations (Figure 1) (Shi et al., 2020). This method provides a microbial mapping technology that can identify various taxa with aid of a microscope. Using ten fluorophores, probes with the same encoding sequence, but different readout sequences, can bind in equal amounts to ribosomal RNA (rRNA) molecules within the same cell. As bacterial cells contain hundreds of 16S rRNA copies, each species can be targeted by encoding probes targeting the same sequence, but flanked by different readout sequences, allowing for the assignment of a unique combination of fluorophores. The fluorescence emission spectra are measured, and a spectral barcode is computed. The spectra recorded on each pixel is averaged, and a machine learning classifier decodes the cell barcodes. The authors were able to achieve single-cell quantitation and found previously undescribed genera in the human oral plaque microbiome (Shi et al., 2020). The authors additionally applied this novel method to study the effect of antibiotics on the spatial organization of the gut microbiome, targeting up to 47 genera using HiPR-FISH, and revealed spatial association disruption between several genera. The authors demonstrate single-cell mapping of complex communities, which allows for bacterial spatial organization questions to be addressed.

Another image-based method described recently by Whitaker et al. (2017) uses a phage promoter system to introduce genomically integratable vectors into *Bacteroides* in a high-throughput fashion, using an adaptation on the Golden Gate cloning method (Figure 1). This method allows for the *in vivo* imaging of fluorescent *Bacteroides*, as the fluorescent proteins were expressed at higher levels than achievable previously using other promoters, such as the 16s rRNA promoter. Importantly, the authors show this does not come at a fitness cost for the bacteria *in vivo*. Their technique allowed for the differentiation of each species at a single-cell level, using unique combinations of GFP and mCherry expression. The authors use this technique to visualize *Bacteroides* in colonic crypts of the mouse gut and show that colonizing a mouse with one isogenic strain of *B. thetaiotaomicron* provides colonization resistance against a sequentially gavaged isogenic strain. This study provides a great example of how new techniques can be used to provide strain-level resolution of spatial heterogeneity. While not a sequencing technique, this method can give insight into where a particular gut microbiota member is localized, and for the use of resolution of localization of several strains of the same microbe, as shown by the authors.

A recently described sequencing-based spatial technology, termed Metagenomic Plot Sampling by Sequencing (MaPS-seq), is an unbiased technique that avoids the use of a microscope that does not involve single cell analysis, and instead analyzes the sequences of microbial cells in their native geographical context (Figure 1) (Sheth et al., 2019). An input sample is first fixed and embedded into a polymer matrix. The matrix is then fractured into

clusters, the cells are lysed, and clusters of desirable size are selected. This process ensures that the clusters contain the genomic DNA immobilized in the original spatial location, preserving this information. The clusters are then encapsulated with barcoded beads, containing the 16s rRNA amplification primers. The primers are cleaved from the beads, genomic DNA is released, and PCR amplification of the 16s V4 region is performed. Droplets are separated, and the library is deep sequenced. The authors applied MaPS-seq to analyze spatial metagenomics of the mouse colon microbiome and found that certain community members aggregate or clump together across the distal colon. They then use MaPS-seq to investigate the spatial organization along the GI tract and found that certain taxa tended to be found close to each other, or co-associate, such as *Lactobacillaceae* and *Lachnospiraceae*. Further investigation into the benefit of co-associations of bacteria that tend to aggregate together spatially in the gut will be illuminating to the field and could help uncover factors necessary for the culture of certain currently unculturable microbes. MaPS-seq also has the potential to profile interactions between bacteria and eukaryotes, such as fungi or epithelial cells, by modifying the capture primers, as noted by the authors.

While further new technique development is necessary, many cutting-edge techniques for capturing spatial information have been developed recently, as described here. In addition to the techniques described that have been applied to the gut microbiota, several spatial transcriptomics tools have been described in the study of mammalian tissues, especially with regards to the brain. Multiplexed mRNA-FISH has been applied to spatial mapping of mRNA molecules, with the washing and adding fluorophores, to image the same section multiple times for different targets, achieving high multiplexity (Goh et al., 2020). Slide capture technology, a technique where mRNA molecules are hybridized to DNA anchors on a slide, followed by reverse transcription and sequencing, has been applied to the mouse brain to reveal responses to traumatic brain injury (Rodriques et al., 2019), and in the study of breast cancer tissues (Vickovic et al., 2019). However, as this method relies on the polyadenylation of mRNA for capture, it cannot be used for the spatial mapping of microbes, as microbes generally do not have polyadenylated mRNAs. Another method described recently that proves more promising for the gut microbiota field, DNA Microscopy, is a microscope-free method by which transcripts are tagged with randomized nucleotides, followed by amplification tags and concatenation of the copies (Weinstein et al., 2019). A computer algorithm then decodes proximities based on these concatenated sequences and infers an image of the original transcripts at cell-level resolution. DNA Microscopy could theoretically be applied to mapping the spatial relationship of microbes in the gut microbiota but has yet to be applied in practice.

Discussion

High-resolution single cell analysis for microbes allow capturing heterogeneity in bacterial populations identifying rare subpopulations of bacteria, which might be missed in traditional bulk sequencing (KuChina et al., 2021). Various methodologies have been developed to isolate single eukaryotic cells which have been optimized for bacterial cells. This allows performing single cell sequencing and is of great value for in-depth characterization of the microbiome as well as host-microbiome interactions (Woyke et al., 2017). Powerful high throughput sequencing of single microbial cells allows researchers to address questions that will provide insights into their genomes and spatial function of gut microbiome in the etiology of gut and other related diseases (Tolonen and Xavier, 2017; Sharma and Thaiss, 2020). Sequencing gut bacteria at the level of single cells better captures the heterogeneity compared to traditional bulk sequencing and enables the study of microbes that make up a small fraction of a population. This could aid with the tracking emergence of drug resistance in bacteria (Tolonen and Xavier, 2017).

Resolving gut microbiome at the single cell level opens the possibility of addressing new scientific questions. A cross sectional study showed that immunocompromised patients, particularly children with sickle cell disease (SCD) receiving penicillin prophylaxis in the cohort, showed differences in alpha diversity and bacterial abundance of the gut microbiota compared to patients not receiving penicillin (Mohandas et al., 2020). Single cell resolution of gut microbiota will provide a closer look at their functional implications in SCD and other immunodeficiency diseases.

Gut microbiota composition and function are heavily influenced by environmental factors, diet, and age. Studies report patterns of gut microbes that not only influence aging but can also predict age-associated decline (Bosco and Noti, 2021). It will be of interest to identify the effect of diet and age on gut microbiome in immunocompromised patients. The technological advancements in resolving gut microbiota at the level of single cells will allow dissecting functional relationships between gut microbiome and immunocompromised host and how it is influenced by intrinsic and extrinsic factors such as age and diet.

In addition to being impacted by host and environmental factors, the gut microbes also influence each other. These inter-microbial interactions can be effectively captured by the genomics tools optimized for microbial communities. This will facilitate studying how the microbes impact each other and shape the gut microbiome (Bäumler and Sperandio, 2016; Coyte and Rakoff-Nahoum, 2019).

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ML: Supervision, Writing – review & editing. BM: Writing – original draft, Writing – review & editing. BMM: Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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The relationship between gut microbiota and insomnia: a bi-directional two-sample Mendelian randomization research

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Introduction: Insomnia is the second most common mental health issue, also is a social and financial burden. Insomnia affects the balance between sleep, the immune system, and the central nervous system, which may raise the risk of different systemic disorders. The gut microbiota, referred to as the "second genome," has the ability to control host homeostasis. It has been discovered that disruption of the gut-brain axis is linked to insomnia.

Methods: In this study, we conducted MR analysis between large-scale GWAS data of GMs and insomnia to uncover potential associations.

Results: Ten GM taxa were detected to have causal associations with insomnia. Among them, class *Negativicutes*, genus *Clostridiuminnocuumgroup*, genus *Dorea*, genus *Lachnoclostridium*, genus *Prevotella7*, and order *Selenomonadalesare* were linked to a higher risk of insomnia. In reverse MR analysis, we discovered a causal link between insomnia and six other GM taxa.

Conclusion: It suggested that the relationship between insomnia and intestinal flora was convoluted. Our findings may offer beneficial biomarkers for disease development and prospective candidate treatment targets for insomnia.

KEYWORDS

gut microbiome, insomnia, sleep disorders, bi-directional Mendelian randomization analysis, relationship

Introduction

Insomnia disorder, defined by self-reported sleep difficulties, is characterized by persistent difficulty initiating or sustaining sleep as well as related daytime dysfunction. With 10% to 20% of the population affected, insomnia is the second most common mental health issue (after anxiety disorder), and it is more common in older people and women. In adults, 5.8% to 20% of the population suffers from insomnia, but the prevalence of insomnia in the elderly ranges from 30% to 48%. Insomnia disorder is among the top 10

reasons for general practitioners' consultations (Lo Yun et al., 2022). It is also a social and financial burden, raising questions about public health. Insomnia affects the balance between sleep, the immune system, and the central nervous system, which may raise the risk of infection, depression, cardiovascular disease, gastrointestinal disorders, and respiratory illnesses. Chronic insomnia contributes to a variety of negative outcomes, including decreased physical and mental health (e.g., cardiovascular disease and stroke), worsened health-related life quality, and poorer mental health (e.g., chronic pain, anxiety, depression, substance misuse, and suicide). Given the severity of the negative impacts of insomnia, identifying risk factors is essential for treatments (Jia et al., 2022; Yao et al., 2022; Gibson et al., 2023).

The intestinal flora, also referred to as the "second genome," has the ability to control host homeostasis, which includes metabolic rate, immune/inflammatory response, and cardiovascular function (Le Chatelier et al., 2013). The gut microbiome (GM) is also linked to neuropsychiatric illnesses, as it may regulate brain function and behavior through the microbiota-gut-brain axis (Iannone et al., 2019; Zhang et al., 2021; Wang Z. et al., 2022). There are variations in GM taxa among people with epilepsy, depression, autistic spectrum disorder, and Parkinson's disease. Recent research has shown that the gut-brain axis is dysregulated in relation to insomnia and that abnormalities in the gut microbiota can make the condition worse. To date, there have been few investigations into the relationship between intestinal flora and insomnia (Qi et al., 2022; Bundgaard-Nielsen et al., 2023; Chalet et al., 2023).

Fortunately, large-scale genome-wide association studies (GWASs) on gut microbiota and insomnia are now available, allowing for a meaningful assessment of association in MR analysis. Through instrumental variables (IV) that are genetic variants strongly related to the exposure of interest, Mendelian randomization (MR) analysis is used to investigate the causal relationship between exposure and outcome. In MR research, single nucleotide polymorphisms (SNPs) are used as instrumental variables (IV) (Burgess and Thompson, 2017; Burgess et al., 2017). SNPs adhere to the principle of random genetic variation assignment at meiosis, which eliminates the influence of confounding factors and the potential impact of reverse causation because genetic variants exist prior to the start of the disease (Lawlor et al., 2008). Therefore, when compared to RCT, MR analysis can more quickly identify the causal relationships between relevant exposure components and outcomes. Currently, no MR studies on insomnia and GM have been undertaken. Here, we conduct an MR analysis through large-scale GWAS summary statistics of GMs and insomnia to uncover potential GM taxa that could support some current findings and offer novel viewpoints on the identification and management of insomnia.

Materials and methods

Study design

The overall flow chart of this study is shown in Figure 1. The three presumptions below must be satisfied by MR studies: (i) IVs are highly linked with exposure variables, (ii) IVs are independent of confounding factors, and (iii) IVs are only associated with outcomes via exposure factors (Burgess et al., 2017). Our results followed the STROBE-MR guidelines (Skrivankova et al., 2021a).

Data sources of the exposure and outcome

From the MiBioGen project, we obtained the gut microbiota statistics summary-level data, the largest genome-wide meta-analysis to date (Kurilshikov et al., 2021). In the MiBioGen project, the 16S rRNA gene sequencing profiles of 18,340 individuals were assembled and evaluated, and 211 GM taxa were identified (from genus to phylum level, including 9 phyla, 16 classes, 20 orders, 35 families, and 131 genera). The GWAS summary statistics for GMs can be found at https://mibiogen.gcc.rug.nl (Swertz and Jansen, 2007; Swertz et al., 2010; van der Velde et al., 2019). Insomnia GWAS summary data were obtained from the UK Biobank Sleep Traits GWAS: Self-report (insomnia associations and sleep duration associations) (https://sleep.hugeamp.org/downloads.html). Insomnia symptoms were self-reported by European-ancestry UK Biobank participants (n=453,379). Participants were asked the question: "Do you have trouble falling asleep at night or do you wake up in the middle of the night?" and were asked to select from responses of "never/rarely", "sometimes", "usually", and "prefer not to answer" in this sample; twenty-nine percent of people self-reported experiencing frequent insomnia symptoms on a regular basis (or "usually") (Gibson et al., 2023).

Identification of IVs

In this MR study, IVs were SNPs that were highly correlated with each GM taxon. We obtained the number of IVs for gut microbiota data with the threshold ($p<5\times10^{-5}$), and the threshold in reverse MR analyses was set under $p<5\times10^{-8}$ for insomnia data. Additionally, we removed SNPs within a window size of 500 kb and a threshold of $r^2<0.1$ to reduce linkage disequilibrium (LD) for gut microbiota data, whereas for insomnia data in reverse MR analysis, the window size was set at kb=10000 and a threshold of $r^2<0.001$. Then, we eliminated palindromic SNPs and SNPs that were not present in the IV results. Finally, to measure the degree of weak instrumental bias, the F-statistic of IVs was computed. If the F-statistic was >10, it was assumed that no bias was caused by weak IVs. The formula for calculating the F-value is $F = \left(\frac{R^2}{1-R^2}\right)\frac{(n-k-1)}{k}$, $R^2 = 2 \times (1 - MAF) \times MAF \times (\beta)^2$ (Pierce et al., 2011).

Statistical methods

The principal MR method for determining causation was the inverse variance weighted random effect (IVW-RE). Based on the meta-analysis principles, the IVW approach is a Wald ratio estimator extension (Pagoni et al., 2019). Methods of MR-Egger, weighted median, simple mode, and weighted mode were carried out for each GM taxon on insomnia (Bowden et al., 2016; Burgess and Thompson, 2017; Davies et al., 2018). If the IVW approach revealed a causal association for that taxon ($p<0.05$), these four MR

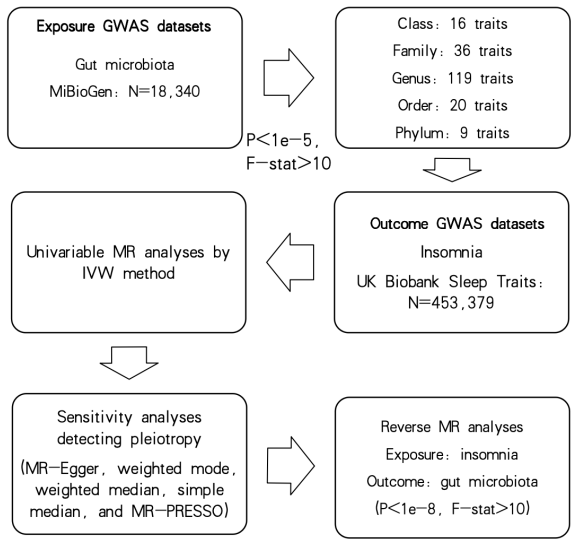

FIGURE 1
Flowchart of current study.

methods were used to supplement the IVW findings. The criterion of the weighted median method is that at least 50% of the SNPs must satisfy the premise that they are valid IVs (Davies et al., 2018; Verbanck et al., 2018). The MR-Egger method provides unbiased estimates even when all selected IVs are multivariate (Burgess and Thompson, 2017). Finally, odds ratios (OR) and 95% confidence intervals (CI) were utilized to present the findings of causal connections. The significance cutoff was established at p<0.05.

Only exposure-outcome pairs that were discovered using all MR techniques and had the same direction were thought to have a causal relationship. We also carried out a number of sensitivity studies to examine the consistency of the causal association. First, horizontal pleiotropy was identified through the MR-Egger and MR-PRESSO tests (Rees et al., 2017; Verbanck et al., 2018; Skrivankova et al., 2021b). Additionally, the leave-one-out and Funnel plots analyses were conducted to evaluate the reliability of the findings. In this study, "TwoSampleMR" and "MR-PRESSO" packages of R software (version 4.3.0) were used to carry out the MR analysis.

To investigate the reverse causality of insomnia (as exposures) on gut microbiota (as outcomes), a reverse MR analysis was carried out for insomnia on each GM taxa. The process followed the same guidelines as the methodology indicated above for the two-sample MR. This bidirectional MR and sensitivity analysis follows the rules of the TwoSample MR and MR-PRESSO packages.

Results

Two-sample Mendelian randomization of gut microbiota (exposure) on insomnia (outcome)

Details of IVs

Under a suggestive significance level of $P<1\times10^{-5}$, 2,284 SNPs were discovered, and three duplicated SNPs (rs10805326, rs2728491, and rs2704155) were deleted. These SNPs were grouped into five categories as final IVs: class, family, order, genus, and phylum. Particularly, there were 200 IVs in 18 classes, 439 IVs in 35 families, 245 IVs in 20 orders, 1,483 IVs in 131 genera, and 112 IVs in 9 phyla. Furthermore, all IVs were shown to be more strongly related to exposure than to outcome ($p_{exposure}<p_{outcome}$), and all F-statistics were greater than 10. Details of the IVs of insomnia are presented in Supplementary Table 1.

MR analysis

First, 211 GM taxa with five methods (IVW-RE, MR-Egger, weighted median, simple mode, and weighted mode) were evaluated using MR analysis to determine their causal relationship with insomnia (Figure 2). The IVW-FE results revealed that 10 GM taxa had a significant association with insomnia. Family *FamilyXIII* (ID: 1957) [OR=0.982 (0.966, 0.997), *p*=0.020], genus *Odoribacter* (ID: 952) [OR=0.976 (0.954,0.999), *p*=0.044], genus *Oscillibacter* (ID: 2063) [OR=0.985 (0.974,0.996), *p*=0.005], and phylum *Verrucomicrobia* (ID: 3982) [OR=0.986(0.974,0.999), *p*=0.032] were related to a lower risk for insomnia, while class *Negativicutes* (ID: 2164) [OR=1.031 (1.016,1.047), *p*=7.53E-05], genus *Clostridiuminnocuumgroup* (ID: 14397) [OR=1.018(1.005,1.031), *p*=0.006], genus *Dorea* (ID: 1997) [OR=1.017(1.001,1.034), *p*=0.039], genus *Lachnoclostridium* (ID: 11308) [OR=1.029(1.007,1.052), *p*=0.009], genus *Prevotella7* (ID: 11182) [OR=1.009(1.002,1.017), *p*=0.017], and order *Selenomonadales* (ID: 2165) [OR=1.031(1.016,1.047), *p*=7.53E-05] were associated with a higher risk of insomnia. Additionally, the findings of Cochran's Q test showed that there was no heterogeneity.

Additionally, four additional methods, MR-Egger, weighted median, simple mode, and weighted mode, were performed to assess the causal effect of these 10 GM taxa on insomnia (Figure 3). The results were consistent with the IVW-FE results. Family *FamilyXIII* (ID: 1957), phylum *Verrucomicrobia* (ID: 3982), genus *Odoribacter* (ID: 952), and genus *Oscillibacter* (ID: 2063) were related with a lower risk for insomnia, while the other six GMs [class *Negativicutes* (ID: 2164), genus *Clostridiuminnocuumgroup* (ID: 14397), genus *Dorea* (ID: 1997), genus *Lachnoclostridium* (ID: 11308), genus *Prevotella7* (ID: 11182), and order *Selenomonadales* (ID: 2165)] showed a higher risk of insomnia. There was no indication of heterogeneity, pleiotropy, or weak instrument bias in the heterogeneity (IVW test and MR-Egger regression), pleiotropy (MR-PRESSO test and MR-Egger regression), or weak instrument bias (F statistic) tests. Additional details are summarized in Supplementary Table 2.

Reverse Mendelian randomization analysis of insomnia (exposure) on gut microbiota (outcome)

IVs were retrieved from GWAS datasets of insomnia in a previous MR analysis of the relationship between gut microbiota

and insomnia, with a significance of P-value at 1×10^{-8}. Forty SNPs were discovered from 2,500 SNPs (SNPs of insomnia GWAS data at $P<1\times10^{-8}$) after removing SNPs with linkage disequilibrium. First, we conducted MR analysis to determine the relationship between insomnia and 10 GM taxa, including the class *Negativicutes* (ID: 2164), family *FamilyXIII* (ID: 1957), genera *Odoribacter* (ID: 952), *Oscillibacter* (ID: 2063), *Clostridiuminnocuumgroup* (ID: 14397), *Dorea* (ID: 1997), *Lachnoclostridium* (ID: 11308), *Prevotella7* (ID: 11182), order *Selenomonadales* (ID: 2165), and phylum *Verrucomicrobia* (ID: 3982). It demonstrated the lack of a causal relationship between insomnia and these 10 GM taxa, which was consistent with our prior MR findings. Additional details are summarized in Supplementary Table 3 and Supplementary Figure S1.

Reverse MR analysis was then used to investigate the other 201 GM taxa for insomnia. According to the IVW-FE results, six GM taxa substantially correlate with insomnia. Insomnia could increase the abundance of the gut microbiota of family *Oxalobacteraceae* (ID:2966) [OR=3.075 (1.453, 6.511), *p*=0.003], genus *Butyrivibrio* (ID:1993) [OR=2.656(1.005, 7.016), *p*=0.049], genus *Clostridiumsensustricto1* (ID:1873) [OR=1.708 (1.085, 2.687), *p*=0.021], and genus *Oxalobacter* (ID:2978) [OR=2.434(1.104,5.370), *p*=0.028], while insomnia could decrease the abundance of genus *Eubacteriumnodatumgroup* (ID:11297) [OR=0.310(0.098,0.961), *p*=0.042] and genus *RuminococcaceaeUCG013* (ID:11370)[OR=0.522 (0.345, 0.791), *p*=0.002]. Furthermore, four additional methods, MR-Egger, weighted median, simple mode, and weighted mode, were performed to assess the causal effect of insomnia on these GM taxa (Figure 4). The outcomes matched those of the IVW in a similar way. In this investigation, neither the IVW test nor the MR-Egger regression showed any evidence of heterogeneity, pleiotropy, or weak instrument bias. The MR-PRESSO test and the MR-Egger regression also showed no evidence of these phenomena. Then, we conducted scatter plots and leave-one-out plots for insomnia on six GM taxa [family

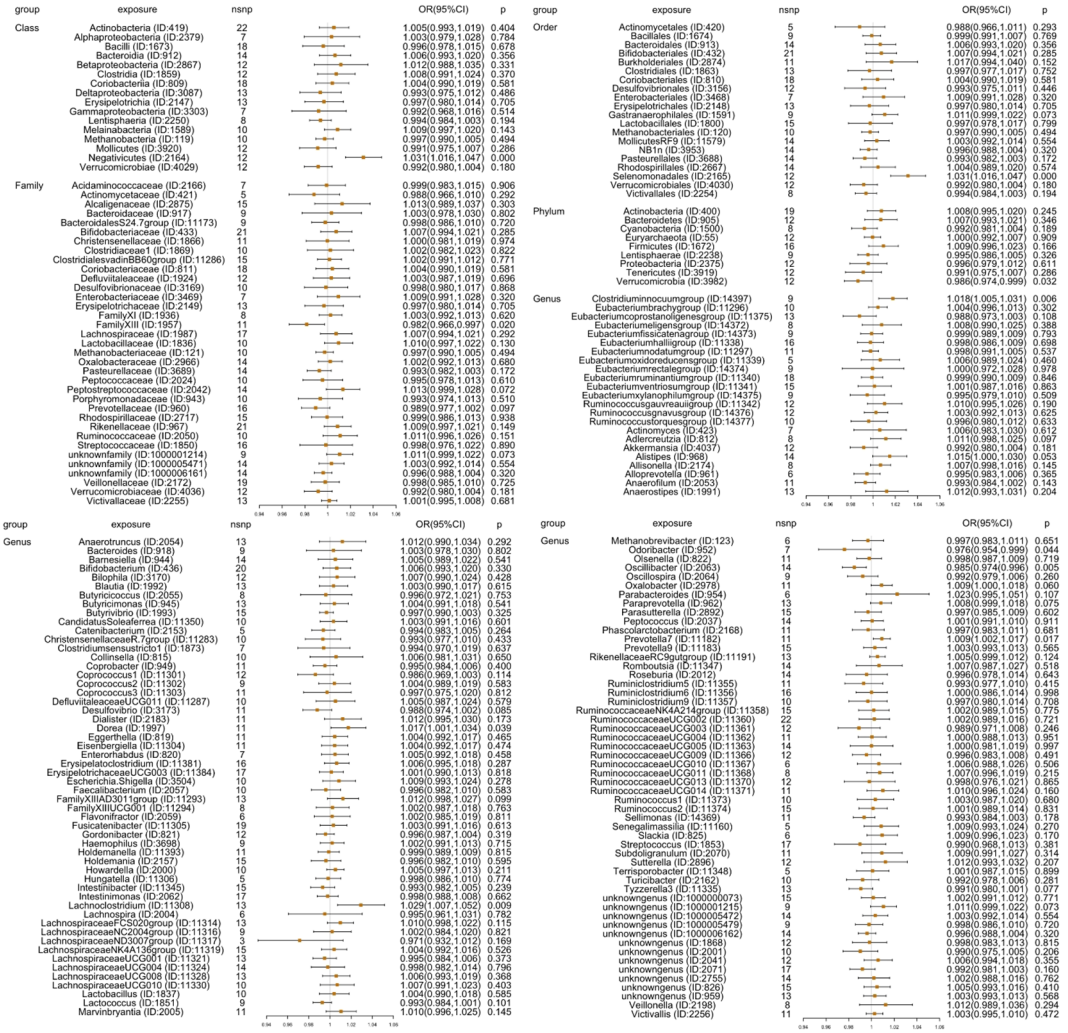

FIGURE 2
Results [OR (95%CI)] for MR analyses for GWAS data of 211 GM taxa (exposure) on insomnia (outcome) through inverse variance weighted random effect (IVW-RE) method: 9 phyla, 16 classes, 20 orders, 35 families, and 131 genera. It showed that 10 GM taxa, including class *Negativicutes* (ID: 2164), Family *FamilyXIII* (ID: 1957), order *Selenomonadales* (ID: 2165), phylum *Verrucomicrobia* (ID: 3982), genus *Odoribacter* (ID: 952), genus *Oscillibacter* (ID: 2063), genus *Clostridiuminnocuumgroup* (ID: 14397), genus *Dorea* (ID: 1997), genus *Lachnoclostridium* (ID: 11308), and genus *Prevotella7* (ID: 11182), had causality with insomnia.

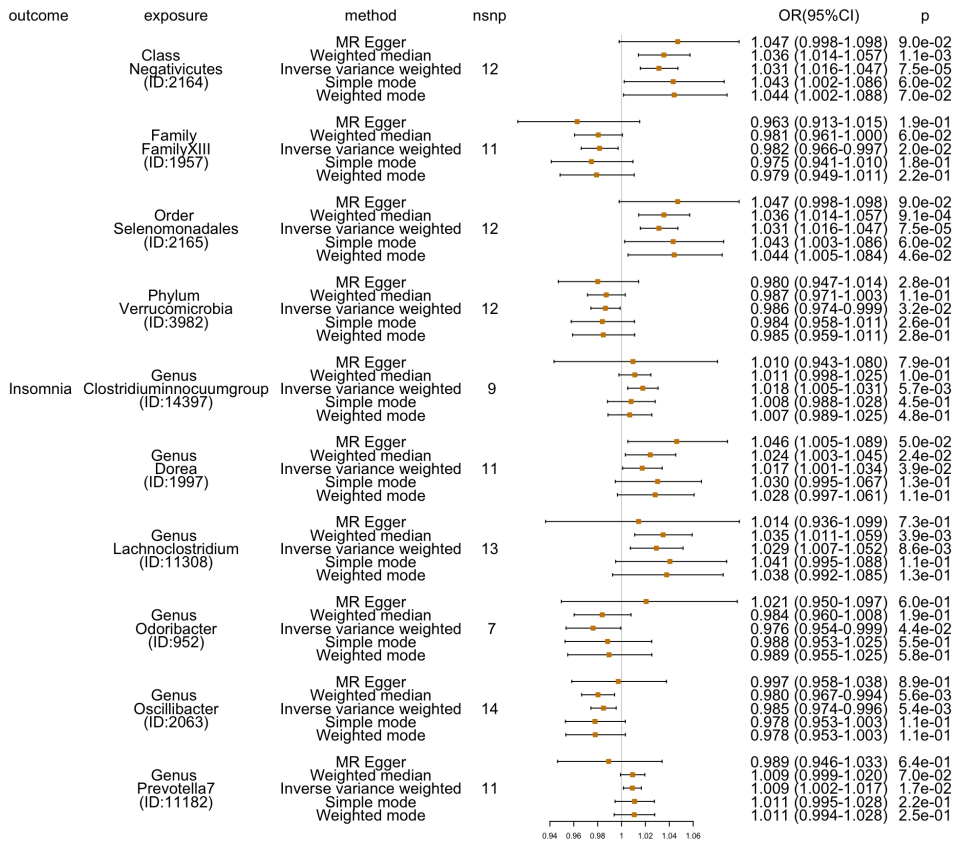

FIGURE 3
Five methods' (MR-Egger, weighted median, inverse variance weighted, simple mode, and weighted mode) results (OR[95%CI]) of MR analyses for 10 GM taxa on insomnia.

Oxalobacteraceae (ID:2966), genus *Butyrivibrio* (ID:1993), genus *Clostridiumsensustricto1* (ID:1873), genus *Oxalobacter* (ID:2978), genus *Eubacteriumnodatumgroup* (ID:11297), and genus *RuminococcaceaeUCG013* (ID:11370)]. Furthermore, the inverse variance weighted, MR-Egger, and weighted median results of the MR Steiger directionality test demonstrated a strong direction from insomnia to the six GM taxa. The robustness of our findings was demonstrated by the leave-one-out sensitivity analysis, which showed that no one SNP drives a causal association (Figure 5). Funnel plots of Inverse variance weighted and MR Egger results excluded a potential bias (Supplementary Figure S2).

Discussion

To the best of our knowledge, this is the first MR investigation using huge GWAS summary-level data to indicate a probable causal connection between gut microbiota and insomnia. This study examined the causative impact of 211 GM taxa (from the class, family, order, genus, and phylum level) on insomnia. In this study design, we checked and confirmed the assumptions of Mendelian Randomization (MR). We discovered 10 GM taxa that are connected to insomnia, and reverse MR analysis revealed that 6 GM taxa may be affected. The gut microbiome's potential protective or contributing effects on insomnia suggest a close connection between the two conditions.

Nowadays, the gut microbiome and its impact on humans are receiving increasing attention. Growing data suggest that the gut microbiota (GM) can control host homeostasis in both health and disease, for example, through the gut-brain axis, gut-lung axis, gut-kidney axis, gut-skin axis, gut-liver axis, and gut-immune axis. Researchers have discovered links between gut microbiota and many diseases, such as autism spectrum disorder, depression, epilepsy, Alzheimer's disease, type 2 diabetes, obesity, chronic obstructive pulmonary disease, atopic dermatitis, COVID-19 illness, psoriasis, and systemic autoimmune diseases. Clinical trials on intestinal flora have shown efficacy in the treatment of disorders such as epilepsy, autism, Alzheimer's disease, inflammatory bowel disease, rheumatoid arthritis, and psoriasis (Iannone et al., 2019; Orru et al., 2020; Mahmud et al., 2022; Mousa et al., 2022; Qin et al., 2022). Recent research has shown that GMs are involved in neuropsychiatric disorders because they affect brain activity and behavior through the microbiota-gut-brain axis (Johnson and Foster, 2018). It has been discovered that disruption of the gut-brain axis is linked to insomnia. In our study, we identified six GMs in the reverse MR. Given that gut microbiota was related to many diseases, these disorders may directly or indirectly arise in the six GMs. The gut microbiota's

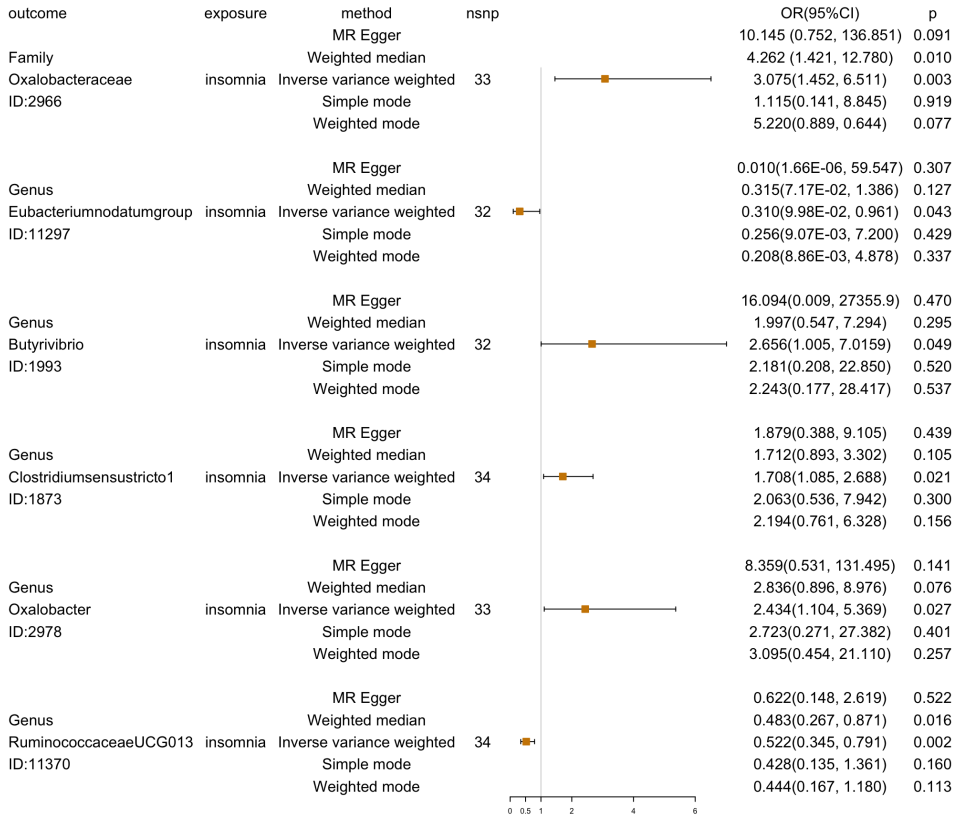

FIGURE 4
Reverse MR analyses results (OR[95%CI]) of five methods (MR-Egger, weighted median, inverse variance weighted, simple mode, and weighted mode) for insomnia on six GM taxa (family *Oxalobacteraceae*, genus *Butyrivibrio*, genus *Clostridiumsensustricto1*, genus *Oxalobacter*, genus *Eubacteriumnodatumgroup*, and genus *RuminococcaceaeUCG013*).

particular metabolites have been reported to be related to insomnia, and alterations in the gut microbiota may worsen the condition. Its molecular mechanism is not yet fully understood (Haimov et al., 2022; Feng et al., 2023).

Insomnia is believed to have a negative impact on the quality of life in adults and the elderly population around the world. In general, insomnia affects 5.8 to 20% of the adult population, whereas it affects 30% to 48% of the elderly population. Insomnia is the result of a complex interaction of behavioral (such as stress, lifestyle, workplace culture, environment, and sleeping arrangements), physiological, and genetic factors. The negative effects of insomnia on many organs lead to abnormal sleep patterns, cognitive performance, and emotional reactions (Li et al., 2020; Jia et al., 2022; Gibson et al., 2023). In addition to contributing to the pathological progression of the immunological, endocrine, and cardiovascular systems, it also causes neuropsychiatric illnesses such as depression, dementia, mania, schizophrenia, and anxiety disorders. The risk of hypertension, diabetes mellitus, arthritis, stomach ulcers, gastroesophageal reflux illness, migraine, depression, obesity, heart attack or stroke, asthma, menstruation issues, obesity, and infection has also been linked to insomnia. These conditions and consequences have a cumulative effect on insomnia. Multiple attempts have been made to build models to interpret and explain the onset and evolution of insomnia; nevertheless, these models are insufficient to represent comprehensive knowledge (Reynolds et al., 2017; Gao et al., 2019; Cai et al., 2021; Jiang et al., 2022; Wang Q. et al., 2022; Zeng et al., 2023).

In this study, through MR analysis of the GWAS database, we investigated whether there is a connection between intestinal flora and insomnia. The findings demonstrated a strong relationship and potential interaction between gut flora and insomnia. Studies have suggested that gut bacteria play a role in the development of insomnia, but the specific mechanism is unknown. Growing evidence points to a critical function for the gut microbiota in the regulation of sleep behavior, both directly and indirectly, as well as a potential role in the pathophysiology and etiology of sleep disorders. It has been found that in older people with insomnia, differences in the composition of the gut microbiota and the abundance of particular genera are associated with poor sleep and poor cognitive function. Studies revealed that insomniacs had considerably higher relative abundances of *Lactobacillus crispatus* and *Streptococcus* compared to healthy controls. Five metabolic pathways, including those for glycerophospholipid metabolism, glutathione metabolism, nitrogen metabolism, aspartate, glutamate, alanine metabolism, and aminoacyl-tRNA production, may be involved in the gut microbiota's ability to cause insomnia (Le Chatelier et al., 2013; Ning et al., 2022). In both human studies and animal models, it has been suggested by researchers that gut bacteria may contribute to sleep issues.

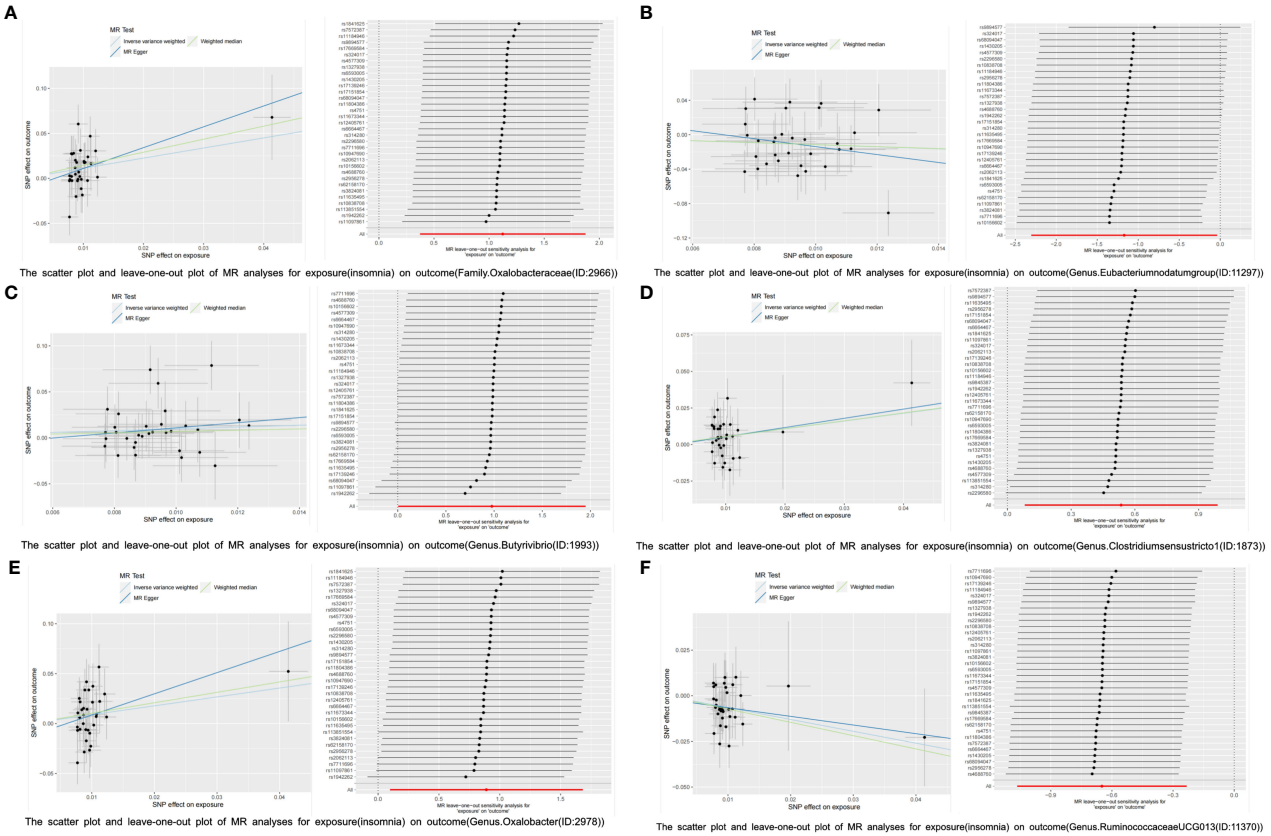

FIGURE 5
The scatter plot and leave-one-out plot of MR analyses for insomnia on six GM taxa. **(A)**: exposure: insomnia, outcome: family *Oxalobacteraceae* (ID:2966). **(B)**: exposure: insomnia, outcome: genus *Eubacteriumnodatumgroup* (ID:11297). **(C)**: exposure: insomnia, outcome: genus *Butyrivibrio* (ID:1993). **(D)**: exposure: insomnia, outcome: genus *Clostridiumsensustricto1* (ID:1873). **(E)**: exposure: insomnia, outcome: genus *Oxalobacter* (ID:2978). **(F)**: exposure: insomnia, outcome: genus *RuminococcaceaeUCG013* (ID:11370).

When compared to the findings of earlier studies, this study's findings exhibit parallels and discrepancies. Pro-inflammatory activation may be one major component causing insomnia. According to studies, chronic sleep deprivation is linked to higher levels of IL-1 and TNF-α in the brain, as well as higher levels of IL-6 in the blood during the day. According to research (Wang Q. et al., 2022), people with acute and chronic insomnia disorders have lower abundances of several anaerobic gut flora taxa, including *Lachnospira*, *Roseburia*, and *Prevotella 9*. We discovered that the genera *Prevotella7* (ID:11182) and *Lachnoclostridium* (ID:11308) are associated with a significant incidence of insomnia. *Prevotella* is a Gram-negative bacterium that helps break down protein and carbohydrate foods. *Prevotella* is frequently believed to have a lower abundance in certain diseases (Ley, 2016). Studies (Feng et al., 2023) also identified that several *Prevotella* (such as *Prevotella amnii*, *Prevotella buccalis*, *Prevotella colorans*, and *Prevotella timonensis*) were associated with changes in inflammatory and metabolite levels, indicating that *Prevotella* may affect sleep by regulating metabolites and promoting inflammation. However, more is not always better; recent human research has showed an increase in *Prevotella* to systemic illnesses such as periodontitis, bacterial vaginosis, rheumatoid arthritis, metabolic problems, low-grade systemic inflammation, and schizophrenia (Bertelsen et al., 2021; Iljazovic et al., 2021). The gut taxon *Lachnoclostridium* is a genus of Gram-positive bacteria, and people with ulcerative colitis and irritable bowel syndrome tend to have higher concentrations of *Lachnoclostridium* (Cai et al., 2022). *Lachnoclostridium* was more prevalent in patients with COVID-19 and was identified by MR analysis as having a high risk of AD (Iannone et al., 2019; Zhang and Zhou, 2023).

In contrast to earlier research, our findings show that the genus *Dorea* (ID:1997) has a high risk of sleeplessness. According to studies (Zhang et al., 2021), patients with major depressive illnesses and sleep disorders had lower levels of *Streptococcus*, *Dorea*, *Barnesiella*, and *Intestinibacter*. *Dorea* bacteria is a member of the thick-walled bacterial porophyllium group, which is widely distributed in the human intestine. By inducing Treg cells and preventing Th17 cell differentiation and function, *Dorea* bacteria can control the intestinal immune response and preserve the stability and integrity of the gut mucous barrier. *Dorea* is more prevalent and is suspected to have an inflammatory effect in patients with multiple sclerosis, inflammatory bowel disease, colorectal cancer, autism spectrum disorders, and obesity. Studies have shown that the composition, diversity, and metabolic activity of the gut microbiota change significantly between healthy individuals and insomniacs. *Bacteroides* and *Clostridiales* are considered to be the two most crucial biomarkers for differentiating

between insomniacs and healthy people. Additionally, the mechanism behind the connection between gut microbiota and insomnia is unclear. Research has found that the microbial ecosystem in the human gut is complex and diverse, and the collaborative relationships between different types of bacteria can disrupt the stability of the microbial ecosystem; the competitive relationship between different bacterial communities helps maintain the stability of the intestinal ecosystem. Rakoff-Nahoum S (Rakoff-Nahoum et al., 2016) evolved cooperation within the *Bacteroidales*, the dominant Gram-negative bacteria of the human intestine. We know little about cooperation within this important ecosystem and studies are few. Our research provides guidance and a foundation for the management of insomnia. This current study is also incomplete, we did not identify GMs and the associated SNPs to understand the meaning of the SNPs to the GM. It will be very meaningful to figure out the function of SNPs to the GMs in the future.

The current study has a number of limitations that need to be mentioned. Firstly, because the study only included participants with primarily European ancestry, there could be already many genomic variations within European ancestry. It may be possible that the SNPs in the host can directly or indirectly cause insomnia. Therefore, in order to strengthen the conclusion, additional research involving participants from diverse parts of the world would be necessary to extend the findings to other groups without constraint. Secondly, a higher permissive threshold ($p<1\times10^{-5}$) was used because there were so few IVs that met the rigorous criteria ($p<5\times10^{-8}$) for screening. Thirdly, self-reporting insomnia symptoms has limitations, including recall bias and lack of granularity; the cases of insomnia in this study were not strictly defined, so future analysis based on strict criteria for insomnia GWAS data is required to strengthen confidence in a conclusion. Fourthly, the GM GWAS data included in this analysis was based solely on 16S rRNA sequencing from genus to phylum level; additional metagenomic and multiomic techniques should target gut microbiota composition at a more precise level to prevent bias. Fifthly, MR analysis relies on three important assumptions mentioned above. In this study, SNPs are used as instrumental variables (IV), GMs as exposure, and insomnia as outcome (GMs>SNPs>insomnia); in reverse MR analysis, insomnia is used as exposure and GMs as outcome (insomnia>SNPs>GMs); considering the complicated links between insomnia and GMs, it may be possible that host SNPs may affect insomnia through GMs; another MR study (GMs as IV, SNPs as exposure, and insomnia as outcome; SNPs>GMs>insomnia) could be conducted to explore the causality between host SNPs and insomnia in the future. Finally, the identified GMs might exist in both insomnia and healthy individuals; therefore, it is necessary to regroup the individuals with or without the GM-associated significant IVs, and intervening with GMs in insomnia research can help strengthen the causal link in the future.

Conclusion

Overall, we detected 10 causal associations after performing an MR analysis on the causal impacts of 211 GM taxa on insomnia. Among them, class *Negativicutes*, genus *Clostridiuminnocuumgroup*, genus *Dorea*, genus *Lachnoclostridium*, genus *Prevotella7*, and order *Selenomonadalesare* were significantly associated with increased insomnia risk. We discovered a causal link between insomnia and six other GM taxa through reverse MR analysis. It suggested that the relationship between insomnia and intestinal flora is convoluted. However, since the current work was based on a GWAS summary-level dataset derived from 16S rRNA sequencing, more in-depth analyses based on more advanced large-scale studies generated from metagenomics sequencing are required. Nevertheless, our findings may offer beneficial biomarkers for disease development and prospective candidate treatment targets for insomnia.

Data availability statement

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

Ethics statement

This study of "The relationship between gut microbiota and insomnia: a bi-directional two-sample Mendelian randomization research" relied on publicly available de-identified data from participant studies that had been authorized by an ethical standards committee. There was hence no need for extra, separate ethical approval for this investigation. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

YL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. QD: Data curation, Supervision, Writing – review & editing. ZL: Project administration, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

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

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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.2023.1296417/full#supplementary-material

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Multidirectional associations between the gut microbiota and Parkinson's disease, updated information from the perspectives of humoral pathway, cellular immune pathway and neuronal pathway

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The human gastrointestinal tract is inhabited by a diverse range of microorganisms, collectively known as the gut microbiota, which form a vast and complex ecosystem. It has been reported that the microbiota-gut-brain axis plays a crucial role in regulating host neuroprotective function. Studies have shown that patients with Parkinson's disease (PD) have dysbiosis of the gut microbiota, and experiments involving germ-free mice and fecal microbiota transplantation from PD patients have revealed the pathogenic role of the gut microbiota in PD. Interventions targeting the gut microbiota in PD, including the use of prebiotics, probiotics, and fecal microbiota transplantation, have also shown efficacy in treating PD. However, the causal relationship between the gut microbiota and Parkinson's disease remains intricate. This study reviewed the association between the microbiota-gut-brain axis and PD from the perspectives of humoral pathway, cellular immune pathway and neuronal pathway. We found that the interactions among gut microbiota and PD are very complex, which should be "multidirectional", rather than conventionally regarded "bidirectional". To realize application of the gut microbiota-related mechanisms in the clinical setting, we propose several problems which should be addressed in the future study.

KEYWORDS

gut microbiota, Parkinson's disease, microbiota-gut-brain axis, inflammatory reaction, neuronal pathway

1 Introduction

Parkinson's disease (PD) is the leading movement disorders and the second leading neurodegenerative disease (NDD), with which patients are expected to be over doubled in the next 20 years (Asakawa et al., 2016b; Qin et al., 2022). The activity of daily living (ADL) and quality of life (QOL) in the advanced patients are significantly affected, however, more officious treatments are under developing. Till now, dopaminergic medicine and surgical therapy like deep brain stimulation (DBS) remain the mainstay treatments against PD (Qin et al., 2022). There has been no revolutionary breakthrough in terms of new therapy since 1967 levodopa and 1993 DBS emerged. It is difficult to obtain a novel but satisfactory treatment against PD because of the extreme complex and multifold pathophysiological mechanisms of PD, which remains unclear by far (Qin et al., 2022). Hence, clarifying the PD related mechanisms has been an urgent task faced by the global PD researchers. Although many researchers have attempted to interpret PD related mechanisms from multidimensions, the substantial pathogenesis (we call it "neurotoxic environment") to cause apoptosis of the dopaminergic neurons in the substantial nigra remains intricate. Aging related neurodegeneration seems to be a reasonable interpretation for many PD related changes. However, the factor of aging cannot elucidate those patients with PD onset in a young age. Many animal models were established according to the known neurotoxins. Unfortunately, none of these models can completely imitate the human' PD (Asakawa et al., 2016a), which implies that neurotoxin might not be a main cause of this "neurotoxic environment". It seems to indicate that the "neurotoxic environment" is made from many factors. PD is the final comprehensive result of the complicated interaction/crosstalk among these factors. Other than the conventional environmental and genetic pathogenic factors, recently, the roles of gut microbiota and the related microbiota-gut-brain axis come into the picture, particularly with the development of the high-throughput sequencing (Tan et al., 2022).

The relationship between gastrointestinal (GI) dysfunction and PD was noticed even by James Parkinson, who first reported PD in 1817 (Tan et al., 2022). It is reported that GI dysfunction is consistently found in 80% early PD patients (Fasano et al., 2015; Killinger et al., 2018). Gut microbiota represents a huge community of bacteria residing in the GI tract (Jia et al., 2021). The total amount of gut microbiota genes is approximately 100 times than that of human genes and is known as the "second genome" of humans (Moeller et al., 2012; Komaroff, 2018). Using the technology like high-throughput sequencing, investigation regarding the microbiota-gut-brain axis stepped into a new era. Recently, a growing number of studies have elucidated the PD-related metabolic changes of gut microbiota are closely associated with the occurrence and development of PD (Shen et al., 2021; Tan et al., 2021). These "impaired" gut microbiota along with their relevant metabolites are believed to interact with the host, mainly via the microbiota-gut-brain axis, and finally become a pathogenic factor of PD (Mayer et al., 2015). Additionally, many later studies suggested that gut microbiota can be considered as the diagnostic/therapeutic biomarkers for PD, or biomarkers to display the PD progression (Schapira, 2013; Neurology, 2016; Nair et al., 2018). However, due to the characteristics of gut microbiota, somewhile it is difficult to make a directed interaction to change the behaviors of the gut microbiota. Hence, development of a medication against PD on the basis of the gut microbiota related mechanisms are quite difficult. Recently, Tan and her colleagues published an insightful study discussing the available evidence regarding the microbiome–gut–brain axis and PD (Tan et al., 2022). This study critically reviewed the complex interaction/crosstalk between the brain and gut via the microbiome–gut–brain axis. Based on this study, we motivated this review focusing on the association between gut microbiota and PD from the perspectives of humoral pathway, cellular immune pathway and neuronal pathway. We attempted to provide the wider and update information on this topic. We believe these take-home- messages will deepen the understanding the roles of gut microbiota in the PD scenario, and will be helpful for developing novel diagnostic/therapeutic targets against PD based on the gut microbiota.

2 Pathological links between PD and Gut microbiota

Clinically, many PD patients suffer from GI dysfunction, this might be the first noteworthy symptoms which drew the attention of the clinicians to investigate the role of GI in PD. Indeed, whether it is the GI dysfunction causes PD, or conversely PD causes GI dysfunction is a chicken-and-egg problem. Although growing evidence elucidated that dysbiosis of gut microbiota plays a pathogenic role in development of PD, where the microbiota-gut-brain axis plays a key role, nowadays, the mainstream opinion is that the association between PD and Gut microbiota is bidirectional (Tan et al., 2022): Certainly, dysbiosis of gut microbiota may lead PD development, and GI dysfunction may be a non-motor symptom in PD. Importantly, dysbiosis of gut microbiota exhibits complicated interactions with PD pathophysiology, and comprehensively drives the PD progress.

2.1 Dysbiosis of gut microbiota in PD subjects

PD patients may experience many symptoms of GI dysfunction, such as constipation, delayed gastric emptying, altered bowel habits, and nausea (Marrinan et al., 2014; Pellegrini et al., 2015; Mrabet et al., 2016). Indeed, these symptoms have been noticed as non-motor symptoms in PD. It has been reported that PD patients are commonly accompanied with and infection of *Helicobacter pylori* infection and peptic ulcer (Rees et al., 2011; Tan et al., 2020). Interestingly, when the patient-derived α-synuclein (α-syn) were stereotactically injected into the striatum or enteric nerves in a non-human primate animal, both injections would cause lesions in the nigrostriatal pathway and enteric nervous system (Arotcarena et al., 2020). This finding indicates that exposure of α-syn may results in

pathological changes, not only in brain, but also in GI. Numerous studies revealed the abnormality of the gut microbiota in PD. Structure and function of the gut microbiota in PD patients are quite different in comparison with the healthy subjects among these studies (Table 1). The heterogeneous results indicate that the changes of gut microbiota are affects by many complicated factors such as age, geographic provenance, dietary habit, research protocols and sequencing methods (Maier et al., 2018; Boertien et al., 2019). Of those, *Akkermansia* was reportedly increased in PD patients (Gerhardt and Mohajeri, 2018; Baldini et al., 2020; Nishiwaki et al., 2020; Vascellari et al., 2020; Zhang et al., 2020). Meanwhile, abundance of *Bifidobacterium* was reportedly increased (Vascellari et al., 2020; Li et al., 2021; Tan et al., 2021; Yan et al., 2021b). *Roseburia* and *Prevotella* were observed to be significantly decreased in PD patients (Nishiwaki et al., 2020; Vascellari et al., 2020; Zhang et al., 2020; Li et al., 2021), and a decrease in *Prevotella* was observed, which was negatively correlated with PD pathogenesis (Hasegawa et al., 2015). Additionally, many studies indicated close association between dysbiosis of gut microbiota and PD-related clinical manifestations like PD severity, duration, motor and nonmotor symptoms (Ren et al., 2020; Zheng et al., 2021).

Put another way, dysbiosis of gut microbiota is also observed in PD animal models (Table 2), albeit all the animal models available nowadays cannot completely imitate the pathophysiological changes in PD patients (Asakawa et al., 2016a). Similar to PD patients, *Akkermansia* was also increased (Gorecki et al., 2019; Yan et al., 2021a; Zhao et al., 2021), whereas abundance of *Prevotella* was reportedly decrease in these animal models (Sampson et al., 2016; Joers et al., 2020; Yan et al., 2021a). Similar to the scenario in Human patients, the changes of gut microbiota in animal models are also influenced by many factors such as neurotoxin used, and administrating approaches, dose, and timing.

Some authors believed that specific gut microbiota might be considered as a potential marker and therapeutic target for PD because the changes of PD-related gut microbiota are closely related to the occurrence and development of PD (Ji et al., 2021). However, due to the heterogeneous nature of these results, no matter in human patients and in animal models, till now, there remains no

TABLE 1 Microbial alterations in patients with PD.

Studies	Country	Participants and intervention	Changes in intestinal microflora		Detection methods
			Decreased	Increased	
Tan et al., 2021	Malaysia	Patients with PD (n=104), together with spouse (n=91) or sibling (n=5) controls free of neurological disorders and living in the same community	Not listed	*Cloacibacillus*, *Catabacter*, *Christensenella*, *Butyrivibrio*, and *Bifidobacterium*	16S rRNA
Qian et al., 2020	China	Patients with PD [37 (47.4%) female; age 67.0 (5.6) years], 75 healthy control subjects [36 (48.0%) female; age 65.3 (7.5) years]	Not listed	King-doms Viruses and Archaea and of the phyla Synergistetes, Verrucomicrobia and Viruses with no name	Shotgun metagenomic sequencing
Yan et al., 2021b	China	Patients with PD (n=20); healthy control subjects (n=20)	*Faecalibacterium*	Alistipes, Rikenellaceae_RC9_gut_group, *Bifidobacterium*, *Parabacteroides*	16S rRNA
Vascellari et al., 2020	Italy	Patients with PD (n=64) and healthy controls (n=51)	*Bacteroides, Blautia, Lachnospira, Butyrivibrio, Roseburia, Pseudobutyrivibrio, Brevibacterium, Dolichospermum, Coprococcus, and Odoribacter.*	*Akkermansia*, *Escherichia*, *Bifidobacterium*, *Streptococcus*, *Clostridium*, *Serratia*, *Veillonella*, *Prosthecobacter*, *Enterobacter, and Slackia.*	16S rRNA
Nishiwaki et al., 2020	Japan	Patients with PD (n=223); healthy control subjects (n=137)	*Faecalibacterium*, *Roseburia, and Lachnospiraceae* ND3007 group	*Genus Akkermansia and family Akkermansiaceae*	16S rRNA
Baldini et al., 2020	Luxembourg	Patients with PD (n=147); healthy control subjects (n=162)	*Paraprevotella*	*Akkermansia*、*Christensenella and Lactobacillus*	16S rRNA
Li et al., 2021	China	Patients with PD (n=69) and controls in three groups ((n=244, comprising 66 spouses, 97 age-matched, and 81 normal samples, respectively)	*Roseburia*	c_Actinobacteria, f_Bifidobacteriaceae, g_Bifidobacterium, o_Bifidobacteriales, p_Actinobacteria f_Lactobacillaceae, and *g_Lactobacillus*	Shotgun metagenomic sequencing
Zhang et al., 2020	China	Patients with PD (n=63), healthy spouses (HS) (n=63)and healthy people (n=74)	*Fusobacterium*, *Prevotella*	*Oscillospira*, *Akkermansia*	16S rRNA

TABLE 2 Microbial alterations in animal PD models.

Studies	Country	Animal models	Changes in intestinal microflora		Detection methods
			Decreased	Increased	
Yan et al., 2021a	China	A53T transgenic monkeys with early PD symptoms	*Prevotella*	*Sybergistetes*, *Akkermansia*, *and Eggerthella lenta*	Shotgun metagenomic sequencing
Sun et al., 2018	China	MPTP-induced PD mice model	Phylum Firmicutes and order Clostridiales	Phylum Proteobacteria, order Turicibacterales and Enterobacteriales	16S rRNA
Zhao et al., 2021	China	MPTP-induced PD mice model	Bacteroidetes phylum	Genera *Akkermansia, Desulfovibrio, Verrucomicrobia* phylum and Verrucomicrobiaceae family	16S rRNA
Joers et al., 2020	USA	MPTP-induced PD monkey model	*Prevotella*	Firmicutes phylum	16S rRNA
Perez-Pardo et al., 2018	Netherlands.	Rotenone-induced PD mouse model	*Actinobacteria* and *Bifidobacterium*	Phyla Bacteroidetes and Firmicutes	16S rRNA
Gorecki et al., 2019	Australia	Human α-syn over-expressing PD mouse model	*Verrucomicrobiae*	*Akkermansia*	16S rRNA
Sampson et al., 2016	USA	Mice that overexpress α-syn	*Lachnospiracae, Rikenellae, Peptostreptococacae and Butyricicoccus* sp	*Proteus* sp., *Bilophila* sp., *and Roseburia sp*	16S rRNA
Lai et al., 2018	China	A neurotoxin PD mouse model induced by chronic low doses of MPTP	*Prevotellacea, Clostridiale*	*Erysipelotrichaceae*	16S rRNA

compelling evidence supporting which gut microbiota can be served as a diagnostic/therapeutic target against PD. In addition, the aforementioned results are derived from investigations with small samples in different experimental conditions. Moreover, they cannot answer the chicken-and-egg problem, namely whether the pathophysiological changes of PD cause dysbiosis of gut microbiota, or *vice versa*. Hence, before we can conclude the roles of gut microbiota in PD, much more deep and rigorous investigations are desired.

2.2 The modulatory role of gut microbiota in neurological functions

The interaction/crosstalk between brain and gut microbiota is increasingly attended. Gut microbiota is living in a relatively balanced environment in a normal human, which may contribute to modulation of the neurological functions such as learning, cognitive and memory (Parashar and Udayabanu, 2017; Cryan et al., 2020). Cryan et al. reported that gut microbiota can regulate intellectual and behavioral development, and is associated with some neurological disorders (Cryan et al., 2019). A number of investigations recently documented that complete absence or severe depletion of the gut microbiota may result in impairments of learning and memory in the hosts (Chu et al., 2019; Yu et al., 2019; Liu et al., 2020). Olson et al. found that co-treatment of ketogenic diet and hypoxia can change the gut microbiota with *bilophila wadsworthia* enriched, then promote cognitive impairment in germfree (GF) mice (Olson et al., 2021). Wu et al. reported that enterococcus faecalis can affect social behavior through discrete neuronal circuits and mediate stress response in GF mice (Wu et al., 2021c).

In the scenario of PD, many studies also elucidated a close association between PD and gut microbiota. Sampson et al. reported that gut microbiota regulates motor deficits and neuroinflammation in α-syn overexpressed mice. Importantly, once the gut microbiota derived from PD patients was transplanted into these models, the neurological deficits were remarkably enhanced (Sampson et al., 2016). Wekerle reported that Escherichia coli and Salmonella typhimurium may induce secretion of the Curli protein and trigger accumulation of α-syn in aged Fischer 344 rats and Caenorhabditis elegans (Wekerle, 2017). Later, Sun et al. found that fecal microbiota transplantation (FMT) resulted in attenuation of microglial activation, decrease of short-chain fatty acids (SCFAs) and caused motor deficits (Sun et al., 2018). Clinically, it has been documented that gut microbiota can influence PD severity (Malkki, 2017; Rani and Mondal, 2021).

These aforementioned reports suggested a close link between PD and gut microbiota from multidimensions. However, direct evidence elucidating PD and gut microbiota remains insufficient. In this regard, it is indispensable to discuss the potential roles of several factors involved in the interactions between PD and gut microbiota.

2.3 Roles of microbiota-gut-brain axis

Recently, Tan et al. reviewed the potential roles of microbiota-gut-brain in the PD scenario (Tan et al., 2022). Indeed, microbiota-gut-brain axis plays a key role in the interactions between gut

microbiota and brain. It is known that at least four systems involved in the modulation of the microbiota-gut-brain axis, namely central nervous system (CNS), autonomic nervous system (ANS), hypothalamic pituitary adrenal (HPA) axis, and enteric nervous system (ENS) (Cryan et al., 2019; Heiss and Olofsson, 2019; Rutsch et al., 2020; Doroszkiewicz et al., 2021). Delivery of inflammatory signals plays a key role in the interaction between brain and gut microbiota (Agirman et al., 2021). A conventional theory is that dysbiosis of gut may induce peripheral inflammation, the inflammatory signals can be delivered into the brain via these pathways thereby cause the inflammatory response in CNS, ultimately form the "neurotoxic environment" in the brain, which might be a pathogenic factor causing PD. However, there is no direct evidence proving that dysbiosis of gut microbiota might cause PD. Several hypothetic pathways were introduced contributing the inflammatory interactions among brain, gut and microbiota, including humoral pathway, cellular immune pathway and neuronal pathway (Agirman et al., 2021). However, these pathways are also problematic in terms of PD pathophysiology (Figure 1).

2.3.1 Humoral pathway

Agirman et al. introduced that dysbiosis of gut microbiota may trigger release of pro-inflammatory cytokines and disrupt the blood brain barrier (BBB) integrity, then make a gateway which may introduce peripheral toxins, and pathogens into brain and cause neuroinflammation (Agirman et al., 2021). Meanwhile, such neuroinflammation may trigger release of glucocorticoid and deteriorate the environment of gut microbiota. These bidirectional regulations create a vicious circle. However, this pathway is mainly based on the BBB damage. If the BBB is relative unbroken, the interactions between CNS and peripheral blood are limited. Albeit a recent report indicated that BBB damage was found in PD with dementia (Wong et al., 2022), there is no direct clinical evidence showing the BBB damage in PD without cognitive impairment, particularly in early stage of PD. Hence, other than the cytokines, liposoluble small molecules might play a more important role in the humoral pathway, such as metabolites in the tryptophan–kynurenine pathway (KP), SCFAs, and bile acids (BAs) (Qin et al., 2022).

2.3.1.1 The roles of tryptophan metabolites

Tryptophan is an essential amino acid which approximately over 95% is metabolized through KP whereas less than 5% is generates 5-HT. Importantly, both metabolites in KP and 5-HT are proven to be closely associated with the PD pathophysiology.

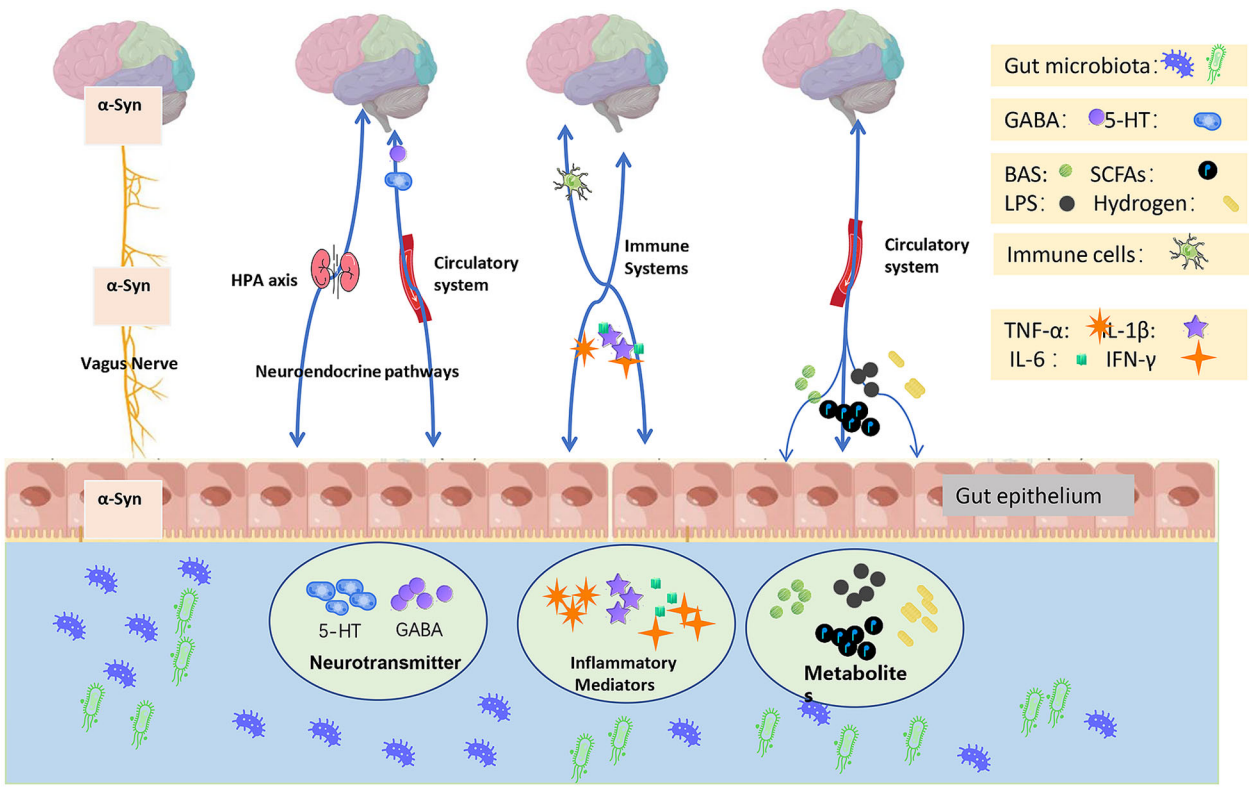

FIGURE 1
Microbiota-gut-brain axis-related PD pathology. Intestinal microbiota along with its derivatives play a role in the pathology of PD. The emerging mechanisms include modulation of the gut environment, maintenance of the ecological balance among the gut microbiota, and effects on the neurotransmitters, inflammatory factors, and related metabolites within the gut. The potential influence pathways were *i)* the vagus nerve conveys α-syn; *ii)* the neuroendocrine pathways primarily involve the HPA axis, and the circulation-mediated neurotransmitters such as serotonin (5-HT) and gamma-aminobutyric acid (GABA); *iii)* the immune system regulates immune cells and mediates the release of inflammatory factors; and *iv)* the circulation system transports BAS, SCFAs, LPS, and hydrogen.

2.3.1.1.1 KP and PD

Recently, we reviewed the role of KP in the pathogenicity of PD (Qin et al., 2022). Both aging and dysbiosis of gut microbiota may activate some rate-limiting enzymes in KP, such as indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO). Activation of KP will result in enhancement of the downstream toxins, such as 3-hydroxykynurenine, 3-hydroxyanthranilic acid quinolinic acid. Although these toxins are mainly produced peripherally, the liposoluble nature with small molecular weight make they are easily cross the BBB and accumulate in the CNS, thereby exhibiting neurotoxic effects. Hence, at the state of BBB remains unbroken, KP may act as an "aging signal deliverer", particularly deliver inflammatory signal from the periphery to the CNS. Close association among KP, α-syn, and PD has been well documented at this review (Qin et al., 2022). However, there remains no direct evidence supporting the activation of KP will cause α-syn deposition and ultimately cause PD (Qin et al., 2022).

2.3.1.1.2 The roles of 5-HT

As a minor production of the tryptophan metabolism, 5-HT cannot directly cross BBB. Hence, the 5-HT in CNS is generated in the CNS in case of BBB is unbroken. Many studies reported the close association between 5-HT and gut microbiota. It is documented that the hippocampal 5-HT and its' main catabolic product levels increase whereas plasma tryptophan, and tryptophan metabolism via KP decrease in the GF mice (Wikoff et al., 2009; Diaz Heijtz et al., 2011). Animal experiments demonstrated that after administration of antibiotics in GF mice, the levels of 5-HT in colon and feces decreased, nevertheless reconstruction of gut microbiota restored 5-HT levels in these animals (Yano et al., 2015; Ge et al., 2017). Desbonnet et al. found that *Bifidobacterium infantis* can increase plasma tryptophan level, which may influence central 5-HT transmission (Desbonnet et al., 2010). These results imply that 5-HT is remarkably affected by the gut microbiota. Some gut microbiota may enhance the peripheral tryptophan level, which can cross BBB, and subsequently increase the tryptophan level in CNS, and finally increase the 5-HT level in CNS. In terms of the connection of 5-HT and PD, Tong et al. found that plasma 5-HT levels in PD patients were lower than those in healthy control, and 5-HT levels were negatively correlated with the severity of non-motor symptoms like depression and pain (Tong et al., 2015). Kotagal et al. found that 5-HT may change Aβ metabolism and reduce the risk of PD related cognitive impairment (Kotagal et al., 2018). Lelieveld et al. reported that 5-HT may influence the sleep disordered breathing in PD (Lelieveld et al., 2012). These findings seem to imply the potential associations between 5-HT and the non-motor symptoms in PD, however, require further verification.

2.3.1.1.3 The roles of tryptophan metabolites SCFAs

SCFAs, such as acetic, propionic, butyric, isobutyric, valeric, and isovaleric are commonly produced by gut microorganisms from fermentation of dietary fiber by gut microorganisms (Cummings et al., 1987; Bergman, 1990; Miller and Wolin, 1996). It was documented that *Bifidobacterium, Clostridia, Lactobacillus, Bacteroides,* and *Faecalibacterium* can produce SCFA (Hold et al., 2003; Chambers et al., 2015; Koh et al., 2016; Yang et al., 2020). SCFAs, serve as the primary nutrients of the colonocyte, play a fundamental role in maintaining intestinal homeostasis and regulating energy metabolism (Cummings and Macfarlane, 1997; Priyadarshini et al., 2018). Recently, the SCFAs were proposed as key mediators of microbiota-gut-brain interactions (Dalile et al., 2019). SCFAs are commonly produced in the gut. They can cross BBB, thereby can regulator the inflammation reactions in both periphery and CNS. Peripherally, SCFAs contribute to regulating intestinal permeability and enhancing the integrity of intestinal barrier. Kim et al. found that SCFAs activate G protein coupled receptor (GPR) 41 and GPR43 on intestinal epithelial cells, lead to the production of chemokines and cytokines, and mediate protective immunity and tissue inflammation in mice (Kim et al., 2013). Butyrate administration can affect the expression of tight junction proteins thereby maintain the integrity of intestinal barrier (Plöger et al., 2012; Wang et al., 2012). Additionally, butyrate ministration can increase the levels of tight junction protein and improve intestinal permeability via GPR109A (a G-protein coupled receptor) (Karunaratne et al., 2020). In CNS, SCFAs display the analogous protective effects on BBB. Braniste et al. reported that administration of Clostridium butyricum (mainly produces butyrate) and Bacteroides thetaiotaomicron (mainly produces acetate and propionate) resulted in decrease of the BBB permeability in the frontal cortex, hippocampus, and striatum, along with upregulation of the occludin expression in GF mice, indicating a protective effect on BBB (Braniste et al., 2014). In addition, SCFAs interacting with immune cells, contribute to reduction of the systemic inflammation, subsequently alleviation of the neuroinflammation (Segerstrom and Miller, 2004; Miller and Raison, 2016; Parada Venegas et al., 2019). Moreover, SCFAs constantly modulate maturation and function of microglia in CNS (Smith et al., 2013; Borre et al., 2014). These findings suggest a neuroprotective effect of SCFAs.

Numerous studies elucidated the effects of SCFAs on PD. Aho et al. found the SCFAs levels in PD patients were lower in comparison with healthy people, which were associated with gastrointestinal inflammation and the development of PD (Aho et al., 2021). Another study found that anti-inflammatory butyrate-producing bacteria such as genera *Blautia, Coprococcus*, and *Roseburia* were lower, whereas pro-inflammatory Proteobacteria of the genus *Ralstonia* were higher in the sigmoid mucosa of the PD patients (vs healthy controls). They therefore verified the involvement of pro-inflammatory dysbiosis of gut microbiota in the PD pathophysiology, particularly in the inflammation-induced α-syn misfolding (Keshavarzian et al., 2015). These human results seem to imply the neuroprotective effects of SCFAs on PD, which were also verified by several *in vitro* studies (Chen et al., 2006; Sechi et al., 2008; Fu et al., 2015; Jang et al., 2017). Evidence from the current animal studies also proved the significant effects of SCFAs on PD subjects. However, the results are controversial among studies. Hou et al. found that administration of sodium butyrate achieved suppression of the neuroinflammation and alleviation of the neurological deficits in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treated mouse PD models (Hou et al., 2021a). Later, they also found that oral administration of gut

microbiota-derived propionate resulted in amelioration of the motor deficits and depletion of the dopamine neurons in 6-hydroxydopamine (6-OHDA) induced mouse PD models (Hou et al., 2021b). Conversely, Cannon et al. found the enhancement of butyric acid level and reduction of isobutyric acid level in a Pink1−/- PD mouse (Cannon et al., 2020). Qiao et al. found that administration of sodium butyrate resulted in deterioration of the motor deficits and neuroinflammatory by increasing the number of microglia and activated astrocytes (Qiao et al., 2020). These contradictory results imply an extremely complex nature of SCFAs under a PD condition. The problem whether SCFAs play a neuroprotective or neurotoxic role remains still requires further investigation.

2.3.1.1.4 The roles of tryptophan metabolites BAs

BAs are synthesized by the hepatic cells and secreted to the intestine through the bile duct. Primary BAs are transformed into secondary BAs under the action of gut microbiota in the terminal ileum and colon (Russell and Setchell, 1992; Ridlon et al., 2006). BAs can cross the BBB. It has been reported that BAs are distributed in the brain in human and animals, of which approximately 20 BAs were identified in the rat brain (Xie et al., 2013; Zheng et al., 2016; Pan et al., 2017). Chenodeoxycholic acid (CDCA) and cholic acid (CA) are the major BAs in the rat's brain (Mano et al., 2004; Zheng et al., 2016). BAs can affect the immune regulation in CNS (Fiorucci et al., 2010). Currently, many studies proved the potential neuroprotective effects of BAs in several neurological diseases. Many studies have demonstrated the potential neuroprotective effects of BAs in various neurological diseases. Furthermore, BAs have been found to be involved in cognitive functions such as learning and memory (Abrams et al., 2006). For instance, intracerebroventricular administration of tauroursodeoxycholic acid (TUDCA) in adult rats significantly enhanced early neurogenesis (Soares et al., 2018). In a mouse model of PD, TUDCA prevented JNK phosphorylation in the midbrain and striatum by regulating Akt signaling and prevented MPTP-induced dopaminergic cell death (Castro-Caldas et al., 2012). Additionally, TUDCA was found to activate Nrf2 to prevent oxidative stress induced by 1-methyl-4-phenylpyridinium and α-syn in an *in vitro* experiment using SH-SY5Y cells (Moreira et al., 2017). TUDCA was also reported to induce mitochondrial autophagy by increasing the expression of PINK1, parkin, and LC3-II/LC3-I in another experiment (Rosa et al., 2017). In a transgenic mouse model of PD, TUDCA reduced striatal cell apoptosis and improved locomotor ability (Keene et al., 2002). Interestingly, TUDCA administration improved cognitive impairment in db/db mice (Liu et al., 2020). Moreover, UDCA was found to have anti-inflammatory potential in the rotenone model of PD, reducing rotenone-induced NF-κB expression and TNF-α levels (Abdelkader et al., 2016). In the study of major depressive disorder, activating the bile acid receptor FXR (farnesoid X receptor) was reported to regulate neuroimmunity and inhibit brain inflammation (Bao et al., 2021). The latest research shows that obeticholic acid (OCA), a derivative of BAs, reduced the expression of neuroinflammatory microglia and IL-1 in the hippocampus (Wu et al., 2021b). Several studies have suggested that BAs may play a role in cognitive dysfunction (Chen et al., 2020b; Jena et al., 2020). In summary, BAs may exert their effects on PD through anti-inflammatory and neuroimmunomodulatory mechanisms.

It is known that BAs and gut microbiota affect mutually. Commonly, BAs are modified by the microorganisms via certain enzymatic reactions. It is easy to understand that the abnormal state of gut microbiota in PD context may affect the modification of BAs. However, the direct evidence remains insufficient. Previous studies reported that several bile salt hydrolase-containing bacteria, such as *Bacteroides* spp., *Bifidobacterium, and Lactobacillus*, were enhanced in PD patients (Spichak et al., 2021; Ghezzi et al., 2022), indicating a complicated association among PD, microbiota, and BAs (Kiriyama and Nochi, 2023), which is still unclear. Wang et al. documented that BAs might play a role as a "star molecule" in the mechanisms of microbiota-gut-brain axis, which involved in the interactions between gut microbiota and PD (Wang et al., 2023). (5) The roles of tryptophan metabolites γ- Aminobutyric acid.

GABA (γ-Aminobutyric acid), also known as 4-aminobutanoic acid, is a non-protein amino acid and the primary inhibitory neurotransmitter in the mammalian central nervous system (Hyland and Cryan, 2010; Vithlani et al., 2011; Muthukumar et al., 2014). Besides the brain tissue, GABA is present in peripheral tissues such as the heart, stomach, small intestine, liver, and kidney (Akinci and Schofield, 1999; Lakhani et al., 2014). GABA possesses antioxidant, anticonvulsant, anti-inflammatory, analgesic, sedative, and anxiolytic properties (Rorsman et al., 1989; Adeghate and Ponery, 2002; Lacerda et al., 2003; Menezes and Fontes, 2007; Ben-Othman et al., 2017), and has been reported to enhance brain and nervous system function, as well as cognitive and memory functions (Yamatsu et al., 2016; Kim et al., 2019a; Inotsuka et al., 2021). Recent studies have found that GABA produced by gut microbiota (*Bacteroides, Parabacteroides, Escherichia species, Lactobacilli*, and *Bifidobacteria*) can participate in neural activities through the microbiota-gut-brain axis (Yunes et al., 2016; Strandwitz et al., 2019). An animal experiment using probiotics found that *L. rhamnosus (JB-1)* can induce region-dependent changes in GABA mRNA expression in the brain through the vagus nerve, and reduce stress-induced corticosterone and anxiety- and depression-related behavior (Bravo et al., 2011). GF mice were found to have significantly reduced levels of GABA in feces and blood, and antibiotics were found to change the level of GABA in feces, suggesting that the microbiota may contribute to the circulating level of GABA (Matsumoto et al., 2017; Fujisaka et al., 2018). A study on *Caenorhabditis elegans* found that GABA produced by Escherichia coli HT115 has a protective effect on neurons (Urrutia et al., 2020). while a previous study on learning in bees reported that the brains of bees colonized with the heritable *Bifidobacterium* strain displayed an increased level of GABA (Wu et al., 2021a). It has been reported in the literature that GABA plays a crucial role in protecting the intestinal barrier and preventing gastrointestinal mucosal inflammation (Xie et al., 2017; Chen et al., 2019). GABA may contribute to regulation of the population and diversity of gut microbiota, such as enhancement of the dominant microorganism populations, enrichment of the microbial community (Chen et al., 2019). In addition, GABA may improve the colon health by enhancement of the levels of acetate, propionate, butyrate and total short-chain fatty acids, along with decrease of

colonic and cecal pH values (Xie et al., 2017). Recent clinical trials and animal studies have shown that GABA plays an important role in the pathogenesis of PD. An experiment examining the GABA levels of PD patients and healthy controls found that the GABA levels in the upper brainstem of PD patients were significantly lower than those of healthy controls (Song et al., 2021b). while a preliminary study also found that a single dose of L-dopa increases upper brainstem GABA in PD (Song et al., 2021c). Some experts suggest that the reduced GABA levels in PD patients may be associated with GABAergic dysfunction (Elmaki et al., 2018). Animal experiments have also reported that polymannuronic acid regulates GABA levels through the microbiota-gut-brain axis in PD mice to prevent the loss of dopaminergic neurons (Dong et al., 2020). Based on the aforementioned information, we can hypothesize that GABA plays an important role in the pathogenesis of PD mediated by the microbiota-gut-brain axis.

2.3.1.2 The roles of LPS

LPS, also known as endotoxin, is an integral structural component of the outer membrane of gram-negative bacteria (Raetz and Whitfield, 2002). Dysbiosis of the microbiota leads to increased intestinal permeability, allowing LPS products to translocate from the intestinal lumen into the host circulation (Rietschel et al., 1994; Tilg et al., 2016). Several studies have found a significant increase in gram-negative bacteria in PD patients, which is closely related to intestinal inflammation (Guo et al., 2015; Nighot et al., 2017; Gorecki et al., 2019). Furthermore, a study found a positive association between the relative abundance of gram-negative Enterobacteriaceae and the severity of postural instability and gait difficulty (Scheperjans et al., 2015). An animal experiment demonstrated that injecting LPS into the rat substantia nigra can activate microglia, trigger the inflammatory response, increase the expression of pro-inflammatory cytokines, and change the activity of oxidative stress markers, inducing the characterization of a PD model (Sharma and Nehru, 2015). Different routes and modes of LPS administration can cause various parkinsonian symptoms. Intraperitoneal injection of LPS can increase α-Syn expression in the large intestine and intestinal permeability (Kelly et al., 2014). The PD model induced by endotoxin can be established by unilateral intranasal drip of LPS. These mice showed progressive hypokinesia, selective loss of dopaminergic neurons (He et al., 2013), decreased dopamine (DA) content in the striatum, and α-Syn aggregates without systemic inflammation and immune response (He et al., 2013). Intra-rectal injection of LPS derived from P. mirabilis can induce inflammation in the nigrostriatal regions of the brain (Choi et al., 2018). Intestinal biopsy of PD patients revealed a close relationship between increased intestinal permeability and intestinal α-syn and LPS (Forsyth et al., 2011). Moreover, a series of *in vitro* and *in vivo* experiments proved the relationship between LPS and intestinal permeability and the associated inflammatory signals (Guo et al., 2013; Nighot et al., 2017; Nighot et al., 2019). Previous research has shown that inflammatory monocytes primed with LPS can internalize α-syn, which in turn facilitates its dissemination from the periphery towards the brain and spinal cord (Peralta Ramos et al., 2019). In conclusion, it is speculated that LPS releases inflammatory factors and induces α-syn accumulation in the intestine, which then reaches the brain through the circulatory system, resulting in PD-related pathological changes.

2.3.1.3 The roles of hydrogen

Hydrogen is a metabolite of bacterial fermentation in the gut and has been shown to have selective antioxidant and anti-inflammatory effects (Levitt, 1969; Ohsawa et al., 2007). Furthermore, it has been found to possess anti-apoptotic and cytoprotective properties, contributing significantly to cell protection (Xie et al., 2010; Dixon et al., 2013). In a rat model of PD, treatment with hydrogen was reportedly neuroprotective and prevented dopaminergic cell loss (Fu et al., 2009). Mice models and PD patients also showed similar findings (Fujita et al., 2009; Yoritaka et al., 2013). An animal experiment demonstrated that drinking hydrogen water and intermittent hydrogen exposure can prevent 6-hydroxydopamine-induced PD in rats (Ito et al., 2012). Moreover, evidence has shown that the generation and oxidation of H2 help maintain the diversity of the gut microbiome (Hylemon et al., 2018). A study discovered that probiotics can promote the intestinal production of hydrogen and improve metabolic syndrome in C57BL/6J mice (Zhao et al., 2019). Analysis of 16S rRNA gene isolates indicated a low abundance of hydrogen-producing bacteria in PD patients (Hasegawa et al., 2015). Gas chromatography measurements showed that compared to the control group, the intestinal hydrogen production of PD patients was 2.2 times lower (Suzuki et al., 2018). A clinical trial found that oral intake of lactulose can increase the concentration of hydrogen in PD patients (Ito et al., 2012). Subsequent studies provided evidence that lactulose may increase hydrogen production by the intestinal microbiota (Ohno et al., 2012). These findings suggest that the abundance of intestinal hydrogen-producing bacteria may be related to the occurrence and development of PD. Therefore, intestinal-derived hydrogen may serve as a novel candidate non-invasive biomarker for diagnosis, prognosis, and treatment response in PD.

2.3.2 Immune systems pathway

It is known that the immune system, no matter innate immune system and adaptive immune system, is closely associated with the gut microbiota. Gut microbiota plays a key role in maintaining intestinal mucosal barrier and contributes to maintenance and development of the immune system (Fernández et al., 2003; Candela et al., 2008; Olszak et al., 2012). Immune function of gut microbiota initiates in the intestine and then regulates systemic immunity during the immune response (Dai et al., 2020). A number of early studies documented that implantation of segmented filamentous bacteria from the small intestine resulted in recovery of the immune function of intestinal B and T lymphocytes (Umesaki et al., 1995; Talham et al., 1999; Umesaki et al., 1999). Later, Forsythe found that implantation of the intestinal microorganism achieved reconstruction of the immune function, which is deficient in a GF mouse (Forsythe et al., 2010).

Nevertheless, immunoreactions in CNS and the peripheral organs are relatively independent under the condition that BBB is unbroken. Due to aforementioned BBB state in PD, here, we only discuss the inherent cellular immune pathway in CNS.

2.3.2.1 Microglia

Microglia are key immune cells in the CNS, which contribute to presentation of antigen, production of cytokines, and activation/regulation of inflammation (Chen et al., 2020a; Jiang et al., 2020). Numerous studies have elucidated that microglia are remarkably affected by changes of gut microbiota. Erny et al. found that the proportions of immature microglia in GF mice increased in GF mice. Interestingly, once the gut microbiota in these GF mice was recovered, the features of microglia were accordingly recovered (Erny et al., 2015). Alternatively, knockout of the microglia-specific gene MafB upregulated the expression of the inflammatory pathways in adult GF mice (Matcovitch-Natan et al., 2016). Song et al. found that dysbiosis of gut microbiota caused suppression of the microglia activation and upregulation of the TLR4/NF-κB inflammatory pathway in the hippocampus of aging rats (Song et al., 2021a). Accordingly, treatment of FMT can alleviate the dysbiosis of gut microbiota and resulted in suppression of the microglia activation (Sun et al., 2018). These results reveal a close interaction between microglia and gut microbiota, even the aforementioned experiments were using the animals which BBB seems to be unbroken. Many authors believed that the bidirectional interactions between microglia and gut microbiota might play a role in the etiology of neurodegenerative diseases (Balducci and Forloni, 2018; Davoli-Ferreira et al., 2021; Wang et al., 2021b; Zheng et al., 2023). In the PD scenario, this hypothesis is also supported by many experiments. Sampson et al. proved that gut microbiota plays a role in the motor deficits, microglia activation and α-syn pathology in a α-syn overexpressed mouse (Sampson et al., 2016). Fang et al. found that using the mouse strain MG1363-pMG36e-GLP-1 expressing glucagon-like peptide-1 to treat MPTP-treated mice resulted in amelioration of the motor deficits, suppression of the microglia activation and inflammation, reduction of the pathogen Enterobacteriaceae, and enhancement of the probiotics Lactobacillus and Akkermansia (Fang et al., 2019). Joers et al. treated the PD monkey models with a novel TNF inhibitor, XPro1595. They found that the diversity of gut microbiota, inflammatory, and the microglial behavior were correspondingly changed (Joers et al., 2020). Sun et al. found that oral administration of Clostridium butyricum achieved improvement of the motor deficits, dopamine neuron depletion, synaptic dysfunction and microglia activation in MPTP treated mice (Sun et al., 2021a). All these experiments verified the close relationship between microglia and gut microbiota under the PD condition from various treatments and animal models. Hence, Claudino Dos Santos et al. recently commented that inflammatory reactions may activate microglia and induce aggregation of α-syn, ultimately cause damage of dopaminergic neurons, where dysbiosis of gut microbiota plays a key role (Claudino Dos Santos et al., 2023). There is no clinical evidence investigating the roles of microglia during the intervention of gut microbiota in PD patients. Importantly, there is no study observing changes of the gut microbiota once the microglia in PD are intervened. The complicated interactions among microglia, PD, and gut microbiota are still intricate, which require further investigation in the future.

2.3.2.2 Astrocytes

As the most abundant cells in CNS, astrocytes also play a role in the regulation of the inflammatory and immune reactivity. Interactions between astrocytes and gut microbiota are also reported. Rothhammer et al. reported that dietary tryptophan is metabolized into aryl hydrocarbon receptor agonists by the gut microbiota, and then act on astrocytes to suppress CNS inflammation (Rothhammer et al., 2016). Zhang et al. found that FMT from the NLRP3 KO gut microbiota significantly ameliorated the depression-like behaviors along with astrocyte dysfunction via suppression of the circular RNA HIPK2 (circHIPK2) expression in animal depression models. They hence proposed a gut microbiota-circHIPK2-astrocyte axis in depression (Zhang et al., 2019). Sanmarco et al. found an astrocyte subset, LAMP1+TRAIL+ astrocytes suppressed the CNS inflammation by inducing T cell apoptosis, while these LAMP1+TRAIL+ astrocytes are maintained by the microbiome-licensed meningeal IFN+ NK cells (Sanmarco et al., 2021). Margineanu et al. reported that the hippocampal expression of astrocyte-neuron lactate shuttle related genes was regulated by the gut microbiota (Margineanu et al., 2020). Another study indicated that greater level of gut microbiome-derived metabolite trimethylamine N-oxide was associated with higher expression of brain pro-inflammatory cytokines and astrocyte activation markers (Brunt et al., 2021). These studies also elucidate the crucial role of astrocytes in the immune modulation in the CNS. In terms of PD, Sun et al. found that FMT protected the neuronal function by inhibition of the neuroinflammation and reduction of astrocytes activation in the substantia nigra (Sun et al., 2018). Another acupunctural study found that acupuncture on PD mice regulated the abundance of gut microbiota and restored the overexpression of astrocytes in striatum and substantia nigra (Jang et al., 2020). By far, the evidence between astrocytes and gut microbiota in PD remains limited.

2.3.2.3 Inflammatory mediators

To the extent known, the modulations between PD and dysbiosis of the gut microbiota are bidirectional. *i)* Dysbiosis of gut microbiota may cause PD-related abnormal inflammatory response. Gut microbiota may regulate immune-related inflammatory factors in the intestine, and subsequently affect the CNS (Shimizu et al., 2011; Huang et al., 2019c; Claudino Dos Santos et al., 2023). Microbiota along with their products can stimulate intestinal epithelial cells and macrophages in the gut, resulting in activation of immune response and the release of inflammatory cytokines, and they can reach the CNS (Banks, 2009; Mayer et al., 2014; Gorky and Schwaber, 2016). At autopsy, it was found that the expression of pro-inflammatory cytokines and chemokines were upregulated in the brain tissue and cerebrospinal fluid of patients with PD, including TNF-α, IL-1β, IL-6 and IFN-γ (Mogi et al.,

1994a; Mogi et al., 1994b). Knockout of the TNF-α receptor gene exhibited neuroprotective effects in mice (Bhaskar et al., 2014). Under the physiological conditions, gut microbiota downregulates IFN-γ expression in astrocytes while upregulates TRAIL expression in astrocytes (Sanmarco et al., 2021). The gut microbiota can produce various toll like receptor (TLR) ligands that can play a pro-inflammatory role in a specific environment (Caputi and Giron, 2018). Thus, any changes of gut microbiota and destruction of the gut epithelial barrier may activate TLRs, and promote inflammatory response in both the gut and brain of PD patients (Terán-Ventura et al., 2014; Dheer et al., 2016; Sun et al., 2018). It has been known that the signal regulation of TLR2 and TLR4 can affect the development of PD (Harms et al., 2018; Pascual et al., 2021). In the intestines of PD patients, enhancement of CD3+ T cells in colon tissue was found along with the immune interactions among TLR4, cytokines, and chemokines (Terán-Ventura et al., 2014). Epidemiological studies also suggested that inflammatory bowel disease is a significant risk factor for the development of PD (Lin et al., 2016; Villumsen et al., 2019; Weimers et al., 2019). Some scholars hypothesized that the alteration of gut microbial flora leads to gastrointestinal system disturbance which cause neuroinflammation by prion α-syn expression and produces PD like symptoms (Sharma et al., 2019). *ii)* PD may have GI dysfunction, which worsen the state of gut microbiota. In PD patients at the early stage, mild intestinal inflammation is commonly developed that triggers a low-level immune response and increases intestinal permeability (Houser and Tansey, 2017). Accordingly, intestinal inflammation and enhanced intestinal permeability are the intestinal characteristics of PD patients (Houser and Tansey, 2017; Perez-Pardo et al., 2019). These issues markedly break the balance of gut microbiota and deteriorate their ecology. Thus, the imbalance of intestinal microecology increases the pro-inflammatory environment in the intestine, which promotes the neuroinflammation of the central nervous system and leads to the development of PD. In this regard, PD and dysbiosis of the gut microbiota may generate a bidirectional vicious circle, and promote the development and progression of PD.

It has been reported that α-syn misfolding might initiate in the gut, and spread to the brain via the vagus nerve (Claudino Dos Santos et al., 2023). This hypothesis provided a plausible "pathway" of the impacts of gut microbiota to the α-syn-based PD pathogenesis (see next section). However, due to the complex nature of interactions among microglia, astrocytes, gut microbiota and enteric glia, we believe that the actual interactions between PD and gut microbiota -related inflammatory reactions are far more complex than "bidirectional". It might be "multidirectional" involved by many known and unknown factors, which indeed warrant further investigation.

2.3.3 Neuronal pathways

2.3.3.1 The vagus nerve pathway

The vagus nerve, comprising the tenth pair of cranial nerves, is the longest and most widely distributed pair of cranial nerves, containing sensory, motor, and parasympathetic nerve fibers (Rosas-Ballina et al., 2011; McVey Neufeld et al., 2019; van der Meij and Wermer, 2021). It serves as a crucial link between gut bacteria and the brain, transmitting signals from various intestinal tracts and being associated with numerous gastrointestinal tracts, nervous system, and immune system diseases (Berthoud and Neuhuber, 2000; Goehler et al., 2000; Powley et al., 2011; Ahmed et al., 2019). The vagus nerve communicates with the brain through multiple synaptic connections with the nucleus tractus solitarius of the brain stem (Biaggioni, 2017). According to reports, when gut microbiota stimulate the afferent neurons of ENS, the ENS and vagus nerve form a synaptic connection and an information transmission pathway, enabling mutual communication and regulation between gut microbiota and the brain (Yu et al., 2020). Studies have also found that sensory neurons of the vagus nerve form various mechanical and chemical sensory terminals along the gastrointestinal tract to receive intestinal-brain signals (Bravo et al., 2011; Perez-Burgos et al., 2013). Additionally, enteroendocrine cells can form synapses with neighboring nerves to assist the vagus nerve in receiving signals from the intestine (Bohórquez et al., 2015; Bellono et al., 2017; Fülling et al., 2019). A large prospective cohort study found that subjects who have undergone complete vagotomy have a low risk of PD (Svensson et al., 2015). A post-mortem study has shown that PD pathology may start in the gastrointestinal tract and then spread through the vagus nerve to the brain (Braak et al., 2006). An animal study found that the central role of Lactobacillus rhamnosus was eliminated after vagus nerve amputation (Fung et al., 2017). Recently, α-syn has emerged as a critical factor contributing to PD pathogenesis (Mor et al., 2017). More evidence has indicated that the vagus nerve is a route by which α-syn pathology can spread both to and from the brain (Pan-Montojo et al., 2012; Matteoli and Boeckxstaens, 2013; Gray et al., 2014; Liu et al., 2017). A new mouse model of PD demonstrates α-syn pathology spreading from the gut to the brain via the vagus nerve (Kim et al., 2019b). Recent advances have suggested that gut bacteria enhance the aggregation of α-syn and other similar proteins, a mechanism that might promote the ability of pathogens to reach the CNS through the vagus nerve (Sampson et al., 2016; Choudhry and Perlmuter, 2017). However, some scholars denied the connection between PD and the vagus nerve (Tysnes et al., 2015). A study using baboons as a model found a possible systemic mechanism in which the general circulation would act as a route for long-distance bidirectional transmission of endogenous α-syn between the enteric and the central nervous systems (Arotcarena et al., 2020). Although accumulated and convincing evidence supports this pathway, its specific mechanisms remains unclear and requires further exploration.

2.3.3.2 The HPA axis pathway

The HPA axis is a crucial component of the neuroendocrine system that regulates various body processes to cope with stress (Smith and Vale, 2006). Under stress, Corticotropin-releasing factor (CRF) is released from the paraventricular nucleus (PVN) of the hypothalamus, which is the main regulator of the HPA axis and induces the release of adrenocorticotropic hormone (ACTH) into systemic circulation (Mastorakos and Zapanti, 2004). Subsequently, the adrenal cortex is stimulated by ACTH and secretes

glucocorticoids (Smith and Vale, 2006). The released glucocorticoids then bind to glucocorticoid receptors (GR), resulting in feedback inhibition (Keller-Wood and Dallman, 1984). In recent years, more and more studies have been conducted to discover a bidirectional communication between the neuroendocrine system and gut microbiota through the HPA axis (Farzi et al., 2018). The CNS can regulate intestinal function through the HPA axis, and the gut microbiota can also regulate gut hormone levels, which affect CNS function through the HPA axis (Sun et al., 2020). An animal experiment using GF mice found that Enterococcus faecalis can inhibit excessive stress response in social interaction by limiting the production of corticosterone levels mediated by the HPA axis, thereby promoting the social activity of mice (Wu et al., 2021c). An experiment using GF and pathogen-free mice demonstrated that maternal isolated early life stress altered the HPA axis in a microbiota-independent fashion (Jiang et al., 2015; Kelly et al., 2016). Additionally, evidence suggests that GF mice can overstimulate the HPA axis under stress, while probiotics can alleviate the response state of the HPA axis in mice (Heym et al., 2019; Huang et al., 2019b). A study from 2013 found that DBS of the subthalamic nucleus can reduce cortisol levels in patients with advanced PD (Seifried et al., 2013). Psychological stress and changes in the hypothalamic-pituitary-adrenal axis were also present in patients with "*de novo*" PD (Ibrahimagic et al., 2016). Increasing numbers of clinical trials and animal experiments showed that the HPA axis was altered and unbalanced in PD patients, leading to a significant increase in cortisol levels (Höschl and Hajek, 2001; Zhang et al., 2017; Grigoruţă et al., 2020). Interestingly, one study found that non-motor symptoms of PD (e.g., mood and anxiety) were significantly associated with hair cortisone levels (van den Heuvel et al., 2020). Hair glucocorticoid levels reflect longer-term HPA-axis function and can provide additional insights into the role of a dysregulated HPA-axis in PD (Wosu et al., 2013; Stalder et al., 2017). In conclusion, although there are few studies on the role of the HPA axis in gut microbiota and PD, we cannot ignore the roles of the HPA axis in the microbiota-gut-brain axis of PD.

3 Treatment of PD Targeted at Gut Microbiota

In the clinical setting, the primary treatment for PD remains dopaminergic medication. Currently, there is no known cure for PD. Although growing evidence supporting the role of gut microbiota in PD, the gut microbiota-based therapy is still under investigation. This section summarizes the emerging preventive and therapeutic interventions that modulate the gut microbiome (Table 3).

3.1 Prebiotics

Prebiotics are organic substances that are not digested and absorbed by the host, but selectively promote the metabolism and proliferation of beneficial bacteria in the body, thereby improving host health. Once they reach the colon, prebiotics are decomposed and utilized, promoting the growth of colonic microbiota, where they play an important role in improving the intestinal microecology and regulating lipid metabolism. Different types of prebiotics include isomalto-oligosaccharide, fructooligosaccharide, galacto-oligosaccharide (GOS), xylooligosaccharide, lactulooligosaccharide, soybean oligosaccharide, and inulin (Slavin, 2013). Although there are only few studies using prebiotics in the treatment of PD, neuroprotective effects of prebiotics were verified. Feeding GOS to rats could elevate the expression of central brain-derived neurotrophic factor (BDNF), N-methyl-D-aspartate receptor subunit (NMDAR), and D-serine (Savignac et al., 2013). In another experiment, neonatal prebiotic (BGOS) supplementation increased the levels of synaptophysin, GluN2A-subunits, and BDNF proteins in the adult rat hippocampus (Williams et al., 2016). Additionally, it was reported that xylooligosaccharide could effectively restore the cognitive function of obese-insulin resistant rats by reducing the activation of microglia through the gut-brain axis (Chunchai et al., 2018). Lactulose was also found to contribute to repairing intestinal barrier injuries, improving gut microbiota imbalances, and reducing post-stroke inflammatory responses in the stroke mice (Yuan et al., 2021). Findings of these studies demonstrated that prebiotics may play an important role in the improvement of neurological functions. Moreover, prebiotics could improve immune function and regulate bowel defecation habits (Jeurink et al., 2013; Meksawan et al., 2016). We speculate that this may be associated with amelioration of the inflammation and gastrointestinal dysfunction in PD. A clinical trial found that a fermented milk containing prebiotic fiber was superior to placebo in improving constipation in PD patients (Barichella et al., 2016). In PD striatum rotenone model mice, prebiotic fibers were found to partially alleviate the motor and non-motor problems caused by rotenone (Perez-Pardo et al., 2017). Additionally, it restored the level of striatal dopamine transporter in PD mice, indicating its neurorestorative properties (Perez-Pardo et al., 2017). In summary, the prebiotics-related mechanisms might lie in improving the composition of the gut microbiota, improving intestinal barrier function, and showing potential beneficial effects on the regulation of the microbiota-gut-brain axis.

3.2 Probiotics

Probiotics are microorganisms that can improve the balance of gut microflora thereby exhibit beneficial effects in the host. The majority of available studies reported that administration of probiotic achieved neuroprotective benefits and alleviation of cognitive impairments (Ji and Shen, 2021). Probiotics can exert beneficial effects via decline of neurotransmitter levels, chronic inflammation, oxidative stress, and apoptosis in NDDs (Westfall et al., 2017). An early study found that regular intake of probiotics significantly improved stool consistency and defecation habits in patients with PD (Cassani et al., 2011). A clinical trial found that a mixture of probiotics (*Lactobacillus acidophilus, Bifidobacterium bifidum, Lactobacillus reuteri, and Lactobacillus fermentum*) ameliorated the clinical symptoms and reduced inflammatory and oxidative responses in PD patients (Tamtaji et al., 2019). *In vitro*

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TABLE 3 Documented gut microbiota-related PD treatments.

Therapeutic method	Names	Research subjects	Changes in intestinal microflora, metabolites, or related factors		Principal results
			Increased abundance	Decreased abundance	
Prebiotics	GOS (Savignac et al., 2013)	Adult male Sprague Dawley rats	Bifidobacteria	Not listed	Elevated central BDNF, NMDAR and D-serine
	BGOS (Williams et al., 2016)	Neonatal rats	Not listed		Increased the levels of synaptophysin, GluN2A-subunits and BDNF proteins
	Xyloolidosaccharide (Chunchai et al., 2018)	High-fat-diet mice	Decreased plasma insulin level, HOMA index, area under the curve of the oral glucose tolerance test, plasma total cholesterol level, and LDL cholesterol level		Restored the cognitive function
	Lactulose (Yuan et al., 2021)	Mice after stroke	Bradyrhizobium, Oceanobacillus, Escherichia, and Leptothrix	Lactobacillus, Clostridium, Flavobacterium, Brachybacterium, and Helicobacter	Repaired intestinal barrier injuries, improved gut microbiota imbalances, and reduced post-stroke inflammatory responses
	Fermented milk containing prebiotic fiber (Barichella et al., 2016)	Patients with PD with Rome III–confirmed constipation	Resulted in a higher increase in the number of total complete bowel movements and in stool consistency, as well as in a larger reduction in the use of laxatives.		Improved constipation in patients with PD
	Prebiotic fibers (Perez-Pardo et al., 2017)	The rotenone PD model	Improved rotenone-induced delayed intestinal transit and reduced rotenone-induced alpha-synuclein and GFAP overexpression in the colon.		Normalized rotenone-induced motor and non-motor abnormalities
Probiotics	Milk drink containing 6.5×109 CFU of Lactobacilus casei Shirota (Cassani et al., 2011)	Patients with PD	Increased in the number of days per week in which stools were of normal consistency and significant reductions in the number of days per week in which patients felt bloated, experienced abdominal pain and sensation of incomplete emptying		Improved stool consistency and defecation habits
	A mixture of probiotics (Tamtaji et al., 2019)	Patients with PD	Decreased movement disorders society-unified parkinson's disease rating scale, reduced high-sensitivity C-reactive protein and malondialdehyde, and enhanced glutathione levels.		Improved the clinical symptoms and reduced inflammation and oxidative response
	Actobacillus and bifidobacterium (Magistrelli et al., 2019)	PBMCs isolated from PD patients	Reduced pro-inflammatory and increased the anti-inflammatory cytokines		Decreased pro-inflammatory cytokines, oxidative stress
	The probiotics consisted of six bacterial strains (Hsieh et al., 2020)	A genetic MitoPark PD mouse model	Reduced the motor impairments in gait pattern, balance function, and motor coordination.		Reduced the deterioration of motor dysfunction
	Bifidobacterium longum BB536 and Lactobacillus rhamnosus HN001(Ilie et al., 2021)	A rotenone-induced PD zebrafish model	An increase of distance swam in the first days of the treatment with a highest value		No statistical significance
	A mixture of probiotics (Alipour Nosrani et al., 2021)	6-hydroxydopamine induced PD rat models	Improved rotation behavior, cognitive function, lipid peroxidation, and neuronal damage		Probiotics had neuroprotective effects
	A mixture of probiotics (Srivastav et al., 2019)	PD models induced by MPTP or rotenone	Downregulated expression of monoamine oxidase B in the striatum		Ameliorated neurodegeneration
	SLAB51 (Castelli et al., 2020)	6-hydroxydopamine induced PD rat models	Modulated the BNDF pathway, increased neuroprotective protein levels and decreased the neuronal death proteins		Confirmed a neuroprotective effect exerted by the probiotics.
	Bacillus subtilis (Goya et al., 2020)				Protected against α-syn Aggregation in C. elegans

(Continued)

TABLE 3 Continued

Therapeutic method	Names	Research subjects	Changes in intestinal microflora, metabolites, or related factors		Principal results
			Increased abundance	Decreased abundance	
		Caenorhabditis elegans model of synucleinopathy	Inhibited α-syn aggregation and clears preformed aggregates in an established Caenorhabditis elegans model of synucleinopathy		
FMT	Fecal bacterial liquid from healthy donors (Kuai et al., 2021)	Patients with PD	*Blautia* and *Prevotella*	*Bacteroidetes*	The intestinal bacterial overgrowth in PD patients returned to normal
	Fecal bacterial liquid from healthy donors (Huang et al., 2019a)	A PD patient	*Lachnoclostridium,* Dialister, Alistipes, and Unidentified-Ruminococcaceae	*Blautia, Coprococcus,* and *Roseburia*	The constipation and tremors were effectively relieved
	Fecal bacterial liquid from healthy donors (Xue et al., 2020)	Patients with PD	Relieved the motor and non-motor symptoms with acceptable safety in PD		Compared with nasointestinal FMT, colonic FMT may be more effective
	Fecal bacteria from healthy mice (Sun et al., 2018)	MPTP-induced PD mice	Firmicutes	Proteobacteria	Protected PD mice by suppressing neuroinflammation and reducing TLR4/TNF-α signaling.

experiments found that probiotics (*Lactobacillus and Bifidobacterium*) inhibited the production of inflammatory cytokines and ROS in PD patients (Magistrelli et al., 2019). In line with the clinical studies, it was documented that long-term administration of probiotics achieved protection of dopamine neurons and amelioration of the motor dysfunction in PD mice (Hsieh et al., 2020). In the rat model of PD induced by 6-hydroxydopamine, administration of probiotics could improve the rotational behavior, cognitive function, lipid peroxidation, and neuronal damage (Alipour Nosrani et al., 2021). In another experiment, it was found that a mixture of probiotics could protect dopaminergic neurons against neurotoxic effects induced by MPTP or rotenone exposure (Srivastav et al., 2019). *In vivo* and *in vitro* experiments demonstrated that SLAB51, a novel probiotic agent, could regulate the BDNF pathway, increase the level of neuroprotective proteins, and reduce neuronal death proteins. Thus, the neuroprotective effects of probiotics were verified (Castelli et al., 2020). In addition, probiotics (*Bifidobacterium longum BB536* and *Lactobacillus rhamnosus HN001*) were found to have tendency to improve the swimming performance and reduce the oxidative stress in a rotenone-induced PD zebrafish model (Ilie et al., 2021). Interestingly, the probiotic *Bacillus subtilis* was reportedly to prevent α-syn aggregation in *Caenorhabditis* elegans models (Goya et al., 2020). Accordingly, probiotics can be considered as an emerging treatment for PD subjects.

3.3 FMT

FMT refers to transfer of functional bacteria from feces of healthy individuals to the intestinal tracts of patients in a specialized manner to regulate the gut microbiota. The objective of FMT is to restore the healthy diversity of gut microbiota and provide successful therapy for certain diseases both within and outside the intestinal tract. Recently, FMT has also been considered as a potential therapy for the treatment of NDDs, particularly for PD (Sun et al., 2021b; Wang et al., 2021a). Kuai et al. found that the abundance of *Blautia* and *Prevotella* increased after undergoing FMT in PD patients, whereas the abundance of *Bacteroides* decreased sharply, and the PAC-QOL scores and Wexner constipation scores significantly decreased (Kuai et al., 2021). Huang et al. found that constipation and tremors were effectively relieved after undergoing the FMT treatments (Huang et al., 2019a). Additionally, Xue et al. found that FMT alleviated the motor and non-motor symptoms in PD patients, and colonic FMT was more preferable than nasointestinal FMT (Xue et al., 2020). Oral administration of specific microbial metabolites to GF mice promoted neuroinflammation and motor symptoms, meanwhile, colonization of α-syn-overexpressing mice with feces from PD patients deteriorated the physical impairments in animals (Sampson et al., 2016). In MPTP-induced PD mice, FMT was found to achieve reducing dysbiosis of gut microbial, decreasing fecal SCFAs, alleviating physical impairments, and increasing striatal DA and 5-HT levels (Sun et al., 2018). These results suggest that FMT the feces of healthy subjects could change the microecology of the intestine, thereby exhibit therapeutic effects in PD subjects.

4 Concluding remarks

The refractory nature of PD treatments, particularly for advanced PD so far, requires better exploration of the novel strategies other than dopaminergic-based treatments. Undoubtedly the gut microbiota-related pathogenesis has been highlighted and become a beacon for the drug development. Although advances in the technologies like high-throughput sequencing, GF animals and GMT make deep investigation of gut

microbiota become feasible, the associations between gut microbiota and PD are still intricate due to the complex nature of interactions among gut microbiota as well as the relative influence factors. The present review summarized the potential the gut microbiota-related mechanisms from the perspectives of humoral pathway, cellular immune pathway and neuronal pathway. Numerous studies have elucidated the potential mechanisms from these different angles. It seems a plausible hypothesis that α-syn might initiate in the gut induced by the dysbiosis of gut microbiota, and then transport into CNS with a "prion-like" manner via several pathways (such as the vagus nerve pathway) (Claudino Dos Santos et al., 2023). However, we still believe the association between gut microbiota and PD is far more complex than "bidirectional", it must be "multidirectional", which require further investigation. So far, there is still a long way before the gut microbiota-related strategy against PD can be actually application in the clinical setting. Future investigation should focus on the following issues: *i)* Clarification of delivery mechanisms of the gut-original α-syn into brain. How to intervene such delivery processes (prevention of PD)? *ii)* Other than the α-syn-related mechanisms, are there any other PD related pathogenic factors involved in the dysbiosis of gut microbiota (such as KP-related liposoluble small-molecule neurotoxins)? *iii)* Development of gut microbiota-based PD biomarkers with low-cost, low-invasion and high specificity is indispensable. *iv)* Establishment of GF animal models in large animals, such as non-human primates, is needed. *v)* Implementation of well-designed clinical trials of GMT, prebiotics and probiotics. More investigations with comprehensive and rigorous experimental design are therefore highly anticipated.

Author contributions

XJ: Conceptualization, Formal analysis, Software, Writing – original draft, Writing – review & editing. QC: Funding acquisition, Writing – original draft. YZ: Funding acquisition, Writing – original draft. TA: Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

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Conflict of interest

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

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Comparing sputum microbiota characteristics between severe and critically ill influenza patients

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Background: Currently, limited attention has been directed toward utilizing clinical cohorts as a starting point to elucidate alterations in the lower respiratory tract (LRT) microbiota following influenza A virus (IAV) infection.

Objectives: Our objective was to undertake a comparative analysis of the diversity and composition of sputum microbiota in individuals afflicted by severe and critically ill influenza patients.

Methods: Sputum specimens were procured from patients diagnosed with IAV infection for the purpose of profiling the microbiota using 16S-rDNA sequencing. To ascertain taxonomic differences between the severe and critically ill influenza cohorts, we leveraged Linear Discriminant Analysis Effect Size (LEfSe). Additionally, Spearman correlation analysis was employed to illuminate associations between sputum microbiota and influenza Ct values alongside laboratory indicators.

Results: Our study encompassed a total cohort of 64 patients, comprising 48 within the severe group and 16 within the critically ill group. Intriguingly, *Bacteroidetes* exhibited significant depletion in the critically ill cohort (p=0.031). The sputum microbiomes of the severe influenza group were hallmarked by an overrepresentation of *Neisseria, Porphyromonas, Actinobacillus, Alloprevotella, TM7x*, and *Clostridia_UCG-014*, yielding ROC-plot AUC values of 0.71, 0.68, 0.60, 0.70, 0.70, and 0.68, respectively. Notably, *Alloprevotella* exhibited an inverse correlation with influenza Ct values. Moreover, C-reactive protein (CRP) manifested a positive correlation with *Haemophilus* and *Porphyromonas*.

Conclusion: The outcomes of this investigation lay the groundwork for future studies delving into the connection between the LRT microbiome and respiratory disorders. Further exploration is warranted to elucidate the intricate mechanisms underlying the interaction between IAV and *Alloprevotella*, particularly in disease progression.

KEYWORDS

influenza virus, severe and critically ill, 16s-rDNA sequencing, bacteria, case control

1 Introduction

Influenza virus infection remains the primary cause of elevated incidence and mortality in respiratory infectious diseases globally (Nair et al., 2010; Iuliano et al., 2018; Li et al., 2019). Effective mitigation strategies, encompassing vaccines and antiviral drugs, play a pivotal role in alleviating the associated health and economic burdens (Cowling and Zhong, 2023; Kumari et al., 2023). However, challenges including vaccine-virus mismatches, suboptimal vaccine coverage, and the intricate interplay of influenza viruses with these countermeasures necessitate urgent exploration of innovative problem-solving approaches (Belongia et al., 2016; Nachbagauer and Palese, 2020; Morens et al., 2023).

In the past decade, modern technology has facilitated comprehensive investigation of host-associated microbiota (Cullen et al., 2020), revealing the pivotal role of microbiota composition in maintaining the equilibrium of healthy individuals (Dominguez-Bello et al., 2019). Conversely, dysregulation of microbial composition can exacerbate pathological conditions, attracting increased attention from research teams worldwide. In comparison with intestinal microbiota, our understanding of the post-influenza virus infection alterations in the respiratory tract microbial community is relatively limited. Nonetheless, the respiratory tract microbiota undeniably plays a pivotal role in shaping the host's immune response and is crucial for effective eradication of invasive viruses.

Several studies have reported discernible differences in respiratory tract microbiota between healthy individuals and patients afflicted by respiratory virus infections (Korten et al., 2016; Kaul et al., 2020). The respiratory tract microbiota not only correlates with influenza symptoms and viral shedding, but also serves as a reliable predictor of influenza susceptibility (Lee et al., 2019a; Lee et al., 2019b; Tsang et al., 2020). Furthermore, investigations have linked the microbial composition in the respiratory tract to outcomes in influenza patients (Jia et al., 2017; Tsang et al., 2020) as well as other respiratory diseases (Haran et al., 2021; Rattanaburi et al., 2022). Simultaneously, research has demonstrated significant shifts in the microbial composition and diversity of the respiratory tract following influenza virus infection (Hanada et al., 2018; Wen et al., 2018; Zhou et al., 2020; Rattanaburi et al., 2022; Hernández-Terán et al., 2023). However, a majority of these investigations have primarily centered on the microbiota of the upper respiratory tract (URT), limiting insights from the lower respiratory tract (LRT) that could potentially enrich our comprehension of the role of microbiota in influenza-associated disease (Hernández-Terán et al., 2023).

In light of these considerations, this study endeavors to employ 16S rRNA gene sequencing to meticulously compare the diversity and configuration of LRT microbiota in severe and critically ill influenza patients. Additionally, we seek to scrutinize the correlation between respiratory tract microbiota and the Ct value, alongside laboratory examinations, thereby attaining a deeper insight into the intricate interplay of respiratory tract microbiota in influenza patients.

2 Materials and methods

2.1 Study subjects

A total of 64 individuals with confirmed influenza were enrolled from December 2018 to January 2020 at Beijing Ditan Hospital, Capital Medical University. Among them, 48 individuals exhibited severe influenza symptoms, while 16 were classified as critically ill influenza patients. Severe in'

M-luenza cases met one or more of the following criteria: (1) persistent fever exceeding 3 days, coupled with intense cough, purulent or hemoptysis sputum, or chest pain; (2) rapid and labored respiration, dyspnea, and cyanosis of the lips; (3) altered cognitive function, including delayed responsiveness, drowsiness, restlessness, or seizures; (4) severe emesis, diarrhea, and signs of dehydration; (5) concurrent pneumonia; (6) considerable aggravation of pre-existing chronic ailments; (7) other clinical conditions necessitating hospitalization. Critically ill influenza patients met one or more of the following conditions: (1) progressive respiratory insufficiency requiring mechanical ventilation; (2) shock; (3) acute necrotizing encephalopathy; (4) multi-organ dysfunction; (5) other grave clinical situations mandating close monitoring and treatment. Exclusion criteria encompassed individuals below 18 years of age and pregnant women.

2.2 Sputum samples collection and DNA extraction

Prior to sample collection, participants were instructed to rinse their mouths, and dentures or braces were to be removed. Sputum was collected following a deep cough. Samples were deemed unacceptable if saliva and food residues accounted for over two-thirds of the sputum volume or if saliva and oral contaminants constituted more than half of the sputum volume. Each sputum sample, obtained from participants upon admission or in the early hours of the second day of admission, amounted to 2 ml. Samples were preserved in sterile containers at -80°C until DNA extraction, which was performed using the MagaBio Pathogens DNA/RNA Purification Kit (BSC75S1E) following the manufacturer's guidelines. The concentration, purity, integrity, and size of DNA were determined through NanoDrop (Thermo Fisher Scientific) and 1.0% agarose gel electrophoresis. Subsequently, the DNA samples were frozen at -20°C for subsequent analyses.

2.3 Identification of influenza A/B and 16S ribosomal RNA Gene sequencing

Influenza A virus (IAV) identification was executed for all sputum samples using the Influenza A/B virus nucleic acid detection kit (PCR fluorescent probe method, Applied Biological Technologies, Beijing, China). The bacterial 16S rRNA gene sequences encompassing the

variable regions V3–V4 were amplified employing the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHV GGGTATCTAATCC-3'), alongside the Q5 High-Fidelity 2X Master Mix (New England BioLabs Inc., Ipswich, MA). The resulting products underwent purification using 0.9× volumes of AMPure XP beads (Beckman Coulter, Inc., Brea, CA). Each sample was quantified using the Qubit 2.0 Fluorometer (ThermoFisher Scientific, Inc., Waltham, MA), pooled with equal input mass, and subjected to further purification using 0.9× volumes of AMPure XP beads (Beckman Coulter, Inc., Brea, CA). The final sequencing pool's concentration was determined by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA) on a Roche 480 LightCycler (Roche, Basel, Switzerland). Subsequent sequencing was conducted by Beijing Novogene Technology Co. employing an Illumina PE 250 on an Illumina Noveseq Sequencer (Illumina, Inc., San Diego, CA).

2.4 Bioinformatics and statistical analyses

High-throughput 16S rRNA sequencing raw fastq files were demultiplexed and subjected to quality filtering using QIIME (version 2022.8.0). Dada2 was employed to truncate the linker sequence, merge the paired ends, eliminate chimeras, and generate amplicon variant sequences (ASVs) for noise reduction. Taxonomic analysis of each 16S rRNA gene sequence was performed using QIIME (version 2022.8.0) and compared against the SILVA rRNA database with a confidence threshold of 70%.

Descriptive statistics were utilized to represent continuous variables as mean (standard deviation [SD]) and categorical variables as frequency (percentage). A comparison of patient characteristics between the severe and critically ill influenza groups was achieved using the t-test or the Wilcoxon rank-sum test (continuous variables), and the Chi-square test or Fisher's exact test (categorical variables). Relative abundance was calculated as the proportion of a specific bacterium relative to the total bacterial count in a given sample. Statistical analyses were carried out using R 4.3.1. A p-value less than 0.05 denoted statistical significance. The Wilcoxon rank-sum test was employed to assess differences in alpha diversity indices between the two groups. Beta diversity was evaluated via principal coordinate analysis (PCoA) to ascertain sample group similarities. Nonparametric multivariate analysis of variance (Adonis) was utilized to test inter-group differences. Linear discriminant analysis effect size (LEfSe) was conducted to identify bacteria accounting for distinctions between the two sample groups, using a logarithmic LDA score threshold of 2.0.

3 Results

3.1 General clinical features of the patients

The study encompassed 48 individuals diagnosed with severe influenza and 16 individuals categorized as critically ill influenza patients in a cross-sectional analysis. None had received influenza vaccination. Essential demographic attributes such as gender, age, smoking habits, and alcohol consumption were effectively matched between the two groups. No discernible differences emerged in underlying conditions (hypertension, diabetes, hyperlipidemia, cerebral vascular disease, heart disease, and chronic pulmonary disease) between the two groups (p=1.000, 0.106, 1.000, 0.427, 0.282, 0.521, respectively). Only one of the 64 patients was first diagnosed in our hospital, and most of the patients had antiviral treatment history before the treatment in our hospital, but there was no significant difference in the history of antiviral treatment and the days from onset to admission between the two groups (p=1.000, 0.764, respectively) (Table 1).

Each sputum and blood sample obtained from participants upon admission or in the early hours of the second day of admission. There were no statistical differences about the duration between the onset of illness and sample collection (p=0.588). Within the critically ill group, elevated levels of white blood cells (WBC), neutrophil percentage (NE%), and neutrophil count (NE#) were observed in comparison to the severe group (p=0.022, 0.005, 0.017, respectively). Conversely, lymphocyte percentage (LY%), lymphocyte count (LY#), monocyte percentage (MO%), basophil percentage (BA%), and basophil count (BA#) exhibited lower values in the critically ill group as opposed to the severe group (p=0.007, 0.045, 0.009, 0.004, 0.016, respectively) (Supplementary Figure 2). Although no statistically significant disparities emerged in C-reactive protein (CRP) and Ct values between the two groups (p=0.251, 0.104, respectively), the critically ill group exhibited heightened CRP levels compared to the severe group, while the Ct value in the critically ill group was lower than that in the severe group. In addition, in this diagnosis and treatment, there was no statistically significant difference in the length of hospital stay between the two groups (p=0.100), with two patients in the critically ill group dying on days 6 and 19 after admission (Table 1).

We conducted a careful review of each patient's medical record system, collating all respiratory pathogens tested during hospitalization except influenza virus. This included: (1) Sputum bacterial, fungal and *Haemophilus* cultures: *Pseudomonas aeruginosa* and *Haemophilus influenzae* were detected in samples from two critically ill patients respectively, and *Klebsiella pneumoniae subsp. Pneumoniae* was detected in the sample from a severe patient. (2) Sputum acid-fast staining: All the samples were negative or did not undergo this examination. (3) *Mycoplasma pneumoniae* antibody (gelatin particle agglutination assay) and *Mycoplasma pneumoniae* nucleic acid test: A total of 37 patient samples were tested for the former (12 showed positive antibodies), but the nucleic acid tests were negative, which may mean that the patients had been infected with *Mycoplasma pneumoniae* but are not currently infected. (4) Blood culture: All samples were negative or not performed.

3.2 Analysis of sputum microbial diversity in the severe and critically ill influenza groups

A total of 10,083,115 filtered high-quality partial reads were generated, averaging 157,548 reads per sample. Rarefaction curves

TABLE 1 General clinical characteristics of influenza patients.

Characteristics	Severe (n=48)	Critically ill (n=16)	statistic	*p*-value
Age (years)	65.38 ± 2.43	70.38 ± 2.69	t=-1.379	0.175
Gender (male/female)	24/24	9/7	χ^2 = 0.188	0.665[&]
Smoking, N (%)	8 (16.67)	2 (12.50)	χ^2 = 0.000	1.000[#]
Drinking alcohol, N (%)	8 (16.67)	1 (6.25)	χ^2 = 0.388	0.533[#]
Hypertension	21 (43.75)	7 (43.75)	χ^2 = 0.000	1.000[&]
Diabetes	7 (14.58)	6 (37.50)	χ^2 = 2.606	0.106[#]
Hyperlipidemia	7 (14.58)	2 (12.50)	χ^2 = 0.000	1.000[#]
CVD	6 (12.50)	4 (25.00)	χ^2 = 0.632	0.427[#]
Heart disease	14 (29.20)	7 (43.80)	χ^2 = 1.158	0.282[&]
Chronic pulmonary disease	12 (25.00)	6 (37.50)	χ^2 = 0.412	0.521[#]
External hospital visits before this visit	47 (97.9)	16 (100)	χ^2 = 0.000	1.000[&]
Antiviral treatment in external hospitals	41 (85.4)	14 (87.5)	χ^2 = 0.000	1.000[&]
WBC (4-10*10^9/L)	6.35 (4.10, 8.86)	8.88 (6.74, 10.81)	Z=-2.295	0.022
NE% (50-70%)	73.94 (64.27, 81.21)	86.36 (76.31, 89.74)	Z=-2.837	0.005
NE# (2-8*10^9/L)	4.74 (2.38, 7.08)	7.32 (5.42, 8.45)	Z=-2.388	0.017
LY% (20-40%)	18.22 (11.59, 24.17)	9.67 (6.64, 13.33)	Z=-2.706	0.007
LY# (1-5*10^9/L)	1.14 (0.73, 1.52)	0.78 (0.56, 1.05)	Z=-2.000	0.045
MO% (3-8%)	7.50 (5.20, 11.18)	4.12 (2.07, 8.37)	Z=-2.628	0.009
BA% (0-1%)	0.20 (0.05, 0.32)	0.00 (0.00, 0.10)	Z=-2.854	0.004
BA# (0-0.1*10^9/L)	0.01 (0.00, 0.02)	0.00 (0.00, 0.01)	Z=-2.407	0.016
CRP (0-6*10^9/L)	54.35 (31.22, 149.85)	117.65 (41.97, 216.60)	Z=-1.147	0.251
Ct value[$]	23.80 (20.40, 29.63)	21.64 (17.98, 25.92)	Z=-1.628	0.104
Hospitalization days of this visit	7.00 (5.00, 9.75)	8.00 (6.00, 10.75)	Z=-1.647	0.100
Days from onset to admission	3.00 (2.00, 5.00)	3.00 (2.00, 5.75)	Z=-0.300	0.764
Days from onset to sample collection	4.00 (2.00, 5.75)	3.00 (2.25, 5.75)	Z=-0.542	0.588

[#]: Continuity correction χ^2 test.
[&]: Pearson's chi-squared test.
[$]: Ct value less than or equal to 36 is considered positive.
CVD, Cerebral Vascular Disease; WBC, White Blood Cell; NE%, Neutrophil percentage; NE#, Neutrophil count; LY%, Lymphocyte percentage; LY#, Lymphocyte count; MO%, Monocytes percentage; BA%, Basophil percentage; BA#, Basophil count; CRP, C-reactive protein.

depicting sequence numbers per sample demonstrated that the mean number of sequences attained a plateau around ~5000 sequence reads (Supplementary Figure 1). This observation indicated comprehensive taxonomy detection within each group, and that 5000 reads sufficed to identify the majority of bacterial community members within the sputum microbiota. Evaluation of alpha diversity (Simpson and Shannon indices) and richness (Chao1, ACE) indicated comparable levels of diversity in the sputum microbiota of both groups (Figure 1A). PCoA of Bray-Curtis matrices revealed no significant differentiation between the two groups, underscoring the similarity in beta diversity (PERMANOVA, pseudo-F: 0.917, p=0.549, Figure 1B).

3.3 Bacterial taxonomic differences between severe and critically ill influenza groups

Upon reaching sequence saturation, relative abundance conversion was executed, retaining phylum and genus-level taxonomies with relative abundance surpassing 1% in any given sample. This process yielded 11 qualified phylum-level and 72 genus-level taxonomies. The top five predominant phyla in the severe group were *Firmicutes* (41.5% of total reads), *Actinobacteriota* (20.8%), *Proteobacteria* (14.0%), *Bacteroidota* (15.2%), and *Fusobacteriota* (4.1%) (Figure 2A, left). The critically

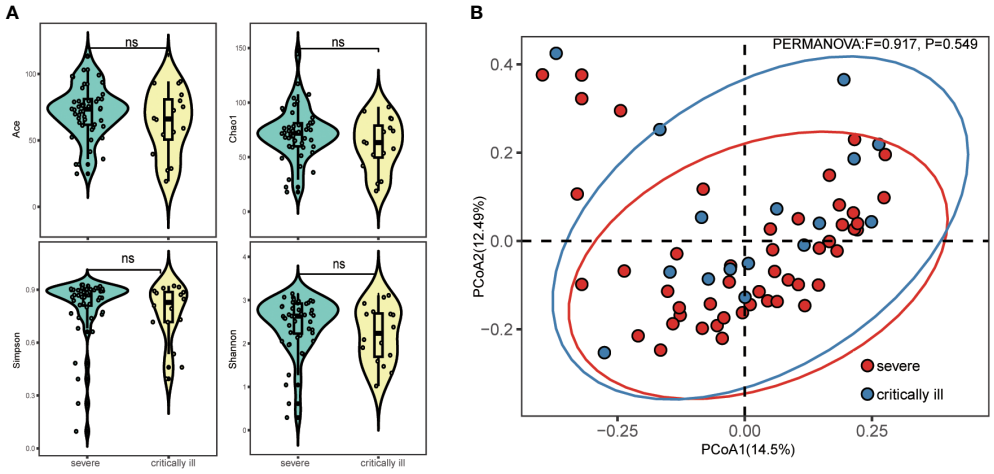

FIGURE 1
Alpha and beta diversity of sputum microbiota in the severe and critically ill influenza patients. **(A)** Alpha diversity (Simpson's index of diversity, Shannon index, Chao1 and ACE) of sputum microbiota in the severe and critically ill groups. **(B)** Beta diversity (Principal coordinates analysis, PCoA) of sputum microbiota in the severe and critically ill groups. No significant difference of bacterial communities between two groups.

ill group exhibited *Firmicutes* (41.8% of total reads), *Actinobacteriota* (20.7%), *Bacteroidota* (10.2%), *Proteobacteria* (20.1%), and *Fusobacteriota* (3.6%) (Figure 2A, left). The remaining phyla constituted relatively lower abundances (less than 1.5%). A phylum-level analysis revealed a statistically significant decrease in *Bacteroidota* ($mean_{critically\ ill}$=0.102, $mean_{severe}$=0.152, p=0.031) within the critically ill group, whereas *Proteobacteria* ($mean_{critically\ ill}$=0.201, $mean_{severe}$=0.140) demonstrated an increase, though without statistical significance (Figure 2A, right). At the genus level, we presented the distribution of the top 10 relative abundance bacteria in the severe and critically ill groups, and found that *Streptococcus* ($mean_{critically\ ill}$=0.281, $mean_{severe}$=0.253) and *Haemophilus* ($mean_{critically\ ill}$=0.090, $mean_{severe}$=0.034) displayed augmentation within the critically ill group, while *Neisseria* ($mean_{critically\ ill}$=0.037, $mean_{severe}$=0.071), *Veillonella* ($mean_{critically\ ill}$=0.062, $mean_{severe}$=0.124), and *Porphyromonas* ($mean_{critical\ illness}$=0.024, $mean_{severe}$=0.053) exhibited reduction (Figure 2B left, Supplementary Figure 3B). Further to screen for differential bacteria, a comprehensive Wilcoxon test was performed on all genera, elucidating significant differences in *Neisseria* and *Porphyromonas* (p=0.012 and 0.037) (Figure 2B, right). Furthermore, within the critically ill group, a decrease was observed in *Alloprevotella*, *Capnocytophaga*, *Clostridia_UCG-014*, and *TM7x* (p=0.048, 0.049, 0.043, 0.020, respectively) (Figure 2B, right).

To ascertain pivotal phylotypes contributing to the differentiation of sputum microbiota between the two groups, LEfSe analysis was executed, setting a threshold of 2. The sputum microbiomes of the severe group were characterized by an abundance of *Neisseria*, *Porphyromonas*, *Actinobacillus*, *Alloprevotella*, *TM7x*, and *Clostridia_UCG-014*, exhibiting ROC-plot AUC values of 0.71, 0.68, 0.60, 0.70, 0.70, and 0.68 respectively (Figures 3A, B).

3.4 Correlation between sputum microbiota and influenza Ct values

16S rRNA gene analysis of sputum samples indicated a prevalence of *Streptococcus* and *Haemophilus* in the critically ill group, and *Neisseria*, *Porphyromonas*, *Actinobacillus*, *Alloprevotella*, *TM7x*, *Clostridia_UCG-014*, *Capnocytophaga*, and *Veillonella* in the severe group. Spearman correlation analysis was performed to investigate the relationship between these genera, Ct value, and laboratory examinations (WBC, NE%, NE#, LY%, LY#, MO%, BA %, BA#, CRP). The outcomes revealed a negative correlation between *Alloprevotella* and Ct value. Additionally, *Haemophilus* and *Porphyromonas* exhibited positive correlations with CRP (Figure 4; Supplementary Figure 3A; Supplementary Table 1). Meanwhile, *Alloprevotella*, *Capnocytophaga* and *Porphyromonas* were negatively correlated with hospitalization days. (Supplementary Figure 4; Supplementary Table 2).

4 Discussion

The respiratory tract, with its distinct microbial communities in various segments, may respond to IAV infection in both similar or distinct ways. While studies often employ mouse models to depict short- or long-term microecological imbalances post IAV infection (Yildiz et al., 2018), clinical cohorts remain underexplored as starting points to decipher post-influenza respiratory tract microbiota changes. Investigations into bacterial microbiota within the URT of influenza patients, spanning oropharynx, nasopharynx, and other segments, have been conducted (Chen et al., 2023). However, limited inquiry has been dedicated to microbial shifts in the LRT following influenza infection. Hence, by employing 16S rRNA gene sequencing, we undertook a comparative analysis of LRT microbiota (sputum)

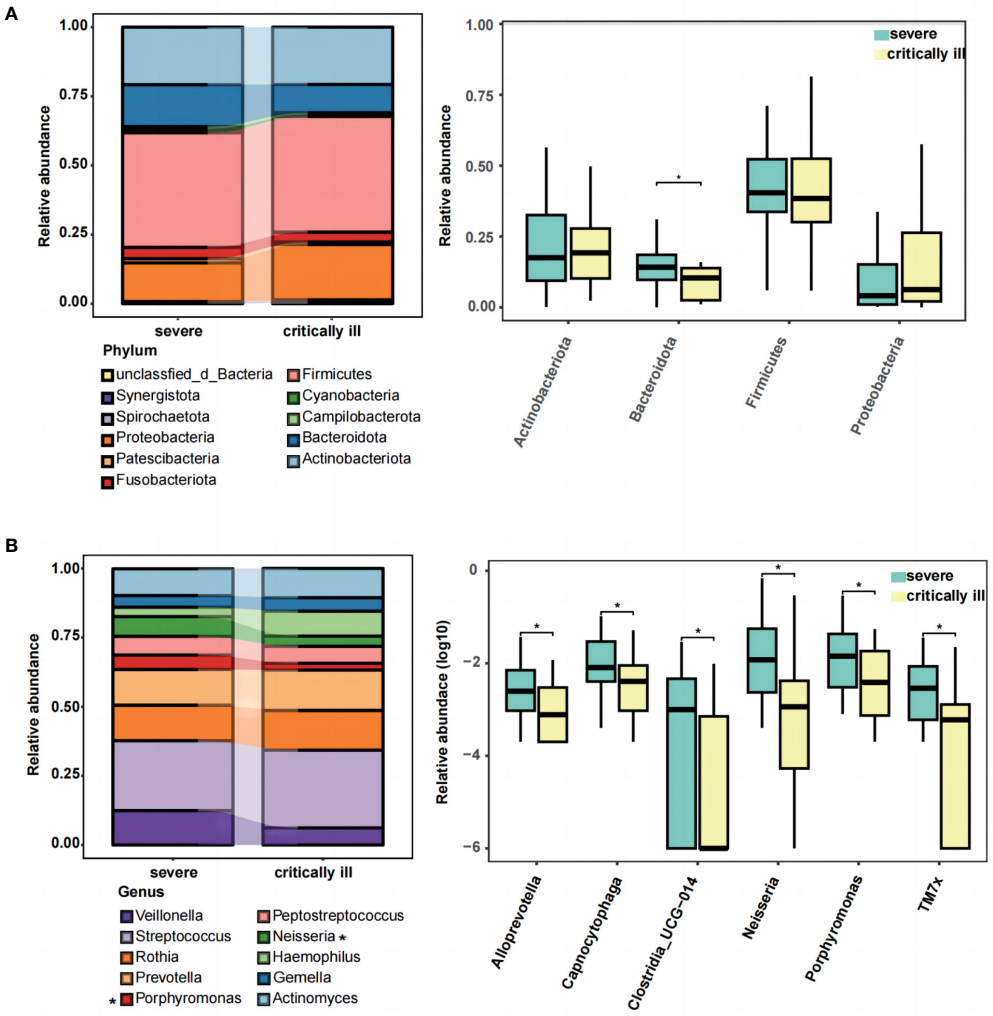

FIGURE 2
The composition and difference of sputum microbial taxa between the severe and critically ill influenza patients at the phylum and genus levels. **(A)** The top ten phylum of bacteria in relative abundance (left), and the Wilcoxon Rank Sum test was performed to detect taxa with significant differences in relative abundances at the phylum levels between the two groups (right). **(B)** The top ten genus of bacteria in relative abundance (left), and the Wilcoxon Rank Sum test was performed to detect taxa with significant differences in relative abundances at the genus levels between the two groups (right). Significant differences are indicated by *p<0.05.

between severe and critically ill patients. Additionally, we assessed the relationship between microbiota and influenza virus Ct values and laboratory examinations.

No marked disparity in alpha and beta diversity was observed in sputum microbiota between severe and critically ill patients in this study. In animal models, IAV-infected mice showed minimal alterations in microbial diversity and richness within the upper and lower respiratory tracts and even different respiratory segments (Planet et al., 2016; Yildiz et al., 2018; Chen et al., 2023). Clinical research, however, has yielded diverse results. For instance, Shannon diversity was significantly lower in influenza A and B groups in comparison to non-influenza groups (nasopharyngeal swab samples) (Rattanaburi et al., 2022), but higher in H7N9 patients relative to healthy controls (oropharyngeal swab samples) (Lu et al., 2017). Among severe pneumonia patients who were influenza virus positive and negative, no statistical difference was observed in any of the Chao1 or Shannon and Simpson indexes (bronchoalveolar lavage fluid specimens) (Zhou et al., 2023). In our study, sputum samples were used as most severe influenza patients can cough and expectorate, thus making it feasible to collect sputum samples in a non-invasive manner. Although influenza A was identified in the samples, influenza subtypes were not distinguished. Hence, these disparities could be attributed to variant viral strains and different respiratory tract sampling sites and methods.

At the phylum level, researcher has previously observed that the URT microbiota of H1N1 influenza patients were mainly composed of *Actinobacteria*, *Firmicutes*, and *Proteobacteria* (Chaban et al., 2013). Our study found that the same applies to LRT microbiota. Notably, *Bacteroidetes* was dominant in the severe group. During IAV infection's acute and recovery stages, *Gammaproteobacteria*, *Firmicutes*, and *Bacteroides* class relative abundance escalates within the LRT (Gu et al., 2019). In line with these findings, our results indicated a prevalence of *Streptococcus* and *Haemophilus* in the critically ill group (although there was no significant difference compared with the severe group), corresponding to *Firmicutes* and

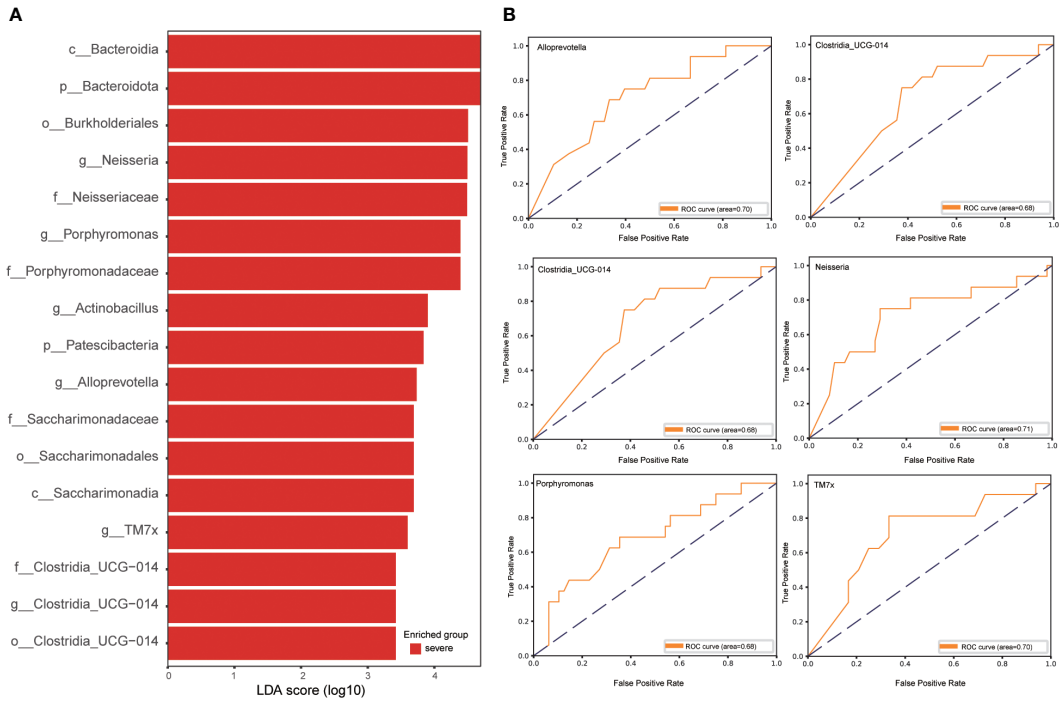

FIGURE 3
Linear discriminative analysis effect size (LEfSe) analysis in the severe and critically ill influenza patients. **(A)** LDA scores indicate significant differences in the microbiota between the severe and critically ill influenza patients. **(B)** Prediction of the key genera in the severe and critically ill influenza patients. Receiver-operating characteristic (ROC) plot for *Neisseria*, *Porphyromonas*, *Actinobacillus*, *Alloprevotella*, *TM7x*, *Clostridia_UCG-014*, area under the parametric curve (AUC) value=0.71, 0.68, 0.60, 0.70, 0.70 and 0.68 respectively.

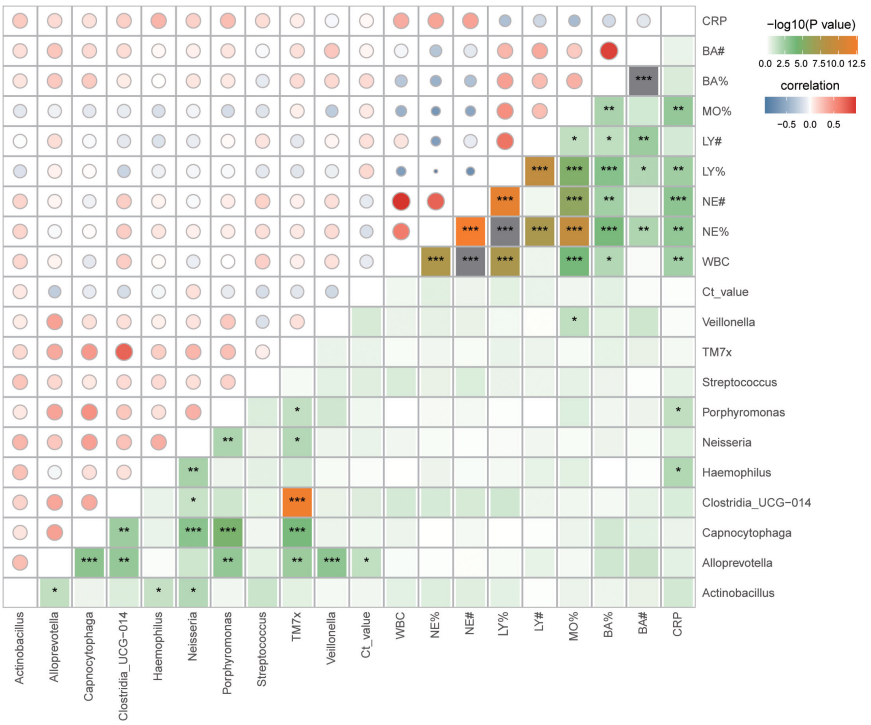

FIGURE 4
Correlation between sputum microbiota and clinical data. Spearman correlation analysis, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. WBC, White Blood Cell; NE%, Neutrophil percentage; NE#, Neutrophil count; LY%, Lymphocyte percentage; LY#, Lymphocyte count; MO%, Monocytes percentage; BA%, Basophil percentage; BA#, Basophil count; CRP, C-reactive protein.

Gammaproteobacteria, respectively. The species of these bacteria are found in the URT of healthy individuals and can potentially cause respiratory infections or influenza virus infections (Ohara-Nemoto et al., 2008; Zhang et al., 2020). Interestingly, significant dissimilarities were evident in specific bacterial taxa, particularly within the severe influenza group. Our research pinpointed variations in *Neisseria*, *Porphyromonas*, *Actinobacillus*, *Alloprevotella*, *TM7x*, *Clostridia_UCG-014*, *Capnocytophaga*, and *Veillonella*. Recently, *Veillonella* has emerged as a noteworthy biomarker for various respiratory viral infections (Li et al., 2023). Dysbiotic microbiota characterized by reduced microbial diversity and elevated abundance of specific bacteria, such as *Streptococcus*, *Pseudomonas*, and *Neisseria*, have been documented in patients with respiratory virus infections (Porto and Moraes, 2021). The link between IAV and *Neisseria* meningitidis disease has been established, and *Neisseria* meningitidis has been found to enhance IAV infection by adhering to human HEC-1-B epithelial cells (Rameix-Welti et al., 2009).

The results of our data showed that there were no statistically significant differences between the two groups in the history of antiviral therapy before the current admission, as well as in the days from the onset of illness to admission, the days from the onset of illness to specimen collection, and the length of hospital stay. However, our Spearman correlation analysis showed that *Alloprevotella* and *Porphyromonas* were negatively correlated with hospitalization days, respectively. In patients with COVID-19, poor clinical outcome was associated with the enrichment of an oral commensal (*Mycoplasma salivarium*) in the lower airways (Sulaiman et al., 2021). These suggest that the characteristics of the microbiota may, to some extent, be indicative of disease progression in patients with respiratory viruses. Our investigation identified elevated WBC, NE%, and NE# in the critically ill group relative to the severe group, coupled with reduced LY % and LY#. Previous research has similarly indicated a higher proportion of neutrophils in critically ill influenza cases compared to non-critically ill cases, with a correspondingly lower lymphocyte proportion (Li et al., 2022). Our findings are in accordance with these observations. Although CRP and Ct values exhibited no statistically significant differences between the groups, the critically ill influenza patients manifested elevated CRP levels and lower Ct values, implying heightened viral loads and more pronounced inflammatory reactions or tissue damage. Remarkably, our Spearman correlation analysis between influenza Ct values and the aforementioned genera exposed a negative correlation with *Alloprevotella*. A prior study revealed associations between nose/throat microbiota and susceptibility to influenza virus infection, particularly in terms of the relative abundances of *Alloprevotella* oligotypes(Lee et al., 2019b). As such, we should pay attention to the changes of *Alloprevotella* in the course of influenza infection and its correlation with the severity of the disease. Our study additionally suggests a potential link between *Alloprevotella* and influenza disease severity. For example, CRP exhibited positive correlations with *Haemophilus* and *Porphyromonas*. In cases of chronic obstructive pulmonary disease exacerbations, higher CRP levels are observed in bacterial infections involving *Haemophilus influenzae* and *Streptococcus pneumoniae* (Gallego et al., 2016). Other investigations have also suggested *Porphyromonas* genus as a potential focus in various pulmonary conditions (Guilloux et al., 2021).

While acknowledging our study's strengths, certain limitations warrant consideration. First, we did not perform testing for other types of respiratory viruses on the sputum samples used in our study. To compensate for this shortcoming to the best of our ability, we meticulously reviewed all tests for respiratory pathogens other than the influenza virus that were conducted during the hospitalization of the patients, and found that the patients were infected with other Gram-negative bacilli, suggesting that influenza virus infection may weaken the immune system and make individuals more susceptible to other bacterial infections. Second, healthy sputum samples are challenging to collect, precluding inclusion of healthy controls. Our study primarily focused on comparing respiratory tract microbiota differences in influenza patients of varying severities. Additionally, our cross-sectional design and limited clinical sample size emphasize the necessity for expanded samples and longitudinal cohort studies. Certain classical limitations of the cross-sectional study design should be acknowledged, such as it not allowing for conclusions of causality. We also employed amplicon-based 16S rRNA gene sequencing, which provides modest taxonomic resolution at the species level. Enhanced classification precision and reproducible metagenomic sequencing are pivotal to validate our approach across multiple longitudinal cohorts and clinical contexts. From the perspective of microbiota, investigating how the results could guide clinical management strategies, such as targeted interventions and personalized treatment approaches should be considered. Manipulating the composition of respiratory tract microbiota in an attempt to impact disease outcomes or treatment responses is also an area of future research. Ultimately, further validation experiments are needed to determine whether these results could be leveraged to delineate future biomarkers that identify patients at risk of progression to critically ill states.

5 Conclusions

The outcomes of this study bestow foundational insights for prospective investigations delving into the intricate interplay between the LRT microbiota and respiratory diseases. Furthermore, additional scrutiny is warranted to unravel the underlying mechanisms governing the interplay between IAV and *Alloprevotella* in disease progression. The innovation encapsulated within this study augments our understanding of the intricate host-microbiota-virus dynamics, accentuating the goal of clinical cohort-driven inquiries into microbiota shifts during viral infections.

Data availability statement

The data presented in the study are deposited in the National Center of Biotechnology Information (NCBI) repository, accession number PRJNA1045822.

Ethics statement

The studies involving human participants were reviewed and approved by Beijing Ditan Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZG: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. YZ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. XZ: Conceptualization, Data curation, Investigation, Project administration, Resources, Writing – review & editing. TL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – review & editing. SS: Conceptualization, Data curation, Investigation, Project administration, Resources, Writing – review & editing. RS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. RJ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1297946/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Rarefaction analysis of bacterial 16S rRNA gene sequences. Pielou and Shannon indexs were used to evaluate whether further sequencing would likely detect additional taxa, indicated by a plateau.

SUPPLEMENTARY FIGURE 2
Comparison of clinical indicators between severe and critically illness influenza patients. **(A)** CRP: C-reactive protein; **(B)** LY%: Lymphocyte percentage; **(C)** LY#: Lymphocyte count; **(D)** MO%: Monocytes percentage; **(E)** NE%: Neutrophil percentage; **(F)** NE#: Neutrophil count; **(G)** WBC: White Blood Cell; **(H)** BA#: Basophil count; **(I)** BA%: Basophil percentage.

SUPPLEMENTARY FIGURE 3
Distribution of *Haemophilus* in severe and critically ill influenza patients **(A)** and its correlation with C-reactive protein (CRP) **(B)**.

SUPPLEMENTARY FIGURE 4
Correlation between sputum microbiota and clinically relevant days.

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Urinary microbiota signatures associated with different types of urinary diversion: a comparative study

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Background: Radical cystectomy and urinary diversion (UD) are gold standards for non-metastatic muscle-invasive bladder cancer. Orthotopic neobladder (or Studer), ileal conduit (or Bricker) and cutaneous ureterostomy (CU) are mainstream UD types. Little is known about urinary microbiological changes after UD.

Methods: In this study, urine samples were collected from healthy volunteers and patients with bladder cancer who had received aforementioned UD procedures. Microbiomes of samples were analyzed using 16S ribosomal RNA gene sequencing, and microbial diversities, distributions and functions were investigated and compared across groups.

Results: Highest urine microbial richness and diversity were observed in healthy controls, followed by Studer patients, especially those without hydronephrosis or residual urine, α-diversity indices of whom were remarkably higher than those of Bricker and CU groups. Studer UD type was the only independent factor favoring urine microbial diversity. The urine microflora structure of the Studer group was most similar to that of the healthy individuals while that of the CU group was least similar. Studer patients and healthy volunteers shared many similar urine microbial functions, while Bricker and CU groups exhibited opposite characteristics.

Conclusion: Our study first presented urinary microbial landscapes of UD patients and demonstrated the microbiological advantage of orthotopic neobladder. Microbiota might be a potential tool for optimization of UD management.

KEYWORDS

bladder cancer, urinary diversion, microbiota, neobladder, Studer

1 Introduction

Bladder cancer is a common urologic malignancy, with estimates of 91,893 new cases and 42,973 deaths in China every year (Siegel et al., 2022). According to the depth of tumor invasion, it can be classified as non-muscle invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC). Considering substantial risks of metastasis and lethality, radical cystectomy (RC) and lymphadenectomy are recommended for the treatment of non-metastatic MIBC, and urinary diversion (UD) inevitably becomes the last but crucial surgical step for such cases. Digestive tracts are usually employed for UD, like stomach, ileum and sigmoid colon, and orthotopic UD (or neobladder, ONB), ileal conduit (IC) and cutaneous ureterostomy (CU) are mainstream UD procedures (Lenis et al., 2020). ONB is believed to be the gold standard for surgical bladder reconstruction following RC though it is performed less often than other UD types (Janssen et al., 2021). Studer, which are the most common orthotopic urinary diversion, provides daytime continence rate up to 92%, respectively. The main advantage of ONB is that on the basis of radical resection of bladder cancer, the normal urinary system anatomy can be approximately simulated. Since ONB procedure basically maintains a normal physiological and anatomical state, postoperative patients do not need an external stoma or carry urine collection bag. They are also likely to accept the adverse psychological stimulation brought by this surgery. Studer is the most common ONB technique which provides a daytime continence rate up to 92% (Sheybaee Moghaddam et al., 2022). As for incontinent UD procedures, Bricker is ranked as the most representative IC technique which has the advantages of shorter operation time and fewer complications compared with ONB. With the advent of robotic surgeries, IC or Briker has seen a proportionally greater increase in utilization as the number of robotic RCs being performed with intracorporeal UD rises (Janssen et al., 2021). In the long run, the advantage of Bricker over incontinent CU is that stents do not need to be replaced regularly, thus reducing the possibilities of infection and renal insufficiency as well as improving patients' quality of life. Nonetheless, CU also shows a wide scope of application owing to relatively limited intraoperative trauma, especially for the elderly and more frail patients (Korkes et al., 2022). Shared decision-making and patient-centered approach should be used when tailoring the UD type.

As for human microorganisms, they are widely distributed in the gut, mouth, skin, bronchus, reproductive tract, organ tissues (Hou et al., 2022). In recent years, with the understanding of microorganisms, it has been found that the contribution of microorganisms to human malignant tumors is as high as 20% (Garrett, 2015).The most prominent examples are Helicobacter pylori, which is linked to stomach cancer, and the high-risk form of human papillomavirus, which is linked to cervical cancer (van Elsland and Neefjes, 2018). The interaction of microorganisms with their hosts is extremely complex, and a variety of molecular mechanisms can be envisaged through which tumor occurrence, tumor progression, and response to anticancer therapies can be influenced. Microorganisms can also induce chronic inflammation, providing a backdrop for tumor development, or trigger an immunosuppressive response that disrupts immune surveillance of cancer (Zitvogel et al., 2017). Finally, microbial metabolism of host metabolites, food components, or foreign substances may produce harmful compounds and may even promote the development of tumors in distant body parts (Roy and Trinchieri, 2017).Studies suggest that dysbiosis of microbiome has been revealed responsible for various urological disorders, such as urgent incontinence, interstitial cystitis, overactive bladder and urinary malignancies (Bhide et al., 2020; Knippel et al., 2021; Lane et al., 2022). Definitely, UD procedures will alter the original composition of urinary microbiome, which may be affected by the symbiotic collection of bacteria, fungi, parasites and viruses that inhabit the surface of our body's epithelial barrier.

Previous or current researches of UD have been mainly focusing on surgical techniques or postoperative functional outcomes. However, urinary microbiological changes after RC with UD have not been illustrated. Here, for the first time, we aim to identify the urinary microbiota signatures for patients with different UD types and preliminarily analyze their associations with functional outcomes.

2 Methods

2.1 Study population and urine sample collection

After approved by the institutional review board, patients who had received RC with UD previously and healthy controls were recruited for the current study between January and May 2023, at Shanghai Tenth People's Hospital. Informed written consent was obtained from each participant for sample collection as well as other procedures and protocols. All UD cases had been histologically confirmed as urothelial carcinoma at RC. Those with a prior history of sexually transmitted infection were excluded. Recent urinary or systemic infection or any use of either antibiotics or probiotics (within 2 months) were also excluded. Healthy individuals volunteered as the control group, none of whom had a history of urinary infection or urologic disease. Clean-catch midstream urine samples of 50 ml from all participants were collected under the strict aseptic procedures. For IC and CU groups, urine samples were drained and collected via sterile ureteral stent and catheter, respectively. Under the guidance of professional biotechnologists,

Abbreviations: ACE, abundance-based coverage estimator; ASV, amplicon sequence variant; COG, cluster of orthologous groups of proteins; CU, cutaneous ureterostomy; IC, ileal conduit; KEGG, Kyoto gene and genome encyclopedia; LEfSe, linear discriminant analysis effect size; MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; ONB, orthotopic neobladder; PCoA, principal coordinate analysis; PERMANOV, permutational multivariate analysis of variance; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; QIIME, quantitative insights into microbial ecology; RC, radical cystectomy; rRNA, ribosomal RNA; UD, urinary diversion.

fractions for microbiota analysis from urine samples were centrifuged at 16,000 g for 10 min, and supernatants were aliquoted and stored at − 80 °C; for further processing. Meanwhile, all participants were asked to complete a structured questionnaire to gather information about socio-demographic characteristics. For UD cases, serum creatinine was detected and ultrasonography was employed to evaluate urinary tract functions (i.e., residual urine, hydronephrosis). Besides, clinicopathologic features were also extracted from the electronic medical record database (Figure 1A). Data collection followed the principles outlined in the Declaration of Helsinki.

2.2 DNA extraction and 16S ribosomal RNA (rRNA) gene amplicon sequencing

Bacterial DNA was isolated from the urine sample using a DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration and integrity were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. PCR amplification of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene was carried out in a 25 μl reaction using universal primer pairs (343F: 5′-TACGGRAGGCAGCAG-3′; 798R: 5′-AGGGTATCTAATCCT-3′). To rule out contamination during amplification, a PCR-negative control with no template DNA was processed. The amplicon quality was visualized using gel electrophoresis. PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA, USA) and quantified using Qubit dsDNA assay kit (Invitrogen, Life Technologies, Grand Island, NY, USA). PCR amplification was then parallel performed in 25 μl of 2 × Phanta Max Master Mix, 2 μl of forward primer (10 μM), 2 μl of reverse primer (10 μM), 50 μl of ddH2O and template DNA. The PCR was conducted under the following conditions: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The concentrations were then adjusted for sequencing, which was performed on an Illumina NovaSeq6000 with two paired-end read cycles of 250 bases each (Illumina Inc., San Diego, CA, USA; OE Biotech Co., Ltd., Shanghai, China).

2.3 Bioinformatic analysis

Raw sequencing data were in FASTQ format. Paired-end reads were preprocessed using cutadapt software to detect and cut off the adapter. After trimming, paired-end reads were filtered low quality sequences, denoised, merged and detect and cut off the chimera reads using DADA2 (Callahan et al., 2016) with the default parameters of QIIME2 (Bolyen et al., 2019) (2020.11). At last, the software exported the representative reads and the amplicon sequence variant (ASV) abundance table. The representative read of each ASV was selected using QIIME2 package. All representative reads were annotated and blasted against Silva database Version 138 (or Unite) (16s/18s/ITS rDNA) using q2-feature-classifier with the default parameters. The microbial diversity in urine content samples was estimated using the α-diversity indices, including abundance-based coverage estimator (ACE), phylogenetic diversity whole tree, Chao1, Shannon (Chao and Bunge, 2002), observed species and Simpson (Hill et al., 2003). The Unifrac distance matrix performed by QIIME software was used for unweighted Unifrac Principal coordinate analysis (PCoA) and phylogenetic tree construction.

2.4 Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical comparisons of continuous variables describing clinical features were made using the Student's t-test, where the data were normally distributed. Chi-square test was used for categorical variables. For bacterial abundance analysis, the data were first normalized and univariate analysis of variance or Kruskal-Wallis test was used for the significant difference in relative abundance of

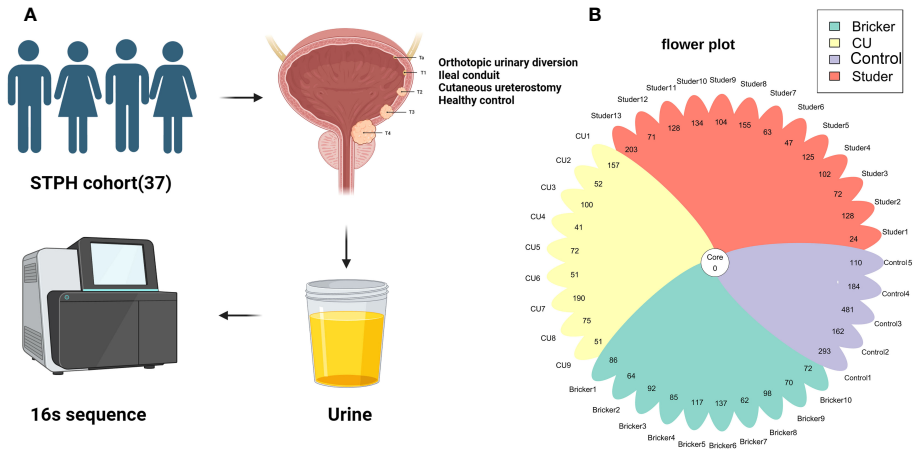

FIGURE 1
(A) Experimental process; **(B)** The number of amplicon sequence variants in 37 samples.

genera. The differences of metabolite abundance in Studer group, Bricker group and CU group were analyzed by student t-test. Logistic regression was used to analyze the associations between clinicopathologic factors and urine microbial diversities. P <0.05 was considered statistically significant, and the P value was corrected by Benjamini-Hochberg FDR.

3 Results

3.1 Subject characteristics

In this study, 16S rRNA gene sequencing was performed on qualified clean midstream urine samples from 5 healthy volunteers and 32 patients who had received RCs and UDs, including 13 Studers, 10 Brickers and 9 CUs. The clinical and demographic characteristics of four groups as well as oncologic features of UD cases are shown in Table 1. There was no statistically significant difference in age, gender, BMI or metabolic comorbidities among these four groups. In terms of UD groups, there was no significant difference in either pathologic T stage at RC or proportion of recurrence. The mean duration from RC to urine sample collection was 48 months for Studer patients, which was significantly longer than other two UD groups (Bricker: 40, CU: 40, P = 0.052).

3.2 Urine microbial diversities of healthy controls and UD individuals

Illumina sequencing of the V3-V4 hypervariable region of 16S rRNA amplicons from all samples yielded 78108 raw reads and 81970 reads after pre-processing (Figure 1A). The sequence counts per sample ranged from 24 to 481 reads. In total, 2543 ASVs were detected and the mean ASVs of urine samples varied among groups, which were 104, 88, 88 and 246 for Studer, Bricker, CU subjects and healthy controls, respectively (Figure 1B). The ASV data volume and categorical information indicated significant differences among groups (Table S1). We evaluated microbial diversities of urine samples, and there was no statistically significant difference in ACE, Chao1, Shannon, observed species or Simpson index among four groups. However, remarkably higher values of these α-diversity indices were unanimously observed in the control group, followed by Studer patients, who were closer to healthy volunteers than counterparts of other UD types (Figure 2). Furthermore, we found that Studer UD type was the only independent clinicopathologic factor favoring urine microbial diversity of UD patients in multivariate regression analyses for both Shannon and Chao1 indices (Tables 2, S2). Interestingly, we also analyzed the associations between urine microbial diversity and Studer ONB functions. As shown in Supplementary Figure S1, urine samples

TABLE 1 Clinicopathologic characteristics of each group.

Variables	Studer n =13	Bricker n = 10	CU n = 9	Control n = 5	P-value
Age (y)	67.5(64.0, 74.0)	71.9(64.0, 79.0)	71. 8(60.0, 85.0)	70.8(63.0, 76.0)	0.2355
Gender					**0.1051**
male	13	8	6	5	
female	0	2	3	0	
BMI	23.9(17.3, 29.8)	22.7(17.8, 26.6)	23.6(18.3, 26.0)	23.9(21.9, 25.3)	0.7845
Hypertension					**0.7640**
Yes	5	5	5	1	
No	8	5	4	4	
Diabetes					**0.7103**
Yes	3	4	2	1	
No	10	6	7	4	
T stage					**0.2869**
T1	4	4	1	\	
T2	7	5	4	\	
T3-4	2	1	4	\	
UD duration (month)	48 (12,120)	40(12,120)	40(12,96)	\	0.0522
Recurrence					**0.3046**
Yes	3	3	4		
No	10	7	5		

Data are presented as average (minimum value, maximum value) for continuous variables or n (%) for counts; BMI, body mass index; n, number of people; \ , not applicable.

from Studer subjects without hydronephrosis or residual urine showed higher α-diversity indices, which were closer to samples from healthy controls.

In this study, PCoA was also used to compare the similarity in the microbial community composition of urine specimens. As shown in Figure 2F, there was no obvious difference in the distribution of bacteria when comparing Studer group with healthy individuals or Bricker group, but a relatively remarkable difference between Bricker group and healthy controls. As for CU subjects, their urine bacteria distribution was significantly different from those of both Studer and Bricker counterparts, let alone the control group. Overall, the PERMANOV test showed that the observed difference was statistically significant (Adonis test, Binary-Jaccard, F=1.4795, P<0.001). Similarly, PCoA based on ONB functions in the Studer cohort also revealed significant results of β-diversity that microbial community composition of urine samples from those without hydronephrosis or residual urine was more similar to the control group (Supplementary Figures S1E, F).

3.3 Urine microbial community distributions of healthy controls and UD individuals

To explore the microbial signature alteration after UD, we evaluated the relative abundance of taxa in different groups. At the phylum level, the majority of dominant bacteria were from Proteobacteria (Studer: 35.9%, Bricker: 50.9%, 67.9% CU: 67.9%, control: 15.5%), Firmicutes (Studer: 30.5%, Bricker: 31.0%, CU: 23.2%, control: 41.8%) and Bacteroidota (Studer: 26.4%, Bricker: 11.9%, CU: 4.8%, control: 31.2%; Figure 3A). At the generic level, three dominants were Enterococcus (Studer: 13.1%, Bricker 14.4%, CU: 24.0%, control: 3.7%), Escherichia-Shigella (Studer: 18.4%,12.7% Bricker: 12.7%, CU: 14.4%, control: 0.1%) and Barnesiella (Studer 14.4%, Bricker: 5.3%, CU: 1.3%, control: 10.2%; Figure 3B). It is worth noting that the Escherichia-Shigella genus was frequently detected in three UD groups, but rarely in the control group. By contrast, Barnesiella was the second most dominant genus in urine samples from both healthy volunteers and Studer subjects, far more often than in samples from Bricker and CU counterparts. Urine microbial communities of different groups at class, order, family and species levels are shown in Figures 3C–F, respectively. Overall, the main urine microflora structures were comparable among different UD types, whereas Studer and CU groups showed the most and least similar to healthy controls, respectively.

To identify specific taxa associated with UD types, linear discriminant analysis effect size (LEfSe) was further performed. As shown in Figure 4, Actinomycetaceae, Solobacterium, Stenotrophomonas, Xanthomonadales and Flavobacteriales were significantly enriched in Bricker group. Abundances of Aggregatibacter, Proteus, Providencia and Erysipelatoclostridiaceae were relatively increased in CU group. Healthy controls were characterized by enrichment of Streptococcus, Muribaculaceae, Gardnerella and Bacteroides, at the generic level. In Studer group, the only abundant genus, Barnesiella, belongs to the family Porphyromonadaceae, within the phylum Bacteroidetes, which was also the most abundant taxa in the control group.

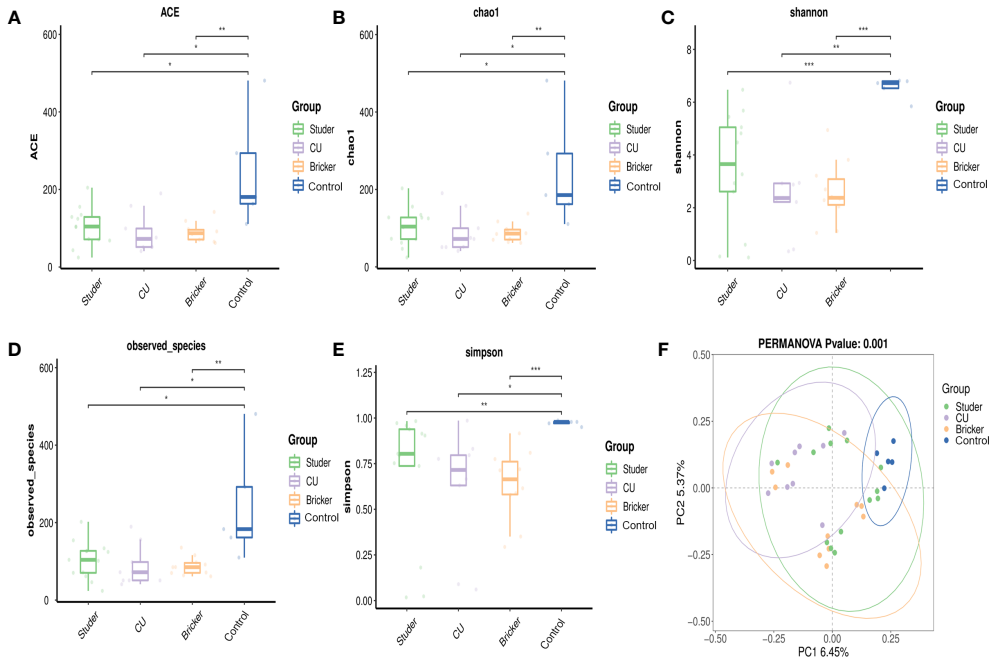

FIGURE 2
α- and β-diversity indices for Studer, Bricker, cutaneous ureterostomy samples and healthy control samples. **(A)** abundance-based coverage estimator index; **(B)** Chao1 index; **(C)** Shannon index; **(D)** observed species index; **(E)** Simpson index; **(F)** principal coordinate analysis based on Adonis test (a dot representing each sample; F = 2.53, P < 0.001). * P <0.05, ** P <0.01, *** P <0.001.

TABLE 2 Logistic regression analysis on α-diversity (Shannon index) for urinary diversion samples.

α-diversity (Shannon index)	B	P	OR	95% CI	
				Upper limit	Lower limit
UD type (Studer vs non-Studer)	-2.627	0.024	0.072	0.007	0.711
Age (y)	-0.136	0.928	0.873	0.046	16.584
Sex (male vs female)	-0.035	0.826	0.966	0.707	1.319
BMI	-0.322	0.806	0.725	0.056	9.447
Recurrence (yes vs no)	-1.091	0.358	0.336	0.033	3.439
Pathological stage (≤T2 vs >T2)	0.044	0.666	1.045	0.855	1.278
UD duration (y)	-0.005	0.792	0.995	0.961	1.31

*The first third of Shannon indices was adopted as cutoff value for regression analysis.

3.4 Microbial functional alterations associated with UD types

To infer functional pathways from microbial community profiles, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) was used, and microbial functional differences between UD patients and healthy controls were investigated using the Kyoto encyclopedia of genes and genomes (KEGG) analysis. In this way, Studer, Bricker, CU and control groups can be clearly distinguished, as shown by Figure 5A. Microbial genes predicted to be significantly enriched in Studer group touched on glycan degradation, N-glycan biosynthesis, sphingolipid metabolism, lysosome, glycosphingolipid biosynthesis as well as neomycin, kanamycin and gentamicin biosynthesis (Figure 5B). Importantly, based on the clustered heat map of KEGG analysis, Studer patients and healthy volunteers shared similar urine microbial gene functions at different level. (Figures 5C, D). For example, Studer and control groups were both significantly enriched in microbial genes associated with development, which were downregulated in Bricker and CU groups. By contrast, negatively functional genes associated with infectious diseases and drug resistance were lowly expressed in Studer and control groups, while both enriched in Bricker and CU groups (Figure 5D).

In addition, we functionally annotated the genes by searching the clusters of orthologous groups of proteins (COG) database. The

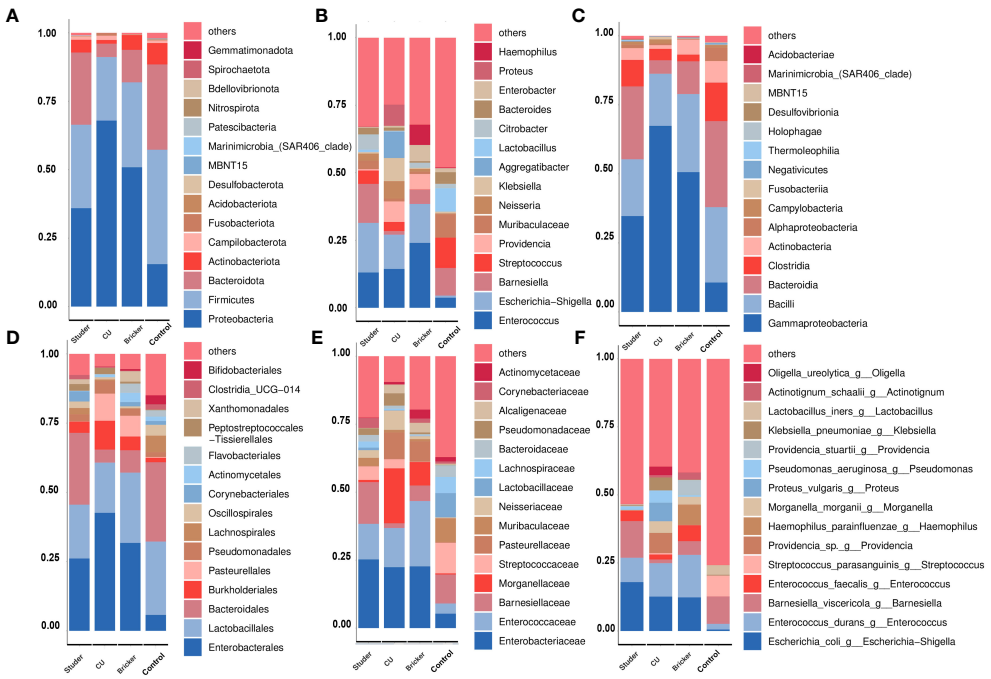

FIGURE 3
Average relative abundances of major taxa represented by bar graphs. **(A)** phylum; **(B)** genus; **(C)** class; **(D)** order; **(E)** family; **(F)** species. Each colored box represents a bacterial taxon and the height of a colored box represents the relative abundance of the organism within the urine samples. Bacterial genera with a relative abundance <1% or those unclassified are grouped as "Others".

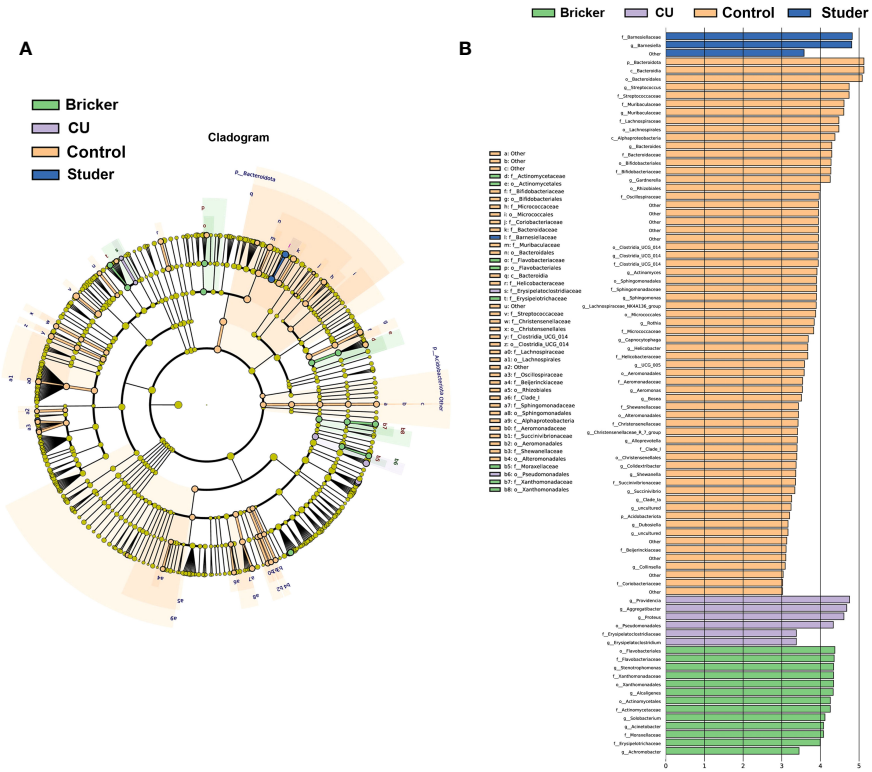

FIGURE 4
Microbial taxa associated with urine samples from patients of urinary diversion and control subjects. **(A)** cladogram representation of the urine microbial taxa associated with urinary diversion and control samples; **(B)** association of specific urine microbiota taxa with different groups by linear discriminant analysis effect size.

first 10 differential COGs were shown, as well as the average abundance of the first 6 differential COGs in each group. Based on the clustered heat map of the COG difference results, we also found Studer patients and healthy volunteers shared many similar urine microbial genes, which were inversely expressed in Bricker and CU groups (Figures 6A–C).

4 Discussion

Reduced microbial diversity has been recognized as a feature in intestinal diseases such as ulcerative colitis, Crohn's disease and colorectal cancer (Lepage et al., 2011; Ahn et al., 2013; Gevers et al., 2014). However, such consistent microbial finding was not confirmed in urinary disorders. Increased microbial diversity was observed in urge incontinence and decreased in interstitial cystitis and overactive bladder, while no significant difference was found in bladder cancer (Zhang et al., 2023). To date, the urine microbial community of patients undergoing UD for bladder cancer has not been adequately studied in comparison to the urine microbiota signatures of bladder cancer. Here, we performed 16s rRNA and bioinformatic analyses to comprehensively characterize the urinary microbial landscapes and potential functional impacts among UD patients. Overall, there were no significant differences among the three groups of UD patients regarding urine microbial diversities, all of which showed remarkably less levels than healthy individuals. However, Studer or ONB surgery, was found to be a factor favoring urine microbial diversity among these patients, showing urinary microbiota signatures most approximate to the control group. In PCoA analysis, Studer group and healthy individuals showed most similar distribution of urine bacteria, whereas CU subjects manifested with totally different urine microbial characteristics. It can be speculated that UD type should be a key factor determining individual urine microbiota. Incontinent UD approaches with abdominal stoma are more liable to affected by exotic microorganism, and this was exacerbated by long-term ureteral stenting among CU patients. ONB procedure would not only mimick the enclosed physiological alignment but also render the microbial environment closest to the original urinary system, thus providing more favorable functional outcomes.

Proteobacteria are considered to be intestinal symbiotic bacteria with pathogenic capacity (Puri, 2019). Increases in intestinal proteobacteria have been reported in colitis-associated colorectal cancer (Quaglio et al., 2022). Proteobacteria may also play an important role in the development of bladder cancer (Liu et al., 2019). Thus, the elevation of Proteobacteria may be considered as unfavorable ecological dissonance, and it was more obvious in Bricker and CU groups, which were less similar to the original alignment than Studer approach. Firmicutes and Bacteroidetes are healthy indicators which vary with age, diet quality and environmental factors. Low flora ratios may be associated with obesity, inflammatory bowel disease, cardiovascular disease and

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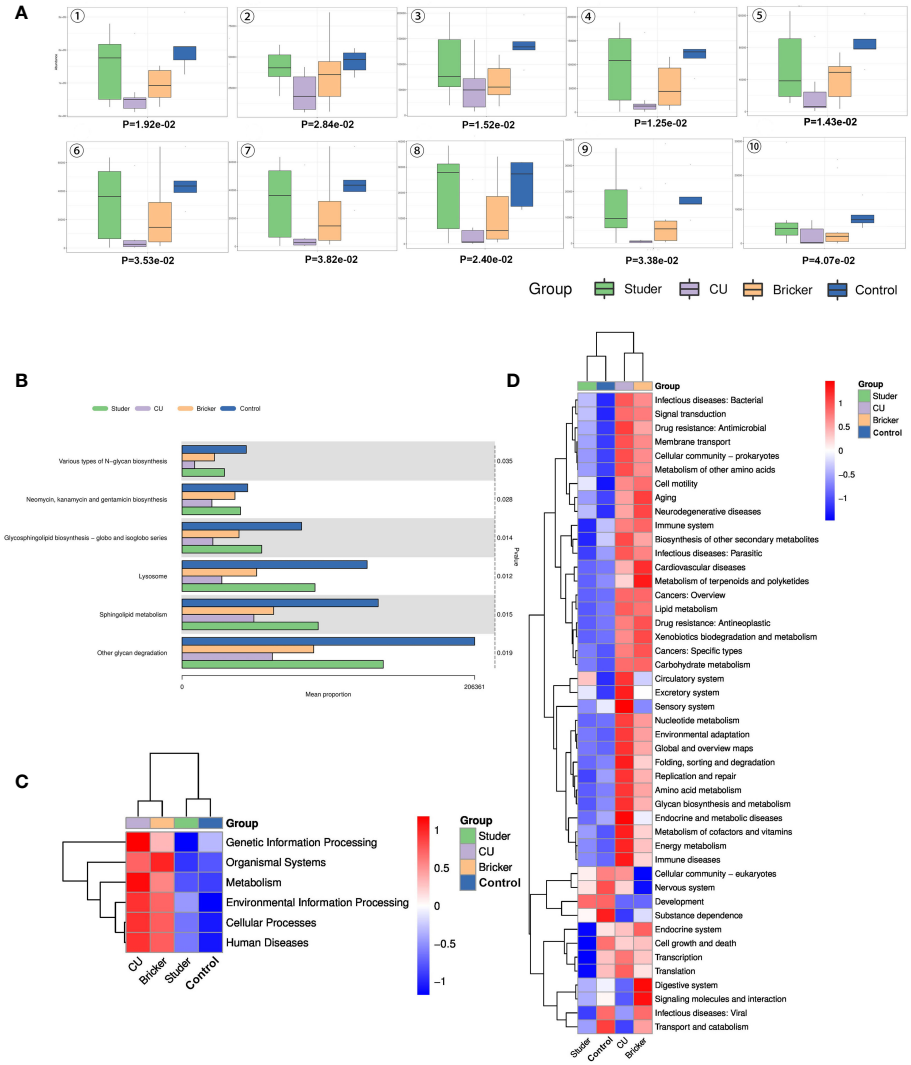

FIGURE 5
Differential KEGG functions among groups. **(A)** box plots of differential KEGG functions: 1. other glycan degradation, 2. neomycin, kanamycin & gentamicin biosynthesis, 3. sphingolipid metabolism, 4. lysosome, 5. glycosphingolipid biosynthesis - globo & isoglobo series, 6. various types of N-glycan biosynthesis, 7. glycosphingolipid biosynthesis - ganglio series, 8. N-glycan biosynthesis, 9. protein digestion & absorption, 10. meiosis - yeast; **(B)** bar charts of top differential KEGG functions; **(C, D)** level 1 and 2 clustering heat maps of KEGG results.

colorectal cancer (Sun and Kato, 2016; Singh et al., 2017; Klement and Pazienza, 2019). Our analysis showed that Firmicutes and Bacteroidetes accounted for 73% of urine bacteria in healthy individuals, 57% in the Studer group, 43% in the Bricker group and 28% in the CU group. Distributions of these major probiotics also indicate that ONB has a clear microbial advantage as the procedure closest to the natural cavity. By contrast, Escherichia-Shigella is one of pathogens that cause intestinal infection (Yacouba et al., 2022), while conflicting results regarding urine Escherichia-Shigella have been reported in patients with bladder cancer (Liu et al., 2019; Hussein et al., 2021). The abundance of Escherichia-Shigella was higher in the Studer group than the other groups. Escherichia-Shigella infection of macrophages and epithelial cells induces a strong inflammatory response and macrophage death (Kotloff, 2022). The definite role of Escherichia Shigella should be further evaluated in UD patients.

This study was based on genera changes to characterize urine ecological disorders, and UD cohorts and healthy controls could be perfectly separated. Within the current sample size, no statistically significant difference of α or β-diversity was observed among sub-cohorts grouped by gender, age, BMI, initial tumor stage, current creatinine or post-UD duration. Interestingly, after dichotomizing the Studer subjects based on urinary functions, those without hydronephrosis or residual urine were closer to the healthy individuals in these microbial indices, implying the potential link of urine microbiota with UD functions. Further studies should be carried out to validate our preliminary finding as well as illustrate related microbial mechanisms.

In our study, LEfSe analysis showed that bacterial groups along the Barnesiellaceae to Barnesiella lineage were enriched in Studer patients. Specific function of such flora remains to be explored. In Bricke and CU groups, there were obvious increases in pathogenic bacteria communities, which resulted in higher possibilities of

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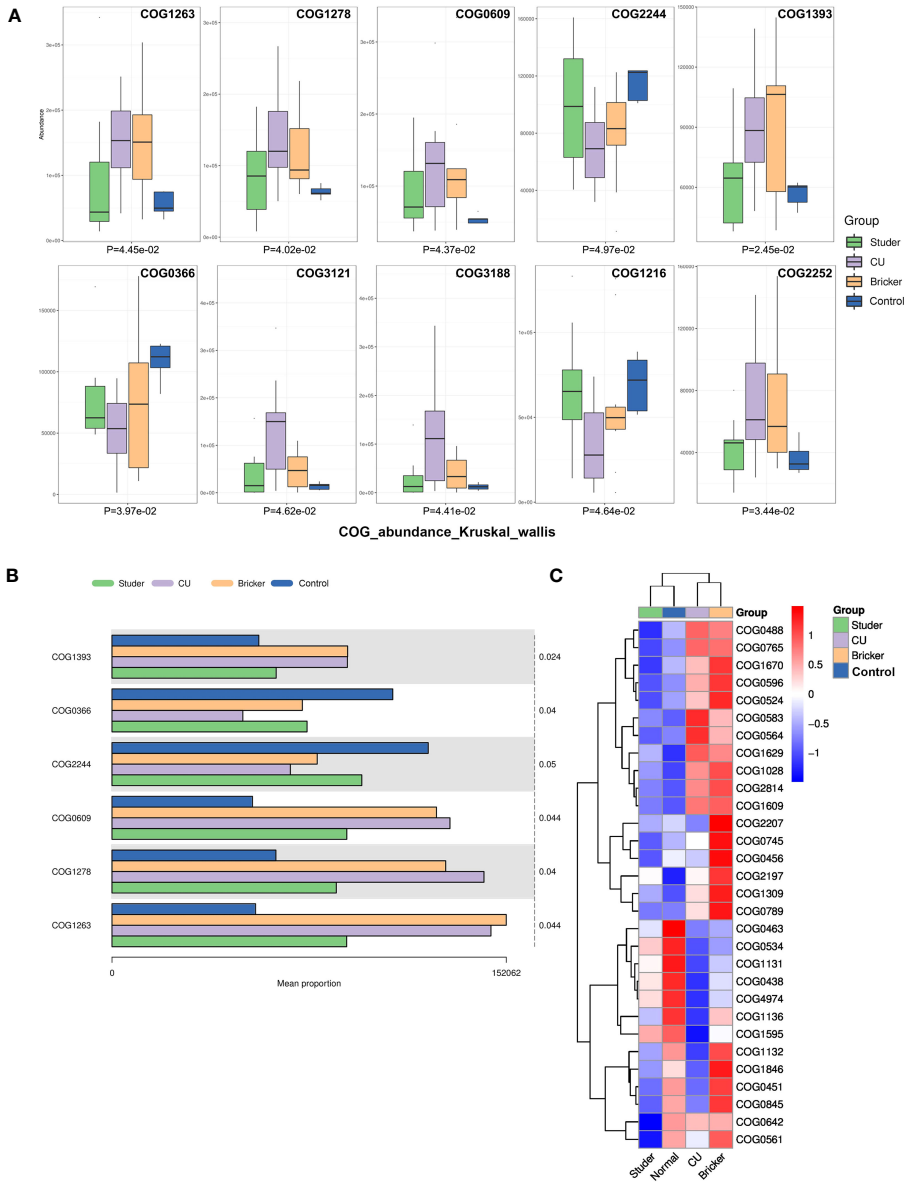

FIGURE 6
Differential clusters of orthologous groups of proteins among groups. **(A, B)** box plots and bar charts of top differential clusters of orthologous groups of proteins; **(C)** clustering heat map of differential clusters of orthologous groups of proteins.

postoperative urinary infections and suboptimal functional outcomes. Solobacterium is considered to be an opportunistic pathogen and important member of oral microorganisms, mainly causing oral diseases (Barrak et al., 2020). Staphylococcus moskii has been reported to cause a variety of infections, such as blood and surgical wound infections (Zheng et al., 2010; Vancauwenberghe et al., 2013). In the Bricker group, high levels of such flora may suggest an increased risk of postoperative infection. Proteus, a member of the Enterobacteriaceae family, is generally considered a low-abundance symbiotic bacteria in the gut and most commonly cited clinically as the cause of urinary infections (Hamilton et al., 2018). In the CU group, we found a significant increase of Proteus, suggesting inevitably higher risk of infections. Overall, these microflora in urine may be a potential biomarker or tool to optimize UD management.

Our study has some limitations. First was the relatively small sample size. Due to the fact that Studer surgery is more commonly performed in men, only male ONB patients were included in this study. This study was also limited to retrospective design that could not determine the causal relationship between the microbiome and post-UD recovery. Hence, prospective studies in larger cohorts are needed. In addition, the ONB or IC mucosa would be better to characterize the microbiota signatures and rule out contamination by confounding microorganisms. Fundamental researches may help elucidate the role of microbiome in different stages after UD.

5 Conclusion

For the first time, we presented comprehensive urine microbial landscapes of UD patients and preliminarily revealed the association of urine microbial features with function outcomes in the current study. Among UD cases, Studer type was the only independent clinicopathologic factor favoring urine microbial diversity, and Studer patients, especially those with perfect ONB functions, exhibit urinary microbiota signatures most approximate to healthy people. Further studies should be carried out to illustrate microbial mechanisms related with the regulation of UD functions.

Data availability statement

The data presented in the study are deposited in the NCBI-Sequence Read Archive (SRA), accession number PRJNA1048651.

Ethics statement

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

Author contributions

YL: Writing – original draft. JZ: Data curation, Writing – review & editing. HC: Writing – review & editing. WZ: Writing – review & editing. AA: Writing – review & editing. SM: Writing – review & editing. XY: Writing – review & editing. TX: Writing – review & editing. YY: Conceptualization, Investigation, Software, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1302870/full#supplementary-material

SUPPLEMENTARY FIGURE 1
α- and β-diversity indices for control and Studer samples. **(A-C)** α- and β-diversity indices for control samples, Studer samples with or without hydronephrosis; **(D-F)** α- and β-diversity indices for control samples, Studer samples with or without residual urine.

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Symbiotic microbial communities in various locations of the lung cancer respiratory tract along with potential host immunological processes affected

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Lung cancer has the highest mortality rate among all cancers worldwide. The 5-year overall survival rate for non-small cell lung cancer (NSCLC) is estimated at around 26%, whereas for small cell lung cancer (SCLC), the survival rate is only approximately 7%. This disease places a significant financial and psychological burden on individuals worldwide. The symbiotic microbiota in the human body has been significantly associated with the occurrence, progression, and prognosis of various diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis. Studies have demonstrated that respiratory symbiotic microorganisms and their metabolites play a crucial role in modulating immune function and contributing to the pathophysiology of lung cancer through their interactions with the host. In this review, we provide a comprehensive overview of the microbial characteristics associated with lung cancer, with a focus on the respiratory tract microbiota from different locations, including saliva, sputum, bronchoalveolar lavage fluid (BALF), bronchial brush samples, and tissue. We describe the respiratory tract microbiota's biodiversity characteristics by anatomical region, elucidating distinct pathological features, staging, metastasis, host chromosomal mutations, immune therapies, and the differentiated symbiotic microbiota under the influence of environmental factors. Our exploration investigates the intrinsic mechanisms linking the microbiota and its host. Furthermore, we have also provided a comprehensive review of the immune mechanisms by which microbiota are implicated in the development of lung cancer. Dysbiosis of the respiratory microbiota can promote or inhibit tumor progression through various mechanisms, including DNA damage and genomic instability, activation and regulation of the innate and adaptive immune systems, and stimulation of epithelial cells leading to the upregulation of carcinogenesis-related pathways.

KEYWORDS

lung cancer, respiratory tract, commensal microbiome dysbiosis, immune mechanisms, chromosome aberration

1 Introduction

Globally, lung cancer remains the leading cause of cancer-related deaths, representing a substantial proportion of total cancer fatalities (18.4%) (Siegel et al., 2022). This disease inflicts significant suffering upon patients and imposes a tremendous burden on society. Although the implementation of health education interventions such as smoking cessation programs and early CT screening, along with advancements in immunotherapy, has led to a reduction in mortality rates among lung cancer patients (Howlader et al., 2020; Giaquinto et al., 2022; Xia et al., 2022), the overall five-year survival rate remains low (Society, 2022). The five-year survival rate for NSCLC is 26%, while for SCLC, it is 7%. Furthermore, the specific mechanisms underlying various subtypes of lung cancer remain unclear, and there is still a limited number of therapeutic targets for clinical translation. These factors impose significant burdens on society's economy and the psychological well-being of patients. Therefore, further exploration of the mechanisms underlying lung cancer and the identification of potential therapeutic targets are of utmost importance.

Traditional beliefs have held that the lower respiratory tract is sterile (Dickson et al., 2016; Huffnagle et al., 2017), as it exhibits lower microbial abundance than other body sites. However, limitations in sampling techniques and the culturing methodology have restricted the identification of bacteria through culture-based methods, resulting in only approximately 1% of bacteria being detectable (Torsvik and Øvreås, 2002; Martiny, 2019). Consequently, many microorganisms remain undetectable (Moffatt and Cookson, 2017). With the continuous development of molecular biology techniques, such as DNA sequencing (Singh et al., 2022), independent of culture-based methods, it has been discovered that the lower respiratory tract of healthy individuals harbors a microbial community. The lung microbiota represents a dynamic assemblage of bacteria, fungi, viruses, and other microorganisms that colonize the lungs through inhalation and different routes (Huxley et al., 1978; Gleeson et al., 1997). Previous research has reported their involvement in the occurrence and progression of various lung diseases, such as asthma, cystic fibrosis, and COPD (Hilty et al., 2010; Silveira et al., 2021; Yagi et al., 2021; Amati et al., 2022; Madapoosi et al., 2022).

The significance of lung microbiota in the occurrence and progression of lung cancer is increasingly recognized by researchers. Previous studies have predominantly focused on investigating the mechanisms underlying gastrointestinal cancers, including gastric cancer and colorectal cancer, concerning the gut microbiota. For instance, Helicobacter pylori (HP) has been identified as a risk factor for gastric cancer (Cheung et al., 2018a; Cheung et al., 2018b; Conteduca et al., 2013), while Fusobacterium nucleatum(Fn) has been implicated in the development of colorectal cancer (Rubinstein et al., 2013; Guo et al., 2020; Liang et al., 2020). Recently, there has been an increasing focus on the lung cancer microbiome. Studies have indicated that the microbial communities in lung cancer patients are dysregulated (Liu et al., 2020; Meng et al., 2023), and specific bacteria or bacterial groups are associated with immune dysregulation in lung cancer (Tsay et al., 2021; Khan et al., 2022). These microorganisms directly or indirectly contribute to the occurrence and development of lung cancer. Extensive research on the lung cancer microbiome highlights its significant role in lung cancer and its potential as a biomarker for early diagnosis and a prognostic indicator for evaluating treatment outcomes.

Currently, comprehensive reviews on the microbial composition in different regions of the lower respiratory tract with lung cancer are lacking. Furthermore, the precise role of specific bacteria in the immune mechanisms of lung cancer remains insufficiently understood. This review provides a comprehensive review of the microbial characteristics in lung cancer patients, focusing on various sample types, including saliva, sputum, BALF, brush samples, and tissue (Figure 1). The review further categorizes the microbial features within each sample type based on different factors such as pathological types, metastasis, PD-L1 treatment, smoking, and coal combustion. It explores the potential immune mechanisms by which the microbial community participates in the early diagnosis and treatment of lung cancer, while also revealing the relationship between respiratory microbiota dysbiosis and regulatory factors such as the environment. By offering a more encompassing overview of lung microbiota, this review is a valuable resource for researchers, facilitating further studies and comparisons. Ultimately, it provides a foundation for a better understanding and improvement of the diagnosis and treatment of lung cancer patients.

2 The microbiota community in the respiratory tract of lung cancer

2.1 Microbiome features of the respiratory tract in lung cancer patients

2.1.1 Differential microbes between lung cancer and healthy controls

2.1.1.1 Upper respiratory tract

Multiple studies on lung cancer currently concentrate on genes with Somatic and Germline variants (Li et al., 2018; Shen et al., 2019). However, genetic factors can only account for 3-15% of hereditary issues (Czene et al., 2002; Dai et al., 2017), while non-genetic environmental factors also play a crucial role, such as respiratory tract symbiotic microbiota (Lee et al., 2016). Although BALF and tissue microbes have considerable research potential, these procedures are invasive. On the other hand, sputum and saliva provide non-invasive means to access respiratory microbiota, making sample collection easier. Therefore, investigating microbiota's predictive and prognostic value in sputum and saliva is of great significance in understanding the association between microbiota and lung cancer.

Human buccal microorganisms comprise more than 600 bacteria, which play distinct functions under different etiologies (Chen et al., 2010; Dewhirst et al., 2010). In various studies, significant differences have been observed between the bacterial compositions of saliva and sputum samples from individuals with lung cancer compared to healthy controls. Xinmin Yan et al.

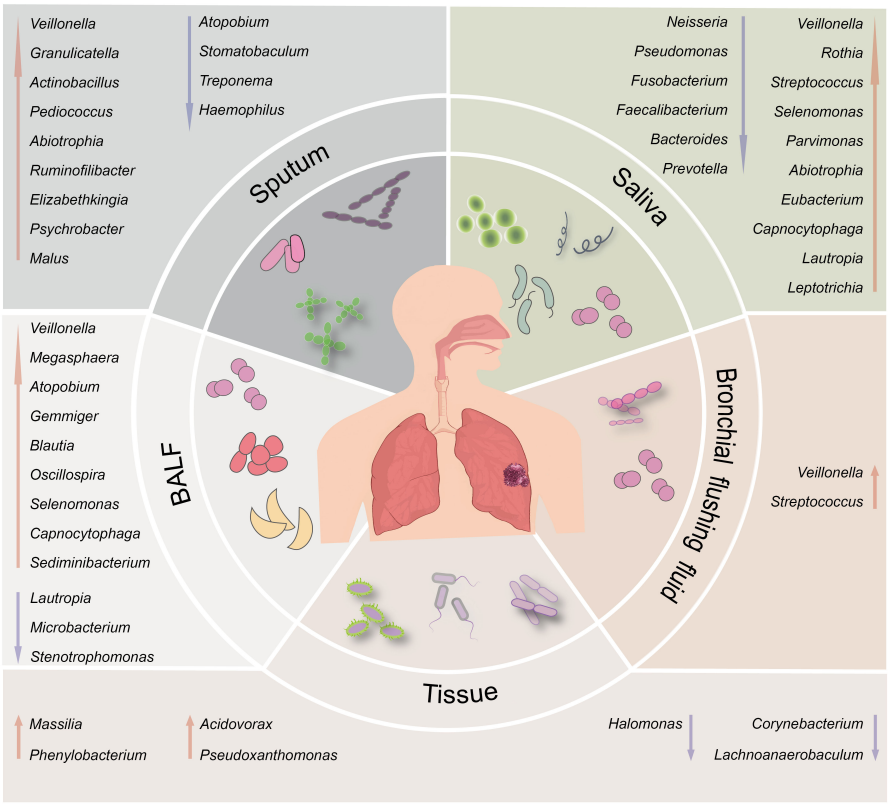

FIGURE 1
Schematic illustration of the differential respiratory microbiota across different anatomical sites between individuals with lung cancer and healthy subjects. The upward red arrows indicate representative microbiota that exhibit significantly higher abundance in the lung cancer group than the healthy control group. In contrast, the downward blue arrows indicate representative microbiota with decreased abundance.

elucidated the intricate association between the salivary microbial community and lung cancer, presenting a comprehensive account for the first time. They found that the phyla Flavobacteriales, Burkholderiales, Campylobacterales, and Spirochaetales, the family Veillonellaceae, and the genera Capnocytophaga, Selenomonas, and Veillonella are prevalent in lung cancer, although Neisseria is less dominant (Yan et al., 2015).A recent study reported similar findings when comparing saliva samples from lung cancer patients and healthy individuals. The authors observed moderate differences at the phylum level between the two groups. At the genus level, Prevotella_7 (Bacteroidetes), Neisseria (Proteobacteria), Streptococcus (Firmicutes), Veillonella (Firmicutes), and Haemophilus (Proteobacteria) were identified as the most prevalent genera in the oral samples (Sun et al., 2023).

Using recycling free-flow isoelectric focusing (RFFiEF) technology, Xiaoteng Jiang and colleagues are concentrating on analyzing salivary bacteria. Neisseria, Pseudomonas, Serratia, Lactobacillus, and Acinetobacter were more prevalent in healthy subjects, while Rothia, Granulicatella, Parvimonas, Abiotrophia, and Eubacterium significantly increased in the lung cancer group. After the qPCR test, Granulicatella considerably rose, whereas Pseudomonas significantly declined (Jiang et al., 2020).

According to research by Weiquan Zhang et al., the presence of the Firmicutes genera Veillonella, Streptococcus, Lautropia, Leptotrichia, Rothia, and Aggregatibacter was considerably more fabulous in the saliva of NSCLC patients than in the control group. Additionally, the relative abundance of Fusobacterium, Prevotella, Bacteroides, and Faecalibacterium decreased overall in the NSCLC group (Zhang et al., 2019).

Simon J. S. Cameron et al. analyzed sputum samples from ten participants, including four with lung cancer and six without. The lung cancer samples exhibited significantly higher levels of Streptococcus viridans, Granulicatella adiacens, Streptococcus intermedius, and Mycobacterium tuberculosis (Cameron et al., 2017).

Elizaveta Baranova et al. analyzed sputum samples from 80 individuals, consisting of 40 cases with squamous cell carcinoma (LUSC) and 40 healthy controls. Their investigation revealed a significant increase in the phylum Firmicutes, as well as the genera Streptococcus, Bacillus, Gemella, and Haemophilus, including the species Streptococcus agalactiae, among those with squamous cell carcinoma. Intriguingly, no variations in the microbiome were observed to pathological stage or smoking status among lung cancer patients (Baranova et al., 2022).

According to Druzhinin et al., sputum from lung cancer patients exhibited unique taxa, including Actinobacillus, Pediococcus, Abiotrophia, Ruminofilibacter, Elizabethkingia, Psychrobacter, and Malus. Among these, Bergeyella showed a significant increase in lung cancer sputum compared to that of healthy controls. Conversely, the genera Atopobium,

Stomatobaculum, and Treponema demonstrated a considerable reduction. The genus Haemophilus was more prevalent in lung cancer, and the species Bergeyella zoohelcum exhibited a considerable increase. Additionally, Atopobium rimae, Treponema amulovorum, and Prevotella (P. histicola and P. sp. oral clone DO014) experienced a considerable decline (Druzhinin et al., 2020).

2.1.1.2 Lower respiratory tract

Lower respiratory tract samples are less susceptible to contamination and are closely associated with lung tissue than saliva and sputum samples. Therefore, studying the correlation between lower respiratory tract bacteria and lung cancer can significantly advance our understanding of this disease's underlying mechanisms. This section focuses on the microbiological distinctions observed in BALF between lung cancer patients and healthy individuals.

In a study by Wang et al., a comparison was made between BALF and salivary bacteria from lung cancer patients (n = 52) and healthy individuals (n = 15), revealing significant differences. The lung cancer group exhibited considerable variations in the levels of Firmicutes and Fusobacteria in their BALF, as well as significantly different levels of Actinobacteria in their saliva, in comparison to the healthy individuals (Wang et al., 2019).

Identifying potential differences between lung cancer and benign samples using microorganisms can aid in distinguishing between suspicious and confirmed cases. In a study conducted by Sang Hoon Lee and colleagues, they examined 28 BALF samples, including 20 from lung cancer patients and eight from individuals with benign illnesses. The researchers found a significant elevation of Veillonella, Megasphaera, Atopobium, and Selenomonas in the lung cancer group (Lee et al., 2016).

The sample size was expanded to include 54 individuals, comprising 32 lung cancer patients and 22 individuals with benign lung disease. The results of this study revealed significant increases in six genera (c: TM7-3, Capnocytophaga, Sediminibacterium, Gemmiger, Blautia, and Oscillospira), while four genera (Microbacterium, Stenotrophomonas, Lautropia, and f: Pseudonadaceae) exhibited significant decreases. These ten genera and three tumor markers (CEA, NSE, and CYFRA21-1) were used to construct a random forest model. The model demonstrated promising predictive capabilities, with an area under the curve (AUC) value of 84.52% for lung cancer, indicating its potential for clinical application (Cheng et al., 2020).

Wen Zeng et al. conducted a study involving BALF samples from 46 NSCLC patients and 29 individuals with benign illnesses. The researchers found that Prevotella, Alloprevotella, and Veillonella were significantly elevated in the lung cancer group. Additionally, positive associations were observed between Prevotella and Megasphaera, Alloprevotella, and Actinomyces. Subcutaneous transplantation and endotracheal instillation experiments were conducted to further investigate the impact of Veillonella parvula. The results showed that subcutaneous transplantation significantly promoted lung cancer, while endotracheal instillation did not lead to tumor formation. These findings suggest that the influence of microorganisms on lung cancer may vary in terms of chronicity, duration, or adequacy. These numerous genera could serve as diagnostic indicators and therapeutic targets (Zeng et al., 2022). For brushing samples, lung cancer patients have a higher abundance of Veillonella and Streptococcus compared to healthy individuals (Tsay et al., 2018). However, it is essential to note that different investigations have reported varying bacterial compositions, which could be attributed to the limited sample sizes. Hence, these findings should be validated through large-scale studies.

2.1.1.3 The biodiversity

The diversity of sputum microorganisms was found to be lower in lung cancer patients compared to healthy controls (Hosgood et al., 2014; Hosgood et al., 2019; Lu et al., 2021). However, V.G. Druzhinin et al. reported no significant change in the diversity of sputum microorganisms between lung cancer patients and healthy individuals (Druzhinin et al., 2020; Druzhinin et al., 2021). These studies highlight notable disparities in β diversity. Furthermore, Salvador Bello et al. found no difference in the α diversity of salivary microbes between lung cancer patients and healthy individuals. Still they observed a substantial difference in the β diversity of salivary bacteria (Bello et al., 2021). However, Weiquan Zhang et al. have presented arguments highlighting significant variations in both α diversity and β diversity between the two groups when considering saliva (Zhang et al., 2019).

There are conflicting findings regarding the microbial diversity in BALF observed in different studies. Sílvia Gomes, Wen Zeng et al. found that the BALF microbial α-diversity in lung cancer patients was higher than in non-lung cancer patients (Gomes et al., 2019; Zeng et al., 2022). However, Sang Hoon Lee et al. reached the opposite conclusion (Lee et al., 2016). Several other studies have also reported no significant differences in microbial α- and β-diversity between lung cancer and benign disease groups in BALF (Tsay et al., 2018; Wang et al., 2019; Cheng et al., 2020; Zhuo et al., 2020). However, Sang Hoon Lee et al. suggested a significant difference in β-diversity between cancer and benign control groups (Cheng et al., 2020; Zeng et al., 2022).

2.1.2 Microbiome under different pathological types

2.1.2.1 Upper respiratory tract

It is essential to distinguish different pathologic types after comparing overall lung cancer with healthy controls. A comparative analysis of salivary microbes in LUAD and LUSC revealed distinct patterns. Specifically, in the saliva of squamous cell carcinoma, Streptococcus and Porphyromonas were significantly lower, while Prevotella showed no significant changes between the two groups (Yan et al., 2015). On the other hand, Streptococcus, Capnocytophaga, and Actinomyces were enriched in the saliva of lung cancer patients. The LSCC group demonstrated a higher enrichment of Capnocytophaga and Actinomyces than the healthy group. Additionally, the SCLC group exhibited a higher concentration of Streptococcus, while Rothia was significantly higher in the adenocarcinoma group (Wang et al., 2019).

In a study conducted by Qixin Leng et al., 25 bacteria in adenocarcinoma and squamous cell carcinoma sputum and tissues were compared using ddPCR. The results revealed significant increases in Capnocytophaga in adenocarcinoma samples and Acidovorax in squamous cell carcinoma samples, with both sputum and tissue demonstrating similar trends. However, adenocarcinoma tissues exhibited exclusive elevations of Haemophilus and Fusobacterium, contradicting previous research indicating Haemophilus accumulation in lung cancer sputum (Lu et al., 2021). Squamous cell carcinoma sputum, on the other hand, contained only Streptococcus and Veillonella. Also, Helicobacter levels were reduced solely in adenocarcinoma sputum (Leng et al., 2021).

Danhui Huang et al. investigated the microbiological variations between sputum and bronchial flushing fluid (BWF) samples and reported the microorganisms of squamous cell cancer and adenocarcinoma, respectively. The levels of Streptococcus (Firmicutes) in sputum were higher in lung cancer patients than in BWF (benign lung disease with fibrosis). Moreover, peripheral lung cancer exhibited higher levels of Streptococcus (Firmicutes) in sputum than central lung adenocarcinoma (Huang et al., 2019), which contradicts earlier research (Yan et al., 2015). Additionally, large-cell carcinoma demonstrated higher levels of Veillonella and Leptotrichia than adenocarcinoma (Druzhinin et al., 2020). Regarding sputum α microbial diversity, no significant differences were found between lung adenocarcinoma and lung squamous cell carcinoma (Huang et al., 2019).

2.1.2.2 Lower respiratory tract

A recent study showed that the populations of Haemophilus parainfluenza, Neisseria subflava, Porphyromonas endodontalis, and Fusobacterium nucleatum were significantly higher in the adenocarcinoma group when compared to squamous cell carcinoma (Jang et al., 2023). In the study conducted by Ke Wang et al., it was found that Veillonella and Capnocellhaga exhibited significantly higher levels in the BALF of patients with lung squamous cell carcinoma (LUSC). Conversely, Lactobacillus showed a marked increase in the BALF of individuals with small-cell lung carcinoma (SCLC) (Wang et al., 2019).

In the study conducted by Minglei Zhuo et al., significant increases in the genera Spiroplasma and Weissella were observed in the BALF of the lung cancer group when compared to the healthy side of the lung affected by cancer (Zhuo et al., 2020). Furthermore, Slvia Gomes et al. examined the BALF samples from individuals with adenocarcinoma, squamous cell carcinoma, and healthy controls. They also used RNA sequencing data from the TCGA database to validate their findings. Sphingomonas, Brevundimonas, Acinetobacter, and Methylobacterium were exclusively found in lung adenocarcinoma (LUAD), while Enterobacter, Morganella, Kluyvera, and Capnocytophaga were predominantly present in lung squamous cell carcinoma (LUSC). The prevalence of a proteobacteria-dominated microbiome in the BALF of squamous cell carcinoma is associated with a progressive decline in survival rates (Gomes et al., 2019).

Danhui Huang et al. conducted a study to identify the microbiological colonies in squamous cell carcinoma and adenocarcinoma and the microbial differences between sputum and bronchial washing fluid (BWF) samples in lung cancer patients. The results showed that BWF samples exhibited a significantly higher abundance of Proteobacteria and a lower abundance of Firmicutes than sputum. Comparing the bronchial washing fluids (BWFs) across various pathological types, Firmicutes and the genera Veillonella, Megasphaera, Actinomyces, and Arthrobacter were found to be more prevalent in NSCLC with no distant metastasis (AD-M0) than in squamous cell carcinoma with no distant metastasis (SC-M0). Conversely, Capnocytophaga and Rothia showed a significant decrease in AD-M1 compared to SC-M1, and no association was observed between these distinct bacteria and smoking status. AD-M1 demonstrated a lower abundance of Firmicutes and Streptococcus in BWF than SCC-M1. Furthermore, SCC-M1 exhibited a higher presence of Veillonella and Rothia than SCC-M0 (Huang et al., 2019).

There is also controversy regarding the diversity comparison among different pathological types. Danhui Huang et al. suggested that microbial diversity is not associated with pathological types (Huang et al., 2019), while Sílvia Gomes et al. found that the α-diversity of microbiota was higher in squamous cell carcinoma compared to adenocarcinoma (Gomes et al., 2019). Interestingly, Erin A Marshall et al. found, through a 10-year follow-up, that there was no significant difference in α-diversity between individuals with a previous history of lung cancer, those who would develop lung cancer in the future, and non-lung cancer patients (Marshall et al., 2022).

2.1.3 The microbiota community associated with lung cancer metastasis

Metastasis of lung cancer is a substantial contributor to death and recurrence in NSCLC (Siegel et al., 2022). Several studies have revealed that microbes play a role in tumor metastasis. In a survey conducted by Hui Lu et al., a comparison of microbes in feces and sputum was performed among 121 participants, including 87 with NSCLC and 34 in good health. The findings revealed that gut microbes were exclusively associated with lung cancer that did not metastasize, whereas sputum microbes showed a connection to lung cancer regardless of metastasis. Higher concentrations of Haemophilus (specifically, Haemophilus parainfluenzae and Haemophilus influenzae) were observed in lung cancer samples compared to healthy controls. Additionally, sputum and feces from individuals with metastatic lung cancer exhibited enrichment of Coriobacteriaceae and Actinomyces. The sputum of advanced metastatic lung cancer showed a higher abundance of Pseudomonas, while Campylobacter was more abundant in both the regular group and advanced metastatic lung cancer. An analysis of a random forest model determined that the sputum model provided better predictive accuracy than intestinal microbes. Hence, sputum microorganisms were more closely associated with the development of lung cancer than intestinal microbes (Lu et al., 2021).

Dan Hui Huang et al. examined 85 sputum samples obtained from newly diagnosed patients with NSCLC. Their analysis revealed that in early-stage lung cancer (stage I and stage II), the phylum Firmicutes and the genera Peptoniphilus, Granulicatella, Hylemonella, Actinobacillus, SMB53, and Gemella were more abundant. On the other hand, in advanced-stage lung cancer

(stage III and stage IV), the phyla Actinobacteria and the genus Actinomyces exhibited more significant enrichment. Compared to the non-metastatic group, the metastatic group revealed significant enrichment of the genera Peptostreptococcus, Peptococcus, Parabacteroides, and Escherichia. Moreover, in the lymph node metastasis group, there was a considerable increase in the genera Parvimonas and Pseudomonas. The EGFR mutant group exhibited significant enrichment of Bacteroidetes and Tenericutes, along with the genera Sharpea, Prevotella, Porphyromonas, Parvimonas, Desulfovibrio, Mycoplasma, Actinobacillus, Dialister, and Eikenella. Compared to stage III lung cancer, only Paludibacter showed a significant increase. The specific airway genus and metabolic function of sputum microbiota were found to undergo changes associated with tumor stage, intrathoracic metastasis, lymph node metastasis, and EGFR mutation (Huang et al., 2022). Porphyromonas endodontalis was significantly more abundant in the sputum of patients with stages I-II than those with stages III-IV. Additionally, patients with metastases exhibited higher levels of Capnocytophaga and lower levels of Atopobium rimae than those without metastasis (Druzhinin et al., 2020).

Significant differences in microbial diversity were observed in sputum samples when comparing lung cancer's early and advanced stages.However, microbial diversity was found to be unrelated to stages III and IV, intrathoracic and lymph node metastases, and EGFR mutation (Huang et al., 2022).

2.2 The relationship between microbiota and the host genome

Lung microbes and genes collaborate to facilitate the onset and progression of lung cancer. In their study, V. G. Druzhinin et al. investigated sputum samples obtained from lung cancer patients and controls. They focused on exploring the connection between microbes and the occurrence of chromosomal mutations. Interestingly, their findings revealed a significant decrease in the genus Atopobium, while Alloprevotella exhibited a significant increase among patients with a high frequency of chromosomal aberrations (CA) (Druzhinin et al., 2020). Moreover, V. G. Druzhinin et al. expanded the sample size to include 66 lung cancer patients and 62 healthy subjects. Their investigation revealed significant increases in Streptococcus, Bacillus, Gemella, and Haemophilus among lung cancer patients. Additionally, they found that Bacteroides, Lachnoanaerobaculum, Porphyromonas, Mycoplasma, and Fusobacterium were associated with the frequency of chromosomal aberrations. Interestingly, the genera Megasphaera and Selenomonas bovis negatively correlated with micronuclei (MN) (Druzhinin et al., 2021).

2.3 The relationship between microbiota and environmental factors

2.3.1 Upper respiratory tract

Lung microorganisms can be influenced by environmental factors such as coal combustion and smoking, which have implications for the development and progression of lung cancer. In his study, H. Dean Hosgood, III examined the impact of microbiota in sputum and buccal samples on lung cancer among never-smoking women. Additionally, the study explored the effect of coal on these associations. The findings revealed that the diversity of bacterial communities in buccal samples was comparable between the case and control groups. However, significant differences were observed in sputum samples. Specifically, Granulicatella, Abiotrophia, and Streptococcus were abundant in lung cancer patients' sputum. Lung microorganisms can be influenced by environmental factors such as coal combustion and smoking, which have implications for the development and progression of lung cancer. In his study, H. Dean Hosgood, III examined the impact of microbiota in sputum and buccal samples on lung cancer among never-smoking women.

Additionally, the study explored the effect of coal on these associations. The findings revealed that the diversity of bacterial communities in buccal samples was comparable between the case and control groups. However, significant differences were observed in sputum samples. Specifically, Granulicatella, Abiotrophia, and Streptococcus were abundant in lung cancer patients' sputum. In smoking-related lung cancer patients, the species Selenomonas bovis, genus Bacteroides, and genus Selenomonas were found to be more abundant compared to never-smoking lung cancer patients.

Conversely, the genus Peptostreptococcus exhibited higher abundance in never-smoking lung cancer patients. Among healthy smokers, the species Bulleidia moorei, genus Granulicatella, and genus Bulleidea were prevalent, whereas the genus Neisseria was abundant in healthy nonsmokers (Druzhinin et al., 2020). For more detailed information, please refer to Table 1.

2.3.2 Lower respiratory tract

Lung cancer and smoking are widely acknowledged to be closely related. However, only a small percentage of smokers, approximately 10-15%, will develop lung cancer. This presents a clinical challenge in predicting which smokers are at risk (Bruder et al., 2018; Herbst et al., 2018). Moreover, smoking has been shown to impair the function of the epithelial barrier, increasing the likelihood of lung resident bacteria infiltrating the lungs and contributing to the onset and progression of disease (Hou et al., 2019; Wong et al., 2020). Therefore, a comprehensive exploration of the interplay between smoking and lung bacteria has the potential to enhance the early detection of lung cancer.

Erin A Marshall et al. conducted a study where 400 bronchial brush samples were subjected to 16S microbiota sequencing. The samples were divided into an exploration group and a validation group. Over 10 years, the participants were closely monitored, and differentiated microbial communities were analyzed using linear differentiation analysis and the establishment of a linear model. The results revealed that patients with higher scores had a greater risk of developing lung cancer, and the onset occurred earlier, indicating the potential of microbiota in predicting the occurrence of lung cancer (Marshall et al., 2022).

Jun-Chieh J Tsay et al. compared oral and bronchial brush samples from individuals with lung cancer (n=39), benign lung

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TABLE 1 Summary of studies of lung cancer-associated microbiome in saliva and sputum.

Year	Reference PMID	Sample Type	Sample Size	Subgroup	Analytical Method (hypervariable regions)	Main Findings
2019	31598405	BWF and sputum	92	BWF: LUAD(n=21), LUSC(n=19) sputum: LUAD(n=37), LUSC(n=15)	16S rRNA sequencing (V3-V4)	1. compared BWF, Streptococcus (Firmicutes) in sputum is higher. 2. Streptococcus in peripheral LUAD was significantly higher than that of central lung adenocarcinoma in the sputum group
2015	26693063	saliva	86	discovery cohort: SCC (n=10), AD(n=10), control(n=10) validation cohort: SCC (n=13), AD(n=28), control(n=15)	16S rDNA sequencing (V3-V6)	1. Phyla Flavobacteriales, Burkholderiales, Campylobacterales, Spirochaetales, and genus Capnocytophaga, Selenomonas, and Veillonella in lung cancer were higher, while phylum Bacteroidales and Neisseria were lower. 2. Streptococcus and Porphyromonas were significantly decreased in the saliva of squamous cell carcinoma
2021	34787462	sputum and stool	121	NSCLC(n=87), health(n=34)	16S rRNA sequencing	1. Compared with the healthy group, Haemophilus was enriched in the lung cancer group. 2. the sputum and feces of the family Coriobacteriaceae and genus Actinomyces were enriched in the metastatic lung cancer group; 3. Pseudomonas had a high abundance in advanced metastatic lung cancer sputum.
2021	33673596	tissue and sputum	237	cohort 1: tissue: tumor (n=31) and paired tissue (n=31); sputum: cancer (n=17), healthy smokers (n=10) validation cohort: sputum:cancer(n=69), healthy smokers(n=79)	ddPCR	1. Acidovorax is overexpressed in squamous cell carcinoma tissues compared to non-squamous and adenocarcinoma tissues. 2. Capnocytophaga was overexpressed in adenocarcinoma tissues, and Haemophilus and Fusobacterium were lower than those in squamous cell carcinoma or paracancerous tissue. 3. Acidovorax, Streptococcus, and Veillonella were overexpressed in the sputum of squamous cell carcinoma, while Helicobacter was underexpressed.
2019	30942501	sputum	90	never smoking smoking: cancer(n=45), control(n=45)	16S rRNA sequencing (V1-V2)	The higher risk of lung cancer is associated with reduced levels of phylum Fusobacteria
2014	24895247	sputum and buccal samples	16	never smoking female: cancer(n=8), control(n=8)	16S rRNA sequencing (V1-V2)	1. Granulicatella, Abiotrophia and Streptococcus in sputum were enriched in lung cancer. 2. Proteobacteria (Neisseria) were enriched in Reshui Village (smoky coal). 3. Bacilli and Streptococcus (tentatively assigned to S. infantis and S. anginosus) were enriched in Laibin Town (smokeless coal). 4. Bacilli species (Streptococcus infantis and Streptococcus anginosus) were enriched in PAH-rich lung cancer samples
2022	35142041	sputum	85	NSCLC:early stage (n=22), advanced stage (n=51), unidentified(n=12)	16S rRNA sequencing (V3-V4)	1. IPaludibacter in stage IV was significantly higher than in stage III. 2. The early stage significantly enriched Firmicutes, genera Peptoniphilus, Granulicatella, Hylemonella, Actinobacillus, SMB53, and Gemella. 3. The AS group significantly enriched the phylum Actinobacteria and genus Actinomyces.
2020	32541778	sputum	34	lung cancer(n=17), control(n=17)	16S rRNA sequencing (V3-V4)	1. The unique genera of lung cancer are Actinobacillus, Pediococcus, Abiotrophia, Ruminofilibacter, Elizabethkingia, Psychrobacter, and Malus. 2. Compared with healthy controls, Bergeyella was significantly increased in lung cancer patients' sputum. Genus Haemophilus was more likely to appear in patients with lung cancer, and Bergeyella zoohelcum was significantly increased. 3. Compared with non-smoking lung cancer, Selenomonas bovis, genera Bacteroides, and Selenomonas are more prevalent in smoking patients, while Peptostreptococcus Zhouea are more prevalent in non-smoking patients. 4. Veillonella and Leptotrichia are higher in large cell carcinoma than LUAD

(Continued)

TABLE 1 Continued

Year	Reference PMID	Sample Type	Sample Size	Subgroup	Analytical Method (hypervariable regions)	Main Findings
2017	28542458	sputum	10	lung cancer(n=4), control(n=6)	16S rRNA sequencing	Streptococcus viridans, Granulicatella adiacens, Streptococcus intermedius, and Mycobacterium tuberculosis were significantly higher in lung cancer samples.
2022	36143401	sputum	80	LUSC(n=40), control(n=40)	metagenomic sequencing	1. Firmicutes, Streptococcus, Bacillus, Gemella, and Haemophilus, species Streptococcus agalactiae, were significantly increased in squamous cell carcinoma. 2. There is no difference between stage and smoking for lung cancer patients
2021	33454779	sputum	128	lung cancer(n=66), control(n=62)	16S rRNA sequencing (V3-V4)	1. There were significant increases in Streptococcus, Bacillus, Gemella, and Haemophilus in lung cancer 2. Bacteroides, Lachnoanaerobaculum, Porphyromonas, Mycoplasma, and Fusobacterium are related to chromosome mutations in patients with lung cancer. 3. Megasphaera genera and Selenomonas bovis increase
2020	32786473	saliva	43	lung cancer(n=22), control(n=21)	rFFiEF	1. six bacterial genera were significantly up-regulated in the lung cancer group, while two were down-regulated. 2. qPCR confirmed that Granulicatella increased significantly in the lung cancer group, while Pseudomonas decreased significantly
2021	35699005	bronchial biopsies, saliva, and faecal samples	41	central lung cancer (n=25), control(n=16)	16S rDNA sequencing (V3-V4)	Streptococcus, Rothia, Gemella, and Lactobacillus abundances can distinguish patients' saliva from controls.
2019	31205521	saliva	59	NSCLC(n=39), control(n=20)	16S rRNA sequencing (V1-V2)	1. Firmicutes and Veillonella, Streptococcus, Lautropia, Leptotrichia, Rothia, and Aggregatibacter in saliva of the NSCLC group was significantly higher than that of the control group. 2. The relative abundance of Fusobacterium, Prevotella, Bacteroides, and Faecalibacterium decreased in the NSCLC group

nodules (n=36), and healthy individuals (n=10). They found that the lung cancer group exhibited an abundance of Streptococcus and Veillonella, the benign lung nodule group showed enrichment of Streptophyta, Moraxellaceae, and Stenotrophomonas. At the same time, the samples from healthy individuals were enriched with Acholeplasma and Acidocella (Tsay et al., 2018). Please refer to Table 2 for more details.

3 The association between intratumoral microbiota and lung cancer

3.1 The intratumoral microbiota characteristics in lung cancer

Studies have described on the association between bacteria in alveolar lavage fluid, lung brush samples, sputum, and saliva and lung cancer (Hilty et al., 2010; Charlson et al., 2011; Erb-Downward et al., 2011; Pragman et al., 2012; Borewicz et al., 2013; Segal et al., 2013; Willner et al., 2013; Dickson et al., 2014; Bassis et al., 2015; Dickson et al., 2015), but due to the invasiveness of lung tissue, difficult to get samples. The low microbial abundance, there are few microorganism studies in lung cancer tissues.

The location of the tumor correlates with its aggressiveness, and the 5-year survival rate after pulmonary lobectomy in the lower lobe is poorer than that of the tumor in the upper lobe (Hayakawa et al., 1996; Inoue et al., 2004; Ou et al., 2007). Rea Bingula et al. analyzed 18 cases of NSCLC saliva, BALF (directly obtained from the resected lung lobe), cancerous tissue, paracancerous tissue, and distant cancer tissue and discovered that the microbiomes in saliva, BALF, and tissue were distinct. The tissues were dominated by Proteobacteria, while BALF and saliva were dominated by Firmicutes. All samples showed an increase in the abundance of Firmicutes in lower lobe tumors and a decrease in Proteobacteria. In addition, depending on the location of the tumor, Actinobacteria and Flavobacteriia have opposing abundances in BAL and extratumor tissues. Although tumor microbiota appears to be least impacted by location, paracancerous tissues exhibit the most susceptibility, with a considerable increase in resemblance to BAL microbiota in the upper lobe (Bingula et al., 2020). See Table 3 for details.

The diversity of microorganisms reflects the complexity of the entire microbiome, and existing research has studied the diversity of microorganisms in lung tissue from various subgroups. The diversity

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TABLE 2 Summary of studies of lung cancer-associated microbiome in lower respiratory tract.

Year	Reference PMID	Sample Type	Sample size	Subgroup	Analytical Method (hypervariable regions)	Main Findings
2019	31598405	Bronchial washing fluid and sputum	92	1. BWF(n=40) and sputum(n=52) 2. SCC-M0(n=7) vs AD-M0(n=7), SCC-M1(n=7) vs AD-M1(n=12)	16S rRNA sequencing (V3-V4)	1. Phylum Firmicutes and genera Veillonella, Megasphaera, Actinomyces, and Arthrobacter in AD-M0 were significantly higher than SCC-M0.2. Capnocytophaga and Rothia in AD-M1 are substantially lower than SC-M1.3. In BWF, Phylum Firmicutes and genera Streptococcus AD -M1 are markedly lower than AD-M0 4. Veillonella and Rothia in SCC -M1 were considerably higher than SCC-M0。
2022	35255902	Bronchial washing fluid	400	1. Cohort 1 (n=230): incident-(n=36), prevalent- (n=12), and no-cancer (n=182) 2. Cohort 2 (n=115): incident-(n=18), prevalent- (n=6), and no-cancer (n=91) 3. Cohort 3 (n=48): incident- (n=5), prevalent- (n=3), no-cancer (n=40)	16S rDNA sequencing, V4	the lung cancer group was strongly correlated with the Bacilli class, Lactobacillales, the Streptococcus genus and its family, and the Paenibacillus genus and its family
2016	27987594	BALF	28	lung cancer(n=20), benign diseases(n=8)	16S rRNA sequencing (V1-V3)	The phylum Firmicutes and TM7, genera (Veillonella, Megasphaera, Atopobium, and Selenomonas) are significantly increased in lung cancer; the abundance of Streptococcus, Alloprevotella, and Porphyromonas was higher in lung cancer patients with a history of smoking
2019	30994108	BALF and saliva	66	lung cancer(n=51), health control(n=15)	16S rDNA sequencing, V4	1. The BALF of LUAD and SCLC microbes at the phylum level are mainly Firmicutes, and at the genus level, they are mainly Pseudomonas. 2. LUSC: mainly Veillonella and Corynebacterium,
2020	32676331	BALF	54	lung cancer(n=32), benign diseases(n=22)	16S rRNA sequencing (V3-V4)	1. lung cancer group vs. benign disease, phylum TM7, six genera (c: TM7-3, Capnocytophaga, Sediminibacterium, Gemmiger, Blautia, and Oscillospira) increased significantly. Phylum Proteobacteria, four genera Microbacterium, Stenotrophomonas, Lautropia, and f: Pseudomonadaceae decreased significantly. 2. Capnocytophaga, Sediminibacterium and c:TM7-3 was significantly correlated with CEA and CYFRA21-1.
2021	34963470	BALF	84	low expression of PD-L1(n=59), high expression of PD-L1(n=24)	16S rRNA sequencing (V3-V4)	1. The phylum Firmicutes and the genus Veillonella dispar (Firmicutes) were significantly higher in the PD-L1 high group, and the phylum Proteobacteria and the genus Neisseria (Proteobacteria) were significantly higher in the PD-L1 low group. 2. phylum Proteobacteria and Bacteroidetes, genus Haemophilus, species Haemophilus influenzae and Neisseria perflava were higher in responders than nonresponders, while genus Veillonella and V. dispar were higher in the responder group
2022	35389889	BALF and PBMC	12	responders for ICIs (n=6), nonresponders(n=6)	16S rRNA sequencing (V3-V4)	Proteobacteria in responders is lower, while Bacteroidetesis is higher than in nonresponders.
2020	32984019	BALF	100	paired samples from cancerous lung(n=50) and the contralateral non-cancerous lung(n=50)	16S rRNA sequencing	phylum Tenericutes, genus Spiroplasma, and genus Weissella increased significantly in the lung cancer group, but phylum Bacteroidetes decreased significantly
2022	35254206	BALF	75	NSCLC(n=46), benign disease(n=29)	16S rRNA sequencing (V3-V4)	1. Prevotella, Alloprevotella, and Veillonella in lung cancer were significantly increased 2. Prevotella and Megasphaera, Alloprevotella and Actinomyces are positively correlated.

(Continued)

TABLE 2 Continued

Year	Reference PMID	Sample Type	Sample size	Subgroup	Analytical Method (hypervariable regions)	Main Findings
2018	29864375	Airway brushings	85	lung cancer(n=39), benign pulmonary nodule(n=36), and health(n=10)	16S rRNA sequencing, V4	1. Streptococcus and Veillonella are enriched in lung cancer.2. Streptophyta, Moraxellaceae, and Stenotrophomonas are enriched in benign pulmonary nodule.3. Acholeplasma and Acidocella are enriched in health
2019	31492894	BALF	103	1. lung cancer(n=49), control(n=54) 2. TCGA: ADC(n=515) and SCC(n=501)	16S rRNA sequencing (V3-V6)	1. Sphingomonas, Brevundimonas, Acinetobacter, and Methylobacterium are only in LUAD. 2. Enterobacter, Morganella, Kluyvera, and Capnocytophaga are mainly in LUSC. 3. Clusters of p _ C1 (Proteobacteria dominate) in SCC cases appear to be associated with a slow decline in survival rate

of tumor tissue is less than that of paired distant cancer tissues (Yu et al., 2016; Peters et al., 2019; Chen et al., 2022; Kim et al., 2022), Advanced lung cancer has less diversity than early lung cancer, which has less diversity than normal tissue (Kim et al., 2022), benign lung tissue is less diverse than cancer tissue (Chen et al., 2022), and the diversity of lung tissue in healthy individuals is greater than that in cancer patients, regardless of tumor tissue or tumor-adjacent tissue (Greathouse et al., 2018). The diversity of lung tissue in healthy individuals is greater than that in cancer patients (Bingula et al., 2020; Kovaleva et al., 2020; Dong et al., 2022; Peters et al., 2022), and there is no significant difference between adenocarcinoma and squamous cell carcinoma tissues (Apopa et al., 2018; Kovaleva et al., 2020). Nathan Dumont-Leblond and colleagues hypothesized that the α diversity of malignant tissues is more significant than that of neighboring tissues (Dumont-Leblond et al., 2021). There were no significant differences between GGO and paracancerous tissue in α and β diversity (Wu et al., 2022). Various studies have found that smoking has inconsistent effects on the biodiversity of tumor tissues. The majority of studies indicate that smoking reduces the α diversity of lung tissue (Liu et al., 2018; Patnaik et al., 2021; Chen et al., 2022); BALF microorganisms are intermediate between saliva and lung cancer tissue, and the α and β diversity of saliva and BALF microorganisms are more similar in patients with high amylase of BALF, possibly due to microinhalation (Patnaik et al., 2021).

3.2 The correlation between alterations in intratumoral microbial communities and tumor progression

3.2.1 Tissue microorganisms of GGO

Low-dose computed tomography (LDCT) is widely employed as the principal tool for lung cancer screening programs globally, and an increasing number of ground-glass opacities (GGOs) are being discovered (National Lung Screening Trial Research et al., 2011). Persistent GGO with a certain morphology is regarded as malignant and most likely an indolent, slowly progressing lung cancer (Chang et al., 2013). Consequently, it is essential to investigate the microecological characteristics of lung tissue with lung ground-glass nodules. Zhigang Wu et al. compared the differences between GGO and contralateral lower respiratory tract microorganisms and lung tissue microorganisms. The genus Rothia was abundant in the contralateral BALF, while the species Faecalibacterium prausnitzii and Bacteroides uniforms were abundant on the GGO side. GGO tissue and adjacent lung tissue have significant differences in microbiota composition at the level of class, order, family, genus, and species; interestingly, the majority of these are enriched in normal lung tissue, with an AUC of 91.05 percent (95 percent confidence interval: 81.93 to 100 percent) produced by 10 different genera, which has proven effective in detecting lung cancer (Wu et al., 2022).

3.2.2 Microbial communities of the area of distant cancer tissues

Guoqin Yu et al. were the first to research the lung microbial characteristics of distant cancer tissues (n = 165) in lung cancer patients. They discovered that the microbial community of lung tissues was distinct from microorganisms in other body areas and comprised a separate bacterial colony. At the phylum level, the dominant microorganisms in distant cancer tissues were Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, whereas at the genus level, the dominant Bacilli were Bacillus and Bacteroides. In distant cancer tissue, the relative abundance of Thermus is greater in adenocarcinoma than in squamous cell carcinoma, whereas Ralstonia is diminished (Yu et al., 2016).

3.2.3 Differentiated microbial communities of lung cancer tissue versus adjacent tissues

Compared to distant metastatic tissues, the microbial differences between lung cancer tissue and adjacent tissue provide a more accurate reflection of lung cancer.Hui Dong et al. discovered Massilia, Phenylobacterium, and Pseudoxanthomonas were enriched in lung cancer tissue; Brevibacillus, Cupriavidus, and Anaerococcus were more prevalent in neighboring tissues; and Brevundimonas, Ruminococcus, and Polaromonas were significantly different between squamous cell and adenocarcinoma carcinomas. The tumor tissues of smokers include more Massilia and Sphingobacterium and fewer Acidovorax, whereas TP53 mutation-positive tumors are rich in Acidovorax and Massilia (Dong et al., 2022). Acidovorax was also found to be more

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TABLE 3 Summary of studies of lung cancer-associated microbiome in Lung tissues.

Year	Reference PMID	Sample Type	Sample size	Subgroup	Analytical Method (hypervariable regions)	Main Findings
2016	27468850	lung tissues	196	tumor(n=31), the area distant from the tumor(n=165)	16S rRNA sequencing, (V3–V5)	1. More proteobacteria, Thermi, and Cyanobacteria exist in the lung tissues than in other body parts. 2. Compared with patients without metastasis, the relative abundance of Proteobacteria in remote cancer tissues of patients with metastasis has significantly increased
2018	30323970	lung tissues	40	emphysema(n=10), tumor(n=11) and both(n=19)	16S rRNA sequencing (V4)	1. Firmicutes and Bacteroidetes increased significantly in the lung cancer group. At the genus level, Prevotella (Bacteroidetes), Bifidobacterium (Actinobacteria), Proteobacteria (primary the genera) of Streptococcus (Firmicutes) and Escherichia/Shigella and Haemophilus (Proteobacteria). 2. Acinetobacter and Acidovorax increased significantly in the emphysema group
2021	32340803	lung tissues, BALF, and saliva	48	recurrence(n=18), no recurrence(n=18)	16S rRNA sequencing, (V3-V4) and RNA Seq	1. Among saliva, Delftia and Bifidobacterium genera are twice as high in patients with recurrence as in non-recurrence patients. 2. The abundance of Staphylococcus was higher in patients with recurrence of lung cancer tissues, and the abundance of Bacillus and Anaerobacillus was lower. There was no difference in non-lung cancer tissue between the two groups. 3. There were differences in the 19 genera between the two groups in BALF.
2022	35479075	lung tissues	53	high-IAP(n=17), low IAP(N=17) and FLC(n=19)	16S rRNA sequencing and RNA-seq	The enrichment microorganisms are divided into opportunistic pathogens, probiotics, and microorganisms that degrade pollutants. The third category involves Sphingomonas, Sphingopyxis, etc., which are helpful for degrading pollutants but may also lead to epithelial damage and chronic inflammation.
2022	35693079	lung tissues	216	AC EGFR+(n=54), AC EGFR-(n=54), SCC (n=54) and adjacent normal tissues(n=54)	16S rRNA sequencing	Stenotrophomonas accounted for most of the NSCLC tissues with recurrence, and Haemophilus influenzae (H. influenzae) was enriched in patients with squamous cell carcinoma.
2022	36091439	lung tissues, BALF	74	diseased BALF(n=11), paired controlvBALF (n=11), GGO(n=26) and distant control (n=26)	16S rRNA sequencing, (V4/V3/ V3-V4/V4-V5)	1. In BALF, Rothia were higher on the healthy side, Faecalibacterium prausnitzii and Bacteroides were higher on the GGO side. 2. At the phylum level, Proteobacteria have a significant difference in tumor tissue and adjacent tumor tissue.
2022	36303210	lung tissues	80	tumor(n=39), the area distant from the tumor(n=41)	16S rRNA sequencing (V4)	1. In tumor tissue, higher abundance of Pseudomonadales, Actinomycetales, and Marmoricola aurantiacus species was associated with poorer survival, especially DFS. 2. the higher abundance of Bacteroidia and Clostridia and Bacteroidales and Clostridiales is correlated with the lower survival rate in normal lung tissues.
2020	32450847	lung tissues, BALF, and saliva	79	Upper lobe T(n=10), Lower lobe T(n=8)/ saliva(n=17), BAL (n=15), T(n=16), PT (n=14), DT(n=17)	16S rRNA sequencing (V3-V4)	1. Proteobacteria were dominant in tissue samples, while firmicutes increased in the BAL and saliva, class Clostridia and Bacilli, respectively. 2. All samples showed an increased abundance of Firmicutes in the upper lobe of the tumor. However, Proteobacteria decreased. 3. clades Actinobacteria and Flavobacteriia showed opposite abundance between the BAL and peritumoral tissues, depending on the location of the lung lobes.
2020	30733306	lung tissues	38	tumor(n=19), the area distant from the tumor(n=19)	16S rRNA sequencing (V4)	1. In tumor tissues, families Koribacteraceae and Lachnospiraceae were associated with reduced recurrence and disease-free survival. 2. lung tumor tissues had a higher Veillonellaceae family abundance. There is a lower abundance of the Cloaciobacter genus and a lower abundance of the Erysipelotrichaceae family.

(Continued)

TABLE 3 Continued

Year	Reference PMID	Sample Type	Sample size	Subgroup	Analytical Method (hypervariable regions)	Main Findings
2021	33891617	lung tissues	58	tumor(n=29), the area distant from the tumor(n=29)	16S rRNA sequencing (V3-V4)	There are no significant differences in bacteria among groups.
2020	32933105	lung tissues	178	tumor(n=89), the area distant from the tumor(n=89)	16S rRNA sequencing (V3-V4)	1. The content of gram-positive bacteria was significantly increased in LUAD. 2. The combination of high bacterial load and increased iNOS expression in the tumor was a favorable prognostic symptom factor, while the combination of high bacterial load and increased $FOXP3^+$ cell number was a marker of poor prognosis.
2018	30127774	FFPE	29	LUAD(n=11), LUSC (n=10), adjacent normal FFPE samples(n=8)	16S rRNA sequencing	1. The comparison of Actinutes and Bacteriodetes in NSCLC tissues significantly differs from that in normal tissues. 2. Firmicutes and Proteobacteria are significantly lower in adenocarcinoma than in squamous cell carcinoma, and Cyanobacteria are significantly higher in adenocarcinoma. Melainabacteria is only found in squamous cell carcinoma
2022	35005565	lung tissues	286	tumor(n=143), the area distant from the tumor(n=143)	16S rRNA sequencing (V3-V4)	1. Massilia, Phenylobacterium, and Pseudoxanthomonas are mainly distributed in tumor tissues, while Brevibacillus, Cupriavidus, and Anaerococcus are more abundant in non-tumor tissues. 2. Brevundimonas, Ruminococcus, and Polaromonas differed between squamous cell carcinoma and adenocarcinoma. 3. More Massilia and Sphingobacterium and less Acidovorax in the tumor tissues of smokers 4. Acidovorax and Massilia were rich in TP53 mutation-positive tumors.
2018	30143034	lung tissues	336	tumor(n=143), the area distant from the tumor (n=144), control(n=49) TCGA: tumor(n=974), normal adjacent(n=108)	16S rDNA sequencing (V3)	Acidovorax showed a higher abundance in squamous cell carcinoma tissues with TP53 mutations

prevalent in lung cancer tissue from TP53-mutated squamous cell carcinoma patients, according to K Leigh Greathouse et al. (Greathouse et al., 2018). The recent study aimed to characterize the taxonomic profiles of the microbiota in oral saliva, cancerous, and paracancerous tissues of Chinese patients diagnosed with lung adenocarcinoma. The Shannon index of cancerous tissues (CT) was significantly higher compared to that of paracancerous tissues (PT) and saliva. The observed increased relative abundance of Promicromonosporacea and Chloroflexi, coupled with the decreased relative abundance of Enterococcaceae and Enterococcus in lung tissues, may potentially be associated with the risk of developing lung adenocarcinoma (Zhou et al., 2023).

Multiple factors, including hereditary and environmental exposures, contribute to the development of lung cancer. COPD patients are three to ten times more likely to develop lung cancer than healthy smokers (Etzel et al., 2008). Despite the fact that there are overlapping susceptibility genes (Young et al., 2008), there are also individual differences. Given that smoking is the most prevalent risk factor for lung cancer (Chaturvedi et al., 2010; Yu et al., 2014; Zhou et al., 2017), Yanhong Liu et al. collected 40 lung tissues (10 pulmonary bullae, 11 lung tumors, and 19 both lung cancer and bullae) to assess if environmental factors such as smoking influence changes in respiratory bacteria. Proteobacteria (genera Acinetobacter and Acidovorax) are more frequent in the emphysema group (Liu et al., 2018). However, the tissue sampled in this study is distant from tumor tissue, and there is a space between the cancer tissue and the sampled tissue.

3.2.4 Lung cancer tissue microbes and recurrence

Lung tissue bacteria are capable of predicting the recurrence of lung cancer. The five-year survival rate for stage IA lung cancer is 83%, whereas the five-year survival rate for stage IIB lung cancer is 53% (Kay et al., 2017), and early detection of lung cancer recurrence can lessen its mortality rate. Current research on lung cancer recurrence focuses on the microenvironment of the tumor, including mutational burden (Owada-Ozaki et al., 2018), immune cell infiltration (O'Callaghan et al., 2015) and local gene expression (Planck et al., 2013; Kratz et al., 2021; Wang et al., 2021). Researchers are gradually becoming aware of the function of lung microorganisms in the occurrence and progression of lung cancer; thus, it is vital to investigate the association between early lung cancer recurrence and lung bacteria. Santosh K. Patnaik et al. compared the lung microorganisms of saliva, alveolar lavage fluid,

and tissue in patients with stage I NSCLC and performed RNA sequencing on lung tissue. They discovered that Delftia and Bifidobacterium, two salivary bacteria, rose considerably in recurrent patients. Staphylococcus was more common in the lung cancer tissues of relapsed patients, but Bacillus and Anaerobacillus were more common in non-recurrent patients. There were no significant differences between the two groups in non-cancerous tissues, but there were differences in 19 genera in the BALF sample. Phingomonas, Psychromonas, and Serratia genera increased in the lung cancer recurrence group, whereas Cloacibacterium, Geobacillus, and Brevibacterium genera decreased. Lower respiratory bacteria are capable of identifying NSCLC patients with surgical recurrence, as indicated by the area under the curve value of 0.77 (Patnaik et al., 2021).

Brandilyn A. Peters et al. investigated the relationship between microbiome and recurrence in tumor and distant cancer tissue specimens (39 lung and 41 distant cancer tissues) from 46 individuals with NSCLC at stage II. There was no difference in α diversity and β diversity between cancerous and noncancerous tissues discovered. However, both α-diversity and β-diversity of the microbiota in lung cancer tissues were found to be associated with disease-free survival (DFS). In contrast, the α-diversity and β-diversity of the microbiota in adjacent tissues showed no association with DFS, recurrence-free survival (RFS), or overall survival (OS). A high abundance of species Pseudomonadales and Actinomycetales and Marmoricola aurantiacus in lung cancer tissues was negatively associated with disease-free survival (DFS). In contrast, high abundance of Bacteroides and Clostridia and orders Bacteroidales and Clostridiales in distant cancer tissues were negatively associated with survival. Survival was favorably linked with ASV of Alphaproteobacteria and Betaproteobacteria, Burkholderiales, Neisseriales, and Mycobacterium vaccae. Marmoricola aurantiacus is abundant in lung cancer tissue from individuals who have a poor prognosis (Peters et al., 2022).

Ock-Hwa Kim et al. evaluated the relationship between microbes and prognosis (recurrence) of lung cancer tissues of various pathological types and discovered that in the tissues of patients with relapsed NSCLC, the genus Stenotrophomonas accounted for the vast majority, whereas H. influenzae was enriched in patients with squamous cell carcinoma (Kim et al., 2022). Gram-positive bacteria are significantly elevated in lung adenocarcinoma, and a high bacterial load in tumors combined with increased iNOS expression is a favorable prognostic symptom factor. In contrast, a high bacterial load in tumors combined with increased $FOXP3^+$ cell numbers is an indicator of a poor prognosis (Kovaleva et al., 2020).

Brandilyn A. Peters et al. demonstrated for the first time the association between normal lung tissue and lung cancer prognosis, discovering that the diversity and overall microbial composition of normal tissue were associated with decreased recurrence-free survival and disease-free survival. The more prosperous family Koribacteraceae was associated with improved recurrence-free and disease-free survival in normal tissues. Still, the Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae families were inversely associated with recurrence-free survival. The diversity and composition of tumor tissue are not associated with relapse-free survival. The richness and diversity of tumor tissue are lower than that of paired normal tissue (Peters et al., 2019).

3.3 The association between intratumoral microbiota and factors like IAP, smoking, and family inheritance

Y. Chen et al. compared the microbiome of lung cancer patients affected by familial lung cancer (FLC) and indoor air pollution (IAP). Additionally, they describe potential links between host gene expression patterns and their microbiome. The research revealed that smoking and IAP significantly reduced the biodiversity of specific OTUs, particularly in normal lung tissue. Enhanced microorganisms include Sphingomonas and Sphingopyxis, which can decompose pollutants but can also induce epithelial injury and promote chronic inflammation. RNA sequencing data emphasize the IL17, Ras, MAPK, and Notch pathways in the FLC and IAP groups, which are associated with carcinogenesis and reduced immune function (Chen et al., 2022).

4 The interaction between lung microbiota and immune therapy

Only approximately 20% of patients with NSCLC derive therapeutic benefits from the anti-PD-1 antibody nivolumab. Hence, the identification of more accurate predictors becomes imperative. In their study, Chufeng Zhang et al. examined and compared the differences and correlations in gut microbes and salivary microorganisms following PD-1/PD-L1 immunotherapy. Interestingly, they observed no significant difference in respiratory microbial alpha diversity between the effective (R) and ineffective (NR) groups. However, there were notable variations in beta diversity and the abundance of Streptococcus (a respiratory flora) was found to be associated with progression-free survival (PFS) (Zhang et al., 2021). In terms of sputum α diversity, no significant difference was observed between immunotherapy-responsive and non-responsive patients, although a significant difference was noted in terms of β diversity (Zhang et al., 2021).

Gaining insight into the relationship between lower respiratory tract bacteria and therapeutic efficacy can enhance our understanding of potential markers that accurately predict the effectiveness of clinical immunotherapy. Previous studies have established a connection between alterations in lung microorganisms and immune evasion mediated by PD-L1-dependent Treg cells (Gollwitzer et al., 2014). Hye Jin Jin Jang et al. investigated the correlation between microorganisms in lung BALF and PD-L1 expression, as well as the association between microbes and the efficacy of immunotherapy. Their findings revealed a higher prevalence of Veillonella dispar (Firmicutes) in the BALF of patients exhibiting high PD-L1 expression, while Neisseria (Proteobacteria) was more commonly found in the BALF of patients with low PD-L1 expression. The immune response group had a higher abundance of Bacteroidetes and a lower abundance of Proteobacteria (Masuhiro et al., 2022). Conversely, the non-response group exhibited higher levels of the phyla Proteobacteria and Bacteroidetes, as well as the genera Haemophilus and species Haemophilus influenzae and Neisseria perflava. Additionally, the non-response group showed a lower presence of Firmicutes.

Hye Jin Jang et al. studied the lower respiratory tract microorganisms in 84 patients, including 59 patients with low PD-L1 expression and 25 patients with high PD-L1 expression. Their findings revealed a higher prevalence of Veillonella, specifically the species V. dispar, in the immune response group (Jang et al., 2021). Consequently, further research should prioritize investigating the mechanisms associated with the genus Veillonella in the immune response.

The responder group to immune checkpoint inhibitors (ICIs) exhibited higher α-diversity compared to the non-responder group, while there was no difference in β-diversity between the two groups (Masuhiro et al., 2022). Hye Jin Jang et al. also reported that there were no significant differences in α- and β-diversity between the two groups with high and low PD-L1 expression levels (Jang et al., 2021).

5 Potential mechanisms through which commensal microorganisms could impact the host's immune system in relation to lung cancer development

There is growing evidence that symbiotic microbial communities of the host are linked to the onset, progression, and therapeutic efficacy of cancer. The current literature focuses on the gut microbiome, but how the pulmonary symbiotic microbiota and the distal gut microbiota are engaged in the malignant transformation of lung cancer cells and how they simultaneously manage the balance between pro-tumor inflammation and anti-tumor immunity are not well understood. We will comprehensively overview the potential immune mechanisms involved in commensal microorganisms across four important topics (Figure 2).

5.1 The microbes inducing DNA damage and genomic instability

Genotoxins and metabolites produced by bacteria possess the capability to directly inflict damage upon the host's DNA and instigate genomic instability via the generation of reactive oxygen or nitrogen species and the activation of innate immune receptors. When the cumulative effects of such damage exceed the host's self-regulation capacity, it can lead to the carcinogenic impacts (Espinoza and Minami, 2018; Goto, 2020; Goto, 2022). Studies have shown that reactive oxygen species produced by Porphyromonas, hydrogen sulfide produced by Clostridium cholephilum, and superoxide dismutase produced by symbiotic bacteria can cause genomic instability and increase susceptibility to lung cancer (Attene-Ramos et al., 2006; Carbonero et al., 2012). Additionally, microbial dysbiosis can lead to increased reactive oxygen species associated with DNA damage (Mao et al., 2018).

The enrichment of bacteria such as Massilia and Acidovorax in the pulmonary system is consistent with the trends observed in DNA recombination and repair pathways (Dong et al., 2022). Folate produced by Lactobacillus and Bifidobacteria promotes the production of 6-methyltetrahydrofolate, which influences DNA methylation (Kothapalli et al., 2005; Hassan and Zempleni, 2006; Rossi et al., 2011; Zempleni et al., 2012). These studies demonstrate that microorganisms can directly or indirectly contribute to DNA damage or genomic instability.

5.2 Activation and regulation of the innate immune system in response to microbial influence

Microbial dysbiosis can lead to the activation of the host's innate immune system and sustained chronic inflammation through the

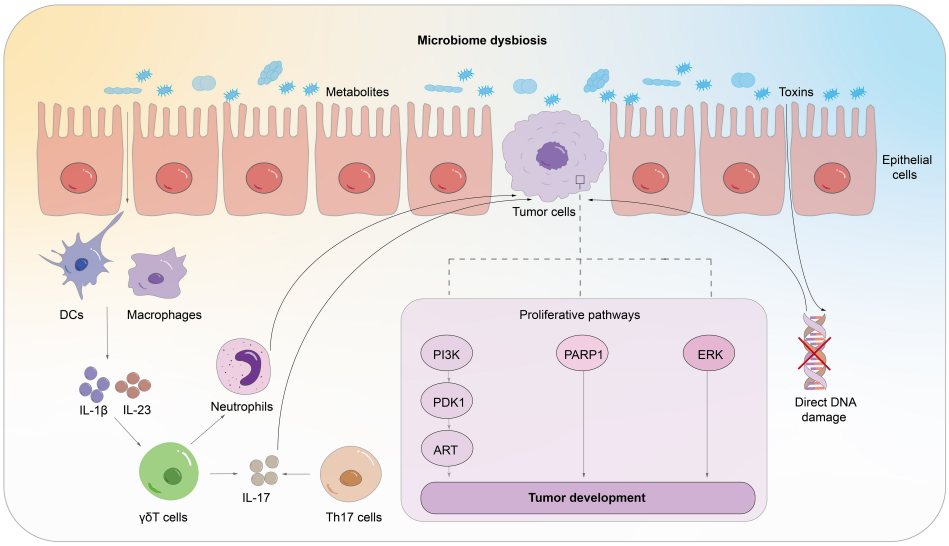

FIGURE 2
The potential mechanism through which commensal microorganisms could impact the host's immune system in lung cancer pathogenesis. The microbiota generates cytotoxicity which induces host cell DNA damage, abnormal activation of proliferation and transformation pathways, and aberrant immunological pathways.

interaction of microbial-associated molecular patterns (MAMPs) with pattern recognition receptors (PRRs) (Evavold and Kagan, 2019; Fitzgerald and Kagan, 2020; Li and Wu, 2021). Antigen-presenting cells, such as alveolar macrophages, dendritic cells, and epithelial cells, can recognize microbial stimulation through PRRs, triggering the expression of immune-related genes and initiating innate and adaptive immune responses (Sellge and Kufer, 2015). Specifically, the activation of Toll-like receptors (TLRs) by microbial products induces the activation of alveolar macrophages and neutrophils (Chang et al., 2014; Segal et al., 2016; Jin et al., 2019). Studies have also demonstrated the upregulation of TLR-4 and TLR-9 expression in lung cancer tissue (Zhang et al., 2009; Goto, 2022). Stimulation of TLR4 using heat-inactivated Escherichia coli has been shown to activate the p38 MAPK and ERK1/2 signaling pathways, leading to adhesive, migratory, and metastatic behavior of non-small cell lung cancer (NSCLC) cells *in vivo* (Chow et al., 2015).

A reduction in microbial diversity hampers the stimulation of antigen-presenting cells, thereby impeding immune responses against tumor antigens (Bingula et al., 2017). Moreover, bacterial overgrowth triggers excessive mucosal immune responses, leading to pro-inflammatory reactions. These reactions, often mediated by the Th17 mechanism, can result in uncontrolled cell proliferation. Excessive activation of the innate immune system results in an expansion of regulatory T cells (Tregs) and M2 macrophages. These immune cells release various molecules, including prostaglandin E2 (PGE2), transforming growth factor-beta (TGF-B), and interleukin-10 (IL-10), which are known for their immunosuppressive properties. Additionally, there is an upregulation of programmed death-ligand 1 (PD-L1), contributing to immune tolerance and tumor evasion (Soroosh et al., 2013; Hussell and Bell, 2014).

5.3 Activation and functional regulation of the adaptive immune system in response to microbes

C.Jin et al. noted that pulmonary symbiotic microorganisms can stimulate the expression of IL-1β and IL-23, thereby inducing the proliferation of $V\gamma6^+$ $V\delta1^+\gamma\delta$ T cells that produce IL-17 and other activated molecules, promoting inflammation and tumor cell proliferation (Jin et al., 2019). Additionally, excessive bacterial overgrowth leads to overactivation of the adaptive immune system and proliferation of Th17 cells, contributing to the occurrence and progression of lung cancer (Chang et al., 2014).

Research has established that certain bacteria present in non-small cell lung cancer (NSCLC) are positively correlated with the presence of Th17 and Th1 cells. This correlation suggests a potential link between the microbiome and immune response in NSCLC (Ma et al., 2017). Pasteurella is significantly positively correlated with cytotoxic CD8+ tumor-infiltrating lymphocytes (TILs) and negatively associated with M2 macrophages. However, Coriobacteriaceae is significantly positively correlated with M2 macrophages and negatively correlated with CD8+ TILs (Zheng et al., 2020). Dysbiosis can trigger the expression of PD-L1 on $CD11b^+CD103^-$ dendritic cells in the lungs and modulate the activation of regulatory T cells (Gollwitzer et al., 2014).

Moreover, short-chain fatty acids (SCFAs) as microbial metabolites (Segal et al., 2017) induce the upregulation of the forkhead box P3 (FoxP3) in $CD4^+$ lymphocytes, leading to the development of regulatory T cells (Treg) and subsequent immune tolerance (Trompette et al., 2014). However, elevated SCFAs levels in the lungs may inhibit the production of interferon-gamma (IFN-γ) by $CD4^+$ and $CD8^+$ T cells, resulting in T cell exhaustion and suppression of cytotoxicity against malignant cells (Segal et al., 2017).

5.4 The interaction between microbes and epithelial cells

The stimulation of epithelial cells by microorganisms can lead to abnormal activation of proliferation and transformation pathways, resulting in carcinogenic effects. The presence of specific microbial species in non-small cell lung cancer (NSCLC) has been found to be associated with the dysregulation of oncogenic transcriptional systems. Research findings indicate that Prevotella, Streptococcus, Veillonella, and tiny Vibrio can stimulate airway epithelial cells, activating the ERK and PI3K signaling pathways. Excitingly, Cyanobacteria-produced microcystins promote cell proliferation by inducing PARP1 overexpression (Apopa et al., 2018).

Stimulation of epithelial cells by nontypeable Haemophilus influenzae (NTHi) leads to the expression of IL17C, which in turn promotes neutrophil infiltration and chronic inflammation and facilitates tumor progression (Jungnickel et al., 2017). Furthermore, interleukin-6 (IL-6) has been shown to promote lung cancer growth by inducing inflammatory responses (Ochoa et al., 2011). Furthermore, CD36 is a crucial link between lung microbiota and cancer. It regulates microcystin processing in the alveoli, increasing PARP1 expression and promoting non-small cell lung cancer development (Apopa et al., 2018).

6 Conclusion

In this review, we evaluated the link between respiratory tract flora and lung cancer, focusing on the characteristics of microbial flora in different locations of lung cancer, including saliva, sputum, BALF, bronchial brushing samples, and tissues, and analyzing the relationship between microbial flora in other parts and genes, environmental factors, pathological types, stages, treatment, and prognosis. The studies on the potential role of microorganisms in the pathogenesis and progression of lung cancer were reported in this article.

So far, the following conclusions can be drawn: Firstly, independent of locations, the microbial composition of lung cancer patients differed considerably from that of healthy individuals, indicating that the microbial flora has the potential to act as a biomarker for the diagnosis and prognosis of lung cancer. Secondly, microorganisms found in the lower respiratory tract, such as BALF, BWF, and tissues, are more suggestive of lung cancer. Finally, in lung cancer patients, pulmonary symbiotic

bacteria directly or indirectly promote or inhibit tumor progression via the pro-inflammatory and anti-inflammatory balance, immune response dysfunction, metabolic pathway, DNA damage, and genomic instability. In conclusion, pulmonary microbiomes may be biomarkers for predicting lung cancer's onset, progression, and prognosis. The causal association between respiratory microbes and lung cancer must be established through more mechanistic research.

The result contradicts between samples reflects the dynamic change of microbiota in carcinogenesis, suggesting that specific bacteria may play different roles at different time points and different host locations (Ramirez-Labrada et al., 2020), which may also be due to the small sample size, regional, dietary, and other factors to resulting in the identical examination of several bacteria. To determine the microbiological characteristics of lung cancer, large-scale microbial investigations on lung cancer with control of intervention factors will be required in the future.

Nowadays, lung cancer is the most lethal tumor, placing a substantial burden on the lives and economies of people all over the world. Therefore, research into the complete pathophysiology of lung cancer, the quest for new therapeutic techniques, and the development of specialized therapeutic medications remain essential for the prevention and treatment of this condition. Due to the intense research interest in the mechanism of action of intestinal microbes on the incidence of colorectal cancer and the effect of pharmacological therapy, the association between pulmonary microbial flora and lung cancer has been increasingly recognized, but it remains a descriptive study. Future studies on the mechanism, prevention, and therapy of microbial involvement are urgently required. Numerous confirmatory cellular and animal experiments must be conducted, which will likely open up new avenues for researching lung cancer disease mechanisms.

Author contributions

JC: Writing – original draft, Writing – review & editing. LZ: Funding acquisition, Writing – review & editing. HW: Supervision, Writing – review & editing.

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Conflict of interest

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

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Causal relationship between the gut microbiota and insomnia: a two-sample Mendelian randomization study

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Background: Changes in the gut microbiota are closely related to insomnia, but the causal relationship between them is not yet clear.

Objective: To clarify the relationship between the gut microbiota and insomnia and provide genetic evidence for them, we conducted a two-sample Mendelian randomization study.

Methods: We used a Mendelian randomized two-way validation method to discuss the causal relationship. First, we downloaded the data of 462,341 participants relating to insomnia, and the data of 18,340 participants relating to the gut microbiota from a genome-wide association study (GWAS). Then, we used two regression models, inverse-variance weighted (IVW) and MR-Egger regression, to evaluate the relationship between exposure factors and outcomes. Finally, we took a reverse MR analysis to assess the possibility of reverse causality.

Results: The combined results show 19 gut microbiotas to have a causal relationship with insomnia (odds ratio (OR): 1.03; 95% confidence interval (CI): 1.01, 1.05; p=0.000 for class. Negativicutes; OR: 1.03; 95% CI: 1.01, 1.05; p=0.000 for order.Selenomonadales; OR: 1.01; 95% CI: 1.00, 1.02; p=0.003 for genus.RikenellaceaeRC9gutgroup). The results were consistent with sensitivity analyses for these bacterial traits. In reverse MR analysis, we found no statistical difference between insomnia and these gut microbiotas.

Conclusion: This study can provide a new direction for the causal relationship between the gut microbiota (class.Negativicutes, order.Selenomonadales, genus.Lactococcus) and insomnia and the treatment or prevention strategies of insomnia.

KEYWORDS

insomnia, gut microbiota, causal relationship, Mendelian randomization, bacterial traits

1 Introduction

Insomnia refers to a sleep disorder characterized by frequent and persistent difficulty falling asleep or maintaining sleep despite appropriate sleep opportunities and sleep environments (Sutton, 2021; Cunnington et al., 2013). The disease is mainly characterized by difficulty in falling asleep, dreaminess, easy awakening, and early awakening and is often accompanied by physical symptoms (pain, nervous, and numbness) and mental disorders (depression, anxiety, and irritability). According to the statistics, more than 30% of the worldwide population experiences one or more symptoms of insomnia (Madari et al., 2021), seriously affecting the lives and work of patients. The pathogenesis of insomnia is very complicated. The occurrence and development of insomnia are closely related to individual factors and various environmental factors. Susceptible factors, inducing factors, and maintaining factors play a very important role (Proserpio et al., 2020).

Recently, increasing evidence shows that changes in the gut microbiota are closely related to host health (Agus et al., 2018; Morrison and Preston, 2016). The microbiota–gut–brain axis has been confirmed to be related to multi-system diseases, such as the nervous system, and participates in the pathogenesis of many mental diseases (Forslund et al., 2017; Cox and Weiner, 2018; Loo et al., 2020). The gut microbiota is called the "second genome" of the human body (Preethy et al., 2022). The ratio of bacteria to human cells is now considered to be closer to 1:1, and the genes it contains are 100 times that of human coding genes. The gut microbiota has been shown to regulate body health and brain function by participating in food digestion and decomposition (Burokas et al., 2017), regulating bile acid metabolism (Burokas et al., 2017), resisting pathogen invasion (Cheng et al., 2019), and participating in immune response (Yang and Cong, 2021). At present, there have been reports about insomnia and the gut microbiota. Thaiss et al. (2016) found that, on the one hand, changing the sleep pattern of mice can change the structure and diversity of their gut microbiota, and on the other hand, changes in the gut microbiota can affect the diurnal fluctuations of host physiology and disease susceptibility. Hua et al. (2020) found that the abundance of some bacteria in autistic children with sleep disorders decreased, which further affected the changes in levels of various neurotransmitters, and which, in turn, may aggravate sleep problems and autism spectrum disorder in children. However, the specific mechanism has not been reported. Therefore, exploring the causal relationship between the gut microbiota and insomnia is particularly important for explaining the incidence, treatment, and prevention of insomnia.

Mendelian randomization study (MR) is a method used in epidemiology to evaluate the relationship between disease exposure factors and disease risk (Sekula et al., 2016; Chen et al., 2021). The genetic variation is used as an instrumental variable to control the bias of causality estimation between disease and exposure factors and eliminate the influence of unmeasured confounders so as to draw strong causal inference. It has been used in the study of many diseases, such as those investigating the relationship between coffee consumption and kidney function (Kennedy et al., 2020), smoking and stroke (Larsson et al., 2019), and rheumatoid arthritis and osteoporosis (Liu et al., 2021). This study is based on pooled data from the Genome-Wide Association Study (GWAS) and explores the causal relationship between the gut microbiota and insomnia using a two-sample MR method.

2 Materials and methods

2.1 Study design process

The exposure data and outcome data in this article are derived from public databases, so this study does not require ethical approval, an informed consent form, or other relevant materials. We used the MR method to analyze the causal relationship between gut microbiota and insomnia using genetic instrumental variables (IVs). The study flow chart is shown in Figure 1.

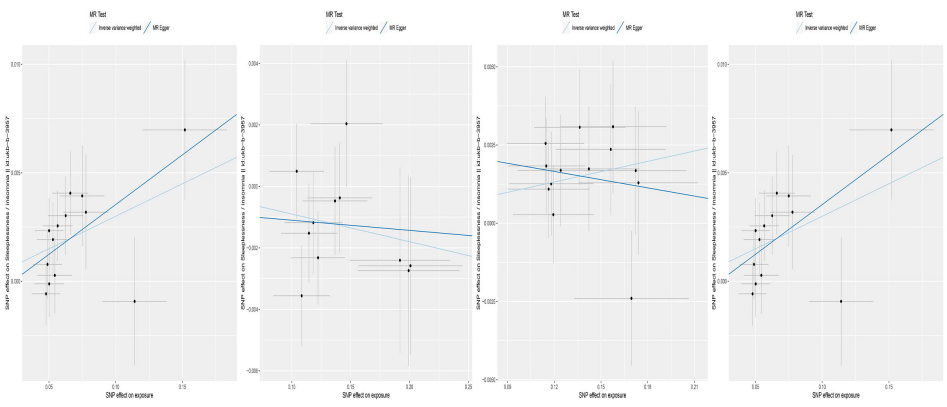

FIGURE 1
The study design of the associations between gut microbiota and insomnia.

2.2 Source of exposure factors

We downloaded the gut microbiota data from https://mibiogen.gcc.rug.nl/, the largest, multi-ethnic genome-wide meta-analysis of gut microbes to date. The data is a collection of 16S rRNA gene sequencing profiles and genotyping data from 18,340 subjects from 11 countries in Asia and Europe by the MiBioGen Consortium. It maps the microbiome signature gene to identify genetic loci that influence the relative abundance or presence of microbial taxa, all of which are in the GWAS database. We diluted all datasets to 10,000 reads per sample to account for differences in sequencing depth and to classify them. The correlation analysis of age, sex, technical covariates, and genetic principal components was carried out. Finally, after eliminating unknown gut microbiotas, all available GWAS summary statistics of 196 bacterial taxa (including 9 Phylums, 16 Classes, 20 Orders, 32 Families, and 119 Genera) were eventually included in the MR analysis.

2.3 Source of outcome factors

We obtained online insomnia data through GWAS (https://gwas.mrcieu.ac.uk/), Dataset: ukb-b-3957, POPULATION: European, sex: male and female, samples size: 462,341, number of SNPs: 9,851,867.

2.4 Selection of IVs

We screened for the statistically significant single-nucleotide polymorphisms (SNPs) as IVs for subsequent studies. After extracting the relevant information, we used the formula [R^2=2×MAF× (1−MAF) ×β^2, F= R^2 (n-k-1)/k (1-R^2), MAF: minor allele frequency of SNPs, n: the sample size, k: the number of IVs employed] to calculate the proportion of variation explained (R^2) and F-statistics. We defined the condition of IVs, which is $p < 1 \times 10^{-5}$, the linkage disequilibrium (LD) threshold at R^2<0.001, and clumping distance = 10,000 kb to ensure IVs were independent of each other (Ning et al., 2022; Johnson et al., 2008). The SNPs with the lowest p-value associated traits were retained and 196 bacterial traits were clustered. Then, with the help of the screened SNPs, the relationship between the gut microbiota and insomnia was identified using different algorithms including the inverse-variance weighted (IVW) method, weighted median method, MR-Egger regression, and the MR pleiotropy residual sum and outlier (MR-PRESSO) test. Finally, IVs were set to ($p<1\times10^{-8}$) in reverse MR analysis.

2.5 Main analysis

We analyzed the relationship between the gut microbiota and insomnia using the IVW method, which provides the most accurate effect estimation for this study. Therefore, the IVW method was used as the main analysis method in this paper. When there is no pleiotropy in IV, the IVW method has high power and accuracy and can obtain the effect estimate with the minimum bias. In this paper, we obtained the IVW mean of SNP ratio estimates by regression of SNP-gut microbiota and SNP-insomnia association (Burgess et al., 2013).

2.6 Pleiotropy test

The IV pleiotropy increases the probability of type I errors in MR analysis, and if it is eliminated directly, it may lead to a weak IV (Burgess and Thompson, 2013). Therefore, we used MR-Egger regression to test for potential horizontal pleiotropy, and if P <0.05, horizontal pleiotropy was present. The MR-Egger regression method tests for potential horizontal pleiotropy, which is a supplementary method in this paper. MR-Egger regression, which weighted linear regression of SNP-gut microbiota and SNP-insomnia effect estimates, provided a valid causal effect estimate even when all SNPs were null instruments. When the direction of the two results is consistent, it is considered a relatively stable causal association.

2.7 Sensitivity analysis

Since the results of the IVW method are susceptible to valid instrumentation and potential pleiotropic effects, we performed sensitivity analyses to assess the robustness of the association, and when the results were consistent, it was indicated that the causal effect was significantly robust. This study also used the weighted median method for sensitivity analysis and comparison with the results obtained from the main analysis. Finally, we performed the MR-PRESSO test, conducting a global test of heterogeneity to identify whether SNPs were present with possible outliers, and we obtained corrected association results after the test.

2.8 Reverse MR analysis

Reverse MR analysis was performed only if all MR methods supported an association between the gut microbiota and insomnia. That is, the causal relationship between insomnia as exposure and the gut microbiota as outcome was assessed. The study sample, method principle, and analysis steps of the reverse MR analysis were the same as those of the forward MR analysis. The direction of the causal effect was further confirmed when the forward MR analysis was statistically significant and the reverse MR analysis was not.

2.9 Statistical analysis

The above analysis was completed using R 4.3.0 and RStudio software, and we conducted relevant research based on the "TwoSampleMR" and "ggplot2" package environments. The evaluation indexes were odds ratio (OR) and 95% confidence interval (95% CI), and P <0.05 was statistically significant.

3 Results

3.1 Main results of the 196 bacterial traits with the risk of insomnia

We analyzed and identified 19 gut microbiotas that were significantly associated with insomnia using the IVW method (Figure 2).

We found that the three gut microbiotas (class.Negativicutes, order.Selenomonadales, genus.RikenellaceaeRC9gutgroup) were significantly positively correlated with insomnia using the IVW method and the weighted-median method, whereas the MR-PRESSO test did not detect outliers (P<0.05), and MR-Egger regression showed no pleiotropy (P>0.05). One gut microbiota (genus.Lactococcus) was significantly negatively correlated with insomnia using the IVW method and the weighted-median method, whereas the MR-PRESSO test did not detect outliers (P <0.05), and MR-Egger regression showed no pleiotropy (P>0.05) (Table 1, Figure 3).

We found that the other 15 gut microbiotas were significantly associated with insomnia using the IVM method, (OR: 0.98; 95% CI: 0.97, 0.99; p=0.039, for class.Verrucomicrobiae; OR: 0.98; 95% CI: 0.97, 0.99; p=0.039, for family.Verrucomicrobiaceae; OR: 1.01; 95% CI: 1.00, 1.02; p=0.017, for genus.Clostridiuminnocuumgroup; OR: 1.01; 95% CI: 1.00, 1.02; p=0.037, for genus.Adlercreutzia; OR: 0.98; 95% CI: 0.97, 0.99; p=0.039, for genus.Akkermansia; OR: 1.01; 95% CI: 1.00, 1.03; p=0.031, for genus.Dorea; OR: 1.01; 95% CI: 1.00, 1.03; p=0.045, for genus.FamilyXIIIAD3011group; OR: 1.02; 95% CI: 1.00, 1.04; p=0.012, for genus.Lachnoclostridium; OR: 1.01; 95% CI: 1.00, 1.03; p=0.020, for genus.Marvinbryantia; OR: 0.98; 95% CI: 0.97, 0.99; p=0.024, for genus.Oscillibacter; OR: 1.02; 95% CI: 1.00, 1.04; p=0.031, for genus.Parabacteroides; OR: 1.01; 95% CI: 1.00, 1.02; p=0.031, for genus.Paraprevotella; OR: 1.00; 95% CI: 1.00, 1.01; p=0.024, for genus.Prevotella7; OR: 0.98; 95% CI: 0.97, 0.99; p=0.038, for phylum.Cyanobacteria; OR: 0.98; 95% CI: 0.97, 0.99; p=0.021, for phylum.Verrucomicrobia); however, this causal relationship was not supported (P>0.05) in the weighted median method results.

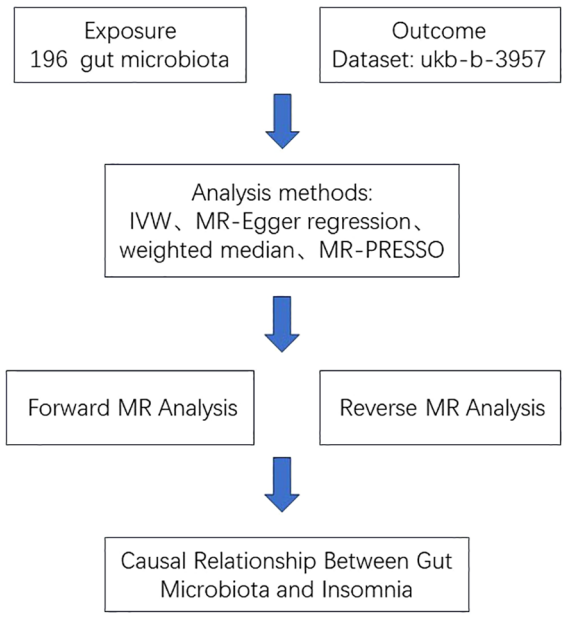

FIGURE 2
Forest plot of the associations between 19 genetically determined bacterial traits with the risk of insomnia.

3.2 The result of reverse MR analysis

We performed reverse MR analysis of the four gut microbiotas. The results showed that we did not find a statistically significant association between Insomnia and any of the three gut microbiotas (class.Negativicutes, order.Selenomonadales, genus.Lactococcus) (Table 2). However, we found a statistically significant association between Insomnia and genus.Rikenellaceae RC9 gut group.

4 Discussion

Insomnia is a sleep disorder characterized by frequent and persistent difficulty falling asleep or maintaining sleep, resulting in inadequate sleep satisfaction. The pathogenesis of insomnia is complex, and it has been previously considered to be related to the abnormality of the GABAergic neuron system (Abad and Guilleminault, 2018), the dysfunction of the hypothalamic–pituitary–adrenal axis (HPA axis) (Dong et al., 2021), the dysfunction of melatonin and its receptor (Cardinali et al., 2012), and the imbalance of central neurotransmitter secretion. The gut microbiota plays an important role in digestion and metabolism and is a hot research topic. It can regulate the central nervous system through multiple neural pathways to affect the sleep and mental state of the host and participate in the occurrence of a variety of neuropsychiatric diseases. At present, many scholars have proposed that the gut microbiota is closely related to nervous system diseases. The gut microbiota can affect host behavior through the immune, neuroendocrine, and vagus pathways of the gut–brain axis, thus regulating the central nervous system bidirectionally (Socała et al., 2021; Góralczyk-Bińkowska et al., 2022; Agirman et al., 2021). Insomnia is an important nervous system disease. Many studies have reported that drug intervention can improve the metabolic abnormalities of enterobacter, enterococcus, brucella, and other bacteria and correct the phenomenon of intestinal flora disorder (Feng et al., 2023; Song et al., 2021; Wang et al., 2022). However, most of the previous studies involved the quantitative detection of intestinal flora, but no conclusion of causality between them was made.

To analyze the causal relationship between insomnia and the gut microbiota, we subsequently took a two-sample MR analysis using data from the GWAS database. The results showed that we identified 19 gut microbiotas associated with insomnia, for example, class.Verrucomicrobiae, family.Verrucomicrobiaceae, genus.Clostridiuminnocuumgroup. Among them, three gut microbiotas (class.Negativicutes, order.Selenomonadales, genus.Lactococcus) were analyzed by forward and reverse MR and various data analysis methods, indicating that there is strong evidence

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TABLE 1 Effect estimates of the associations of Insomnia with four gut microbiotas in the MR analysis.

Gut microbiome	Methods	N.SNPs	OR	95% CI	p-value	Intercept p-value
class.Negativicutes	Inverse-variance weighted	13	1.03	1.01-1.05	0.000	
	Weighted median	13	1.04	1.02-1.07	0.000	
	MR-PRESSO test	13	1.03	1.02-1.05	0.001	
	MR-Egger	13				0.105
order.Selenomonadales	Inverse-variance weighted	13	1.03	1.01-1.05	0.000	
	Weighted median	13	1.04	1.02-1.07	0.000	
	MR-PRESSO test	13	1.03	1.02-1.05	0.001	
	MR-Egger	13				0.105
genus.Lactococcus	Inverse-variance weighted	11	0.99	0.98-0.99	0.034	
	Weighted median	11	0.98	0.97-0.99	0.045	
	MR-PRESSO test	11	0.99	0.98-0.99	0.033	
	MR-Egger	11				0.879
genus.RikenellaceaeRC9gutgroup	Inverse-variance weighted	13	1.01	1.00-1.02	0.003	
	Weighted median	13	1.01	1.00-1.02	0.001	
	MR-PRESSO test	13	1.01	1.00-1.01	0.001	
	MR-Egger	13				0.714

of a causal relationship between insomnia and that the three gut microbiotas are closely related to the risk of insomnia. However, genus.Rikenellaceae RC9 gut group and insomnia are mutually causal.

Class.Negativicutes is a gram-negative bacteria characterized by two cell membranes and is a common strictly anaerobic double-walled bacteria. Its potential to produce specific metabolites has not been fully studied. At present, class.Negativicutes is closely related to diseases such as COVID-19 (Song et al., 2023), low birthweight infants (Warner et al., 2016), and obesity (Hu et al., 2022). In this study, it was found that there was a significant causal relationship between class.Negativicutes and insomnia. Although there was no report on this exposure factor and outcome index in relevant studies and reports, we believed that it may play an important role in the occurrence and development of insomnia. In the future, we will focus on observing the changes of this flora in insomnia, so as to provide positive or negative proof for the causal relationship.

Order.Selenomonadales has also been identified as a risk factor for insomnia. Existing studies have shown that the bacteria play a role in Alzheimer's disease (Zhuang et al., 2018), Idiopathic Nephrotic Syndrome (He et al., 2021), and autism spectrum disorder (Sun et al., 2019). Xia et al. showed that melatonin could effectively improve the changes of intestinal flora in the host, in which Selenomonadales was significantly reduced (Xia et al., 2022). We hypothesize that this order.Selenomonadales may play a crucial role in the progression of insomnia.

Genus.Lactococcus is a gram-positive bacterium of the family streptococcaceae of lactobacillus and is the only negatively

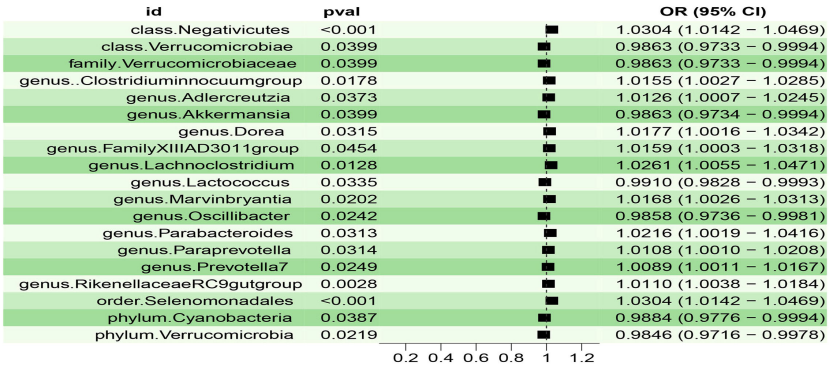

FIGURE 3
Scatter plot of the associations of genetic variants with four bacterial traits and the risk of Insomnia.

TABLE 2 Effect estimates of the associations of Insomnia with four gut microbiotas in the reverse MR analyses.

Gut microbiome	Methods	N.SNPs	OR	95% CI	p-value	Intercept p-value
class.Negativicutes	Inverse-variance weighted	32	1.37	0.94-1.99	0.099	
	Weighted median	32	1.42	0.84-2.38	0.189	
	MR-Egger	32				0.964
genus.Lactococcus	Inverse-variance weighted	31	0.63	0.26-1.54	0.313	
	Weighted median	31	0.70	0.22-2.29	0.558	
	MR-Egger	31				0.604
genus.RikenellaceaeRC9gutgroup	Inverse-variance weighted	30	0.39	0.15-0.99	0.046	
	Weighted median	30	0.30	0.08-1.18	0.085	
	MR-Egger	30				0.357
order.Selenomonadales	Inverse-variance weighted	32	1.37	0.94-1.99	0.099	
	Weighted median	32	1.42	0.85-2.34	0.182	
	MR-Egger	32				0.964

correlated gut microbiota. In clinical microbiology laboratory specimens, it can be isolated from blood, urine, eye cultures, and more commonly from blood cultures of patients with endocarditis. A study has shown that the genus Lactococcus is closely related to insomnia in children (Nebbia et al., 2020).

This study has some advantages. Firstly, this study is based on the GWAS data with the largest sample size in the world, without animal and clinical trials, and is the most relevant study on the relationship between insomnia and the gut microbiota. Secondly, our identification of three gut microbiotas by MR analysis could provide evidence for subsequent studies and help provide new approaches to the targeted treatment of insomnia. Thirdly, the use of MR analysis to explore the association between insomnia and the gut microbiota can avoid the bias caused by reverse causality and unknown confounding, and the different MR methods give consistent causal effects, indicating the robustness of the results.

This study also has some limitations. Firstly, the population included in this study was European, and the lack of data on other ethnicities may have an impact on our causal relationship. Secondly, bacterial classification is only analyzed at the genus level, lacking more professional analysis such as species and strains. Thirdly, MR analysis of the two samples failed to group the two sexes and to understand whether there was a difference in virulence between the sexes.

5 Conclusion

The causal relationship between insomnia and the gut microbiota was evaluated by MR analysis.

Three gut microbiotas (class Verrucomicrobiae, family Verrucomicrobiaceae, genus Clostridium innocuum) play a key role in the progress of insomnia, which provides a new research direction for the pathogenesis and treatment of insomnia.

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

This article does not require ethical approval.

Author contributions

QW: Data curation, Investigation, Software, Writing – original draft. TG: Writing – original draft. WZ: Writing – original draft. DL: Writing – original draft. XL: Writing – original draft. FC: Writing – review & editing. JM: Writing – review & editing.

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Conflict of interest

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

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Gallbladder microbial species and host bile acids biosynthesis linked to cholesterol gallstone comparing to pigment individuals

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Gallstones are crystalline deposits in the gallbladder that are traditionally classified as cholesterol, pigment, or mixed stones based on their composition. Microbiota and host metabolism variances among the different types of gallstones remain largely unclear. Here, the bile and gallstone microbial species spectra of 29 subjects with gallstone disease (GSD, 24 cholesterol and 5 pigment) were revealed by type IIB restriction site-associated DNA microbiome sequencing (2bRAD-M). Among them (21 subjects: 18 cholesterol and 3 pigment), plasma samples were subjected to liquid chromatography–mass spectrometry (LC-MS) untargeted metabolomics. The microbiome yielded 896 species comprising 882 bacteria, 13 fungi, and 1 archaeon. Microbial profiling revealed significant enrichment of *Cutibacterium acnes* and *Microbacterium* sp005774735 in gallstone and *Agrobacterium pusense* and *Enterovirga* sp013044135 in the bile of cholesterol GSD subjects. The metabolome revealed 2296 metabolites, in which malvidin 3-(6''-malonylglucoside), 2-Methylpropyl glucosinolate, and ergothioneine were markedly enriched in cholesterol GSD subjects. Metabolite set enrichment analysis (MSEA) demonstrated enriched bile acids biosynthesis in individuals with cholesterol GSD. Overall, the multi-omics analysis revealed that microbiota and host metabolism interaction perturbations differ depending on the disease type. Perturbed gallstone type-related microbiota may contribute to unbalanced bile acids metabolism in the gallbladder and host, representing a potential early diagnostic marker and therapeutic target for GSD.

KEYWORDS

cholesterol gallstone, gallbladder microbiota, 2bRAD-M, bile acids metabolism, LC-MS metabolomics, multiomics

Introduction

Cholelithiasis (also referred to as gallstone disease, GSD) is common worldwide. Individuals with GSD have an increased risk for gallbladder and liver disease and an increased level of inflammation (Ryu et al., 2016; Arrese et al., 2018; Rodríguez-Antonio et al., 2020). GSD can profoundly impact one's life and work performance due to the pain symptom. However, the underlying mechanisms contributing to gallstones influenced by the gallbladder microbiome remain unclear. Approximately two-thirds of gallstones are the cholesterol type, and the rest are mainly pigment stones (Portincasa and Wang, 2012). Cholesterol stones are usually yellow-green in color and are predominantly composed of cholesterol while pigment stones are brown-dark in color and are made of pigment bilirubin (Kose et al., 2018). Microbiota and host metabolism variances among the different types of gallstones remain largely unclear. Thus, understanding such variances may provide microbial and physiological insights into the formation of different types of gallstones.

The human microbiome, also known as the second genome, can influence metabolic homeostasis through microbiota–host interactions in physiological and pathological conditions (Fan and Pedersen, 2021). Normally, the dynamically stable microbiota plays a vital role in sustaining the balance of host inflammation, thus positively regulating phycological functions including immune responses (Belkaid and Hand, 2014). However, the microbiota inhabiting the gallbladder of humans is substantially under-studied. Our prior studies of the bacterial and fungal fractions of the gallbladder microbiome in GSD revealed substantial trans-kingdom interactions between various microbial taxa at the genus level (Hu et al., 2023). Importantly, high taxa resolution will reveal finer details and the distribution pattern of opportunistic pathogens in the gallbladder microbiota. However, obtaining accurate, species-resolution, landscape-like taxonomic profiles from bile and gallstone samples is challenging due to their low-biomass. Given that, a cost-effective sequencing strategy—type IIB restriction site-associated DNA microbiome sequencing (2bRAD-M)—has been proposed for assessing such challenging samples (Wang et al., 2012; Sun et al., 2022). The ability to profile low-biomass microbiomes at the species level is pivotal to uncovering the crucial roles of the gallbladder microbiota underlying human health.

Furthermore, metabolomic profiles (metabolomics) provide a comprehensive readout of human (patho)physiology and advance our understanding of disease etiology (Ramautar et al., 2013; Gonzalez-Covarrubias et al., 2022). This promising approach is central to disease prevention and diagnosis since the human blood metabolome mirrors the overall physiological state (Johnson et al., 2019). However, the blood metabolomic profiles of individuals with cholesterol or pigment GSD remain unknown.

Herein, the present study for the first time further explores the profile of the gallbladder microbiota at the species level and its links with the plasma metabolites by integrating microbiome (2bRAD-M sequencing) and metabolome (untargeted LC-MS metabolomics), respectively. This wealth of multi-omics data provides an opportunity to investigate associations between the gallbladder microbiome, health, and the type of gallstone.

Materials and methods

Subjects and sample collection

We recruited 29 individuals with gallstone (gallbladder-stone) and chronic cholecystitis in the present study (Table 1; Supplementary Table 1). Their age ranged from 21 to 82 years, averaging 42.4 years. Most were female (22/29), and 24 of 29 had cholesterol stones (the remaining subjects had pigment-type stones).

The gallstone and bile samples were collected after gallbladder removal surgery (laparoscopic cholecystectomy) as previously described (Hu et al., 2023). In brief, bile samples (2–5 mL) were immediately collected from each patient after gallbladder removal. Stones (3–5 pellets) were washed in sterile stroke-physiological saline solution prior to collection from removed gallbladder. All samples were strictly sterilely collected and stored at −80°C until further use. In total, 56 samples (27 paired bile and gallstone samples and 2 individual bile samples) were acquired for the microbiota analysis. Blood was also collected to assess plasma metabolites. Twenty-one plasma samples were assessed using an untargeted metabolomics approach (Table 1; Supplementary Table 1; Figure 1A).

Microbial DNA extraction, library preparation, and sequencing

The genomic DNA of bile (200 μL) and stone (approximately 200 mg) microbiota were extracted using a QIAamp DNA Mini Kit and QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) respectively following the manufacturer's instructions. DNA concentrations were detected using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were stored at −80°C until required for experiments. The 2bRAD libraries were prepared according to previous studies and purified with the QIAquick PCR purification kit (QIAGEN, Germany) (Lam et al., 2022; Sun et al., 2022). Microbial sequencing was conducted on an Illumina Novaseq

TABLE 1 Demographic and clinical details of GSD subjects in current multiomics study.

Data	Factor	Value
Microbiome	Sample size	29
	Age (year)[a]	42.4 (21-82)
	Male/Female	7/22
	Cholesterol/Pigment	24/5
	Bile/Gallstone	29/27
Metabolome	Sample size	21
	Age (year)[a]	41.9 (23-82)
	Male/Female	5/16
	Cholesterol/Pigment	18/3

[a]mean (min-max).

PE150 platform by OE Biotech Co., Ltd. (Shanghai, China). Briefly, DNA (1 pg-200 ng) was digested with 4 U of the enzyme *BcgI* (NEB) for 3 h at 37°C. Subsequently, the adaptors were ligated to the DNA fragments performed by combining 5 μL of digested DNA with 10 μL of a ligation master mix containing 0.2 μM each of two adaptors and 800 U T4 DNA ligase (NEB) at 4°C for 12 h. Then, ligation products were amplified, and PCR products were subjected to 8% polyacrylamide gel. Bands of approximately 100 bp were excised from the polyacrylamide gel, and the DNA was diffused from the gel in nuclease-free water for 12 h at 4°C. Sample-specific barcodes were introduced by PCR with platform-specific barcode-bearing primers. Each 20 μL PCR contained 25 ng of gel-extracted PCR product, 0.2 μM of each primer, 0.3 mM dNTP, 1×Phusion HF buffer and 0.4 U Phusion high-fidelity DNA polymerase (NEB). All adaptor and primer sequences used in this study can be found in Supplementary Table 2.

Microbial data processing, diversity analysis and taxonomic assignment

Raw reads (only R1 reads were used in the current study) were filtered to extract the digested fragments ("enzyme reads") using the *BcgI* restriction enzyme recognition site. Clean reads were obtained from enzyme reads using two criteria. First, reads with >8% unknown bases (N) were filtered; second, reads with of low-quality bases exceeding 20% (Q-value < 30) were removed. Microbial diversity was analyzed using the Shannon (Shannon, 1997), Simpson (Simpson, 1949), and Chao1 (Chao, 1984) indices (alpha diversity) and Bray-Curtis distance (Sørensen et al., 1948) (beta diversity). The 2bRAD-M pipeline (https://github.com/shihuang047/2bRAD-M) was applied to perform the taxonomic profiling using a unique 2bRAD tag database (2b-Tag-DB) comprising taxa-specific *BcgI*-derived tags identified from 173,165 microbial genomes.

Metabolomics profiling of plasma samples

A quality control (QC) sample was obtained by mixing and blending equal volumes (10 μL) of each plasma sample to estimate the mean profile representing all the analytes encountered during analysis. A Dionex U3000 UHPLC/QE plus quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA) was used for both ESI positive and ESI negative ion modes. An ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm) was used. All the samples were stored at 4°C during the analysis. The binary gradient elution system consisted of (A) water (containing 0.1% formic acid, v/v) and (B) acetonitrile (containing 0.1% formic

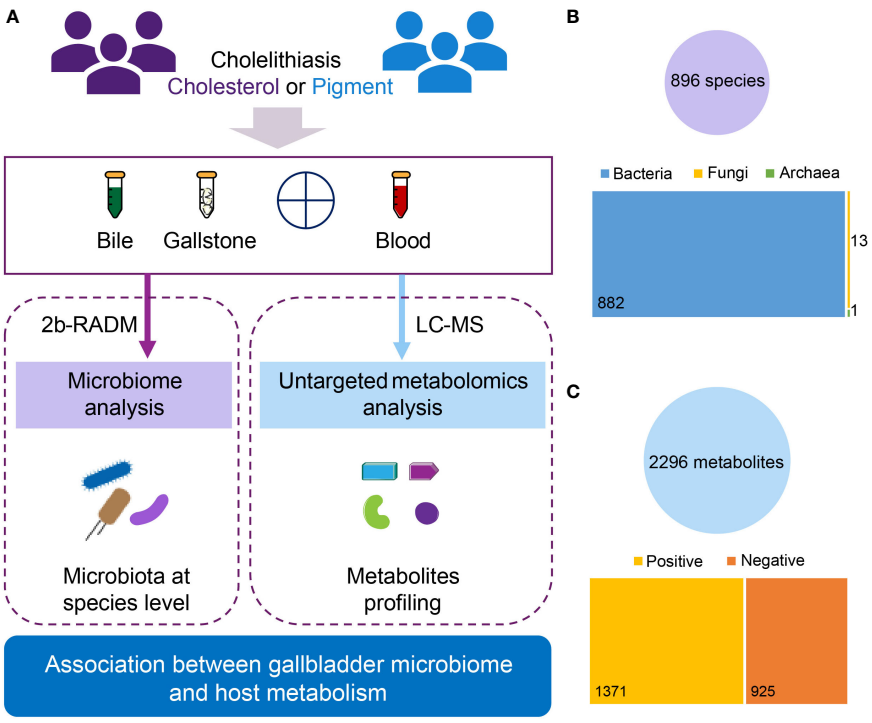

FIGURE 1
Overview of the current multiomics study. **(A)** Diagram of study design. A total of 29 subjects diagnosed with cholesterol (n = 24) or pigment (n = 5) GSD were assessed. For the gallbladder microbiota analysis, bile and gallstone samples were collected from all patients, yielding 56 samples (27 paired bile and gallstone samples, and 2 bile samples only) for 2bRAD-M. For the plasma metabolomic analysis, plasma samples from 21 (18 cholesterol and 3 pigment) of those 29 individuals were used for LC–MS. **(B)** Overview of microbial data characterized by 2bRAD-M. In total, 896 species (882 bacteria, 13 fungi, and 1 archaeon) were identified from 54 bile and gallstone samples (2 samples were removed during quality control). **(C)** Overview of metabolic data characterized by LC–MS. In total, 2296 metabolites (1371 positive and 925 negative compounds) were identified from 21 plasma samples.

acid, v/v) and separation was achieved using the following gradient: 0.01 min, 5% B; 2 min, 5% B; 4 min, 30% B; 8 min, 50% B; 10 min, 80% B; 14 min, 100% B; 15 min, 100% B; 15.1 min, 5% and 18 min, 5% B. The flow rate was 0.35 mL/min and column temperature was 45°C. The injection volume was 2 μL. The mass resolution was set at 70,000 for the full MS scans and 17,500 for HCD MS/MS scans and collision energy was set at 10, 20 or 40 eV.

LC–MS data processing

The raw LC–MS data were processed using Progenesis QI V2.3 (Nonlinear, Dynamics, Newcastle, UK) for baseline filtering, peak identification, integral, retention time correction, peak alignment, and normalization. Metabolite identification was conducted based on the precise mass-to-charge ratio (M/z), secondary fragments, and isotopic distribution using the Human Metabolome Database (HMDB), Lipidmaps (V2.3), Metlin, EMDB, PMDB, and self-built databases (Shanghai Lu-Ming Biotech Co., Ltd., Shanghai, China) for the qualitative analysis. The extracted data were then further processed by removing peaks with missing values (ion intensity = 0) in over 50% of the groups and replacing the zero value with half of the minimum value and screening according to the qualitative results of the metabolite. Metabolites with scores below 36 (out of 60) points were removed. Finally, a data matrix (2296 metabolites) was generated by combining the positive and negative ion data and analyzed by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) (Pang et al., 2021).

Statistical analysis

Wilcox test and Adonis (PERMANOVA) were used for the microbial alpha and beta diversity analyses, respectively. A linear discriminant analysis (LDA) effect size (LEfSe) was employed for the microbial biomarker analysis. The strategy "one-against-one" (stricter) was chosen for LEfSe. Metabolomics data were statistically analyzed using MetaboAnalyst (V5.0). Metabolites with a variable importance in projection (VIP) score ≥ 1.0 with a P value < 0.05 were considered significant. Classical univariate receiver operating characteristic (ROC) curve analysis was used to identify potential metabolite biomarkers and evaluate their performance. The significance cutoff was set at $P < 0.05$. Other statistical cutoffs are individually indicated in each figure where appropriate.

Results

Overview of gallbladder microbiota data and plasma metabolomics profiling

Overall, we identified 896 species by 2bRAD-M of both bile and gallstone samples, including 882 bacteria, 13 fungi and 1 archaeon (Figure 1B). Additionally, 2296 metabolites were characterized by LC-MS metabolomics. Among these metabolites, approximately 60% (1371/2296) were positive compounds, whereas 925 were negative (Figure 1C; Supplementary Figure 1A). Lipids and lipid-like molecules, Organic acids and derivatives, and Organoheterocyclic compounds were the top 3 superclasses of detected metabolites (Supplementary Figure 1B). Fatty acyls, carboxylic acids and derivatives, organooxygen compounds, steroids and steroid derivatives, and prenol lipids were the top 5 metabolic classes (Supplementary Figure 1C). Amino acids, peptides, and analogues, fatty acids and conjugates, and fatty acid esters were the main subclasses (Supplementary Figure 1D).

Gallstone type-specific changes to gallbladder microbiota are dependent of sample type

We first examined microbial diversity to explore the microbial differences in the gallbladder of subjects with cholesterol and pigment-type GSD. The rarefaction curve of detected microbial species in the gallbladder microbiota reached saturation as the sample number increased, indicating that the gallbladder microbiota in our population captured most gallbladder microbial members (Figure 2A). Generally, the microbial alpha diversity in bile samples was higher than that in the gallstone samples (Figures 2B–D). Although the Shannon (Figure 2B, $P = 0.21$ in bile, $P = 0.15$ in gallstone) and Chao1 (Figure 2D, $P = 0.73$ in bile, $P = 0.55$ in gallstone) indices showed no significant change between cholesterol and pigment GSD subjects regardless of the sample type, a marked difference was present in the gallstone versus bile sample based on the Simpson index (Figure 2C, $P = 0.16$ in bile, $P = 0.04$ in gallstone). Moreover, the microbial diversity in bile from pigment GSD individuals was significantly higher than that in gallstones from cholesterol GSD subjects (Figures 2B–D). The microbial beta diversity based on a principal coordinates analysis (PCoA) of the Bray-Curtis distance showed distinct clustering of bile and gallstone samples from all subjects (Figure 2E, $P = 0.001$), and cholesterol GSD subjects (Figure 2E, $P = 0.0005$).

2bRAD-M revealed a large group of microbial species across 20 phyla in the gallbladder, including bacteria, fungi, and archaea (Supplementary Figure 2A). A total of 896 species were identified (Figure 1B). *Meiothermus silvanus*, *Acinetobacter johnsonii*, *Agrobacterium pusense*, *Vulcaniibacterium thermophilum*, *Escherichia flexneri*, *Streptococcus agalactiae*, *Cutibacterium acnes*, *Streptococcus mutans*, *Klebsiella pneumoniae*, and *Dietzia maris* were the dominant microbial species (Supplementary Figure 2B). We further accessed the microbial signatures associated with type of gallstone by LEfSe (Figure 3; Supplementary Figure 3). The highly abundant species *Meiothermus silvanus* (genus *Meiothermus*) was substantially enriched in the gallstone of pigment GSD subjects (LDA (log 10) = 4.85, $P = 0.0347$). *Cutibacterium acnes* (genus *Cutibacterium*, LDA (log 10) = 4.39, $P = 0.00458$) and *Microbacterium sp005774735* (LDA (log 10) = 3.56, $P = 0.0351$) were biomarkers of gallstone in cholesterol GSD subjects. Ten species, including *Tepidimonas fonticaldi* (LDA (log 10) = 4.03, $P = 0.0408$), *Micrococcus endophyticus* (LDA (log 10) = 3.82,

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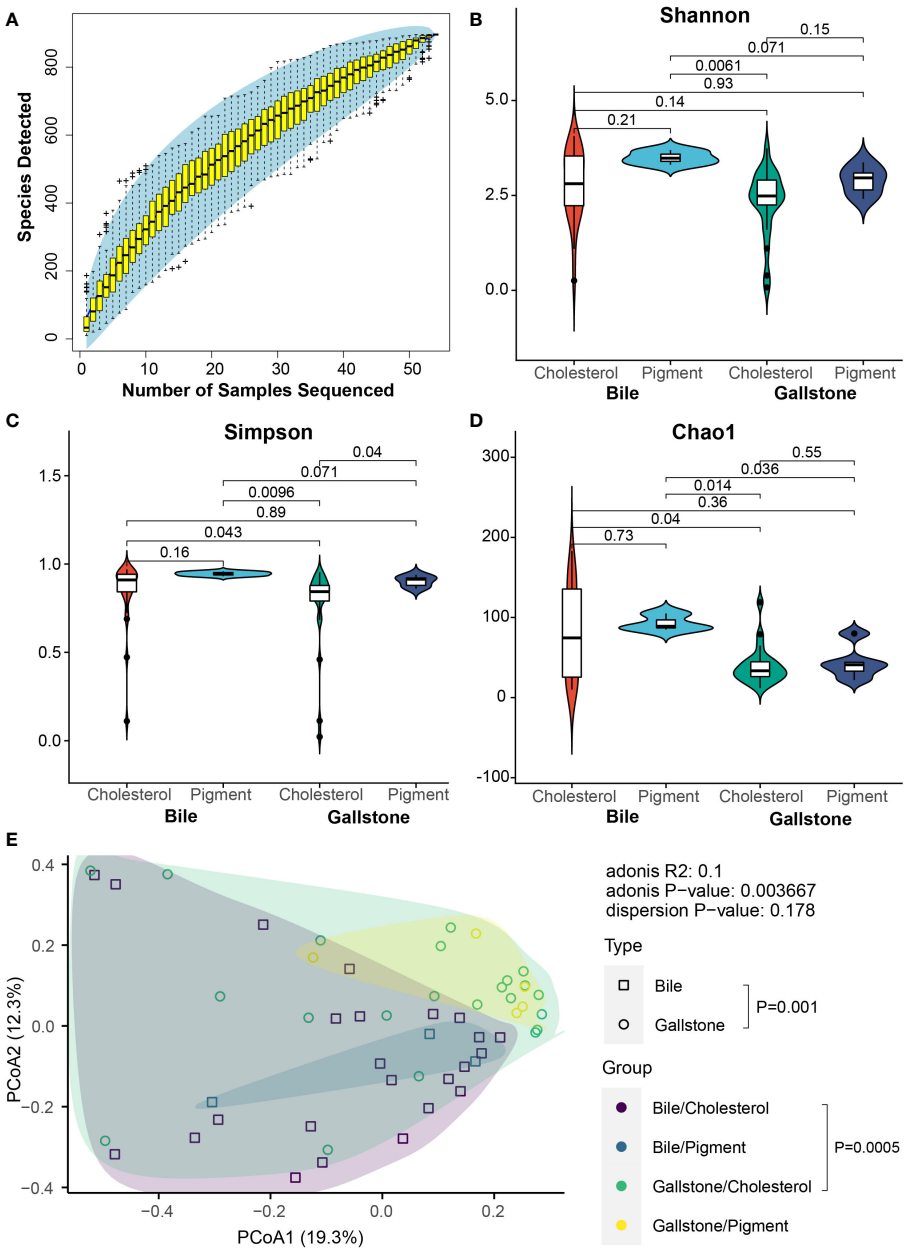


FIGURE 2
Alteration of microbial diversity between cholesterol and pigment GSD patients depends on sample type. **(A)** Coverage of members in the gallbladder microbiota. Rarefaction curve of detected microbial species in the gallbladder microbiota reaches saturation as the sample number increases. **(B–D)** Gallbladder microbial alpha diversity among cholesterol/bile (n = 24), cholesterol/gallstone (n = 22), pigment/bile (n = 3) and pigment/gallstone (n = 5) groups by Shannon **(B)**, Simpson **(C)**, and Chao1 **(D)** indices. The statistical analysis was performed using the Wilcoxon test. **(E)** Principal coordinate analysis (PCoA) of the Bray–Curtis distance among cholesterol/bile (n = 24), cholesterol/gallstone (n = 22), pigment/bile (n = 3) and pigment/gallstone (n = 5) groups. The statistical analysis was performed using a pairwise PERMANOVA (Adonis) test.

P = 0.0418), and *Methylobacterium rhodesianum* (LDA (log 10) = 3.69, *P* = 0.00319), were enriched in the bile samples from pigment GSD subjects, while *Agrobacterium pusense* (LDA (log 10) = 4.66, *P* = 0.000453), and *Enterovirga sp013044135* (LDA (log 10) = 3.29, *P* = 0.0251) were enriched in the bile samples from cholesterol GSD subjects. These results demonstrate that alterations in the gallbladder microbiota differ in bile and gallstone samples from subjects with different gallstone types.

Significant alterations in plasma metabolites between cholesterol and pigment GSD individuals

Untargeted LC-MS metabolomics was performed to assess the metabolic profiling differences between cholesterol and pigment GSD subjects. In total, 2296 metabolites were detected in the plasma of cholesterol and pigment GSD subjects. Partial least squares discriminant analysis (PLS-DA) confirmed the large difference

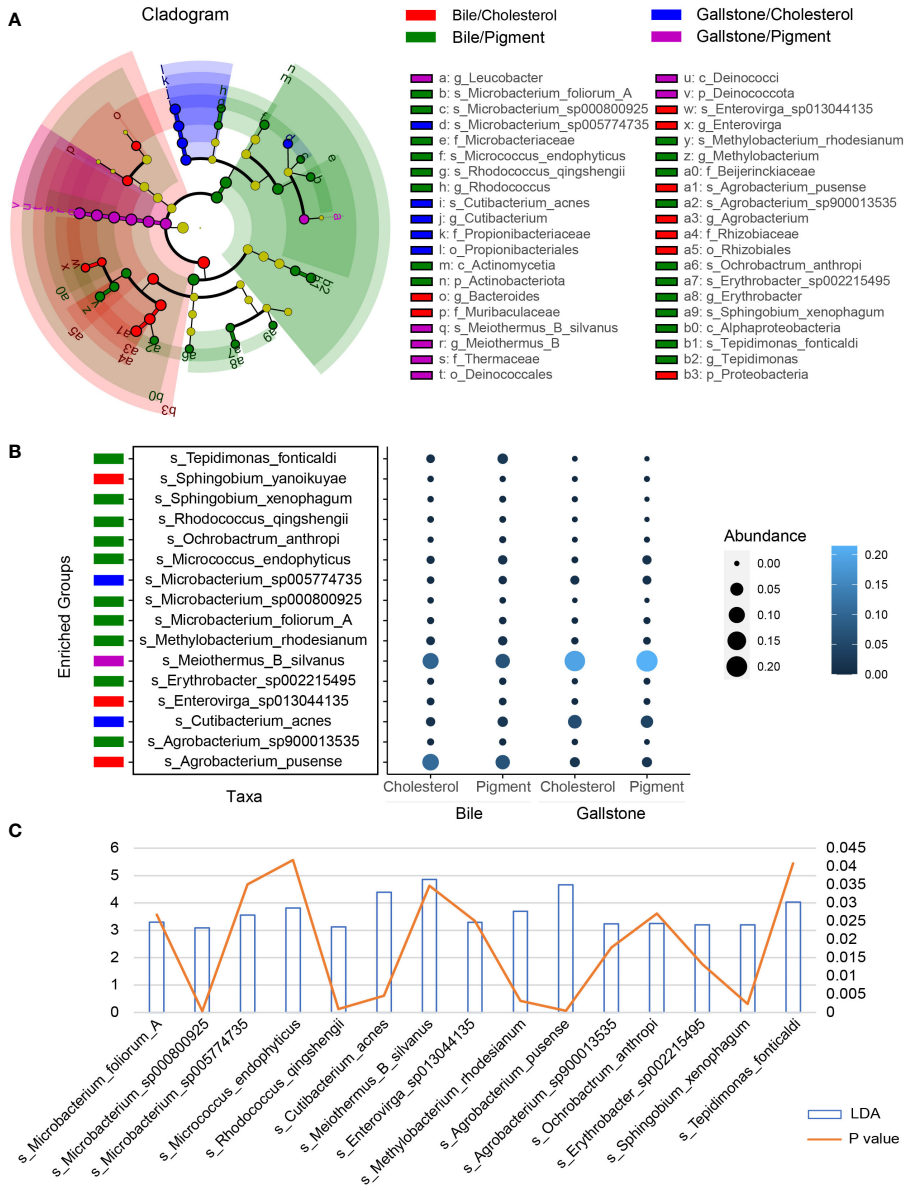


FIGURE 3
Gallstone type-related changes in gallbladder microbial species depends on sample type. **(A)** LEfSe cladogram showing the differing abundance of microbiota among cholesterol/bile (n = 24), cholesterol/gallstone (n = 22), pigment/bile (n = 3) and pigment/gallstone (n = 5) groups. The diameter of each circle is proportional to the abundance (LDA score (log 10) > 3.0). **(B)** Bubble plot showing the representative microbial species markers among the four groups. **(C)** Bar and line graphs showing the LDA scores (log 10) and *P* values of these representative species.

observed in the plasma metabolome of cholesterol or pigment GSD subjects (Figures 4A). The top 15 metabolites detected by PLS-DA between cholesterol and pigment GSD subjects are shown in Figure 4B. Metabolites malvidin 3-(6''-malonylglucoside) (0.64_1157.2640m/z, Figure 4B; Supplementary Figure 4A), 2-Methylpropyl glucosinolate (4.76_356.0497m/z, Figure 4B; Supplementary Figure 4B), and ergothioneine (7.16_269.0596m/z, Figure 4B; Supplementary Figure 4C) were enriched in plasma from cholesterol GSD subjects, whereas 2-hydroxy-9Z,12Z-Octadecadienoic acid (15.23_279.2315m/z, Figure 4B) was enriched in pigment GSD subjects (VIP score > 2). Moreover, the differential metabolites volcano plot showed that 17 metabolites, including 15.23_279.2315m/z and 15-octadecene-9,11,13-triynoic acid (6.02_248.1643m/z, Figure 4C, Supplementary Figure 4D) were enriched in pigment GSD subjects, while 15 metabolites, including malvidin 3-(6''-malonylglucoside), 2-Methylpropyl glucosinolate, and ergothioneine, were enriched in cholesterol GSD subjects (Figure 4C; Supplementary Figure 4; Supplementary Table 3, $|\text{Log2(FC)}| > 1$, $P < 0.05$).

Metabolite set enrichment analysis (MSEA) was performed using MetaboAnalyst to assess the functions of altered metabolites between cholesterol and pigment GSD subjects. Bile acid biosynthesis ($P = 0.00712$) is significantly enriched in cholesterol GSD individuals while caffeine metabolism ($P = 0.00307$) is markedly enriched in pigment GSD individuals (Figures 5A, B; Supplementary Figures 5A, B). In addition,

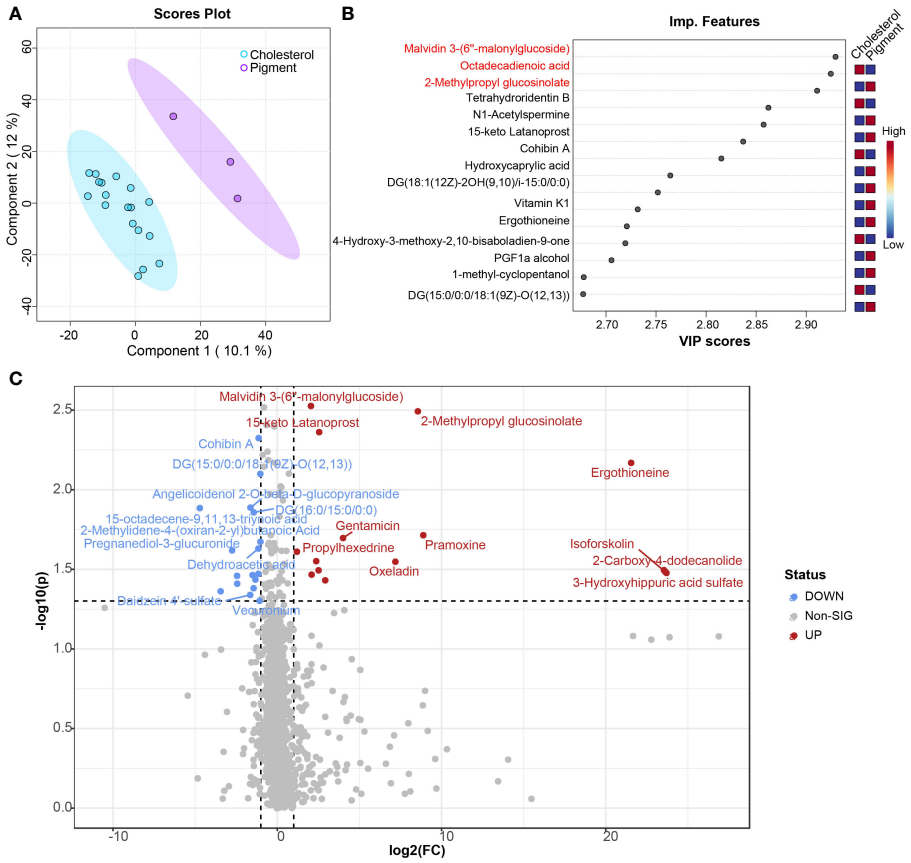


FIGURE 4
Comparison of plasma metabolome between cholesterol and pigment GSD subjects. **(A)** Scores plot of results from partial least squares–discriminant analysis (PLS-DA). **(B)** Top 15 important metabolic features between cholesterol (n = 18) and pigment (n = 3) GSD patients detected by PLS-DA ranked by variable importance projection (VIP) scores. **(C)** Volcano plot analysis of different metabolites between cholesterol (n = 18) and pigment (n = 3) GSD patients. Fold change (FC) > 2, *P*-value threshold < 0.05. Red points represent metabolites with increased abundance in cholesterol GSD patients; blue points represent metabolites with increased abundance in pigment GSD patients.

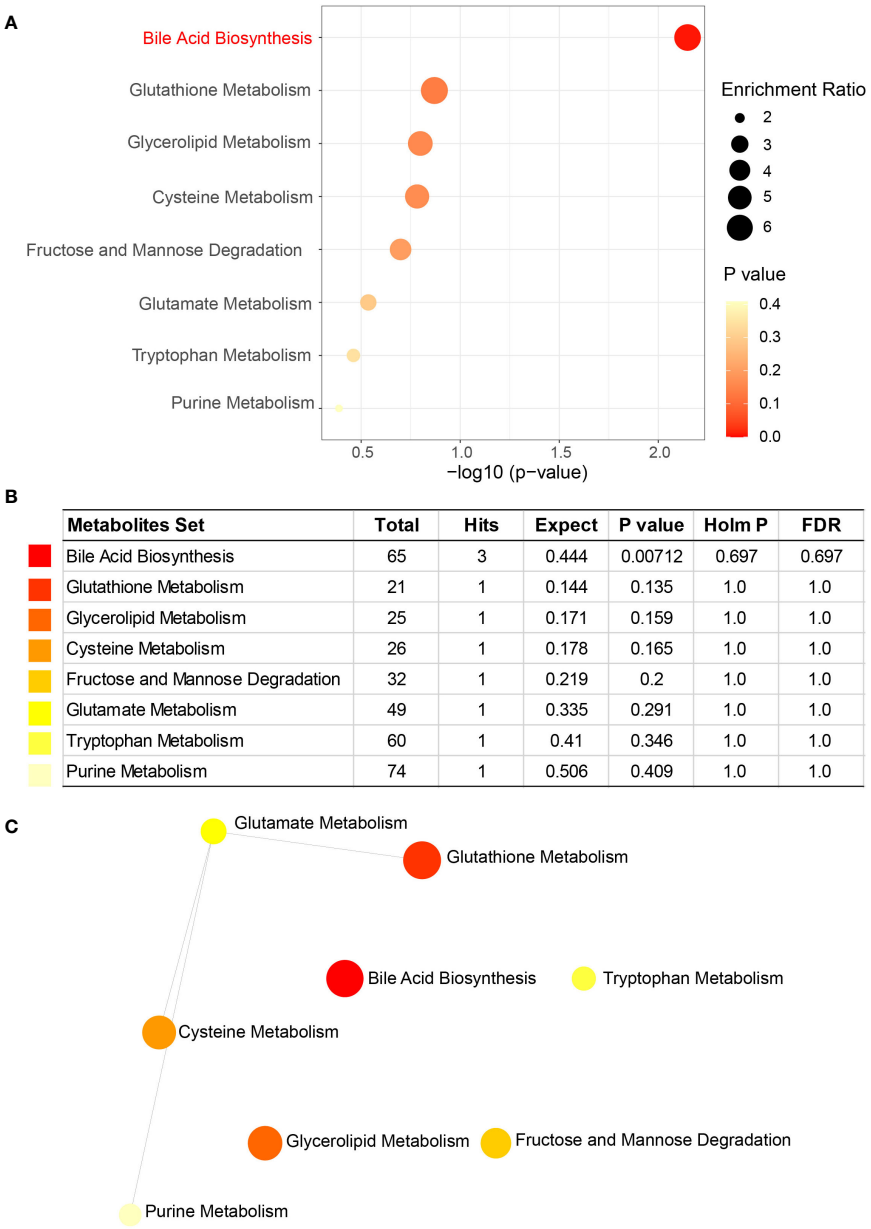
dysfunction of some amino acids (cysteine, glutamate, and tryptophan) metabolism was found in cholesterol GSD subjects although the difference was not significant (Figures 5A, B). The three amino acids and purine (metabolism) are connected to a network in cholesterol GSD subjects, as shown in Figure 5C. Altogether, these results imply that cholesterol GSD is linked to dysfunctional bile acid metabolism.

Potential plasma metabolites biomarkers for distinguishing cholesterol gallstone from pigment

To further extract whether the metabolites can serve as biomarkers distinguish cholesterol or pigment gallstone from gallstone subjects, ROC curve analysis was performed using plasma metabolite sets in current study. Metabolite malvidin 3-(6"-malonylglucoside) which is enriched in cholesterol individuals can be a potential biomarker for cholesterol gallstone (Figure 6A, AUC = 0.88). Two plasma metabolites 2-hydroxy-9Z,12Z-Octadecadienoic acid (Figure 6B, AUC = 0.981) and DG (18:1 (12Z)-2OH(9,10)/i-15:0/0:0), Figure 6C, AUC = 0.963) are identified as potential biomarkers of pigment gallstone. These results indicate that the plasma metabolites could be biomarkers for distinguishing different types of gallstones.

Perturbed gallbladder microbiota–host metabolism interactions are dependent on sample type

A Spearman correlation analysis of representative metabolites (metabolites that altered significantly, related to Supplementary Table 3) and microbial species was performed to further probe the relationships between the gallbladder microbiota and plasma metabolites. A total of 1024 connects comprising 508 positive and 516 negative correlations were obtained from the bile and gallstone samples (Figure 7A). In bile (Figures 7B, C left), *Enterovirga sp013044135* was significantly positively correlated with DG (14:1 (9Z)/17:1(9Z)/0:0) [iso2] (12.02_573.4507m/z, *rho* = 0.478, *P* = 0.0328, Supplementary Table 4) and vecuronium (13.57_575.4662m/z, *rho* = 0.497, *P* = 0.0257, Supplementary Table 4). *Agrobacterium pusense* was significantly positively correlated with pramoxine (7.02_311.2324m/z, *rho* = 0.481, *P* =



Metabolites Set	Total	Hits	Expect	P value	Holm P	FDR
Bile Acid Biosynthesis	65	3	0.444	0.00712	0.697	0.697
Glutathione Metabolism	21	1	0.144	0.135	1.0	1.0
Glycerolipid Metabolism	25	1	0.171	0.159	1.0	1.0
Cysteine Metabolism	26	1	0.178	0.165	1.0	1.0
Fructose and Mannose Degradation	32	1	0.219	0.2	1.0	1.0
Glutamate Metabolism	49	1	0.335	0.291	1.0	1.0
Tryptophan Metabolism	60	1	0.41	0.346	1.0	1.0
Purine Metabolism	74	1	0.506	0.409	1.0	1.0

FIGURE 5
Metabolite set enrichment analysis (MSEA) of enriched metabolites in cholesterol GSD subjects. **(A)** Key pathways related to cholesterol gallstones. **(B)** Details of key pathways associated with cholesterol gallstones. **(C)** Network of key pathways enriched in cholesterol gallstones. Enrichment Ratio computed by Hits/Expected, where hits = observed hits; expected = expected hits.

0.0333, Supplementary Table 4), oxeladin (8.93_353.2795m/z, *rho* = 0.466, *P* = 0.0381, Supplementary Table 4), and gentamicin (8.93_500.3035m/z, *rho* = 0.488, *P* = 0.0291, Supplementary Table 4). *Micrococcus endophyticus* was significantly positively correlated with dihydroisomorphine-6-glucuronide (6.72_464.1910m/z, *rho* = 0.504, *P* = 0.0236, Supplementary Table 4) and 6.73_462.1779m/z (*rho* = 0.532, *P* = 0.0158, Supplementary Table 4). *Microbacterium sp000800925* was significantly positively correlated with formyl-5-hydroxykynurenamine (11.08_253.0833m/z, *rho* = 0.608, *P* = 0.0044, Supplementary Table 4). *Sphingobium xenophagum* was markedly negatively correlated with N-goshuyoyl lysine (7.53_370.3060m/z, *rho* = -0.550, *P* = 0.0119, Supplementary Table 4), pramoxine (7.02_311.2324m/z, *rho* = -0.510, *P* = 0.0216, Supplementary Table 4). In gallstone (Figures 7B, C right), *Erythrobacter sp002215495* was significantly positively correlated with 5 alpha-Pregnan-3 beta, 20 beta-diol 20-sulfate (8.44_399.2216m/z, *rho* = 0.700, *P* = 0.000596, Supplementary Table 5), pregnanediol-3-glucuronide (9.33_495.2973m/z, *rho* = 0.684, *P* = 0.000886, Supplementary Table 5), and daidzein 4'-sulfate (6.91_314.9964m/z, *rho* = 0.457, *P* = 0.0430, Supplementary Table 5), but markedly negatively correlated with L-cis-Cyclo(aspartylphenylalanyl) (6.06_245.0920m/z, *rho* = -0.632, *P* = 0.00277, Supplementary Table 5). The results indicate that the gallbladder microbiota is closely correlated with plasma metabolites.

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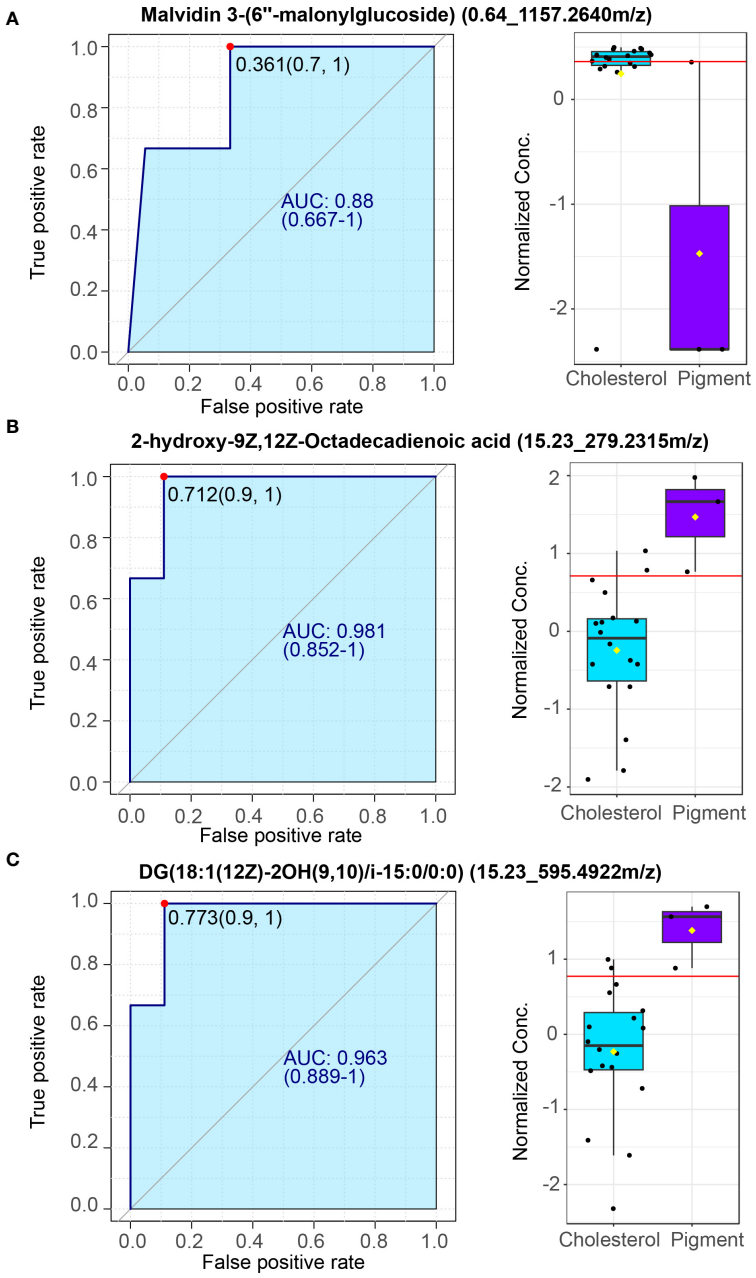

FIGURE 6
Potential plasma markers of cholesterol and pigment gallstones. **(A)** Plasma marker malvidin 3-(6''-malonylglucoside) for cholesterol gallstones. **(B, C)**. Plasma markers 2-hydroxy-9Z,12Z-Octadecadienoic acid **(B)** and DG (18:1(12Z)-2OH(9,10)/i-15:0/0:0) **(C)** for pigment gallstones. Left: Receiver operating characteristic (ROC) curve. Right: normalized concentrate of plasma markers between cholesterol (n =18) and pigment (n = 3) GSD patients.

Discussion

Mounting evidence exists for the importance of the microbiota in the development of many diseases. However, microbiota alterations in the gallbladder of cholesterol and pigment gallstone subjects remain unexplored. Thus, studies characterizing the gallbladder microbiota in GSD and identifying microbial therapeutic targets at the species level are highly warranted. Accordingly, we use 2bRAD-M sequencing to show for the first time that the gallbladder species-level microbiota is distinct between cholesterol and pigment GSD subjects. These data expand our current insight into the potential role of gallbladder microbiome profiling in GSD.

Little is known about the gallbladder microbiome and its role in cholesterol or pigment GSD. Previously, no studies have described species-level changes in the diversity and structure of the human gallbladder microbiome in cholesterol versus pigment GSD. As we previously reported, the microbiota in bile samples is larger than that in gallstone samples (Hu et al., 2023). The microbiota in cholesterol gallstone exhibited the lowest diversity in current

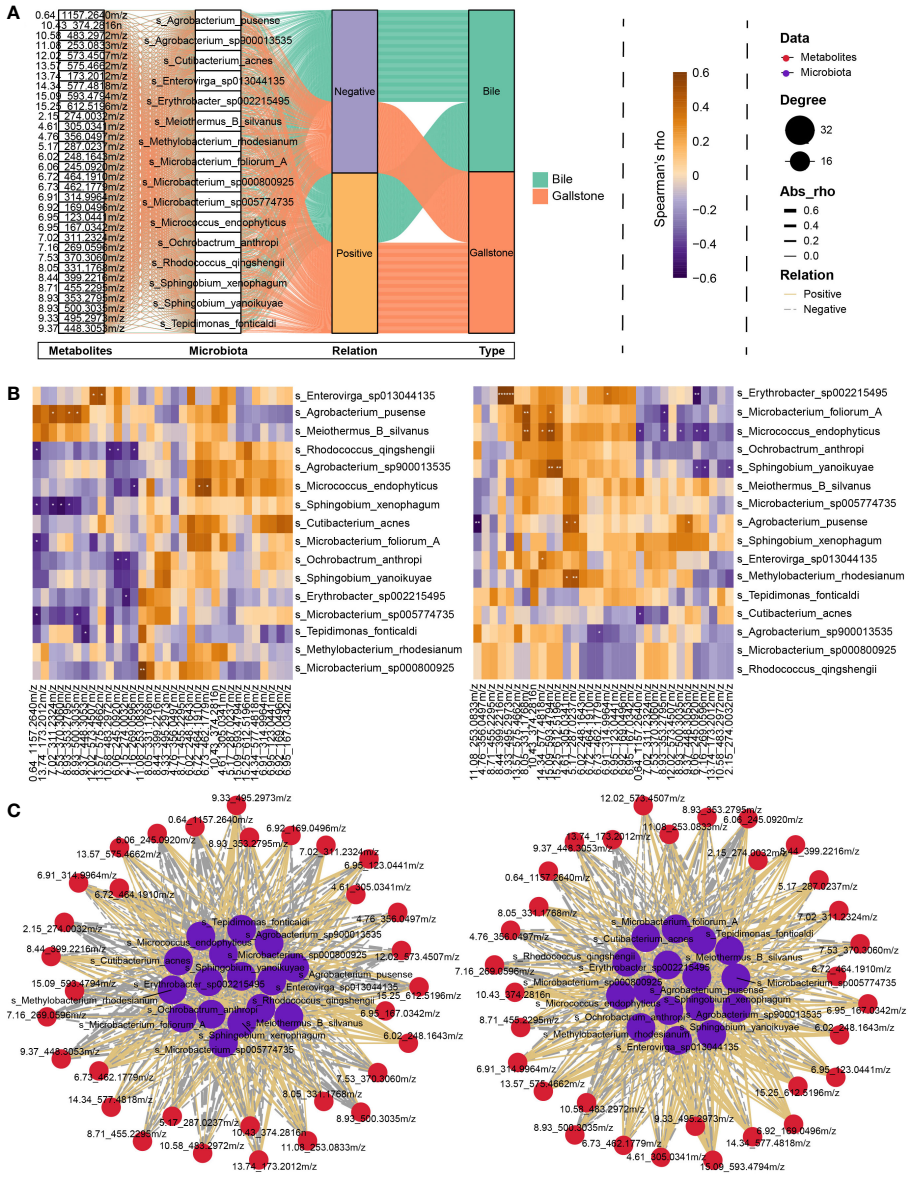

FIGURE 7
Interplay between gallbladder microbial species and plasma metabolites. **(A)** Sankey diagram showing an overview of the relationship between representative microbiota and metabolites. Significantly altered metabolites, microbial species, relationship, and sample type are shown from left to right. **(B)** Heatmap of the Spearman correlation between representative microbiota and metabolites in bile (Left) and gallstone (Right) related to panel **(A)**. The color indicates the Spearman's *rho* value. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. **(C)** Network of correlation between representative microbiota and metabolites in bile (Left) and gallstone (Right) samples related to panel **(B)**. Node sizes are presented as degrees. The thickness of the edge represents the absolute Spearman's *rho* value. Solid edges represent positive correlation, and dotted lines represent negative correlation.

study, consistent with previous findings that cholesterol stones rarely exhibit bacterial signatures (Stewart et al., 1987). The biofilm forming bacterial species *Meiothermus silvanus* that requires sulfur compounds to grow is abundant in the gallbladder, especially in pigment gallstones (Chung et al., 1997). Evidently, Kose et al. found that bacterial carbohydrate and sulfur metabolism were more abundant in pigment gallstones using a metagenomics approach (Kose et al., 2018). Many pathogenic species including *Acinetobacter johnsonii*, *Streptococcus agalactiae*, *Streptococcus mutans*, and *Dietzia maris* are greatly abundant in the gallbladder of GSD subjects (Supplementary Figure 2). In addition, *Agrobacterium pusense* (*Rhizobium pusense*), the chief human pathogen within the *Agrobacterium* spp., is also a predominant species in the gallbladder of GSD subjects (Aujoulat et al., 2015). *Vulcaniibacterium thermophilum* can degrade chlorinated phenols and other aromatic compounds and is also one of the most abundant species in the gallbladder (Wu et al., 2021). *Escherichia flexneri* is a waterborne and foodborne bacterial pathogen. Bile salts have been reported to promote strengthened *E. flexneri* biofilm formation and stimulate virulence factor expression (Nickerson et al., 2017). *Cutibacterium acnes* commonly colonizes human skin and, can also colonize medical devices due to its biofilm-forming ability (Ahle et al., 2023). *Klebsiella pneumoniae* typically colonizes human mucosal surfaces of the oropharynx and gastrointestinal

tract and can display high degrees of virulence and antibiotic resistance when entering the body (Ashurst and Dawson, 2023). Overall, disordered gallbladder microbial ecology might contribute to the pathogenesis of gallstone and could be a target for controlling pathogenic microbiota and preventing gallstone formation.

Metabolomics is a powerful way to assess the potential role of metabolites on disease. However, the metabolic disorders of people with cholesterol and pigment gallstones remain unclear. Here, we performed untargeted metabolomics of plasma samples to delineate the metabolic variance in plasma samples between cholesterol and pigment GSD individuals. Distinct metabolic profiling was observed between the two gallstone types, indicating that different metabolic dysfunctions might contribute to the formation of different types of gallstones (Figure 4). Ergothioneine, synthesized only by some microorganisms, is present at a high level in the plasma of subjects with cholesterol gallstones. However, it is suggested that a deficiency in blood/plasma could be associated with the disease onset or progression (Halliwell et al., 2018). Thus, this finding could potentially be explained by plasma level differences, which were not assessed here and require further study. However, ergothioneine levels are lower in pigment GSD subjects than in cholesterol GSD subjects (Figure 4). Plasma metabolites can potentially be assessed to quickly diagnose many diseases, such as Parkinson's disease and nonalcoholic fatty liver disease (Masoodi et al., 2021; Pathan et al., 2021). Flavonoids, such as malvidin 3-(6"-malonylglucoside), are closely related to cholesterol gallstones, while 2-hydroxy-9Z,12Z-Octadecadienoic acid is a potential diagnostic marker for pigment gallstones (Figure 6). Although we have identified potential plasma metabolite biomarkers, rigorous validation in large cohort is warranted.

The altered metabolite dataset enrichment analysis revealed that bile acid biosynthesis is closely related to cholesterol gallstones (Figure 5). Thirty years ago, Berr and coworkers reported that cholesterol GSD is characterized by disordered bile acid metabolism (Berr et al., 1992). A recent study has linked the gut microbiota, bile acids, and biliary cholesterol with the formation of cholesterol gallstones (Hu et al., 2022). Altogether, these evidences suggest that bile acids play a vital role in cholesterol GSD. The detailed changes in the bile acids profiling of cholesterol GSD patients require further investigation using a targeted metabolomics approach.

Accumulating metabolomic evidence suggests that microbiota affect host metabolism. Therefore, we further explored the correlations between gallbladder microbial species and plasma metabolites. We found a strong association between gallbladder microbiota and plasma metabolites. Importantly, the interactions between the microbiota and plasma metabolites differed substantially between bile and gallstone samples (Figure 7). Notably, as we reported previously, the gallbladder microbiota of GSD patients differed substantially between bile and gallstone samples (Hu et al., 2023). The current study provides further evidence that the bile and gallstone microbiota pronouncedly differ, and bacteria are the most abundant microbial type in the gallbladder of GSD patients (Figure 3; Supplementary Figure 2). Thus, even the same species has a different correlation with plasma metabolites between bile and gallstones, such as *Erythrobacter sp002215495* and *Agrobacterium pusense*. This finding indicates that microbiota may differ in different environments and, thus, influence the host metabolism. However, further research is required to elucidate the potential interactions found in the current study.

Moreover, we explore the power for using plasma metabolites to discriminate the two gallstone types. Our results show that some metabolites including malvidin 3-(6"-malonylglucoside) (AUC = 0.88) are very powerful biomarkers for distinguishing the types of gallstones (Figure 6). This, in turn, opens new avenues for metabolite-based diagnostics and even therapeutics that benefit from the easy accessibility of the blood for metabolites monitoring and manipulation. As mentioned above, microbiota can affect host metabolism in both health and disease status by derived microbial compounds (Fan and Pedersen, 2021). Our integrated microbiome and metabolome census analyses also suggest that gallbladder microbiota may affect the plasma metabolites although cause-and-effect study is further needed. Together, our finding implies that plasma metabolite as well as gallbladder microbiota could jointly serve as diagnostic markers for different types of gallstones.

Our study has some limitations. First, this was a cross-sectional study lacking dynamic patient follow-up. Therefore, a longitudinal study on the dynamic evolution of metagenomic and metabolomic profiles in GSD and basic research studies investigating the potential underlying mechanisms are needed. Second, the current study does not involve a control group of healthy subjects. Indeed, as we discussed previously, control bile microbiota could be obtained from liver transplant donors when applicable (Molinero et al., 2019; Hu et al., 2023). Healthy plasma metabolic profiling is also absent. Since this is a cross-sectional study with a wide dispersion of ages, it is difficult to find adequately matched controls. Accordingly, the levels of the thousands of plasma metabolites are strongly influenced by an individual's genetics, diet, and gut microbial composition (Chen et al., 2022). Moreover, a reference map of potential determinants for the human serum metabolome has also suggested the importance of host genetics, gut microbiome, clinical parameters, diet, and lifestyle (Bar et al., 2020). However, we could not match the details of interested metabolites found in our study from these studies. This phenomenon confirms that the metabolic profile of the current study is a unique, person-specific signature. Thus, the aim of this study was to excavate the alterations and potential applications of gallbladder microbiota and plasma metabolomics between cholesterol and pigment GSD patients, especially focusing on cholesterol GSD. Last, the limited sample size especially in pigment GSD group might limit the significance of the MSEA results. Thus, large cohort concerning this topic is warranted in the future. Regardless, our results shed light on the importance of bile acids metabolism in cholesterol GSD.

Conclusions

We identified distinct changes in the gallbladder microbiota and functional alterations in bile acid biosynthesis pathways during cholesterol gallstone disease progression. In addition, the intricate

interactions among several GSD-related species and the altered plasma metabolites are closely associated with GSD progression. Our multiomics analyses identified potential mechanisms underlying the microbiota–gallbladder–bile acids axis in GSD progression and uncovered promising evidence for novel targets for GSD diagnosis and intervention. Moreover, the sample type (such as bile)-related changes in gallbladder microbiota combined with plasma metabolite differences provide a potential avenue for noninvasively diagnosing gallstone types; however, the findings require validation in a large population.

Data availability statement

Sequencing data for all samples have been deposited in the Genome Sequence Archive GSA, http://bigd.big.ac.cn/gsa database in the BIG Data Center, Chinese Academy of Sciences, under accession code CRA010801. LCMS metabolomics datasets raw and processed files are available on the EMBL-EBI MetaboLights repository under the MTBLS7736 identifier https://www.ebi.ac.uk/metabolights/MTBLS7736. Other data supporting this study's findings are available from the corresponding author upon reasonable request. The analysis methods and software used in this article are all open source, and no new methods or algorithms were generated.

Ethics statement

The studies involving humans were approved by The Institutional Ethics Review Board of the Third People's Hospital of Chengdu. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

XZ: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. JH: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. YL: Data curation, Investigation, Resources, Writing – review & editing. JT: Data curation, Investigation, Resources, Writing – review & editing. KY: Data curation, Investigation, Resources, Writing – review & editing. AZ: Investigation, Resources, Writing – review & editing. YJL: Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. TZ: Conceptualization, Funding acquisition, Project administration, Validation, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1283737/full#supplementary-material

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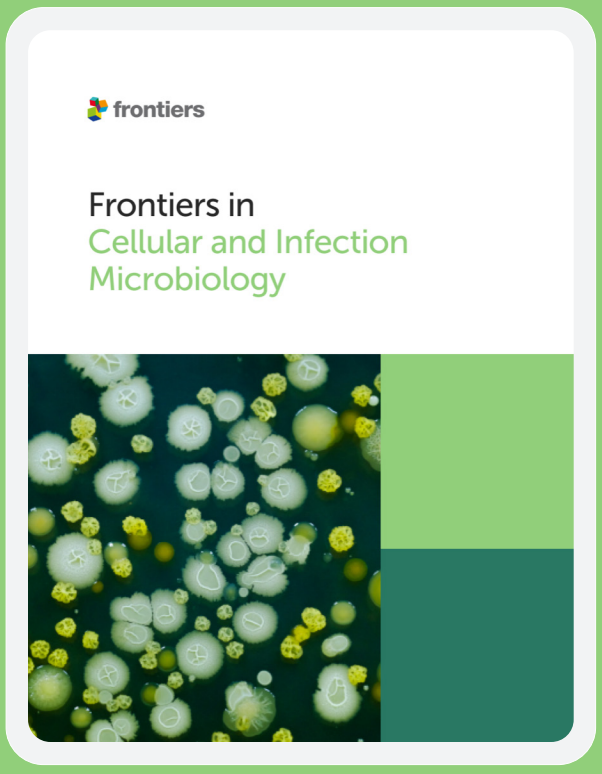

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