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The microbiome in the development of gastrointestinal diseases

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The microbiome in the development of gastrointestinal diseases

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Editorial: The microbiome in the development of gastrointestinal diseases

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Editorial on the Research Topic
The microbiome in the development of gastrointestinal diseases

The human microbiome, a complex and dynamic ecosystem composed of trillions of microorganisms residing in various body sites, plays a critical role in maintaining health and homeostasis. Recent research has increasingly focused on the gut microbiome (GM) due to its significant influence on gastrointestinal (GI) health and its involvement in the development of various GI diseases. This editorial synthesizes findings from 16 manuscripts, including 11 original research articles, 3 reviews, 1 mini-review, and 1 systematic review, authored by a diverse group of 109 researchers from countries including China, Croatia, Finland, France, Germany, India, Iran, Kazakhstan, Poland, Portugal, Romania, Slovakia, and Sweden. Collectively, these studies highlight the intricate relationships between gut microbiome composition and several GI disorders, including colorectal cancer (CRC), inflammatory bowel disease (IBD), diverticular disease, small intestinal bacterial overgrowth (SIBO), and metabolic dysfunction-associated fatty liver disease (MAFLD) (Figure 1). The evidence presented reveals how dysbiosis (microbial communities' imbalance) can contribute to inflammation, impaired immune responses, and altered metabolic functions that predispose individuals to these diseases. Furthermore, new insights into the gut-brain axis are revealing how GM can influence not only local intestinal health, but also systemic conditions affecting other organs. Interventions aimed at modulating the microbiome composition and/or function by prebiotics, probiotics, or dietary changes have shown promise in alleviating symptoms and improving treatment outcomes in patients with GI diseases. As our understanding of the GM role expands through this extensive body of work in multiple international contexts, it becomes increasingly clear that targeting the microbial balance may offer innovative strategies for the prevention and effective management of GI diseases.

The study by Magnan et al. investigates the relationship between GM, bacterial translocation, and acute GI injury in critically ill patients with septic shock. The study involved 60 adults over seven days and assessed changes in GM diversity and their correlation with clinical outcomes. Results show a significant decrease in bacterial diversity and richness from day 0 to day 7, with lower alpha diversity associated with higher SOFA

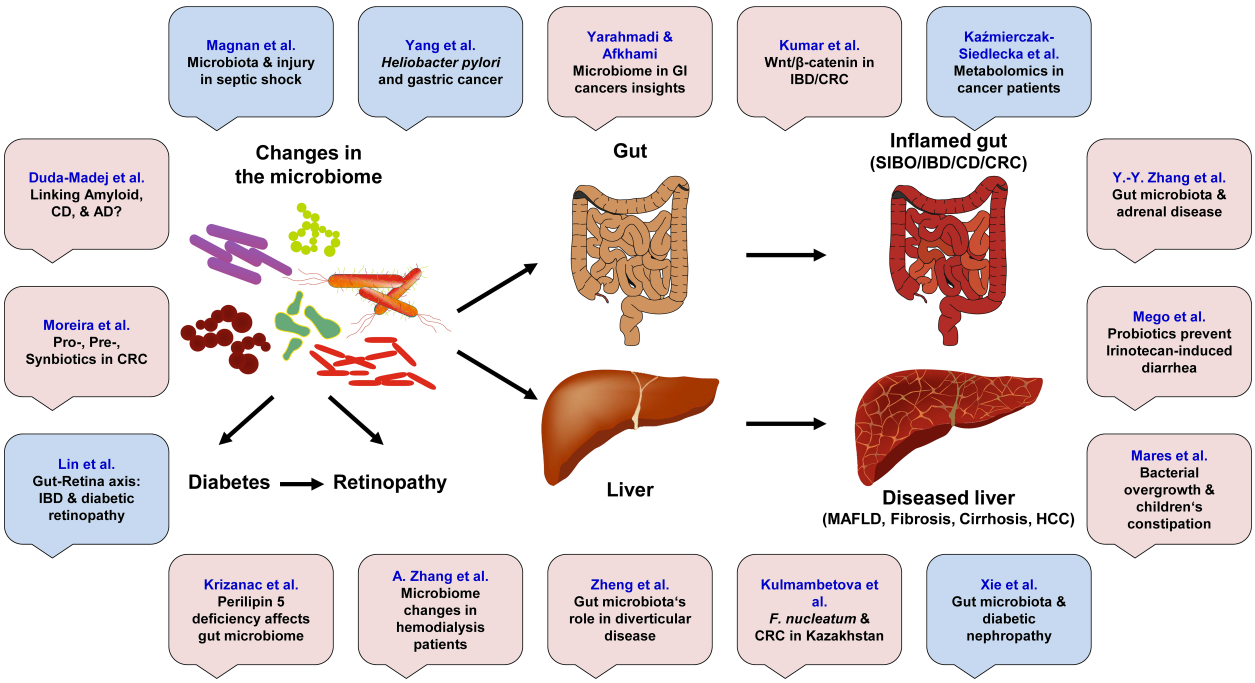

FIGURE 1
This Research Topic contains 11 original articles (marked in bright red) and 5 review article (marked in light blue). The articles highlight the relationships between changes in the gut microbiome and several gastrointestinal disorders, including colorectal cancer (CRC), inflammatory bowel disease (IBD), diverticular disease, small intestinal bacterial overgrowth (SIBO), metabolic dysfunction-associated fatty liver disease (MAFLD), and hepatocellular carcinoma (HCC). These changes can also be associated with diabetes, retinopathy, Alzheimer disease (AD) and many other disorders.

scores. An increase in *Enterococcus* species was observed alongside a decrease in beneficial bacteria such as *Bifidobacterium*. In addition, increased levels of bacterial translocation were observed at both admission and day 7 compared to healthy controls (HC), suggesting that gut inflammation may promote bacterial translocation into the circulation. Mortality analysis revealed that non-survivors had lower GM diversity on admission. Certain genera such as *Mogibacteriaceae* were more abundant in non-survivors, while others such as *Escherichia* decreased over time. The increase in *Enterococcus* during hospitalization correlated with worse outcomes. The study concludes that dysbiosis and bacterial translocation significantly influence acute GI severity and mortality risk in septic shock patients, suggesting further exploration of therapeutic strategies targeting GM to improve patient outcomes.

Yang et al. present a bibliometric analysis of research related to *Helicobacter pylori* (HP) and gastric cancer (GC) from 2003 to 2022. Their study aims to assess scientific output, identify influential papers, summarize current knowledge, and explore emerging trends in the field. A total of 1,970 papers were retrieved, showing an increasing trend in publications over the years. China and Japan emerged as the leading contributors, with Vanderbilt University notable for its high output. Key authors include Richard M. Peek Jr. and Maria B. Piazuelo, both from Vanderbilt University. The journal "*Helicobacter*" published the most papers, while "*Gastroenterology*" had the highest number of citations. The analysis highlights relevant themes such as the HP role in gastric tumorigenesis, its pathogenesis in relation to GC and the mechanisms by which HP affects GC development. Emerging areas for future research include autophagy, GM interactions, implications for immunotherapy, exosome functions, epithelial-mesenchymal transition, and γ-glutamyl transpeptidase. The findings underscore that HP is a major risk factor for GC through mechanisms involving inflammation and immunity modulation. HP eradication may prevent early GC stages and improve treatment outcomes. In conclusion, this study provides valuable insights into the global landscape of HP/GC research suggesting potential directions for future investigations to address gaps in knowledge of their interplay.

The review by Yarahmadi and Afkhami highlights the significant link between GM and the development of GI cancers, which account for a third of new cancer cases worldwide. The authors discuss how perturbations in the GI microbiota may influence cancer progression, with some bacteria being cancer-promoting and others being protective. Recent studies suggest that alterations in GM composition are associated with several GI malignancies, including colorectal, gastric, liver and esophageal cancers. The review highlights the relevance of understanding these microbial communities and their interactions with the host immunity as potential avenues for cancer prevention and treatment strategies. The authors explore how GM can affect the efficacy of cancer therapies such as chemotherapy, immunotherapy, and radiotherapy. They report that dysbiosis can lead to inflammatory responses that exacerbate cancer progression and specific bacteria, such as *Fusobacterium nucleatum* (*F. nucleatum*), have been implicated in chemoresistance in CRC. Additionally, the authors

discuss emerging research on non-bacterial components of the microbiome, including viruses and fungi, which also play a role in GI cancers. For example, certain viral infections have been associated with an increased cancer risk. In conclusion, this comprehensive analysis underscores the dual GM role in both facilitating and inhibiting GI carcinogenesis, while suggesting that modulation of these microbial communities may improve therapeutic outcomes for patients with GI cancers.

In their mini-review, Kumar et al. explore the relationship between GM, its secondary metabolites, and the regulation of the Wnt/β-catenin signaling pathway in the context of IBD and CRC. GI cancers represent a significant public health burden, with rising incidences associated with GM dysbiosis. The authors discuss how secondary metabolites produced by gut microbes, such as short-chain fatty acids (SCFAs) and bile acids, play a critical role in maintaining intestinal homeostasis and regulating inflammation-driven tumorigenesis. Dysbiosis can lead to altered levels of these metabolites, resulting in immune cells' activation that contributes to chronic inflammation and increased cancer risk. The review emphasizes the relevance of the Wnt/β-catenin pathway in CRC progression; in detail its dual role in modulating inflammation and promoting cell proliferation. It highlights that microbial metabolites such as butyrate inhibit this pathway, suggesting potential therapeutic avenues for treating CRC through dietary or probiotic interventions. Additionally, the authors discuss how bile acids interact with nuclear receptors such as the farnesoid X receptor to influence both bile metabolism and the Wnt signaling pathway. This interplay provides opportunities for novel therapeutic strategies targeting these mechanisms to attenuate IBD-related inflammation and CRC development. In conclusion, understanding the interactions between gut-derived metabolites and Wnt signaling may provide new treatments approaches for GI cancers while minimizing the side effects associated with conventional therapies. The review calls for further research into these relationships in order to develop effective combinatorial therapies aimed at improving treatment outcomes for patients with IBD and CRC.

The research article by Kaźmierczak-Siedlecka et al. investigates the gut metabolome in patients with gastric cancer (GC) (n=4) and CRC (n=8) prior to initiation of anticancer treatments. The study aims to explore potential differences in metabolite profiles that could serve as biomarkers for these cancers. Stool samples were collected from 12 patients, and untargeted metabolomics was performed using mass spectrometry to analyze a wide range of metabolites. The results revealed distinct metabolic profiles, with higher levels of certain metabolites found predominantly in CRC patients compared to those with GC. Notably, metabolites such as deoxyguanosine, uridine, L-phenylalanine, and 3-indoleacetic acid were significantly elevated in CRC patients. The analysis revealed a more homogeneous metabolic profile among GC patients compared to the diverse profiles observed in CRC patients. This suggests that tumor localization may influence the GM activity and so its metabolites' production. The authors acknowledge the limitations due to the small sample size but emphasize that these preliminary findings pave the way for further research into untargeted metabolomics as a non-invasive tool for early detection and monitoring of GI cancers. Future studies are planned to assess the impact of anti-cancer treatments on these metabolic profiles. In conclusion, this study highlights the potential role of gut-derived metabolites as biomarkers for discriminating between GC and CRC, while highlighting the need for larger studies to validate these findings.

The review by Duda-Madej et al. explores the potential links between Crohn's disease (CD), a chronic inflammatory bowel condition, and Alzheimer's disease (AD), a common neurodegenerative disorder. Both diseases are characterized by complex pathomechanisms involving genetic, environmental, immunological, and microbiological factors. Recent evidence suggests that chronic inflammation in conditions such as CD may increase the risk of AD developing. The authors highlight the gut-brain axis as a critical pathway linking these two diseases, where GM influences neuroinflammatory processes and amyloid aggregation associated with AD. Specifically, they discuss how GM dysbiosis can lead to increased permeability of the intestinal barrier, allowing pro-inflammatory substances to enter the systemic circulation and potentially reach the brain. The review emphasizes the role of amyloid proteins produced by both human cells and gut bacteria, especially bacterial amyloid peptides (curli fimbriae) from certain bacteria that mimic human amyloids. These bacterial amyloids may contribute to neuroinflammation and amyloid-beta aggregation in AD. Furthermore, the authors note that alterations in the GM composition may affect immune responses and metabolic processes associated with both CD and AD. They call for further research into microbial metabolites as potential therapeutic targets for the treatment of both diseases. In conclusion, this review suggests a significant relationship between CD and AD through shared inflammatory pathways and microbial influences. Understanding these links may lead to novel strategies for the prevention and treatment of neurodegenerative diseases rooted in GI health.

In a systematic review by Moreira et al. the therapeutic potential of GM modulation by prebiotics, probiotics, and synbiotics in patients with CRC is discussed. The review follows PRISMA guidelines and includes 24 randomized controlled trials (RCTs) assessing the effects of these supplements on CRC treatment outcomes, focusing on surgical recovery, chemotherapy, and radiotherapy side effects. The authors found that supplementation significantly improved surgical outcomes by decreasing postoperative complications, such as infections and GI symptoms like diarrhea. The results showed that patients who received probiotics or synbiotics had a faster return to normal gut function and shorter hospital stays than control groups. In detail, specific strains such as *Lactobacillus rhamnosus* and *Bifidobacterium lactis* were often associated with positive outcomes. However, the evidence regarding the optimal formulations, such as strain combinations, dosages, and administration duration, remains limited due to high heterogeneity between trials. In addition, the review highlights that while some trials reported benefits of probiotic supplementation during chemotherapy or radiotherapy, others showed no significant improvements. The authors emphasize the need for more RCTs with larger sample sizes and standardized

protocols to further clarify the effectiveness of these interventions. In conclusion, this systematic review suggests that pre-, pro-, and synbiotic supplementation may offer beneficial effects for CRC patients undergoing treatment by improving recovery and alleviating treatment-related side effects. Future research should focus on optimizing these interventions to improve clinical outcomes in CRC management.

The research article by Lin et al. investigates the potential association between IBD and diabetic retinopathy (DR) using Mendelian randomization (MR) and mediation analysis. The study uses genome-wide association study (GWAS) data to explore causal relationships, focusing on IBD subtypes, ulcerative colitis (UC) and CD, and their association with DR. The results indicate a significant negative correlation between UC and DR risk, suggesting that increased inflammation in IBD may affect retinal health. Conversely, the authors suggest that DR may reduce the CD incidence. Mediation analysis identified circulating inflammatory proteins, in particular fibroblast growth factor 21 (FGF21), phosphatidylcholine, and triglycerides, as mediators in these relationships. Elevated FGF21 levels are associated with both microvascular complications in diabetes and intestinal inflammation, highlighting its potential role as a biomarker for DR. The authors emphasize the relevance of understanding the gut-retina axis, noting that dysbiosis in DR may affect systemic inflammation and lipid metabolism, influencing both conditions. They acknowledge limitations such as the focus on participants of European ancestry in the GWAS data, which may affect generalizability. In conclusion, this research provides insights into common pathways between IBD and DR, suggesting that therapeutic strategies targeting these pathways may improve outcomes for patients with both conditions. Further studies are warranted to explore these relationships more comprehensively in diverse populations.

Mares et al. investigate the relationship between SIBO and constipation in pediatric patients. SIBO is characterized by an abnormal increase in bacteria in the small intestine, leading to symptoms ranging from mild GI discomfort to more serious problems such as malabsorption. The authors conducted a thorough literature search and included 79 studies that investigated the prevalence, diagnosis, and treatment of SIBO in children. They highlighted the challenges of diagnosing SIBO due to variations in methodology and lack of standardized criteria, with particular emphasis on breath tests using glucose or lactulose as substrates. The findings suggest that SIBO is common in children with functional GI disorders, although rates vary widely depending on study design. The review notes a strong association between methane production during breath testing and constipation, although results are inconsistent between studies. Treatment strategies for SIBO typically include antibiotics, dietary changes, and probiotics, but research into pediatric applications remains limited. The authors emphasize the need for well-designed studies with larger sample sizes to establish clearer diagnostic criteria and effective treatment protocols tailored for children. In conclusion, although there is evidence linking SIBO to constipation in children, further research is needed to clarify these associations and improve clinical management strategies. The article calls for future research to focus on standardized diagnostic methods and to explore dietary interventions or probiotic therapies as potential treatments for pediatric SIBO.

In their original article, Xie et al. investigated the potential causal relationship between GM and diabetic neuropathy (DN) using MR. The study aimed to clarify how GM changes may influence the DN development and vice versa. Data from GWAS were used, focusing on non-Finnish Europeans for IBD and the FinnGen project for DN. The authors used various MR methods, including inverse-weighted variance analysis, to determine causal relationships while assessing pleiotropy and heterogeneity. The results showed significant associations between specific GM taxa and DN. Elevated levels of the *Christensenellaceae R-7*, *Ruminococcaceae UCG013*, and *Eggerthella* groups were associated with an increased DN risk, whereas *Peptococcaceae and Eubacterium coprostanoligenes* showed protective effects. Reverse MR analysis revealed that elevated levels of *Anaerofilum*, *Dorea*, *Lachnospiraceae UCG-010*, *Ruminococcus 2*, and order NB1n may also contribute to an increased risk of DN. The study highlights the importance of understanding the gut-retina axis in relation to metabolic diseases such as diabetes. It suggests that GM-derived metabolites could serve as non-invasive diagnostic or therapeutic targets for early DN detection. In conclusion, this research provides valuable insights into the complex interactions between GM and DN and supports the hypothesis that these factors are causally related. Further investigations are needed to elucidate the underlying mechanisms and to explore potential clinical applications in the treatment of DN by GM modulation.

The article by Zhang et al. examines the association between GM and three adrenal disorders: adrenocortical insufficiency (AI), Cushing's syndrome, and hyperaldosteronism (HA). Using data from GWAS, the authors used a bi-directional MR approach to investigate these associations. The study identified several bacterial taxa associated with AI, such as *Deltaproteobacteria* and *Desulfovibrionaceae*, which were found to have protective effects against AI. On the other hand, certain families including *Porphyromonadaceae* were associated with an increased AI risk. *Acidaminococcaceae* was identified as a protective factor for Cushing's syndrome, while *Methanobacteria* and *Lactobacillaceae* played a protective role in hyperaldosteronism. Conversely, genera such as *Parasutterella* and *Peptococcus* were associated with an elevated risk for HA. Sensitivity analyses confirmed the reliability of these results, showing no significant horizontal pleiotropy or heterogeneity between the instrumental variables used in the MR analysis. The inverse MR analysis showed no significant causal relationships from adrenal disease to GM. The authors suggest a potential causal relationship between specific gut microbial taxa and adrenal disease, which may provide new diagnostic opportunities and focus on GM modulation. However, they acknowledge limitations related to sample diversity and the exclusion of many single nucleotide polymorphisms during their analysis, a call for further research to validate these findings in diverse populations.

The study by Mego et al. evaluates the probiotics' effectiveness in reducing irinotecan-induced diarrhea in CRC patients with colostomies. The analysis combines data from two clinical trials involving 279 patients, who were randomized to receive either

probiotics or a placebo during their chemotherapy regimen. The results show that while the overall incidence rates of grade 3/4 diarrhea did not differ significantly between groups, subgroup analyses showed that patients with colostomies who received probiotics had a significantly lower incidence of any diarrhea (25.7% *vs*. 51.2%, p=0.028) and no cases of severe diarrhea compared to those who received placebo. Probiotic use was also associated with reduced use of anti-diarrheal medication, although this finding was not statistically significant. The study highlights the potential probiotics' benefits specifically for CRC patients with colostomies undergoing treatment with the topoisomerase inhibitor irinotecan, and suggests that maintaining a healthy GM could help to reduce GI toxicity associated with chemotherapy. Despite these promising findings, the authors acknowledge limitations such as the variability of probiotics' formulations and the lack of preclinical testing before human trials. They call for further research into different probiotic strategies and the mechanisms underlying the observed effects to optimize treatment outcomes for CRC patients experiencing chemotherapy-related side effects. Overall, the study points to a critical area for future investigation regarding GM modulation in cancer treatment.

In their original article, Krizanac et al. investigated the role of perilipin 5 (PLIN5) in regulating GM during the development of metabolic dysfunction-associated fatty liver disease (MAFLD) and its progression to hepatocellular carcinoma (HCC). Using mouse models, the study investigated how PLIN5 deficiency affects the GM composition when mice are fed a Western diet. The authors observed significant changes in microbial diversity, with an increased abundance of beneficial taxa such as *Lactobacillus* in *Plin5*-deficient mice compared to wild-type controls. Additionally, the study found that a Western diet exacerbated these microbial changes and specific bacterial taxa associated with metabolic pathways relevant to liver health were identified. *Deltaproteobacteria* and *Desulfovibrionaceae* were associated with protective effects against liver disease, while certain other taxa were associated with an increased disease risk. The results suggest that *Plin5* plays a critical role in shaping the GM composition, thereby influencing metabolic processes associated with liver disease. By highlighting the interactions between dietary factors, genetic predisposition, and GM, this research opens avenues for potential therapeutic strategies targeting *Plin5* to modulate gut flora and mitigate the progression of liver disease. In conclusion, the study highlights the relevance of understanding how *Plin5* deficiency affects GM dynamics within MAFLD and subsequent progression to HCC, and emphasizes future research directions focusing on human studies to further explore these relationships for therapeutic applications.

The original paper by Zhang et al. investigates the GM profiles in maintenance hemodialysis (MHD) patients who suffer from constipation. They aim to understand how changes in gut flora may contribute to GI symptoms commonly observed in these patients. Fecal samples were collected from 45 participants, including 15 with MHD-related constipation, 15 without constipation, and 15 healthy controls. The authors analyzed differences in GM composition between the groups. The results showed that the MHD constipation group had reduced microbial diversity compared to non-constipated MHD patients and healthy controls. At the genus level, *Enterococcus* and *Escherichia-Shigella* were dominant in constipated patients, while beneficial taxa such as *Bifidobacterium* and *Faecalibacterium* were less abundant. The analysis suggested that certain potentially pathogenic bacteria may exacerbate inflammation and contribute to constipation. Additionally, pathways involved in pyruvate metabolism and flavonoid biosynthesis were enriched in constipated patients, suggesting a metabolic dysregulation associated with their condition. The study highlights a potential link between GM composition and inflammatory responses that may influence gut function. In conclusion, this research provides insights into how GM changes iome may influence constipation in MHD patients. It highlights the need for further investigation into specific bacterial taxa and their role in GI health to develop targeted therapeutic strategies, such as fecal microbiota transplantation (FMT), for the management of constipation associated with hemodialysis.

Zheng et al. investigated the causal relationship between GM and intestinal diverticular disease using a bidirectional two-sample MR approach. The study used genetic instrumental variables from a genome-wide association study involving 5,959 participants to assess the GM effect on diverticular disease, including 5,193 cases and over 457,000 controls sourced from the IEU Open GWAS project. The analysis revealed significant associations between specific microbial taxa and the risk of developing intestinal diverticular disease. In detail, increased levels of *Caryophanales*, *Paenibacillaceae*, *Herbinix*, *Turicibacte*r, and *Staphylococcus fleurettii* were associated with a higher risk of the disease. Conversely, *Chromatiales* and *Arcobacter* showed protective effects. In addition, reverse MR analysis did not reveal any significant causal relationships from diverticular disease back to GM. The findings underscore that variations in GM composition may influence the onset and progression of diverticular disease. This research highlights the importance of understanding how GM changes may contribute to GI disorders such as diverticulosis. It suggests potential avenues for personalized treatment strategies targeting specific microbial populations to prevent or effectively treat diverticular disease. In conclusion, this study provides insights into the relationship between GM and diverticular disease, while highlighting the need for further research in diverse populations to validate these findings and more fully explore the underlying biological mechanisms.

The original research article by Kulmambetova et al. investigates the prevalence of *F. nucleatum* and its association with CRC in patients in Kazakhstan. The study included 83 patients with histologically confirmed CRC, from whom 249 biopsy specimens were collected, including carcinoma tissue (CT), adjacent normal tissue (AT), and distant normal tissue (NT). Using quantitative real-time polymerase chain reaction, the authors detected *F. nucleatum* along with other CRC-associated bacteria such as *Bacteroides fragilis*, *Escherichia coli*, and *Streptococcus gallolyticus*. The results showed a significantly higher *F. nucleatum* prevalence in CT compared to AT and NT, with detection rates of 43.4%, 27.7% and 24.1%, respectively (p=0.02). The frequency of *F. nucleatum* was significantly higher in tumors

located distally in the colon and was associated with larger tumor size and higher consumption of processed meat. Additionally, although no significant correlations were found between *F. nucleatum* infection and various clinical characteristics such as age or sex, the study highlights its potential as a marker for CRC diagnosis due to its association with tumor progression. In conclusion, this study underscores the role of *F. nucleatum* in the CRC pathogenesis in Kazakhstani patients and suggests that it may serve as a valuable diagnostic biomarker for CRC management, warranting further investigation into its mechanisms and implications for cancer development.

The collection of reviews and original research articles highlights the significant role of the GM in various health conditions, particularly in relation to GI and metabolic diseases. Studies investigating the associations between GM and conditions such as CRC, diabetic neuropathy, diverticular disease, and complications arising from maintenance hemodialysis provide compelling evidence that microbial composition can influence disease development, progression, and treatment outcomes. For example, the systematic review of pre-, pro- and synbiotic supplementation suggests that these interventions may improve surgical outcomes and reduce chemotherapy-related side effects in CRC patients. Similarly, research into *F. nucleatum* shows its prevalence in CRC tissues and its potential as a diagnostic marker for this malignancy. Furthermore, studies using MR demonstrate causal relationships between GM changes and various diseases, reinforcing the concept that specific microbial taxa may either contribute to or protect against diseases such as DN and diverticular disease. Overall, these findings underscore the relevance of understanding the dynamics of the gut microbiome and its interactions with host physiology. They suggest that targeting GM profiles through dietary modification or probiotic therapies may offer promising avenues for prevention and management strategies in various health contexts. This growing body of evidence highlights the need for continued research into the complex interplay between GM, diet, inflammation, and disease in order to develop effective therapeutic approaches aimed at restoring microbial balance for improved health outcomes. In particular, potential confounding factors such as diet, lifestyle, genetic predisposition and environmental exposures play a critical role in shaping the GM composition and function. A more nuanced understanding of how these elements interact with microbial communities can help elucidate their collective impact on health outcomes and disease susceptibility.

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Role of gut microbiota and bacterial translocation in acute intestinal injury and mortality in patients admitted in ICU for septic shock

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Introduction: Sepsis is a life-threatening organ dysfunction with high mortality rate. The gut origin hypothesis of multiple organ dysfunction syndrome relates to loss of gut barrier function and the ensuing bacterial translocation. The aim of this study was to describe the evolution of gut microbiota in a cohort of septic shock patients over seven days and the potential link between gut microbiota and bacterial translocation.

Methods: Sixty consecutive adult patients hospitalized for septic shock in intensive care units (ICU) were prospectively enrolled. Non-inclusion criteria included patients with recent or scheduled digestive surgery, having taken laxatives, pre- or probiotic in the previous seven days, a progressive digestive neoplasia, digestive lymphoma, chronic inflammatory bowel disease, moribund patient, and pregnant and lactating patients. The primary objective was to evaluate the evolution of bacterial diversity and richness of gut microbiota during seven days in septic shock. Epidemiological, clinical and biological data were gathered over seven days. Gut microbiota was analyzed through a metagenomic approach. 100 healthy controls were selected among healthy blood donors for reference basal 16S rDNA values.

Results: Significantly lower bacterial diversity and richness was observed in gut microbiota of patients at Day 7 compared with Day 0 ($p<0.01$). SOFA score at Day 0, Acute Gastrointestinal Injury (AGI) local grade, septic shock origin and bacterial translocation had an impact on alpha diversity. A large increase in Enterococcus genus was observed at Day 7 with a decrease in

Enterobacterales, Clostridiales, Bifidobacterium and other butyrate-producing bacteria.

Discussion: This study shows the importance of bacterial translocation during AGI in septic shock patients. This bacterial translocation decreases during hospitalization in ICUs in parallel to the decrease of microbiota diversity. This work highlights the role of gut microbiota and bacterial translocation during septic shock.

KEYWORDS

acute intestinal injury, bacterial translocation, evolution, gut microbiota, metagenome, septic shock

1 Introduction

Septic shock is the cause of 10 to 30% admission to intensive care units (ICUs) with mortality rates ranging from 35 to 40% (Sakr et al., 2018; Vincent et al., 2018; Vincent et al., 2019). The symptomatology is dominated by the presence of organ failures in which the intestine plays a major role (Fiddian-Green, 1988). Impaired perfusion and oxygenation of gastrointestinal tissues is classically reported despite restoration of hemodynamic parameters and systemic oxygenation after vascular filling and administration of vasopressors (Temmesfeld-Wollbrück et al., 1998). This damage induces an acute gastrointestinal injury (AGI) that can occur very early in critical illness, with a major influence on the prognostic of critically ill patients (Mutlu et al., 2001; Reintam Blaser et al., 2013; Zhang et al., 2018). Pathophysiologic mechanisms linking gut microbiota with AGI are probably multifactorial. Proposed mechanisms mainly include alterations in permeability of intestinal mucosal, increase of the host immune system due to general inflammation and activation of antigen presenting cells (Clark and Coopersmith, 2007; Reintam Blaser et al., 2013; Zhang et al., 2018). This is especially due to the specific ICU environment such as antibiotic therapy, vasopressors, mechanical ventilation and parenteral nutrition with their associated deleterious effect on the intestinal barrier. Gut microbiota dysbiosis is a hallmark of septic shock with reduction in gut microbiota diversity in ICU patients compared to healthy controls (Zaborin et al., 2014; McDonald et al., 2016; Ojima et al., 2016; Lankelma et al., 2017; Wan et al., 2018; Yin et al., 2019; Liu et al., 2020). However, none of these studies had identified low gut bacterial diversity as an independent risk factor for mortality in ICU patients with septic shock.

During AGI, an "intestinal crosstalk" takes place between the intestinal epithelium, the intestinal immune system and the gut microbiota (Niu and Chen, 2021). In critical illness, the loss of this interrelation causes systemic manifestations due to intestinal inflammation, local gut permeability and an increased permeability favorable to bacterial translocation, representing a major cause of multiple organ dysfunction syndrome (Clark and Coopersmith, 2007). Indeed, bacterial translocation is the process in which viable and/or bacterial elements cross the gastrointestinal barrier to reach the systemic circulation, disseminating microorganisms in the body (Sandler and Douek, 2012). Principal mechanisms promoting bacterial translocation are increased permeability of the intestinal mucosal barrier, deficiencies in host immune defenses and an imbalance (dysbiosis) of the diversity of gut microbiota (Balzan et al., 2007).

The gut microbiota lives in symbiosis with the body and plays a major role in progression of diverse diseases (Schmidt et al., 2018; Shanahan et al., 2021; Siwczak et al., 2021; Chen et al., 2022; Deng et al., 2022). A diverse and balanced gut microbiota strengthens the host's immunity to intestinal and systemic pathogens, and the dysbiosis of this microenvironment is linked to increased susceptibility to sepsis (Chen et al., 2022). Previous studies have observed that sepsis and its treatment severely affect the gut microbiota (Shimizu et al., 2011; Wolff et al., 2018; Agudelo-Ochoa et al., 2020). Dysbiosis due to pathogenic microorganisms plays a key role in the sepsis, resulting in the loss of commensal gut species (Wolff et al., 2018). Moreover, the abundance of *Enterococcus* spp. acts as a prognostic marker for patients with septic complications and an increased mortality (Shimizu et al., 2011; Agudelo-Ochoa et al., 2020). However, gut microbiota involvement in AGI is not yet known.

In this study, we aimed to describe the evolution of gut microbiota profiles and AGI in patients admitted to ICU for septic shock and to highlight the role of microbiota composition as a contributing factor on poor patient outcome.

2 Materials and methods

2.1 Study design

The present prospective single center observational study was conducted according to the Declaration of Helsinki and the French law (World Medical Association, 2013; Toulouse et al., 2023). It was

approved by a national ethic committee (CCP Ouest III; registration number n°2018-A02193-53) and was registered on ClinicalTrial.gov (NCT03861325). Before inclusion, the patients or representatives were informed of the study and his(her) rights to oppose to the use of their data.

2.2 Population

Sixty consecutive adult patients (≥ 18 years) admitted to ICU for septic shock (Nîmes University Hospital (France)) between July 2019 and September 2020 were prospectively enrolled. Septic shock was defined according to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) (Singer et al., 2016). It corresponds to a sepsis in which underlying circulatory and cellular metabolism abnormalities were profound enough to substantially increase mortality, referred to as a state of persisting hypotension despite administration of vasopressors to maintain mean arterial pressure greater than 65 mmHg, plus elevated serum lactate >2 mmol/L despite adequate fluid resuscitation. Non-inclusion criteria were: 1) a previous or scheduled digestive surgery; 2) the use of laxatives, pre- or probiotic in the previous 7 days; 3) a progressive digestive neoplasia, digestive lymphoma, chronic inflammatory bowel disease (Crohn's disease, etc.); 4) moribund patient, or patients with a care withdrawal or withholding decision; 5) pregnant and lactating patients; 6) and patients already included in a recent interventional trial.

2.3 Outcomes

The patients were followed-up and we determined the early mortality corresponding to death at Day 7 and the late mortality to death at Day 28. To understand the potential impact of the evolution of gut microbiota composition, the studied population was classified in two groups: survivors (alive at Day 28) and non-survivors (died at Day 28).

For the group of healthy controls, 100 subjects were included among healthy blood donors from the French Blood Establishment (EFS, Montpellier, France). Before blood donation, the healthy controls completed a questionnaire to ensure the absence of health problem. These controls were used to determine the basal level (cut-off) of the 16S rDNA marker performed in this study.

2.4 Clinical, biological and therapeutic data

Baseline clinical values were recorded at ICU admission and included the following data: age, sex, weight, associated comorbidities and septic shock origin. Treatments given within 7 days were systematically recorded as well as mechanical ventilation requirement, digestive symptomatology and organ failure. Tolerance to enteral nutrition was notified at Day 3 and 7. The Simplified Acute Physiology Score II (SAPS-II) (Le Gall et al., 2005) and the Sepsis Organ Failure Assessment (SOFA) score (Singer et al., 2016) were calculated within 24 h of admission and daily recorded for 7 days, respectively. SOFA scores ≤7, between 8 and 13 and ≥14 were considered as low, moderate and high, respectively (Elke et al., 2018). The AGI local score was defined in 3 stages: AGI score 0 in patients with no symptoms and low intra-abdominal pressure; AGI score 1 in intermediate patients, with low intra-abdominal pressure but some mild symptoms; AGI score 2 in patients with severe AGI (high pressure and/or more severe symptoms). Then, a clustering method was used to categorize patients into 5 AGI grades according to the guidelines published by the European Society of Intensive Care Medicine (ESICM): AGI grade 0: normal gastrointestinal function, AGI grade I: an increased risk of developing gastrointestinal dysfunction or failure, AGI grade II: gastrointestinal dysfunction, AGI grade III: gastrointestinal failure, and AGI grade IV: marked gastrointestinal failure with severe impact on distant organ function (Reintam Blaser et al., 2012).

2.5 Sample collection and gut microbiota analysis

Stool and blood samples (EDTA anticoagulant tubes) were collected at ICU admission (Day 0) and at Day 7. Fecal samples were stored directly at -80°C within two hours until further processing, whereas blood samples were immediately centrifuged (1,200g; 12 min), aliquoted and stored at -80°C.

Stool DNA extractions were performed from 250 mg of fecal material using the QIAcube automatic extractor (Qiagen, Courtaboeuf, France) with the DNeasy® PowerSoil Pro® kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. A minimum volume of 50 µL at a minimum concentration of 2.5 ng/µL, measured by the QUBIT® 3.0 fluorometric (Thermo Fisher Scientific Waltham, MA, USA) with the QuantiFluor dsDNA system® kit from the same supplier, was used. Negative extraction controls were performed using sterile water for each extraction run.

Metabarcoding analysis of DNA extracts from stool samples was performed by next-generation sequencing in collaboration with Genoscreen© company (Lille, France). The 16S rDNA genes of the hypervariable V3-V4 regions were amplified for the amplicon libraries preparation according to the Metabiote® protocol of Genoscreen©. Positive controls (artificial bacterial community composed of 15 bacterial strains and 2 archaeal strains) and negative controls were also integrated. Library sequencing was performed on a MiSeq run (Illumina Inc., San Diego, CA, USA) 2x250 base pairs chemistry. After validation of a quality control of the obtained sequences, demultiplexing was performed by CASAVA (Illumina®, Paris, France) software using the PERL script ConfigureBclToFactq. A quality filter (pre-processing) and reassembly of the reads using the FLASH tool (Magoc and Salzberg, 2011) were carried out according to the parameters optimized by the company Genoscreen©. Metabiote® Online v2.0 protocol was partially based on the QIIME v 1.9.1 software (Caporaso et al., 2010). After the pre-processing steps, the full-length 16S rDNA sequences go through a step where chimeric sequences are detected and eliminated (in-house method based on the use of Usearch 6.1).

Next, a clustering step was performed to group similar sequences with a defined nucleic identity threshold (97% identity for genus-level affiliation on the targeted region of the 16S rDNA gene) with Uclust v1.2.22q (Edgar, 2010) using an open reference and full linkage operational taxonomic units (OTU) creation process, ultimately creating groups of sequences or OTUs. The most abundant sequence in each OTU was then considered the reference sequence of its OTU and was taxonomically compared to a reference database (Greengenes database, version 13_8; www.greengenes.gov) using the RDP classifier v2.2 method, a naive Bayesian classifier that provided taxonomic assignments from a domain to a genus, with confidence estimates for each assignment (Wang et al., 2007). OTU rarefaction curves were calculated to ensure satisfactory sequencing effort to describe the microbial diversity of each sample.

2.6 Bacterial translocation

The bacterial translocation was evaluated by the quantification of 16S rDNA at Day 0 and Day 7. DNA was extracted from plasma samples with the QIAcube® automatic extractor (Qiagen, Courtaboeuf, France), from 200 µL of plasma using the QIAamp MinElute ccfDNA® kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. DNA was eluted in a final volume of 80 µL. A negative extraction control was systematically extracted in parallel. Real-time qPCR was performed in Taqman technique using the Light cycler 480 II® thermal cycler (Roche diagnostics) and the LC Fast Start DNA MasterPLUS HybProbe® master mix (Roche diagnostics) in a volume of 20 µL, in 96-well plates. Primers and "Taqman" probe used targeted the V5 hypervariable region of the 16S gene as previously described by (Kramski et al., 2011). Absolute quantification analysis was performed using LightCycler® 480 Software (Roche diagnostics), version 1.5, based on a standard curve created from serial dilutions using the provided synthetic DNA. The result was expressed as a Crossing point (Cp). The Cp corresponds to the cycle of the PCR where the detection of fluorescence bends exponentially. This Cp was converted to copies/µL using the standard curve. All experiments were performed in triplicate.

2.7 Statistical analysis

As this work corresponded to a pilot study, no sample size calculation was performed. The statistician (F.S.) considered that 60 patients was sufficient to analyze data considering the published studies (Shimizu et al., 2011; Schmidt et al., 2018; Wolff et al., 2018; Agudelo-Ochoa et al., 2020; Shanahan et al., 2021; Siwczak et al., 2021; Chen et al., 2022; Deng et al., 2022). Statistical analysis was performed with R software version 4.1.0. The clustering algorithm to classify patients in 3 AGI grades (absence of grade 0 and low number of patients in grade IV that were integrated in a grade III-IV) over seven days was established as follows: for each level, a reference profile (centroid) was determined: group 0 took the score AGI value 0 at each time, group 1, the value 1 at each time and group 2, the value 2 at each time. Then, Euclidean distances were calculated between AGI profiles of each patient and the reference profiles of the groups so that each patient was assigned to their closest group. Phyloseq and vegan R packages were used for Metagenomics analyses. The distribution of OTUs and the composition of microbial communities were analyzed by determining their relative abundance at phylum and genus levels. α-diversity represented by Shannon and Chao-1 scores while β-diversity was assessed using Principal Coordinate Analysis (PCA) with Bray-Curtis dissimilarity indices. PCA was also used to show discrimination between groups according to a selection of differentially abundant bacteria. The association between gut microbiota composition and late mortality (survivors vs non-survivors) was assessed by Mann-Whitney test. The area under the receiver operating characteristic (ROC) curve (AUC) was used to illustrate the discriminatory power of bacteria according to the survivors at day 28.

3 Results

3.1 Characteristics of population

Table 1 and Figure S1 show patient baseline characteristics. At day 28, 16 patients have died (late mortality = 26.7%). Among them, 6 patients died within the first week (early mortality = 10.0%). There was no statistical significant difference between survivors and non-survivors at Day 7 and Day 28 (late mortality), except a higher SAPS II score in non-survivors group (62.8 ± 19 versus 51.5 ± 16.8, p=0.0012).

3.2 Evolution of community richness, diversity and structure of the gut microbiota in septic shock patients

Gut microbiota data was available in all 60 patients at ICU admission (Day 0) and at Day 7. The assembly parameter applied at 97% nucleic identity allowed the assembly of full-length 16S rDNA sequences of 85.45% on average and representing a total of 2,865,340 reads and 29,847 full-length 16S rDNA sequences per sample on average.

Community richness and diversity were estimated by Chao-1 and Shannon scores, respectively. In all population samples combined (Day 0 and Day 7), the median Shannon score was 2.78 [1.97-3.27] and a median Chao-1 score was 93 [62.50-121.25] (Figure 1A). There was a high disparity in sample diversity with a Shannon score varying from 0.10 to 4.19. Sample diversity and richness at Day 7 were significantly decreased compared to those on Day 0, estimated by Shannon score (2.97 at Day 0 vs 2.63 at Day 7; p=0.0045) and Chao-1 score (102 at Day 0 vs 86 at Day 7; p=0.0029) (Figure 1B). The alpha diversity evolution between D0 and D7 showed a decrease of the Shannon score (-0.75 [-1.90;-0.02]) and Chao-1 score (-28 [-62.00; 1]).

Finally, the PCA divided gut microbiota of patients into two homogeneous groups. The microbiota had lower variability at Day 0, with much higher variability at Day 7 (Figure S2).

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TABLE 1 Patient baseline characteristics.

Characteristics	Total (n=60)	Non-survivors (n=16)	Survivors (n=44)	p-value
Age, years	75 (67-80)	79.5 (79-80)	74.5 (67.3-80.8)	0.21
Sex ratio, Female/Male	25/35	6/10	19/25	1
BMI, kg/m^2	27.3 (23.4-30.1)	27.3 (23.9-28.3)	27.3 (23.4-30.8)	0.56
SOFA score	8.5 (7-10)	10 (8.5-10)	8 (7-10)	0.39
AGI grade 0	0	0	0	
I	5	0	5	
II	34	13	21	0.12
III	14	2	12	
IV	3	1	2	
Comorbidities				
Coronaropathy	13 (21.7)	6 (37.5)	7 (15.9)	0.09
Heart insufficiency	7 (11.7)	4 (25.0)	3 (6.8)	0.07
Arterial hypertension	41 (68.3)	11 (68.8)	30 (68.2)	1
Diabetes mellitus	20 (33.3)	3 (18.7)	17 (38.6)	0.22
Chronic renal failure	11 (18.3)	3 (18.7)	8 (18.1)	1
Alcoholism	4 (6.7)	2 (12.5)	2 (4.5)	0.29
Hepatic cirrhosis Child A-B	1 (1.7)	1 (6.3)	0 (0)	0.27
COPD	7 (11.7)	2 (12.5)	5 (11.3)	1
Cancer solid/hemopathy	4 (6.7)	1 (6.3)	3 (6.8)	1
Sources of infection				
Pulmonary	21 (35.0)	8 (50.0)	13 (29.5)	0.22
Urinary	16 (26.7)	1 (6.3)	15 (34.1)	0.06
Digestive	8 (13.3)	1 (6.3)	7 (15.9)	0.67
Skin and Soft Tissue Infections	5 (8.3)	2 (12.5)	3 (6.8)	0.60
Other	7 (11.7)	2 (12.5)	5 (11.3)	1
Unknown origin	3 (5.0)	2 (12.5)	1 (2.3)	0.17
Previous hospitalization <3months	21 (35.0)	5 (31.3)	16 (36.4)	0.77
Previous antibiotherapy <3months	14 (23.3)	2 (12.5)	12 (27.3)	0.31
Mortality				
Day 7	6 (10)	6 (37.5)	0 (0)	not applicable
Day 28	16 (26.7)	16	0	not applicable

BMI, Body Mass Index; COPD, Chronic Obstructive Pulmonary Disease.

3.3 Taxonomic composition of the gut microbiota and their evolution

The evolution of taxonomic composition of the gut microbiota of patients in septic shock are detailed in Figure S3, and Tables S1, S2.

At the phylum level, the Firmicutes and Bacteroidota were increased between Day 0 and Day 7 (Mean: 72.58% ±23.17 vs 79.61 ±21.93, p=0.043; 5.96 ±9.95 vs 9.8 ±15.82, p=0.929, respectively), whereas the Proteobacteria and Actinobacteriota were significantly decreased (12.21 ±18.57 vs 4.18 ±0.85, p=0.0009; 8.88 ±14.81 vs 5.9 ±11.96, p=0.014) (Table S1). The increase of Firmicutes was mainly due to a strong abundance of *Enterococcus* and, to a lesser degree, of *Christinensenella* and *Staphylococcus*. In contrast, among the Clostridiales order of Firmicutes, some OTUs within the Tissierellacea family (e.g., *Finegoldia*, *Peptoniphilus*, *1855D* genera), Lachnospiraceae (e.g., *Blautia, Lachnospira, Coprococcus, Clostridium, Dorea, Roseburia* genera), and Ruminococcaceae (e.g, *Ruminococcus*,

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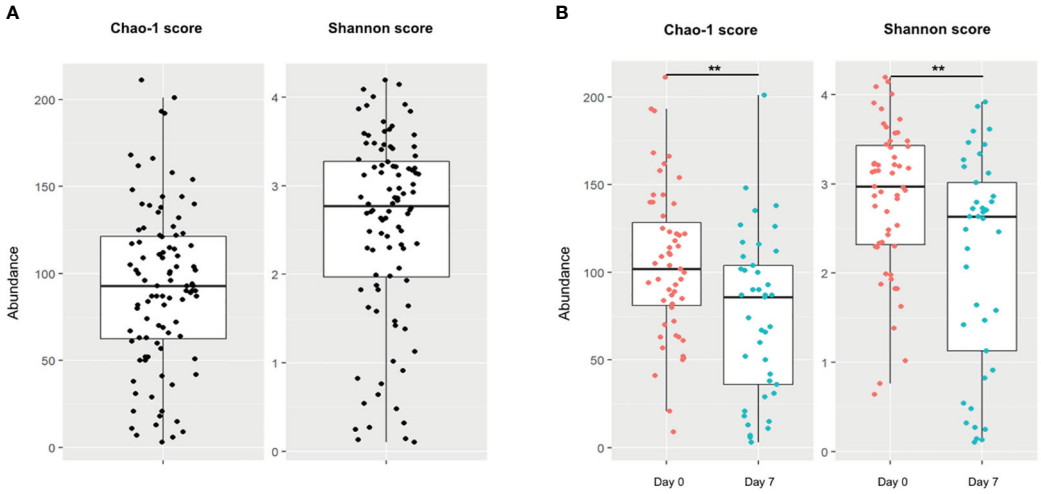

FIGURE 1
Alpha diversity of patient population. Boxes represent the IQR between the first and third quartiles; the horizontal line represents the median. **(A)** Chao-1 and Shannon scores on global population. **(B)** Comparison of Chao-1 and Shannon scores on global population between Day 0 and Day 7. (**, p <0.01).

Butyricicoccus) were significantly decreased at Day 7 (p<0.05). The same trend was observed for *Sarcina, Dialister* and *Anaerofustis*. Among Proteobacteria and Actinobacteriota phyla, some Enterobacterales (e.g., *Escherichia*, *Shigella* genera) and *Bifidobacterium* were also in a significant lower abundance at Day 7 compared to Day 0 (p=0.041). Finally, two Bacteroidales (*Parabacteroides* and *Alistipes*) were significantly abundant at Day 7 compared to Day 0 (p=0.019 and p=0.045, respectively) (Table S2).

A selection of bacteria genera, in particular *Enterococcus*, *Christinensenella*, *Staphylococcus, Parabacteroides* and *Alistipes* discriminated fecal samples at Day 7 compared to Day 0 on the PCA graphic (Figure S4).

3.4 Relationship between clinical indicators and evolution of gut microbiota

The impact of different clinical parameters was evaluated on alpha diversity and its evolution over the seven days (Table 2). At Day 7, Chao-1 score (richness) was higher in patients with a pulmonary origin of sepsis compared to the others (p=0.02) (Figure 2A). There was a higher diversity of the microbiota at admission in patients with high SOFA score (p=0.02) (Figure 2B) associated with a significantly high Shannon score (3.68 at Day 0 vs 2.68 at Day 7; p=0.017). Late mortality and SAPS II score did not have any impact on microbiota diversity in our population, despite the gut microbiota diversity of survivors (at Day 28) being lower compared to the diversity of the non-survivors (Table 2).

An association between some genera and AGI grade was observed. At admission, *Claocibacillus* was more present in AGI grade I and II (p=0.003, q=0.713), *Oisenella* and *Parabacteroides* in AGI grade I (p=0.017, q=1, respectively) and *Gallicola* in AGI grade III-IV (p=0.022, q=1). At Day 7, *Butyricicoccus* was decreased in all AGI grades (p=0.0066, q=0.859), and *Klebsiella* in AGI grade I and III-IV (p=0.0126, q=1), whereas this genus increased in AGI grade II (Table S3). Comparison between Day 0 and Day 7 identified some variations in the genera repartition: patients with AGI grade I presented a significant increase of *Blautia, Dialister* and *Ruminococcus* whereas at AGI grade II and III-IV, we observed a decrease of these species but also *Anaerostipes* and *Dorea*.

The association between SAPS II score and genera showed that at inclusion, the severe score of SAPS II was associated with the significant detection of *Gardnerella, Clostridium* and *Collinsella* (p=0.03, q=1). At Day 7, the moderate SAPS II score was associated with an increase of *Enterococcus*, *Alistipes*, and *Roseburia* (p<0.05, q=1) (Table S3).

Interestingly, a severe SOFA score was observed in presence of *Akkermansia, Blautia, Commamonas, Gardenerella, Faecalibacterium, Butyrivibrio, Parabacteroides* and *Alloscardovia*, whereas SOFA score was less severe in patients harboring *Eggerthella* in their gut microbiota (p<0.05; q=1). At Day 7, the severity of SOFA was associated with the presence of *Peptococacceae, Proteus*, and *Blautia* (p<0.04; q=1). Finally, the evolution of the genera between D0 and D7 was correlated with the significant decrease of *Akkermansia* and *Gardenella*, and an increase of *Clostridiaceae* in patients with severe SOFA score (p<0.04; q=1) (Table S3).

Finally, some genera were associated with the origin of septic shock. Indeed, *Streptococcus* was associated with gut origin, *Methanobrevibacter* with pulmonary origin, *Enterococcus* with gut and urine origin and *Klebsiella* with other origins (Figure S5).

3.5 Impact of AGI on gut microbiota and bacterial translocation

As the link between gut inflammation, dysbiosis and BT has been previously proposed (Mutlu et al., 2001), we determined BT by qPCR of 16S rDNA. Using control patients, a cut-off of BT

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TABLE 2 Impact of clinical parameters on alpha diversity (Shannon and Chao-1 score) and its evolution between Day 0 and Day 7.

Parameters		Alpha diversity				Alpha diversity evolution	
		Day 0		Day 7			
		Shannon score median	Chao-1 score median	Shannon score median	Chao-1 score median	Shannon score median	Chao-1 score median
Mortality (at Day 28)	Survivor	2.66	87	2.46	60	-0.765	-29
	Non-Survivor	3.16	122	3.02	101	-0.581	-26
	p-value	***0.03***	***0.001***	*0.08*	*0.08*	*0.99*	*0.72*
Septic shock origin	Pulmonary	3.2	118	3.2	104	-0.331	-18.4
	Digestive	1.93	73	2.03	56	-0.533	-27
	p-value	*0.08*	*0.10*	*0.07*	***0.02***	*0.33*	*0.71*
AGI grade	I	2.99	104	2.63	90	0.806	27
	II	2.91	96	2.71	87	-0.759	-23
	III-IV	3.17	106	1.42	38	-1.7	-67
	p-value	*0.69*	*0.86*	*0.10*	*0.08*	***0.015***	***0.004***
AGI score	0	2.85	84.5	3.02	100	0.238	14
	1	3.21	106	2.55	87	-0.961	-27.5
	2	3.12	105	1.96	63	-1.1	-44
	p-value	*0.34*	*0.31*	*0.21*	*0.23*	***0.007***	***0.009***
SOFA score	Low	2.68	95	2.03	49	-0.664	-44
	Moderate	2.92	102	2.61	86	-0.772	-23
	High	3.68	149	2.9	93.5	-0.666	-41
	p-value	***0.02***	*0.11*	*0.36*	*0.41*	*0.924*	*0.521*
IGS II	Low	2.87	102	2.49	52	-0.581	-38
	Moderate	2.96	110	2.62	87	-0.773	-28
	High	3.22	100	2.85	94	-0.527	-24.5
	p-value	*0.37*	*0.56*	*0.66*	*0.37*	*0.935*	*0.738*

AGI, acute gastrointestinal injury. Statistical significance was tested using the Mann-Whitney-Wilcoxon test. Bold values represent statistically significant differences ($p<0.05$).

corresponding to a value >12.25 copies/µL was determined. The median plasma 16S rDNA detected in the septic shock patients was 15.70 copies/µL at admission and 19.57 copies/µL at Day 7, significantly higher than in healthy controls (8.96 copies/µL) ($p<0.01$) (Table 3).

Shannon score was significantly correlated to a high BT (>12.25 copies/µL) at admission ($p=0.03$) (Figure 2D). There was a strong association between the AGI grades and the richness and diversity of the gut microbiota of the patients. The patients with an AGI grade 3 had a significantly greater reduction of gut microbiota diversity than those with an AGI grade 2, whereas patients with an AGI grade 1 had an increase of their microbiota diversity (Shannon score; $p=0.015$) (Figure 2C). The same trends were observed with AGI scores at admission (Table 2).

Moreover, we observed that AGI score at admission and Day 7 also significantly influenced BT (15.7 and 15.97 for score 0 vs 19.97 and 25.59 for score 2, respectively; $p=0.077$).

3.6 Relationship between mortality and evolution of gut microbiota

The alpha diversity of the gut microbiota was significantly lower in non-survivors at admission: Shannon score (2.66 vs 3.16, $p=0.03$) and Chao-1 score (87 vs 122, $p=0.013$) (Figure S6; Table 2). However, the evolution of the alpha diversity of gut microbiota at Day 7 was not correlated with poor evolution of septic shock ($p=0.08$) (Table 2).

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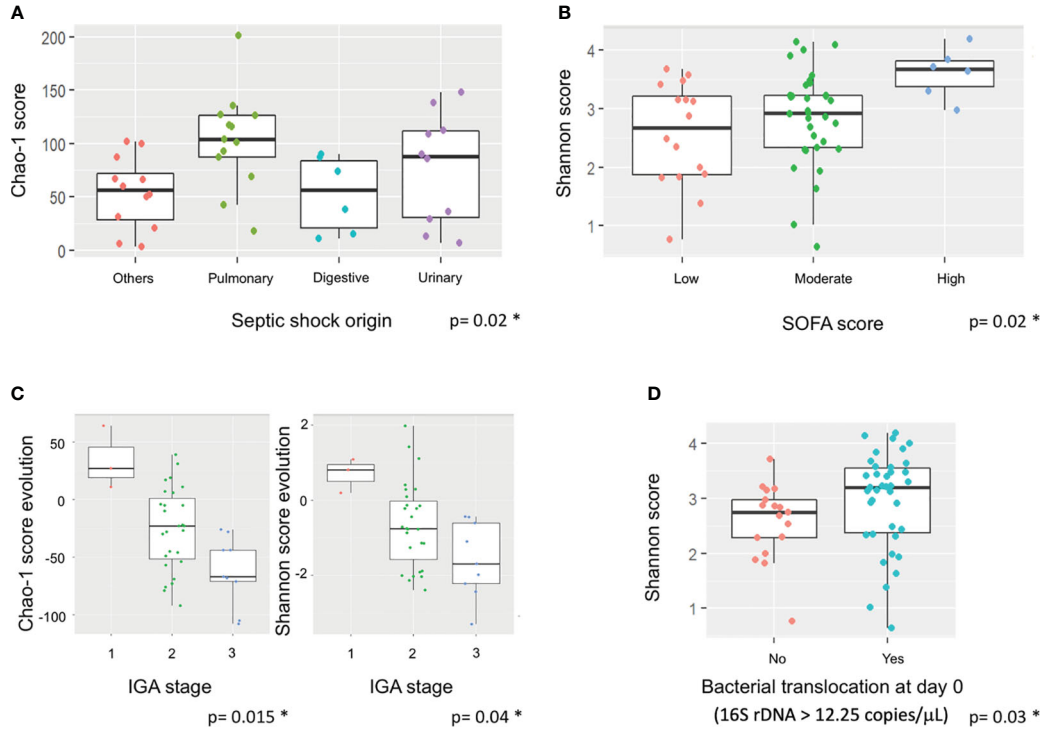

FIGURE 2
Impact of septic shock origin, SOFA score, AGI grades and bacterial translocation on alpha diversity and its evolution. Boxes represent the IQR between the first and third quartiles; the horizontal line represents the median. Statistical significance was tested using the Mann-Whitney test. **(A)** Impact of septic shock origin on Chao-1 score at Day 7. **(B)** Impact of SOFA score at Day 0 on Shannon score at admission. **(C)** Impact of IGA grade change in richness and diversity. **(D)** Impact of BT at admission on Shannon score at admission. * corresponds to significant statistical difference with the p value.

There was a significantly higher abundance of *Mogibacteriaceae, Robinsoniella, Klebsiella* and *Proteus* at admission in the non-survivor vs survivor groups (p<0.03, q=1) (Table S4). In the survivors, the gut microbiota had no variation in genera during the first 7 days (Figure S6). In the non-survivors, there was a significant decrease of *Escherichia* (p=0.03), and to a lesser extent, *Morganella* (p=0.029), *rc4-4* (p=0.023) and *Robinsoniella* (p=0.029) and the significant increase of *Pseudomonas* (p=0.029), *Christensenellaceae* (p=0.038) and to a lesser extent, *Enterococcus* (p=0.007), and *Actinomyces* (p=0.015) (Figure S7; Table S4).

The heat map of Area under the ROC Curve (AUC) between relative abundance at genus level and vital status at Day 28 showed that an increase of *Enterococcus, Pseudomonas, Clostridiaceae* and *Actinomyces* and a decrease of *Proteus* and *Escherichia* were significantly associated with mortality (p<0.05; q=1) (Figure 3). There was no significant difference of BT at admission between survivors and non-survivors (21.9 ± 13.85 copies/µL vs 26.7 ± 45.28, respectively; p=0.403).

TABLE 3 Determination of bacterial translocation by the quantification (qPCR) of 16S rDNA in septic shock patients and healthy controls.

	Septic shock patients n=59		Healthy controls n=100	*p*-values
	D0	D7	D0	
Total	15.70 (10.71-27.06)	19.57 (11.36-30.55)	8.96 (8.14-9.86)	<0.001
AGI score 0	15.70 (8.59-25.08)	15.97 (8.86-3.61)	–	
AGI score 1	15.87 (13.19-28.05)	23.46 (17.92-25.45)	–	0.077
AGI score 2	19.97 (10.58-38.48)	25.59 (15.41-33.9)	–	
Survivor at D28	14.75 (9.90-18.55)	17.57 (10.75-28.94)	–	0.403
Non-survivors	16.80 (11.13-28.40)	24.89 (19.45-31.92)	–	

Statistical significance was tested using the Kruskal Wallis test (for the total) and a mixed ANOVA text (for AGI score). Bold values represent statistically significant differences (p<0.05). All data are expressed by median and IQR.
The bacterial translocation was evaluated according to the AGI score and patient mortality. Data are expressed as copies/µL.

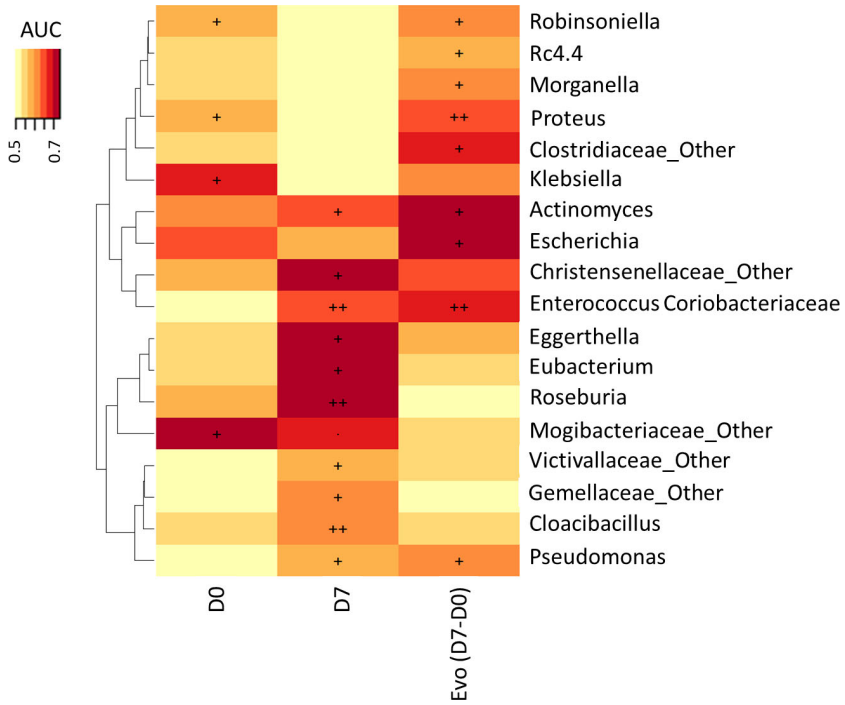

FIGURE 3
Association of vital status at Day 28 with microbiota genus levels at Day 0 and Day 7 and change between Day 0 and Day 7. The heat map of Area under the ROC Curve (AUC) between abundance at genus level and vital status at Day 28 (., $p < 0.10$; +, $p < 0.05$; ++, $p < 0.01$).

4 Discussion

The present study confirmed the decrease of richness and diversity of the gut microbiota in septic shock patients between inclusion and Day 7. It confirms an increase of Firmicutes and Bacteroidota, as previously noted (Shimizu et al., 2011; Zaborin et al., 2014; Liu et al., 2020). The increase of these phyla was directly linked to the increase of *Enterococcus*, *Christinensenella* and *Staphylococcus* among Firmicutes, and *Parabacteroides* and *Alistipes* among Bacteroidota. In parallel, there was a decrease of Proteobacteria and Actinobacteria. Among this last phyla, *Bifidobacterium* genus is an important member of the human gut bacterial population throughout life, with health benefits associated with anti-inflammatory properties (Khokhlova et al., 2012; O'Callaghan and van Sinderen, 2016) and decreased intestinal permeability (Underwood et al., 2015). *Bifidobacterium* supplementation decreases intestinal LPS levels and improves the barrier properties of the intestinal mucosa in mice (Griffiths et al., 2004; Wang et al., 2006). Indeed, in germ-free mice colonized with human gut microbiota, increased levels of *Bifidobacterium* were associated with decreased BT to the systemic circulation (Romond et al., 2008).

Several studies have shown differences in gut microbiota composition in septic shock (Shimizu et al., 2011; Wan et al., 2018; Yin et al., 2019; Agudelo-Ochoa et al., 2020; Liu et al., 2020), whereas the influence of AGI and BT elements from the gut microbiota on inflammation/infection status remains unclear (Clark and Coopersmith, 2007). Here, we clearly highlighted a gut dysbiosis and BT present in patients with a severe AGI (grade 3) (Figure 2; Tables 2, 3). According to the AGI grade at admission, the present study highlighted a great diversity of the gut microbiota with low BT values at grade I, whereas a low diversity and high BT values were observed at grade III-IV. This finding reinforces the idea that gut inflammation could help select some phyla or genera and that gut permeability favors BT. In this context, *Oisenella* and *Parabacteroides* were preferentially detected at AGI grade I and *Gallicola* at AGI grade III-IV at admission. Interestingly, an increase of *Blautia, Dialister* and *Ruminococcus* was observed at AGI grade 1, representing species that could protect against gut inflammation. Moreover, the decrease of *Anaerostipes* and *Dorea* at AGI grade III-IV could also participate in this protection. Among these genera, *Blautia, Dialister* and *Ruminococcus* belong to short chain fatty acids (SCFA) producers of Ruminococcaceae and Lachnospiraceae families (Louis and Flint, 2017). SCFAs have anti-inflammatory, anti-cancer, and anti-oxidant properties and prevent intestinal permeability. These metabolites have previously been shown to be significantly lower in critical patients than in healthy controls (O'Keefe et al., 2011).

Enterococcus includes opportunistic microorganisms that enhance the virulence of pathogens (Lavigne et al., 2008), and the host immunity and inhibit overgrowth of opportunistic pathogens by producing SCFAs (Zhao et al., 2018) and bacteriocins (Brandão et al., 2010). Recently, Liu et al. described an enterotype mainly composed of *Enterococcus* in patients with sepsis associated with a lower occurrence of septic shock, speculating that *Enterococcus* could be a protective biomarker in their population (Liu et al., 2020). Interestingly, in our population, *Enterococcus* had low abundance in the gut microbiota at admission, confirming its possible protective role against septic shock. However, this genus was significantly correlated with severe SAPS II score and was significantly increased

in non-survivors between Day 0 and Day 7, as previously observed (Shimizu et al., 2011; Agudelo-Ochoa et al., 2020). This result suggests that *Enterococcus* has probably been selected during hospitalization (due to the different drugs used) and corroborates the idea that this genus must be associated with a worsening prognostic marker of septic shock. We also observed that *Enterococcus* was preferentially isolated in septic shock from gut and urine origins, suggesting that the intestine reservoir is essential in the disease. Altogether, our results indicate that, while predominant *Enterococcus* may have a protective role at admission, their increase during ICU hospitalization could represent a worsening prognosis. The clear origin of the emergence and colonization of the intestine by enterococci during hospitalization must be determined to combat septic shock-related mortality. *Enterococcus* was not the only genus linked to the late mortality of the patients. *Pseudomonas, Clostridiaceae* and *Actinomyces* were also significantly increased in the gut microbiota of non-survivors, whereas *Proteus* and *Escherichia* were significantly decreased (Figure 3). The decrease of these two last genera can be correlated with the decrease of Proteobacteria phylum. *Pseudomonas, Clostridiaceae* and *Actinomyces* were not associated with AGI grades, and may have been acquired during hospitalization. *Pseudomonas* is a well-known hospital bacterium, particularly present in ICU, affecting immunocompromised patients (Kang et al., 2003). *Clostridiaceae* and *Actinomyces*, two intestinal commensal bacteria, were selected in the gut. They represent opportunistic bacteria that can cause hospital-acquired infection in damaged epithelia where they reside in 'microniches' with low oxygen, favoring anaerobic growth, especially in the deepest layers. ICU management, particularly the use of antibiotics, is the main driver of this selection. Antibiotic use often causes gastrointestinal adverse events, and is usually attributed to change in the composition and diversity of gut microbiota (Lama et al., 2019). The presence of genera identified as non-protective factors also suggests that only one genus or species in gut microbiota are frequently unable to participate alone in the gut inflammation. It is probably the addition of some genera or species that must establish networks of interspecies interconnection modifying the intestine crosstalk. Finally, BT was not significantly correlated with patient outcome, despite gut dysbiosis. We hypothesize that the determination of the BT on admission was either too late or too early in the septic shock process, explaining the higher mortality of our patients after the seventh day.

The main limitation of our study was the single center recruitment that could bias the interpretation of the results and limit its generalization. However, our population was homogenous and notably in their geographical location avoiding some ethnic variations important in gut microbiota analysis. Moreover, some results supported those obtained previously in other teams.

In conclusion, our study highlighted the importance of association between AGI, dysbiosis and BT in patients with septic shock, and reinforces the link between dysbiosis and mortality. This gut inflammation was associated with dysbiosis where potential bacteria differed significantly over time in patients with septic shock. Our results suggest that intestinal and environmental bacteria present in ICU are involved in the AGI severity and the mortality, perpetuating the chronicity of the systemic inflammation. The control of this inflammation remains an objective in the ICU management. Moreover, understanding the mechanisms between inflammation and intestinal bacteria could help to develop future therapeutic strategies in septic shock by targeting the intestinal microbiota.

Data availability statement

The data presented in the study are deposited in the NCBI/NLM repository (Bioproject), accession number PRJNA1034825.

Ethics statement

The studies involving humans were approved by Comité de Protection des Personnes Ouest III (France). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CM: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. TL: Data curation, Formal analysis, Investigation, Resources, Validation, Writing – review & editing. FS: Formal analysis, Methodology, Software, Validation, Writing – review & editing. RT: Data curation, Formal analysis, Writing – review & editing. CD-R: Data curation, Formal analysis, Writing – review & editing. CR: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing. J-YL: Investigation, Project administration, Supervision, Validation, Visualization, Writing – review & editing. PM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing. J-PL: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft.

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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 author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1330900/full#supplementary-material

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Cross-talk between *Helicobacter pylori* and gastric cancer: a scientometric analysis

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Background: *Helicobacter pylori* (HP) is considered a leading risk factor for gastric cancer (GC). The aim of this article is to conduct bibliometric and visual analysis to assess scientific output, identify highly cited papers, summarize current knowledge, and explore recent hotspots and trends in HP/GC research.

Methods: A bibliographic search was conducted on October 24, 2023, to retrieve relevant studies on HP/GC research between 2003 and 2022. The search terms were attached to HP and GC. The main data were from the Web of Science Core Collection (WoSCC). Data visualization was performed using Biblioshiny, VOSviewer, and Microsoft Excel.

Results: In HP/GC research, 1970 papers were retrieved. The total number of papers (Np) in HP/GC was growing from 2003 to 2022. China and Japan were in the leading position and made the most contributions to HP/GC. *Vanderbilt University* and the *US Department of Veterans Affairs* had the highest Np. The most productive authors were Peek Jr Richard M. and Piazuelo M Blanca. *Helicobacter* received the most Np, while *Gastroenterology* had the most total citations (TC). High-cited publications and keyword clustering were used to identify the current status and trends in HP/GC research, while historical citation analysis provided insight into the evolution of HP/GC research. The hot topics included the effect of HP on gastric tumorigenesis and progression, the pathogenesis of HP-induced GC (HP factors), and the mechanisms by which HP affects GC (host factors). Research in the coming years could focus on topics such as autophagy, gut microbiota, immunotherapy, exosomes, epithelial-mesenchymal transition (EMT), and gamma-glutamyl transpeptidase (GGT).

Conclusion: This study evaluated the global scientific output in HP/GC research and its quantitative characteristics, identified the essential works, and collected information on the current status, main focuses and emerging trends in HP/GC research to provide academics with guidance for future paths.

KEYWORDS

***Helicobacter pylori*, gastric cancer, hotspots and trends, high-cited papers, bibliometrics**

1 Introduction

Gastric cancer (GC) remains a global public health concern (Sung et al., 2021), with the fifth most common cancer and the fourth leading cause of cancer-related death worldwide (Thrift et al., 2023). *Helicobacter pylori* (HP) infection is the main pathogenic factor for GC, which plays an essential regulatory role in GC incidence, development, and treatment. Approximately 4.4 billion individuals had HP infection worldwide in 2015 (Hooi et al., 2017). Multiple studies (Hatakeyama, 2004; Correa and Houghton, 2007; Peek et al., 2010; Polk and Peek, 2010; Wroblewski et al., 2010; Hatakeyama, 2014; Amieva and Peek, 2016; Navashenaq et al., 2022) have shown that HP can induce GC by stimulating intracellular inflammatory signals, modulating inflammatory and immune responses, inducing DNA damage and cellular proliferation, and generating carcinogenic bacterial toxins involved in cancer progression. Moreover, HP modifies the efficacy of anti-tumor drugs (Deng et al., 2022; Oster et al., 2022b). HP eradication can contribute to preventing the carcinogenesis and progression of GC and supporting the prevention and treatment of GC (Yan et al., 2022; Li D. et al., 2023).

Bibliometrics has been effectively applied in medical research to visualize hot topics and track the evolution of knowledge in specific fields. Several studies have carried out the bibliometric analysis of HP, such as high-cited papers in HP research (Bang et al., 2019), states and hotspots in HP research (Wang et al., 2023) and HP resistance research (Li M. et al., 2023). Over the past two decades, research on the relationship between HP and GC has steadily increased. However, there is currently a lack of quantitative investigation into this link. This paper aims to conduct a bibliometric analysis of HP/GC-related papers published in the past two decades, focusing on identifying the characteristics of the crosstalk between HP and GC. The study visualized indicators such as hotspots, topics, authors, and institutions in HP/GC research, providing researchers with a fundamental understanding of the interplay between HP and GC, and assisting scholars in better grasping the dynamic changes and trends in HP/GC-related research.

2 Materials and methods

2.1 Data source and search strategy

The WoSCC includes the most renowned and influential academic publications in natural science (Bang et al., 2019; Li M. et al., 2023; Wang et al., 2023), making it an ideal data source for our research. All search results were retrieved from the WoSCC database on October 24, 2023, using the advanced search method with the keywords "Helicobacter pylori" and "stomach cancer" and their corresponding synonyms. The synonyms for Helicobacter pylori and gastric cancer were retrieved from the MeSH Database in PubMed. The search strategy is shown in Table 1 and Figure 1. The screening standards comprised (1): the publication period was from January 1, 2003 to December 31, 2022 (2): the categories included "Article" and "Review". Finally, 1,970 papers containing 1,674 articles and 296 reviews were acquired. The search and data extraction were carried out independently by two researchers (SY and SH) and saved in text format.

2.2 Data analysis and parameter query

The Bibliometrix R package and its Web applications Biblioshiny, VOSviewer, and Microsoft Excel 2019 are used for scientometric analysis. Bibliometrix (https://www.bibliometrix.org/) and VOSviewer provide a range of scientometric analysis tools for building and visualizing bibliometric networks (Bang et al., 2019; Li M. et al., 2023; Wang et al., 2023). Machine learning is used to assess the distribution of various components, such as annual production, most relevant journals/authors/affiliations/countries and their local impact by H-index or total citation (TC), and annual production over time, main funding agencies, country scientific output and collaboration network, historical direct citation network, highly cited papers and high-impact factor (IF) papers, common keywords and their cluster analysis. The impact and value of scientific papers can be assessed through citation analysis. By analyzing the number of publications in a specific field and historical direct citation networks, we can gain insight into the historical development of the field. By conducting a correlation analysis between authors and countries, one can discover potential collaborations between projects. The JCR quartile and IF were defined by the "2022 Incites Journal Citation Report".

3 Results

3.1 Scientific output

In HP/GC research, 1970 papers were retrieved. Annual production can reflect the research trend in a field. Figure 2 lists the annual number of papers (Np) in HP/GC research from 2003 to 2022. From 2003 to 2011, the annual Np remained stable. From 2012 to 2022, the annual Np increased in waves. Before 2013, the Np was less than 100 but more than 50 per year. Since 2013 (except 2015), more than 100 papers had been

TABLE 1 Search query and refinement procedure.

Set	Results	Refinement
1	4364	Step1: TI = ("Tumor*" OR "Tumour*" OR "Cancer*" OR "Neoplasia*" OR "Neoplasm*" OR "Carcinoma*" OR "Malignanc*" OR "Oncolog*" OR "Adenocarcinoma*" OR "Carcinogen*" OR "Oncogen*") AND TS = ("Gastric" or "Stomach") Step2: TI= (Helicobacter pylori or H.pylori) Step3: 1 AND 2
2	2456	Refined by DOCUMENT TYPES: (ARTICLES OR REVIEW ARTICLES)
3	1970	Refined by PUBLICATION YEARS: (2003–2022)

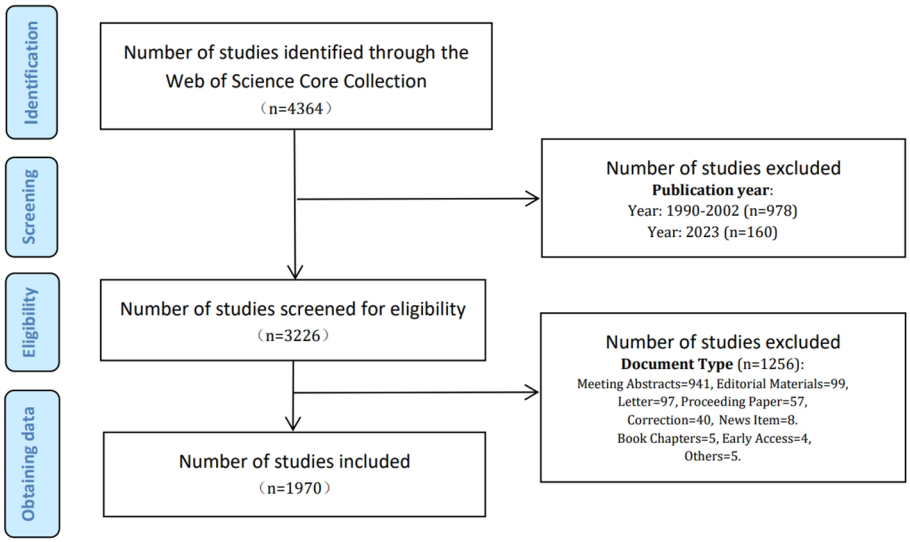

FIGURE 1
Flow chart of literature screening in HP/GC.

published each year. In addition, the cumulative scientific output in HP/GC research was increasing from 2003 to 2022, and the growth trend remained stable.

3.2 Main journals output

The papers involved 564 journals. Table 2 shows the top 10 journals in terms of output, with *Helicobacter* being the most productive (n = 96), followed by *World Journal of Gastroenterology* (n = 95), *PLoS One* (n = 50), *International Journal of Cancer* (n = 45), and *Gastric Cancer* (n = 39). Figure 3A illustrates the annual Np of the top 10 journals, with *Helicobacter* maintaining its position as the most productive journal in 2022. Figure 3B summarizes the cumulative Np of the top 10 journals. The Np of these journals was 463, accounting for about 24.11% of the total output, indicating their excellent production capacity. The TC indicates the significance of journals, and the H-index evaluates their academic impact. Table 3 shows the top ten most cited journals, with *Gastroenterology* at the top, followed by *International Journal of Cancer*, *Gut*, *World Journal of Gastroenterology*, and *Helicobacter*. In terms of H-index, *International Journal of Cancer* (H-index = 30) and *World Journal of Gastroenterology* (H-index = 30) ranked the top, followed by *Helicobacter* (H-index = 28) and *PLoS One* (H-index = 24).

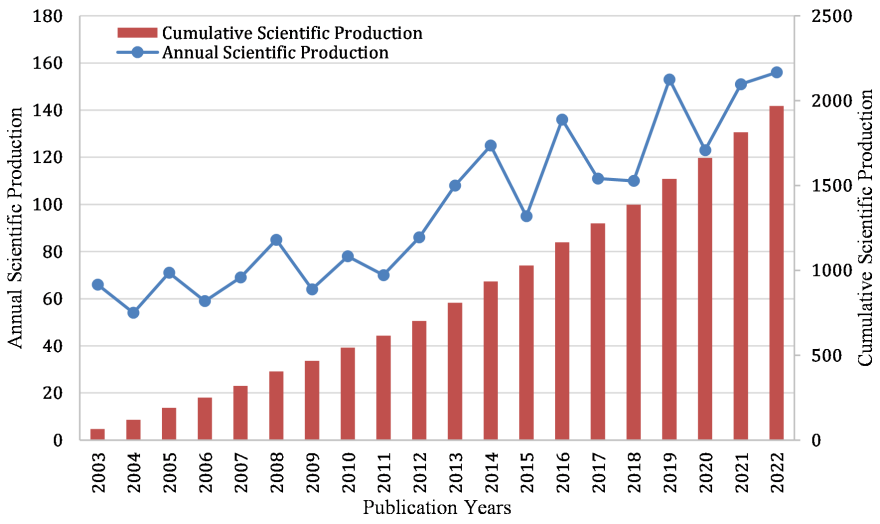

FIGURE 2
Annual and cumulative scientific output in HP/GC.

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TABLE 2 The top 10 productive journals in HP/GC.

Rank	Journals	Np	TC	H-index	IF	JCR	Countries
1	Helicobacter	96	2390	28	4.4	Q2	UK
2	World Journal of Gastroenterology	95	2839	30	4.3	Q2	USA
3	PLoS One	50	1396	24	3.7	Q2	USA
4	International Journal of Cancer	45	3048	30	6.4	Q1	Switzerland
5	Gastric Cancer	39	1018	18	7.4	Q1	Japan
6	Journal of Gastroenterology and Hepatology	32	938	17	4.1	Q2	Australia
7	Oncotarget	29	765	16	—	—	USA
8	Gastroenterology	26	4204	23	29.4	Q1	USA
9	Asian Pacific Journal of Cancer Prevention	26	368	12	—	—	Korea
10	Gut	25	2993	23	24.5	Q1	UK

3.3 Main authors output

Table 4 lists the ten most prolific authors and their TC and H-index. Peek Jr Richard M (n = 50), Piazuelo M Blanca (n = 32), Yamaoka Yoshio (n = 30), Romero-Gallo Judith (n = 27), and Tsukamoto Tetsuya (n = 25) took the top five places. Peek Jr Richard M had the highest Np and H-index, and the highest TC, showing his significant influence. Figures 4A, B respectively show the annual output of the top 10 authors and their collaboration network, with Peek Jr Richard M, Piazuelo M Blanca, Romero-Gallo Judith, Correa Pelayo and Wilson Keith T from *Vanderbilt University* having the closest cooperative relationship (a cooperative group). Moreover, other academic groups included Yamaoka Yoshio team from *Oita University*, Hatakeyama Masanori team from *University of Tokyo*, and Kim Na Young team from *Seoul National University*.

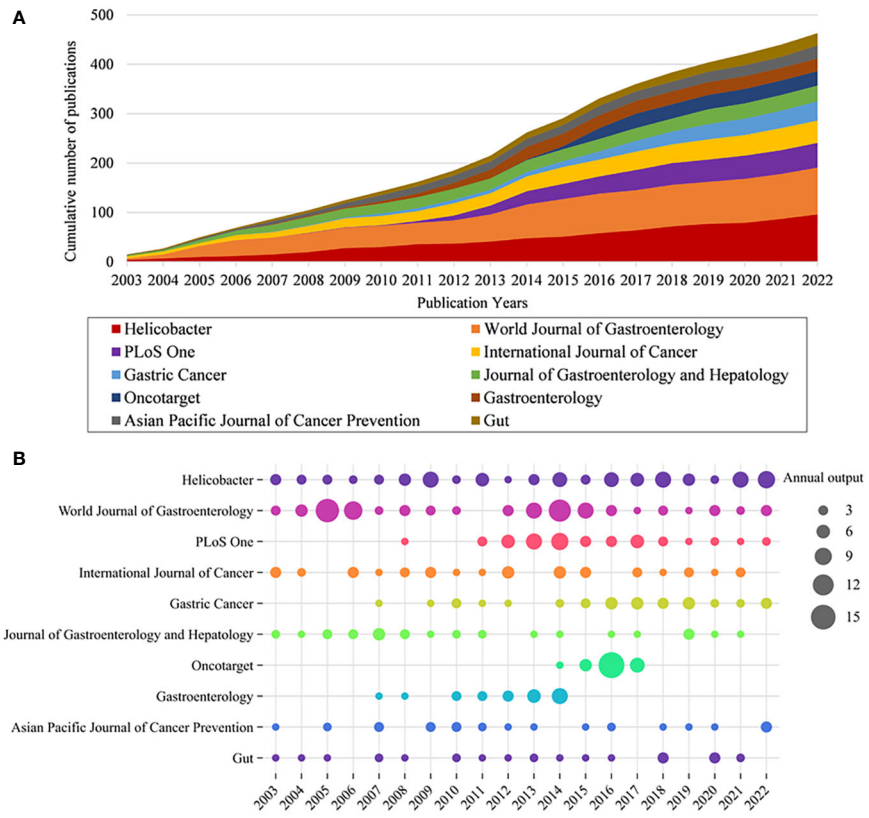

FIGURE 3
(A) Cumulative scientific output of the top 10 most prolific journals in HP/GC. **(B)** Annual production of the top 10 most prolific journals in HP/GC (the size of the circle represents the number, and the larger the circle, the higher the output).

TABLE 3 The top 10 local impact journals in HP/GC.

Rank	Journals	TC	Journals	H-index
1	Gastroenterology	4204	International Journal of Cancer	30
2	International Journal of Cancer	3048	World Journal of Gastroenterology	30
3	Gut	2993	Helicobacter	28
4	World Journal of Gastroenterology	2839	PLoS One	24
5	Helicobacter	2390	Gastroenterology	23
6	PLoS One	1396	Gut	23
7	Proceedings of the National Academy of Sciences of the United States of America	1396	Gastric Cancer	18
8	Nature Reviews Cancer	1314	Journal of Gastroenterology	18
9	Cancer Letters	1198	Journal of Gastroenterology and Hepatology	17
10	Journal of Gastroenterology	1087	Alimentary Pharmacology & Therapeutics	16

3.4 Major countries/regions and institutions output

Table 5 shows that most articles were published by authors from China (n = 571), Japan (n = 420), and the United States (n = 354), accounting for approximately 68.27% of the total output. Figure 5A presents a representation of the country scientific output and primary collaboration networks, highlighting that China had the closest ties with the USA. Figure 5B shows the annual Np of the top 10 countries. Table 5 reveals the top 10 productive institutions, with *Vanderbilt University*, *Seoul National University*, *US Department of Veterans Affairs*, *Veterans Health Administration*, and *University of Tokyo* ranking among the top five. Figure 5C displays their annual Np. Figure 5D draws the main funding agencies such as *National Natural Science Foundation of China* (n =218), *United States Department of Health Human Services* (n =180), the *National Institutes of Health* (n =175), the *Ministry of Education Culture Sports Science and Technology* (n =107), and *Japan Society for The Promotion of Science* (n =90) mainly from China, the USA, and Japan, indicating that their strongly support for HP/GC research.

3.5 Analysis of cited papers in HP/GC research

3.5.1 Top 20 most cited articles in HP/GC research

High-cited articles are a valuable indicator in bibliometrics, reflecting extremely high academic importance and influence in a field. Table 6 presents a list of the top 20 high-cited original research papers.

In clinical research, several studies (Wong et al., 2004; Fukase et al., 2008; Wu et al., 2009; Lee et al., 2013; Choi et al., 2018) published in world-famous journals have shown that eradication of HP can prevent the occurrence of GC and significantly decrease the development of GC. Among them, two studies (Fukase et al., 2008; Choi et al., 2018) showed that patients with early GC treated with HP had a lower incidence of metachronous GC. A longitudinal cohort study (Ohata et al., 2004) showed that there is a strong positive correlation between the degree of gastritis caused by HP and the development of cancer, especially for intestinal GC, indicating that severe gastritis with extensive intestinal metaplasia is a major risk factor for GC. A prospective case-control study

TABLE 4 The top 10 productive authors in HP/GC.

Rank	Authors	Np	TC	H-index	Affiliations	Countries
1	Peek, Richard M.	50	5,220	32	Vanderbilt University	USA
2	Piazuelo, Maria B.	32	1,643	22	Vanderbilt University	USA
3	Yamaoka, Yoshio	30	1,314	17	Oita University	Japan
4	Romero-Gallo, Judith	27	1,667	20	Vanderbilt University	USA
5	Correa, Pelayo	25	2,031	22	Vanderbilt University	USA
6	Tsukamoto, Tetsuya	25	1,242	17	Fujita Health University	Japan
7	Wilson, Keith T.	24	2,108	18	Vanderbilt University	USA
8	Malfertheiner, Peter	23	964	17	University of Munich	Germany
9	Hatakeyama, Masanori	22	2,473	19	University of Tokyo	Japan
10	Wu, Ming-Shiang	22	1,800	15	National Taiwan University	China

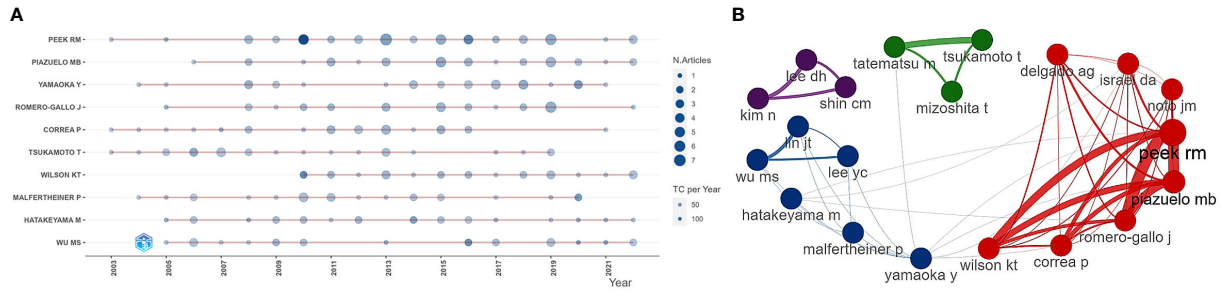

FIGURE 4
(A) Annual output of the top 10 most productive authors in HP/GC (the size of the circle represents output, with larger circles indicating higher output; the color depth of the circle indicates annual citations, with darker colors indicating more citations). **(B)** Co-authorship network of the 20 most productive in HP/GC (each node represents an author; each color represents a collaborative group; each line represents a coordination relationship, with thickness indicating collaboration intensity).

(Kamangar et al., 2006) showed that HP was an important risk factor for non-cardia GC, but negatively correlated with the risk of cardia GC. It is speculated that the decrease in the prevalence of HP may lead to a decrease in the incidence of non-cardia cancer and an increase in the incidence of cardia cancer in Western countries. Interestingly, a study (Ye et al., 2004) showed that HP infection may be not related to the risk of gastric cardia adenocarcinoma but reduce the risk of esophageal adenocarcinoma.

Moreover, in terms of diagnosis, the combination of serum pepsinogen and anti-HP antibody provides a good predictive marker for the development of GC (Watabe et al., 2005). Immunoblotting is more sensitive for detecting anti-HP antibodies than ELISA (Plummer et al., 2015). In treatment, an intervention trial (Ma et al., 2012) showed that garlic and vitamin treatments were associated with non-statistically significant reductions in GC incidence and mortality. On the contrary, a multicenter study (Cheung et al., 2018) showed that long-term use of proton pump inhibitors was associated with an increased risk of GC even after HP eradication. Furthermore, an animal study (Ohnishi et al., 2008) showed that transgenic expression of HP CagA induced gastrointestinal and hematopoietic tumors. A study (Franco et al., 2005) showed that β-catenin nuclear accumulation was increased in gastric epithelium collected from gerbils infected with HP carcinogenic strains. A comparative study (Maekita et al., 2006) showed that HP infection can induce CpG island methylation to varying degrees, and the methylation level of specific CpG islands seems to reflect the cancer risk of HP-negative individuals. In addition, a study (Rhead et al., 2007) has shown that the vacuolating cytotoxin A (VacA) is the main determinant of HP virulence, and the VacA i region is an important determinant of the virulence of HP and the best independent marker of VacA-related pathogenicity. A basic study (Lofgren et al., 2011) showed that the lack of commensal microbiota in HP-infected INS-GAS mice can reduce gastritis and delay intraepithelial neoplasia.

3.5.2 Top 10 most cited reviews in HP/GC research

A review can provide timely guidance for scholars with a large amount of information, including research development, existing problems, and future trends. Table 7 shows the top 10 most cited reviews, mainly from *Nature Reviews Cancer* (n = 2) and *Gastroenterology* (n = 4). Two review articles (Hatakeyama, 2004; Hatakeyama, 2014) outlined the oncogenic mechanisms of the HP CagA protein and the signals emitted by CagA

TABLE 5 The top 10 productive countries and institutions in HP/GC.

Rank	Countries	Np	TC	H-index	Institutions	Np	TC	H-index
1	China	571	15,837	57	Vanderbilt University	80	7,407	43
2	Japan	420	17,167	68	Seoul National University	66	1,897	26
3	USA	354	19,078	72	Us Department of Veterans Affairs	49	4797	26
4	South Korea	182	4,831	37	Veterans Health Administration	49	4,133	32
5	Germany	116	4,755	41	University of Tokyo	48	2,753	27
6	Iran	99	1,957	24	National Institutes of Health USA	42	2,184	22
7	Italy	87	3,076	34	Baylor College of Medicine	40	2,708	24
8	England	59	3,779	33	German Cancer Research Center DKFZ	39	1,090	20
9	Brazil	58	970	18	Helmholtz Association	39	1,090	20
10	India	58	1,185	20	Shanghai Jiao Tong University	39	1,129	17

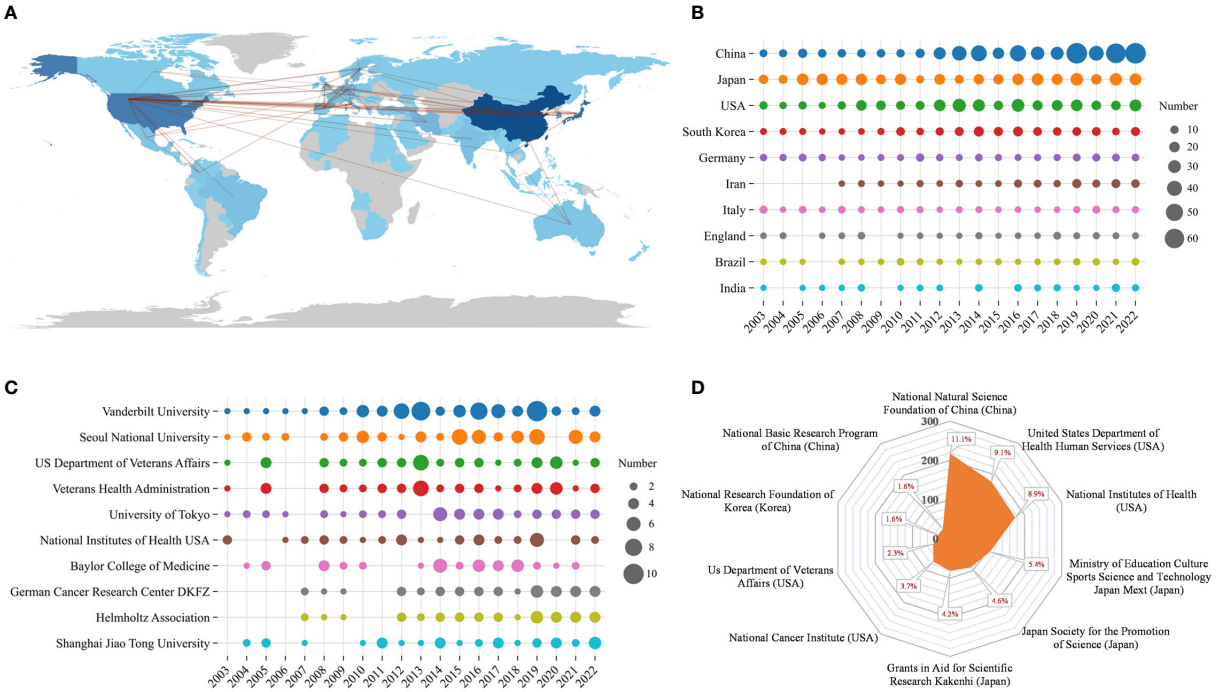

FIGURE 5
(A) Scientific production of various countries and their main international collaboration network in HP/GC. **(B)** Annual production of the top 10 productive countries in HP/GC. **(C)** Annual scientific production of the top 10 productive institutions in HP/GC. **(D)** The top 10 funding agencies and their source countries in HP/GC.

TABLE 6 The top 20 most cited original research in HP/GC.

Rank	Title	First author	Year	Journals	IF	JCR	TC
1	Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China -: A randomized controlled trial	Wong, BCY	2004	JAMA-J. Am. Med. Assoc.	120.7	Q1	1064
2	Effect of eradication of Helicobacter pylori on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial	Fukase, K	2008	Lancet	168.9	Q1	883
3	Global burden of gastric cancer attributable to Helicobacter pylori	Plummer, M	2015	Int. J. Cancer	6.4	Q1	575
4	High levels of aberrant DNA methylation in Helicobacter pylori: Infected gastric mucosae and its possible association with gastric cancer risk	Maekita, T	2006	Clin. Cancer Res.	11.5	Q1	498
5	Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse	Ohnishi, N	2008	Proc. Natl. Acad. Sci. U. S. A.	11.1	Q1	421
6	Progression of chronic atrophic gastritis associated with Helicobacter pylori infection increases risk of gastric cancer	Ohata, H	2004	Int. J. Cancer	6.4	Q1	392
7	Activation of β-catenin by carcinogenic Helicobacter pylori	Franco, AT	2005	Proc. Natl. Acad. Sci. U. S. A.	11.1	Q1	381
8	Helicobacter pylori Therapy for the Prevention of Metachronous Gastric Cancer	Choi, IJ	2018	N. Engl. J. Med.	158.5	Q1	379
9	Fifteen-Year Effects of Helicobacter pylori, Garlic, and Vitamin Treatments on Gastric Cancer Incidence and Mortality	Ma, JL	2012	J. Natl. Cancer Inst.	10.3	Q1	314
10	Predicting the development of gastric cancer from combining Helicobacter pylori antibodies and serum pepsinogen status: a prospective endoscopic cohort study	Watabe, H	2005	Gut	24.5	Q1	313

(Continued)

TABLE 6 Continued

Rank	Title	First author	Year	Journals	IF	JCR	TC
11	A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer	Rhead, JL	2007	Gastroenterology	29.4	Q1	294
12	Long-term proton pump inhibitors and risk of gastric cancer development after treatment for Helicobacter pylori: a population-based study	Cheung, KS	2018	Gut	24.5	Q1	271
13	Helicobacter pylori infection and gastric atrophy: Risk of adenocarcinoma and squamous-cell carcinoma of the esophagus and adenocarcinoma of the gastric cardia	Ye, WM	2004	JNCI-J. Natl. Cancer Inst.	10.3	Q1	262
14	Opposing risks of gastric cardia and noncardia gastric adenocarcinomas associated with Helicobacter pylori seropositivity	Kamangar, F	2006	JNCI-J. Natl. Cancer Inst.	10.3	Q1	253
15	Lack of Commensal Flora in Helicobacter pylori-Infected INS-GAS Mice Reduces Gastritis and Delays Intraepithelial Neoplasia	Lofgren, JL	2011	Gastroenterology	29.4	Q1	244
16	Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer	Chan, AOO	2003	Gut	24.5	Q1	244
17	The benefit of mass eradication of Helicobacter pylori infection: a community-based study of gastric cancer prevention	Lee, YC	2013	Gut	24.5	Q1	240
18	Early Helicobacter pylori Eradication Decreases Risk of Gastric Cancer in Patients With Peptic Ulcer Disease	Wu, CY	2009	Gastroenterology	29.4	Q1	217
19	Regulation of gastric carcinogenesis by helicobacter pylori virulence factors	Franco, AT	2008	Cancer Res.	11.2	Q1	212
20	Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells	Toller, IM	2011	Proc. Natl. Acad. Sci. U. S. A.	11.1	Q1	211

abnormalities integrated into direct carcinogenic damage and genetic instability. CagA-mediated gastric carcinogenesis is carried out through a hit-and-run mechanism. In the process of long-term infection with CagA-positive HP, the carcinogenic effect of CagA is replaced by a series of genetic or epigenetic changes compiled in precancerous lesions. Two meta-analyses (Fuccio et al., 2009; Lee et al., 2016) showed that HP eradication treatment may reduce the risk of GC. Several review articles (Correa and Houghton, 2007; Polk and Peek, 2010; Wroblewski et al., 2010; Wang et al., 2014; Amieva and Peek, 2016) discussed the various factors of HP-induced GC, including host immune response, polymorphism, changes in the apical junction complex, strain-specific bacterial components, specific host-microbe interactions, and the influence of environmental factors such as dietary components and essential micronutrients, as well as the gastrointestinal microbiota. A review (Graham, 2015) described

TABLE 7 The top 10 most cited reviews in HP/GC.

Rank	Title	First author	Year	Journals	IF	JCR	TC
1	Helicobacter pylori and Gastric Cancer: Factors That Modulate Disease Risk	Wroblewski, LE	2010	Clin. Microbiol. Rev.	36.8	Q1	896
2	Helicobacter pylori: gastric cancer and beyond	Polk, DB	2010	Nat. Rev. Cancer	78.5	Q1	742
3	Oncogenic mechanisms of the Helicobacter pylori CagA protein	Hatakeyama, M	2004	Nat. Rev. Cancer	78.5	Q1	572
4	Pathobiology of Helicobacter pylori-Induced Gastric Cancer	Amieva, M	2016	Gastroenterology	29.4	Q1	501
5	Carcinogenesis of Helicobacter pylori	Correa, P	2007	Gastroenterology	29.4	Q1	493
6	Association Between Helicobacter pylori Eradication and Gastric Cancer Incidence: A Systematic Review and Meta-analysis	Lee, YC	2016	Gastroenterology	29.4	Q1	490
7	Helicobacter pylori-induced gastric inflammation and gastric cancer	Wang, F	2014	Cancer Lett.	9.7	Q1	458
8	Helicobacter pylori CagA and Gastric Cancer: A Paradigm for Hit-and-Run Carcinogenesis	Hatakeyama, M	2014	Cell Host Microbe	30.3	Q1	313
9	Meta-analysis: Can Helicobacter pylori Eradication Treatment Reduce the Risk for Gastric Cancer?	Fuccio, L	2009	Ann. Intern. Med.	39.2	Q1	285
10	Helicobacter pylori Update: Gastric Cancer, Reliable Therapy, and Possible Benefits	Graham, DY	2015	Gastroenterology	29.4	Q1	269

the mechanism of HP in the development of GC, reliable treatment and possible benefits.

3.6 High-IF papers in HP/GC research

High-impact Factor (IF) papers are considered a crucial metric for assessing the research quality and influence of scholars, playing a pivotal role in advancing the development of a discipline. High-IF papers tend to captivate the attention and citation of international peers. As high-cited papers tend to be published in high-IF journals, we searched for the articles in high-IF (IF>30) journals (Table 8).

In original articles, there were 14 papers were extracted. Among them, *Cell Host & Microbe* (n = 3), Annals *of Oncology* (n = 3), and *Lancet* and its sub-journals (n = 3) had the most publications, followed by *New England Journal of Medicine* (n = 2), *BMJ* (n = 1), *JAMA* (n = 1), and *Journal of Clinical Oncology* (n = 1). Some high-cited papers have been described above (Wong et al., 2004; Fukase et al., 2008; Choi et al., 2018). In addition, several research found that HP eradication therapy can reduce the risk of GC in HP-infected patients with a family history of GC in first-degree relatives (Choi et al., 2020). HP treatment was associated with a statistically reduced risk of GC death and incidence of GC (Li et al., 2019). The screening and eradication of HP can reduce the burden of GC in high-risk populations in Chinese adults (Yang et al., 2021). Moreover, a prospective study (Meimarakis et al., 2006) showed that HP may be seem as a prognostic indicator after curative resection of gastric carcinoma. Three articles (Hayashi et al., 2012; Tsugawa et al., 2012; Imai et al., 2021) analyzed the carcinogenic mechanism of HP CagA, including the inhibition of autophagic degradation, the pathogenic signal enhancement, and genomic instability. The Eurogast-EPIC study found that 93.2% of GC patients were positive for HP infection (González et al., 2012). The interleukin-1B gene (IL-1B), interleukin-1 receptor antagonist gene (IL-1RN), and PPARγ Pro12Ala polymorphism act in HP-associated gastric adenocarcinoma (Ruzzo et al., 2005; Prasad et al., 2008). Regular use of nonsteroidal anti-inflammatory drugs may be a feasible method to prevent GC, especially in patients with HP infection (Wu et al., 2010).

In review articles, total seven high-IF reviews were published between 2004 and 2019, mainly from *Nature Reviews Cancer* (n = 2), *BMJ* (n = 1), *Clinical Microbiology Reviews* (n = 1), *Cell Host & Microbe* (n = 1), *Physiological Reviews* (n = 1), and *Annals of Internal Medicine* (n = 1). Among them, several high-cited review articles (Hatakeyama, 2004; Polk and Peek, 2010; Wroblewski et al., 2010; Hatakeyama, 2014) had described that the relationship between bacterial virulence factors VacA and CagA protein, outer membrane

TABLE 8 The high impact factors papers in HP/GC.

No.	Doi	Type	Journals	First Author	Year	TC
1	10.1001/jama.291.2.187	Article	JAMA-J. Am. Med. Assoc.	Wong, BCY	2004	1064
2	10.1128/CMR.00011-10	Review	Clin. Microbiol. Rev.	Wroblewski, LE	2010	896
3	10.1016/S0140-6736(08)61159-9	Article	Lancet	Fukase, K	2008	883
4	10.1038/nrc2857	Review	Nat. Rev. Cancer	Polk, DB	2010	742
5	10.1038/nrc1433	Review	Nat. Rev. Cancer	Hatakeyama, M	2004	572
6	10.1056/NEJMoa1708423	Article	N. Engl. J. Med.	Choi, IJ	2018	379
7	10.1016/j.chom.2014.02.008	Review	Cell Host Microbe	Hatakeyama, M	2014	313
8	10.7326/0003-4819-151-2-200907210-00009	Review	Ann. Intern. Med.	Fuccio, L	2009	285
9	10.1136/bmj.g3174	Review	BMJ-British Medical Journal	Ford, AC	2014	219
10	10.1056/NEJMoa1909666	Article	N. Engl. J. Med.	Choi, IJ	2020	182
11	10.1152/physrev.00039.2009	Review	Physiol. Rev.	Peek, RM	2010	162
12	10.1016/j.chom.2012.10.014	Article	Cell Host Microbe	Tsugawa, H	2012	161
13	10.1136/bmj.l5016	Article	BMJ-British Medical Journal	Li, WQ	2019	129
14	10.1200/JCO.2009.26.0695	Article	J. Clin. Oncol.	Wu, CY	2010	111
15	10.1016/j.chom.2012.05.010	Article	Cell Host Microbe	Hayashi, T	2012	110
16	10.1093/annonc/mdr384	Article	Ann. Oncol.	González, CA	2012	85
17	10.1016/S1470-2045(06)70586-1	Article	Lancet Oncol.	Meimarakis, G	2006	82
18	10.1093/annonc/mdi184	Article	Ann. Oncol.	Ruzzo, A	2005	56
19	10.1016/S2468-2667(21)00164-X	Article	Lancet Public Health	Yang, L	2021	50
20	10.1016/j.chom.2021.04.006	Article	Cell Host Microbe	Imai, S	2021	44
21	10.1093/annonc/mdn055	Article	Ann. Oncol.	Prasad, KN	2008	24

proteins, inflammation, host immune response, environmental factors and HP-mediated GC. HP eradication treatment may reduce the risk of GC (Fuccio et al., 2009). Apart from the above high-cited reviews, there were three high-IF reviews worthy of attention. Peek RJ et al (Peek et al., 2010). further depicted that the role of host innate immune system including gastric epithelial cells and immune cells in HP-induced GC. A meta-analysis (Ford et al., 2014) showed that HP eradication therapy may reduce the incidence of GC in healthy asymptomatic infected Asian individuals.

3.7 Keywords in HP/GC research

3.7.1 High-frequency keywords

We examine keywords to pick out the hot topics in HP/GC research. In this paper, a total of 5,811 keywords included 3,176 author's keywords and 2,635 keywords plus were acquired. The common author's keywords (Figure 6A) included "*Helicobacter pylori*", "gastric cancer", "CagA", "diet", "eradication", "intestinal metaplasia", "gastritis", "stomach neoplasms", "gastric carcinoma", "atrophic gastritis", "inflammation", "carcinogenesis", "gastric adenocarcinoma", "VacA", "gastric carcinogenesis", "apoptosis", "stomach cancer", "prognosis", "prevention", "meta-analysis", "cancer", etc. The common keywords plus (Figure 6B) included "infection", "expression", "risk", "carcinoma", "association", "cancer", "CagA", "eradication", "intestinal metaplasia", "epithelial-cells", "prevalence", "atrophic gastritis", "activation", "adenocarcinoma", "population", "cells", "inflammation", "carcinogenesis", etc.

3.7.2 Time series analysis of keywords

Keyword time series analysis in the bibliometric analysis can observe the changes of keyword frequency and co-occurrence relationship over time, so as to reveal the development trend and hot spot changes of research topics. By means of trend topic module in the biblioshiny of Bibliometrix, we analyze the time evolution of keywords. As we can see in Figures 6C, D, the hot author's keywords in recent years include "autophagy", "microbiota", "lncRNA", "early gastric cancer", "EMT", "exosomes", "mortality", "gut microbiota", "immunotherapy", and so on. The hot keywords plus include "mechanisms", "metastasis", "features", "endoscopy", "metabolism", "microbiota", "target", "gamma-glutamyl-transpeptidase", "suppressor-cells", and so on.

3.7.3 Cluster analysis of keywords

According to the extracted keywords, the co-occurrence network between them is constructed, and the relationship between them is established according to the situation that the keywords appear in the same literature at the same time. Based on the co-occurrence keywords, the cluster analysis was conducted to assess the links of the keywords, and the cluster analysis results were shown in Figure 7.

Cluster 1 (green nodes) concerned the links between HP and digestive system disease (such as chronic atrophic gastritis, MALT lymphoma, peptic ulcer, intestinal metaplasia, dysplasia, gastric cancer). HP may cause gastric mucosal inflammation in patients, such as chronic superficial gastritis, chronic atrophic gastritis. It can also cause peptic ulcer, including gastric ulcer, duodenal ulcer, etc.

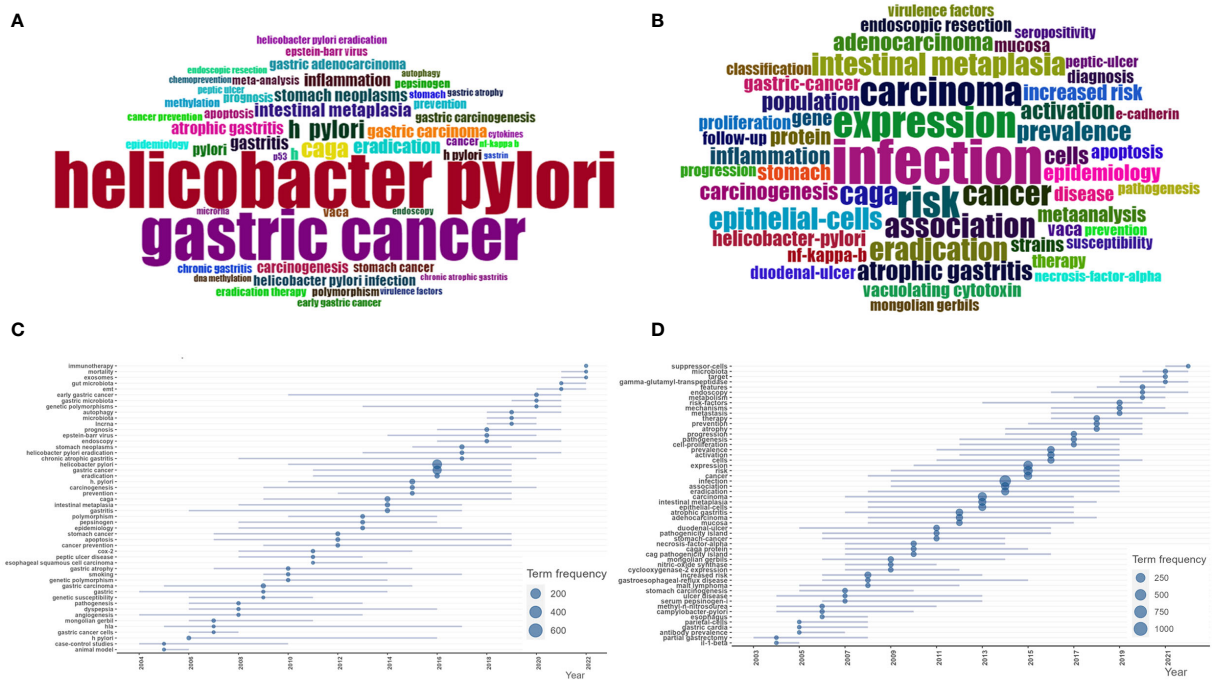

FIGURE 6
(A) Common author keywords in HP/GC. **(B)** Common keywords Plus in HP/GC. **(C)** Common author keywords evolution trends in HP/GC. **(D)** Common keywords Plus evolution trend in HP/GC.

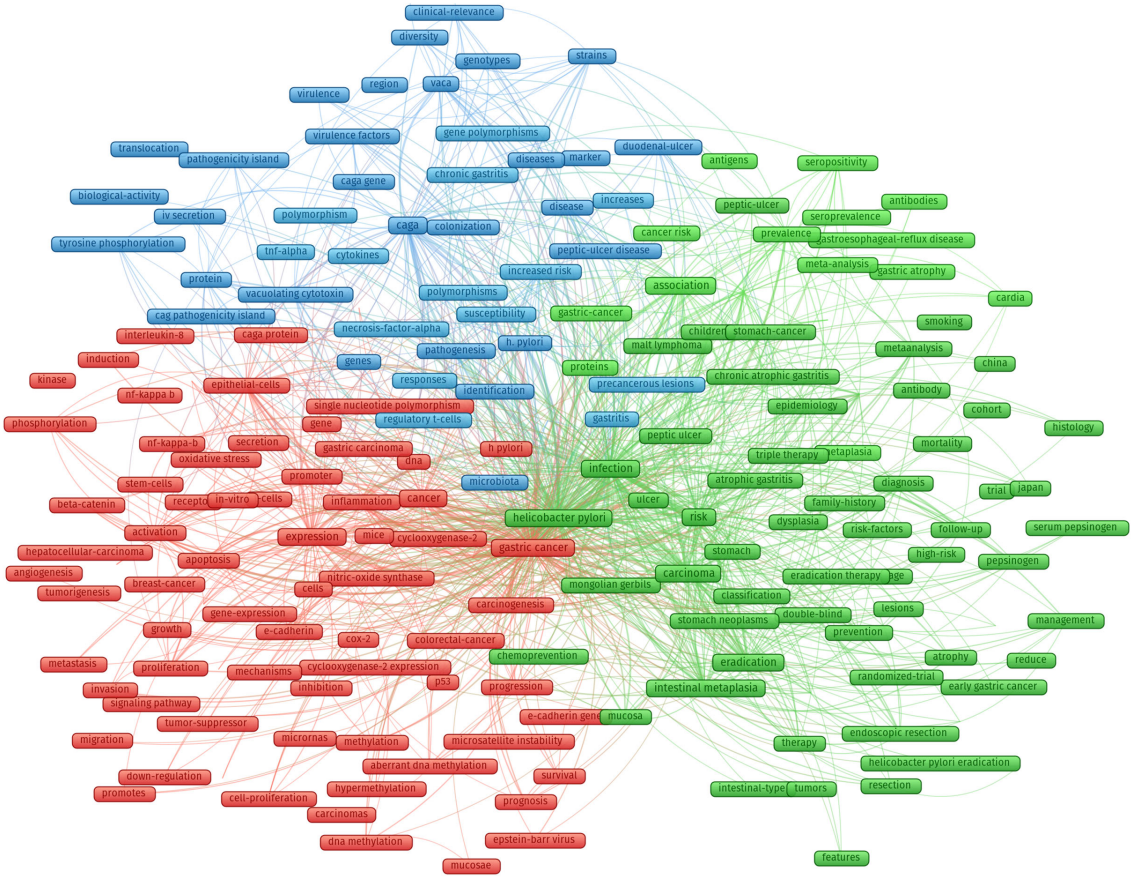

FIGURE 7
Cluster analysis of all common keywords in HP/GC (different colors mean different clusters, the size of the circle indicates the frequency of occurrence of keywords).

With the severity of the disease, HP will gradually destroy the gastrointestinal wall, causing the onset of GC.

Cluster 2 (blue nodes) focused on the pathogenesis of HP-induced GC, especially HP-related virulence factors, including CagA and its pathogenicity island (CagPAI), IV secretion, tyrosine phosphorylation, VacA and gene polymorphism, and precancerous lesions. CagA and VacA are the main virulence factors of HP. CagA is an outer membrane protein with strong immunogenicity, which is present in the cytoplasm of HP and eventually transported into host cells through Type IV secretion system. VacA is a vacuolating toxin produced by HP, which can induce vacuolation and inhibit immune response.

Cluster 3 (red nodes) focused on the mechanisms by which HP affects GC, especially host factors, including inflammation (nf-kappa-b, interleukin-8), β-catenin, E-cadherin, immunity (such as regulatory T cells and intestinal epithelial cells), gene-expression, stem cells, nitric-oxide synthase, p53, COX2 and oxidative stress. Inflammation and immune factors play a key role in the pathogenesis of HP infection. Inflammatory response is one of the important causes of gastric mucosal injury, and also promotes the proliferation and apoptosis of gastric mucosal cells, and ultimately forms tumors.

4 Discussions

In 1994, the World Health Organization (WHO) classified HP as type I carcinogen for GC. Since then, HP/GC research has received increasing attention from scholars. Over the past two decades, given the growing understanding of HP and evidence that HP is a modulator that can influence the occurrence and progression of GC, and alter the outcome of GC treatment, numerous studies have been conducted on the link between HP and GC. Therefore, we mainly focus on literature from the past two decades to better track the status and trends in HP/GC research.

4.1 Analysis of document issuance in HP/GC research

From the annual Np view, from 2003 to 2011, HP/GC research gradually gained steady attention, and the number of articles published each year was more than 50. Since 2011, the research had gradually increased and the annual number of publications was more than 100. Regarding the journals, our study showed that *Helicobacter*, *World Journal of Gastroenterology* and *PLoS One*

ranked in the top three in the Np, *International Journal of Cancer* and *World Journal of Gastroenterology* had the highest H-index, and the *Gastroenterology* had the most TC. *Helicobacter* is a broad-caliber journal that covers the full spectrum of HP research, and promotes communication between the fields of gastroenterology, microbiology, vaccine development, and laboratory animal science. In addition, it is worth noting that the top 20 high-cited and high-IF articles were published mainly in *NEJM* (Choi et al., 2018; Choi et al., 2020; Usui et al., 2023) and *Lancet and its sub-journals* (Meimarakis et al., 2006; Fukase et al., 2008; Yang et al., 2021), followed by *JAMA* (Wong et al., 2004), *BMJ* (Li et al., 2019), *Gut* (Watabe et al., 2005; Lee et al., 2013; Cheung et al., 2018; Oster et al., 2022b), *Gastroenterology* (Wu et al., 2009; Yan et al., 2022; Li D. et al., 2023), and so on. They mainly focused more on clinical research, while *PNAS* (Franco et al., 2005; Ohnishi et al., 2008) focused more on basic experimental research. These prestigious journals are more likely to publish top studies in the next year. *Nature Reviews Cancer* (Hatakeyama, 2004; Polk and Peek, 2010; Thrift et al., 2023) and *Gastroenterology* (Correa and Houghton, 2007; Graham, 2015; Amieva and Peek, 2016; Lee et al., 2016) had the most influential reviews, showing that them can publish more reviews in the next year. These original articles and review articles deserve the attention of researchers, as they often represent significant research achievements in the field, through which we can gain insight into the latest advances in the current research area and identify research directions.

These papers came mainly from China and Japan, followed by the United States, Korea and Germany. China had the largest Np, followed by Japan, which may be due to high incidence of HP (Hooi et al., 2017) and GC (the incidence of GC in China accounts for almost half of the world) (Etemadi et al., 2020) and the high attention and support of the countries. The top 10 institutions were from China, the United States and Japan, showing their good scientific productivity. In China, *Peking University* and *Shanghai Jiao Tong University* published the most papers. *University of Tokyo* and *Oita University* in Japan, and *Vanderbilt University* and *US Department of Veterans Affairs* in the United States made important contributions to HP/GC research. Most of the top 10 authors were from *Vanderbilt University* and *Seoul National University*, which are the world-class research universities. Peek Richard M, a gastroenterologist from *Vanderbilt University*, had most Np and the highest H-index and TC, and had devoted himself to the study of HP, especially the tumorigenesis and pathobiology of HP-induced GC such as activation of β-catenin, virulence factors, innate immunity, microRNAs, iron deficiency and regulation of p53 (Peek et al., 2010; Polk and Peek, 2010; Wei et al., 2010; Wroblewski et al., 2010; Noto et al., 2013; Amieva and Peek, 2016). Latterly, he increasingly focused on the role of gastric microbiome (Noto and Peek, 2017), hydrogen metabolism (Wang et al., 2016) and bile acid metabolism (Noto et al., 2022) in the HP-induced GC. Yamaoka Yoshio from *Oita University* had long been engaged in HP virulence factors and the link between GC and HP infection in East Asian populations (Binh et al., 2017; Sugimoto et al., 2020). Piazuelo M Blanca and Romero-Gallo Judith from *Vanderbilt University* had published many high-cited papers (working closely with Peek Richard M) (Wei et al., 2010; Noto et al., 2013), and the former found the nutraceutical electrophile scavenger 2-hydroxybenzylamine can attenuate GC development caused by HP (Gobert et al., 2023).

4.2 Historical cited papers in HP/GC research

The historiography analysis revealed several classic papers (deserve special attention), annotated with their local citation score (LCS) and global citation score (GCS) in Supplementary Material S1.

In 2004, Wong et al. (2004) showed that eradication of HP significantly reduced the development of CG in HP carriers without precancerous lesions. Hatakeyama (2004) et al. described the oncogenic mechanisms of the HP CagA protein. In 2005, Franco et al. (2005) indicated that HP-induced dysregulation of beta-catenin-dependent pathways may explain the augmentation in HP-induced GC. In 2006, Kamangar et al. (2006) further showed that HP is a strong risk factor for non-cardia GC but is inversely associated with the risk of cardia GC. In 2007, Correa (Correa and Houghton, 2007) reviewed the carcinogenesis of HP, which begins with early inflammation, progresses through metaplasia and atypical hyperplasia, and ultimately leads to cancer development. In 2008, Fukase et al. (2008) found that HP should be eradicated after endoscopic resection of early GC to prevent the development of metachronous GC. Ohnishi et al. (2008) found that HP CagA protein transgenic expression can induce gastrointestinal and hematopoietic tumors in mice. In 2009, a meta-analysis (Fuccio et al., 2009) showed that HP eradication therapy may reduce the risk of GC. Wroblewski et al. (2010) discussed that the main virulence determinants of HP strains and the correlation between these factors and different clinical outcomes after HP infection. In 2010, Polk et al (Polk and Peek, 2010). summarized the possible mechanism of HP leading to GC. A review (Wroblewski et al., 2010) showed that HP virulence factors, host factors, and environmental factors can affect the occurrence and development of GC. In 2012, Ma et al. (2012) showed that HP treatment significantly reduces the incidence of GC. Inversely, Maehata et al. (2012) found that eradication of HP did not reduce the incidence of metachronous GC. HP should be eradicated before the progression of gastric mucosal atrophy. In 2013, Lee et al. (2013) showed that HP eradication led to a significant reduction in gastric atrophy, but at the cost of increased esophagitis. In 2014, Wang et al. (2014) described the pathophysiological mechanism of HP-induced gastric inflammation and GC. In 2016, Amieva et al (Amieva and Peek, 2016). further described the pathology of HP-induced GC. A meta-analysis (Lee et al., 2016) showed that eradication of HP can effectively reduce the incidence of GC, and the protective effect is greater in individuals with a higher baseline risk of GC. In 2018, a study (Choi et al., 2018) showed that patients with early GC receiving HP treatment had a lower incidence of metachronous GC, and the degree of gastric atrophy was more improved than baseline.

4.3 Research status and hotspots in HP/GC research

This paper found that the current hot topics were mainly concentrated in the effect of HP on tumorigenesis and treatment of GC and the possible mechanisms of HP involved in GC.

4.3.1 Effect of HP on gastric tumorigenesis and progression

Some studies showed that HP can promote the progression and carcinogenesis of GC. In a study of 114 histologically confirmed GC cases from eastern Libya, the total HP infection rate was 63.2%, particularly for intestinal-type gastric adenocarcinoma (71.7%) (Elzouki et al., 2012). The infection rate of HP in the GC group was significantly higher than that in the non-GC group, and patients with HP infection had a higher risk of non-cardia GC than those without infection (Binh et al., 2017). There is a strong positive correlation between the degree of gastritis caused by HP and the development of GC, especially in intestinal gastritis, and the progression of chronic atrophic gastritis with HP infection increased the risk of GC (Ohata et al., 2004). Correa outlines the histological progression of HP infection from the precancerous cascade to cancer (Correa and Houghton, 2007). Furthermore, HP is a strong risk factor for non-cardia GC but is inversely associated with the risk of cardia GC (Kamangar et al., 2006). HP infection after endoscopic resection may increase the risk of metachronous GC development (Kim et al., 2014).

Consequently, eradication of HP significantly reduced the risk of developing GC in patients without precancerous lesions, providing additional evidence that HP affects the early stages of GC (Wong et al., 2004). In the East Asian population at high risk of GC, HP eradication effectively reduced the risk of GC regardless of the history of cancer (Sugimoto et al., 2020). Early HP eradication is associated with decreased risk of GC in patients with peptic ulcer diseases (Wu et al., 2009). Several meta-analyses (Fuccio et al., 2009; Lee et al., 2016) also showed that eradication of HP infection was associated with a reduced incidence of GC. In addition, a 2020 double-blind study (Choi et al., 2020) reported that HP eradication therapy can reduce the risk of GC in HP-infected patients with a family history of GC in first-degree relatives. For metachronous GC, a study (Choi et al., 2018) showed that patients with early GC who received HP eradication therapy had a lower incidence of metachronous GC and greater improvement in gastric atrophy grading than patients treated with placebo. A meta-analysis demonstrated that HP eradication can reduce the occurrence of metachronous GC in patients who underwent endoscopic resection (Yoon et al., 2014).

4.3.2 Pathological mechanisms of HP-induced GC

HP-induced GC is the result of a complex interaction between bacterial virulence factors, the host inflammatory response and environmental impact (Noto et al., 2013). HP contributes to gastric carcinogenesis through bacterial virulence factors and metabolites, chronic inflammation, host immunity, barrier disruption, alterations of cell proliferation and cell invasion and apoptosis, and so on (Peek et al., 2010; Wang et al., 2014).

First of all, CagA and VacA are the main virulence factors of HP. CagA and VacA can trigger inflammation and carcinogenesis (Hatakeyama, 2004). Cag A enters gastric epithelial cells via the bacterial type IV secretion system. Notably, CagA is known for its variation, which may influence the potential of different HP strains to promote GC (Hatakeyama, 2014). HP CagA triggers BRCAness to induce genomic instability, which may underlie the development of bacterial GC (Imai et al., 2021). Phosphorylated activated CagA interacts with a variety of host proteins in target cells and continuously activates the abnormal expression of multiple carcinogenic signaling pathways (Yong et al., 2015). Likewise, the risk of gastric cardia and non-cardia adenocarcinoma is much higher in CagA-positive HP infection than in CagA-negative infection (Carlosama-Rosero et al., 2021). VacA can not only induce vacuolization of gastric epithelial cells, but also stimulate apoptosis (Polk and Peek, 2010). Capurro et al. (2019) found that VacA targets the lysosomal calcium channel TRPML1 to disrupt the lysosomal transport, and thereby hijack the lysosomal and autophagy pathways, allowing HP to escape the role of antibiotics, and ultimately survive in the stomach and continuously stimulate host cells.

In addition, inflammation promotes the progression of HP-associated GC, which is supported by the higher incidence of GC in gastritis patients, especially in patients with intestinal metaplasia and dysplasia. HP-associated GC occurs primarily through the inflammatory-cancer pathway. Specifically, HP-induced inflammation leads to a high renewal rate of gastric endothelial cells, high levels of reactive oxygen species and nitrogen in the microenvironment, and an increased likelihood for DNA damage and somatic mutations (Graham, 2015). HP infection can up-regulate many pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α, NF-κB (El-Omar et al., 2000; Polk and Peek, 2010; Wang et al., 2014), and inflammatory mediators facilitate cell proliferation, mutagenesis, oncogene activation, and angiogenesis. IL-1β and TNF-α are proinflammatory acid-suppressive cytokines that are elevated in HP-colonized gastric mucosa (Wroblewski et al., 2010). NF-κB and IL-8 are considered important mediators of gastric pathophysiology in the development of inflammation (Wang et al., 2014). CagA can induce IL-8 expression through NF-κB activation (Polk and Peek, 2010). Activation of NF-κB and up-regulation of IL-8 in gastric epithelial cells are considered important mechanisms of HP-induced carcinogenesis (Brandt et al., 2005).

Furthermore, the effect of HP on GC acts through manipulating host immune systems (Wroblewski et al., 2010). HP and cancer immunomodulatory stromal cells can mediate the immune response to promote tumorigenesis (Navashenaq et al., 2022). HP not only produces immune tolerance by inhibiting T cell function, but also can modify the structure of LPS to evade the recognition of Toll-like receptors (TLRs) pattern recognition receptor family molecules to achieve the purpose of immune escape (Peek et al., 2010). TLRs act in T cell activation, promoting innate immune response and immune tolerance during HP infection (Zhang et al., 2023), which are considered to be the core defects leading to

inflammation and cancer development. HP infection can up-regulate the expression of PD-L1 in GC cells by activating the p38 mitogen-activated protein kinase pathway, inhibiting the proliferation of T cells and inducing the differentiation of naive T cells into Treg cells, thereby avoiding immune surveillance and promoting immune escape that ultimately leads to carcinogenesis (Deng et al., 2022).

Other than that, HP infection also disrupts adhesion junctions by inducing the translocation of membrane E-cadherin, β-catenin, and p120 to the cytoplasm of epithelial cells (Wroblewski and Peek, 2013). Strains isolated from patients with lower ferritin levels induce significantly higher levels of IL-8 than strains isolated from patients with the highest ferritin levels, suggesting that iron deficiency in the host increases the virulence of HP and the risk of GC (Noto et al., 2013). HP can inhibit tumor suppressor gene p53 via activating AKT1 to lead to phosphorylation and activation of Human Double Minute 2, which is a potential mechanism for the risk of GC in HP infected individuals (Wei et al., 2010). A study (Tsugawa et al., 2012) provided a molecular link between HP and GC through the specific accumulation of CagA in GC stem-like cells. A recent study (Usui et al., 2023) further demonstrated that HP infection increased the risk of GC associated with germline pathogenic mutations in homologous recombination genes, providing further insight into the gene-environment interaction in the progress of GC.

4.3.3 Emerging new paradigms of HP-induced GC

Notably, some emerging research, such as gut microbiota, immunotherapy, autophagy, exosomes, EMT and GGT may be the focus in the next few years. We discuss the latest hot keywords as follows.

Gut microbiota (GM): GM has been shown to promote the development of HP-associated GC. The lack of commensal microbiota in HP-infected INS-GAS mice can reduce gastritis and delay intraepithelial neoplasia (Lofgren et al., 2011). A vivo study confirmed that limited colonization of gastric flora other than HP can induce the formation of gastric mucosal tumor lesions in INS-GAS mice (Lertpiriyapong et al., 2014). A study found that the changes in GM may be involved in the progression of gastric lesions related to HP infection and provide clues for future evaluation of microbial alterations after HP eradication (Gao et al., 2018). HP may affect the GM through continuous crosstalk with the host immune system. The diversity, composition and function of GM changed after HP infection (Cui et al., 2022). Successful eradication of HP may restore the gastric microbiota to a state similar to that of uninfected individuals and show a beneficial effect on the GM (Guo et al., 2020).

Immunotherapy: Recent studies have shown that HP infection can adversely affect the tumor immune microenvironment and tumor immunotherapy. The overall survival (OS) and progression-free survival (PFS) of HP-positive cancer patients treated with immune checkpoint inhibitors, such as gastric cancer, melanoma and non-small cell cancer patients, were significantly reduced (Che et al., 2022; Oster et al., 2022b; Tonneau et al., 2022), but the specific mechanism is unknown. Some scholars have suggested that HP may reduce the efficacy of immunotherapy by changing the composition of intestinal flora and tumor immune microenvironment, affecting tumor immune response, but there is still a lack of relevant direct evidence (Oster et al., 2022a).

Autophagy: Autophagy is a cell degradation mechanism and may be triggered by HP. Autophagy can mediate ER stress and inflammation in HP-related GC (Mommersteeg et al., 2022). HP-suppressed autophagy promotes the intracellular survival and persistence of pathogens, and also produces an environment conducive to carcinogenesis (Greenfield and Jones, 2013). HP-induced downregulation of p14ARF tumor suppressor gene leads to inhibition of autophagy in infected cells in a p53-independent manner (Horvat et al., 2018). In addition, reactive oxygen species-induced autophagy degradation of HP CagA is specifically inhibited in cancer stem cell-like cells (Tsugawa et al., 2012). HP infection may promote autophagy in human GC cells through Nrf2-mediated heme oxygenase upregulation (Paik et al., 2019).

Exosomes: Exosome is a small extracellular vesicle. Extracellular vehicles (EVs) play an important role in the evolution of malignant tumors because of the genetic material they carry. HP EV is abundant in gastric juice of patients with gastric cancer, which can induce gastric inflammation and may even induce GC, mainly through the selective uptake of gastric epithelial cells to produce inflammatory mediators (Choi et al., 2017). HP infection can induce the up-regulation of activated mesenchymal-epithelial transition factor in exosomes and the tumor-promoting effect on tumor-associated macrophages (Che et al., 2018). Exosomes have been shown to deliver not only various types of genetic information, mainly miRNAs, but also CagA.

Epithelial-Mesenchymal Transition (EMT): EMT is an important part of the invasion, metastasis and multidrug resistance of GC, and it is also one of the key factors for GC. HP CagA promotes EMT in gastric carcinogenesis via triggering oncogenic YAP pathway (Li et al., 2018). The up-regulation of MMP-7 by pathogenic HP is partly dependent on gastrin, and may indirectly increase the level of soluble heparin-binding epidermal growth factor through EMT, which plays a role in the development of GC (Yin et al., 2010). HP infection may trigger TGF-β1-induced EMT pathway and the emergence of GC stem cells, and eradication of HP may prevent the carcinogenesis of GC by inhibiting these two pathways (Choi et al., 2015).

Gamma-glutamyl-transpeptidase (GGT): GGT, an established virulence factor of HP with immunomodulatory properties, can degrade extracellular glutathione, produce reaction products, and increase DNA damage in gastric cells. HP-induced loss of gastric cell survival and viability may be attributed to secreted bacterial GGT activity (Valenzuela et al., 2013). GGT secreted by HP can activate Wnt/β-catenin signaling pathway to promote the occurrence of GC (Meng et al., 2021). A recent study (Baskerville et al., 2023) shows that HP-induced glutathione degradation occurs through an oxidation-independent mechanism driven by the bacterial enzyme GGT, which enhances the ability of bacteria to obtain nutrients from the host.

4.4 Limitations of the research

Our study has some potential limitations. Firstly, only the papers indexed in WoSCC database were searched and included, which may not cover all relevant studies from multiple databases worldwide, leading to possible incompleteness of the results. Secondly, current bibliometric tools cannot analyze all contents of papers, resulting in some concrete information being overlooked. High-cited and high-IF paper analysis helped compensate for this disadvantage and limitation. Thirdly, since this study only focused on the current stage of published papers, some newly published papers with significant impact may have been cited less. With the rapid development of research, more papers will become available for analysis.

5 Conclusions

Over the last 20 years, interest in HP/GC research has increased. China and Japan were in the leading position and contributed the most to HP/GC research. *Vanderbilt University* and the *US Department of Veterans Affairs* had the maximum Np. The most productive authors were Peek Jr Richard M. and Piazuelo M. Blanca. *Helicobacter* received the most Np, while *Gastroenterology* had the most TC. HP affects the onset and development of GC, as well as the prognosis and effectiveness of treatment of GC. Eradication of HP can not only prevent early GC and metachronous GC but also improve the clinical efficacy of GC treatment. HP may contribute to gastric carcinogenesis through virulence factors, bacterial metabolites, chronic inflammation, and host immunity. Therefore, relevant interventions may represent the next breakthrough in the prevention and treatment of HP-induced GC. Understanding the underlying mechanisms of the links between HP and GC is a fascinating area of research. As HP/GC research advances, gut microbiota, immunotherapy, autophagy, exosomes, EMT, and GGT may emerge as new areas of focus. In summary, this study provides a comprehensive overview of the global status of HP/GC research, enabling scholars to gain a better understand its development trends and identify potential areas for further investigation.

Data availability statement

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

Author contributions

SY: Conceptualization, Data curation, Formal analysis, Investigation, Software, Visualization, Writing – original draft. SH: Conceptualization, Data curation, Investigation, Software, Visualization, Writing – original draft. HY: Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing. XZ: Funding acquisition, Methodology, Project administration, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1353094/full#supplementary-material

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The role of microbiomes in gastrointestinal cancers: new insights

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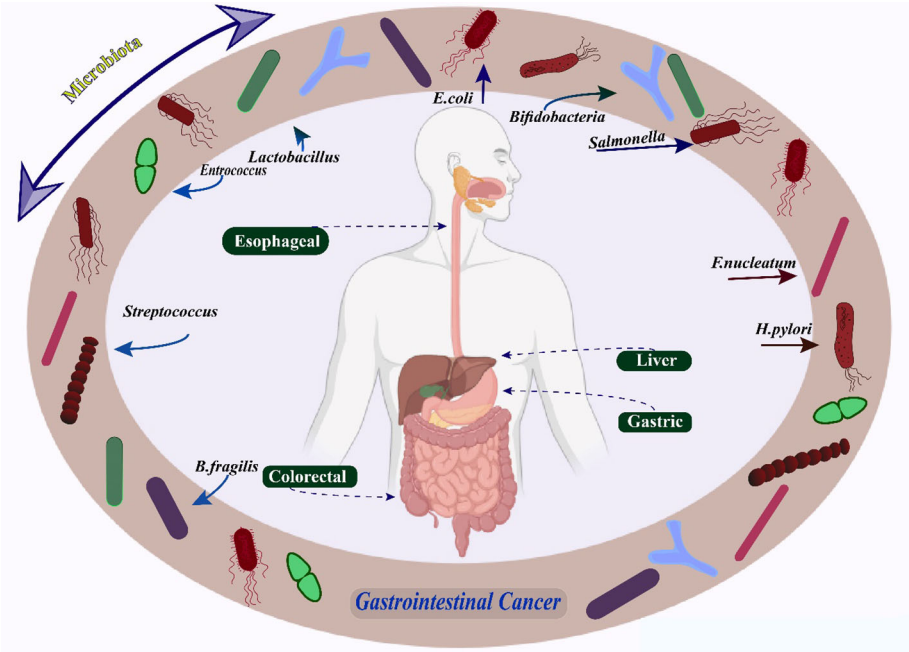
Gastrointestinal (GI) cancers constitute more than 33% of new cancer cases worldwide and pose a considerable burden on public health. There exists a growing body of evidence that has systematically recorded an upward trajectory in GI malignancies within the last 5 to 10 years, thus presenting a formidable menace to the health of the human population. The perturbations in GI microbiota may have a noteworthy influence on the advancement of GI cancers; however, the precise mechanisms behind this association are still not comprehensively understood. Some bacteria have been observed to support cancer development, while others seem to provide a safeguard against it. Recent studies have indicated that alterations in the composition and abundance of microbiomes could be associated with the progression of various GI cancers, such as colorectal, gastric, hepatic, and esophageal cancers. Within this comprehensive analysis, we examine the significance of microbiomes, particularly those located in the intestines, in GI cancers. Furthermore, we explore the impact of microbiomes on various treatment modalities for GI cancer, including chemotherapy, immunotherapy, and radiotherapy. Additionally, we delve into the intricate mechanisms through which intestinal microbes influence the efficacy of GI cancer treatments.

KEYWORDS

gastrointestinal cancer, gut microbiome, microbiota, cancer therapy, inflammation



GRAPHICAL ABSTRACT
Microbiomes in Gastrointestinal Cancers.

Introduction

Gastrointestinal (GI) malignancies constitute approximately one-third of all newly diagnosed cancer cases globally and pose a significant public health challenge. Colorectal cancer (CRC), Gastric cancer (GC), liver cancer, and esophageal cancer are the most commonly observed GI malignancies across the globe (1, 2). Since GI malignancies have been on the rise over the past 5 to 10 years, there is a severe health risk to people due to this trend. The past decade has witnessed the substantiation of the role played by genetic and epigenetic factors in the development of cancer. This has been achieved through the extensive genomic and transcriptome sequencing endeavors undertaken by multiple multinational research initiatives (3). According to current studies, 2.7 million individuals worldwide die from GI cancer yearly, with 4 million cases diagnosed worldwide (4–6). Although GI cancers display a diverse array of biological attributes, several shared risk factors have been discerned. These include pro-tumor genetic mutations, excessive intake of alcohol, smoking, adherence to the Western diet, exposure to radioactive stimuli, and disturbance of the GI microbiota's homeostasis (7). Furthermore, the disruption of the typical GI environment has been associated with the onset of GI malignancies due to the emergence of pro-tumoral fibrosis and the occurrence of significantly potent local or systemic inflammatory and immunological reactions (8–10). In addition to these risk factors, certain disorders are strongly linked to the origin of GI cancers. For instance, it has been found that GI cancer and diabetes are related. One of the most used anti-hyperglycemic medications, metformin, has been demonstrated to lower the incidence rate of GI malignancies in diabetic individuals (11, 12). The comprehension of the role of bacteria in cancer development is significantly restricted compared to the knowledge we have about viruses causing oncogenesis. However, it is feasible to consider that gaining a better understanding of the long-lasting effects of changes in the composition of the GI microbiota may have the potential to contribute to the progress of preventive strategies against cancer. Moreover, bacteria have the potential to indirectly facilitate the process of carcinogenesis through the alteration of both systemic and local immune reactions. These immune responses play a crucial role in progressing GI tract malignancies (13). The GI tract of humans harbors a vast number of microorganisms that work in conjunction with the host to uphold both wellness and illness. The intricate web of interactions between the GI microbiome and the host gives rise to intimate connections that span various components of human physiology, such as the metabolic, immunological, and neuroendocrine systems (14). These creatures are dynamic and subject to influences from medications, food, lifestyle, genetics, and the environment (15). Researchers have discovered that the influence of gut microorganisms extends beyond the confines of the intestines, affecting a range of conditions including pancreatic disease and hepatic disease, in addition to intestinal diseases such as Inflammatory bowel disease (IBD) and CRC (16). Shortly following the moment of birth, the microbiota initiates the process of establishing residence within the GI tract, subsequently maintaining its presence throughout the entirety of an individual's lifespan (17). However, it can vary dynamically in response to nutrition, environmental stresses, lifestyle choices, antibiotics, and other medications (18).

Increasing proof suggests that the microbial population residing in the digestive system holds immense potential to thwart the growth of cancerous cells while also possessing the ability to enhance the potency of chemotherapy and immunotherapy treatments (19). The gut microbiota is accountable for producing short-chain fatty acids (SCFAs), which bestow advantageous effects on the human body. These SCFAs are generated through the metabolic breakdown of dietary fiber, as well as the synthesis of vitamins B and K2. Additionally, the gut microbiota metabolizes various chemicals, such as sterols and exogenous substances, while also playing a role in regulating immunological function (20).

This study aimes to investigate the primary impacts of intestinal microbiota on the initiation and advancement of GI cancers, along with the potential utilization of these microorganisms as a sophisticated approach to discern and manage these ailments, as expounded upon in this comprehensive analysis.

Microbiome in health and gastrointestinal cancer

The analysis of the microbial populations found in various human environments, such as the GI tract, mouth, skin, and vaginal area, requires applying advanced sequencing techniques that can process large amounts of data (21). The utilization of sophisticated sequencing methodologies, encompassing amplicon and shotgun metagenomic sequencing, has significantly transformed our comprehension of the human microbiome by delineating the bacteria linked to either optimal well-being or pathological conditions (22). When the physical condition of an individual is in a state of good health, the gut microbiota engages continuously and regularly with the host organism to sustain a state of balance within the intestines (23). However, maintaining such balance is challenging since the host's genetic makeup and several exogenous variables, including nutritional consumption and antibiotic usage, have a direct impact on the microbiome (24–26). Dysbiosis, the alteration in both composition and functionality within the microbiome, can occur when there is a persistent disturbance in the stability of the microbial community. This alteration may cause various disorders, including cancer (27, 28). In a dysbiotic microbiome, various pathogenic occurrences are encountered, including a modification in the assortment of microorganisms, a decrease in beneficial commensals, and the proliferation of pathobionts. All of these occurrences can impact the formation of tumors, either in the vicinity of the GI tract or at a more remote location, such as the pancreas and liver (29, 30). The GI system harbors the highest abundance of commensal microorganisms among all the regions of the human body. Variable parts of the digestive system have varying levels of commensal microbial diversity and abundance (31). While a multitude of bacteria belonging to the phyla *Firmicutes*, *Proteobacteria, Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and are frequently observed within the gut, certain bacterial species seem to be confined to specific regions (31, 32). Each area or organ's microbial population is related to host characteristics, including pH, oxygen saturation, bile acids, and nutritional bioavailability (33, 34).

GI cancer and gut microbiota

The thorough analysis of microbial populations in the host's environment has been extensively explained as a result of the rapid advancements achieved in next-generation high-throughput sequencing (NGS) (35, 36). Dysbiosis leads to the stimulation of inflammatory components within the GI mucous membranes, which encompasses the liberation of nitric oxide (NO), the presence of oxidative stress, the creation and excretion of pro-inflammatory cytokines, as well as the activation of cyclooxygenase 2 (COX-2). Dysbiosis also causes microecological alterations (27, 37). The detrimental effects of microbial metabolites on extra-intestinal organs can manifest in various pathways, such as the gut-liver axis and the gut-brain axis, thereby impairing their optimal functioning (38, 39). Dysbiosis is believed to be most comprehensible when viewed through carcinogenesis, representing a continuous divergence of the host microbiota from a state of harmony and equilibrium that supports and, or upholds various cancer phenotypes (40, 41). The maintenance of well-balanced gut microbiota is crucial for promoting a healthy lifestyle. At the same time, an imbalance in this microbial community, known as dysbiosis, can lead to inflammatory consequences that accelerate cancer progression (42).

The role of the microbiome in colorectal cancer

Despite an increasingly widespread acceptance of colonoscopy screening, colorectal cancer (CRC) remains the third most commonly occurring cancer and the primary contributor to cancer-related deaths in both male and female populations within the United States (43). In 2019, a forecast indicated that there would be an estimated 51,020 deaths and approximately 145,600 fresh instances of CRC. Additionally, while the occurrence and fatality rate of CRC has experienced a gradual decrease in the past few decades in individuals aged 65 and above, a distinct trend has emerged among individuals under 50, for whom conventional screening methods have not been recommended (44). The evolution of CRC has been comprehensively examined over recent decades via migration and prospective cohort studies, illustrating the significant influence of nutritional and lifestyle determinants (43). According to estimations, it has been noted that modifiable risk factors, namely excessive weight or obesity, excessive alcohol consumption, smoking, high consumption of red meat, physical inactivity, and inadequate intake of dietary fiber, whole grains, or other beneficial nutrients, play a significant role in approximately 50% to 60% of newly reported cases of CRC in the US (43). The microbiome, which encompasses bacteria, viruses, fungi, and an array of diverse organisms, possesses the ability to regulate the condition of well-being, and modifications to it can contribute to the emergence and progression of ailments. There exists an increasing corpus of scholarly investigation indicating that alterations in the constitution of the intestinal microbiota contribute to the genesis and progression of CRC using the

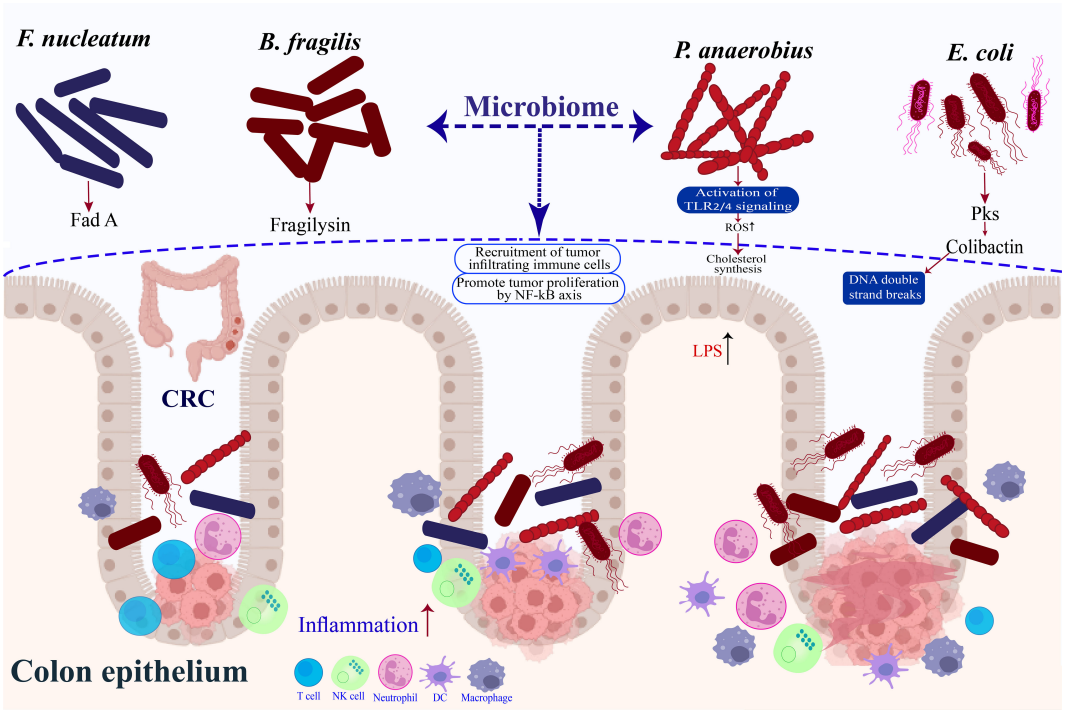


FIGURE 1
Microbes associated with risk of colorectal cancer.

impact of environmental factors that pose a risk (45, 46). This could be the case due to the microbiome's effect on metabolism and immune response (47). Hence, manipulating the intestinal microbial community could potentially serve as a constituent of strategies aimed at averting CRC (48, 49). Research has demonstrated discrepancies in the composition of the intestinal microbiomes between individuals afflicted with CRC and those deemed healthy (controls). Additionally, certain microbial species have been identified as exhibiting increased or diminished presence within the gut microbiomes of CRC patients. Therefore, to improve screening techniques, alterations in the microbiome can be utilized as biomarkers in the early detection of CRC (49). The colon is home to 70% of the human microbiome (50). Individuals who encounter antibiotics early on exhibit an increased propensity to develop colorectal adenoma during their later years (51). The microbiota in the GI tract plays a crucial role in converting the dietary components into metabolites that can either promote or suppress the growth of tumors. The development of CRC is subsequently influenced by these metabolites (52). Over 2000 different bacteria species are thought to exist in the human gut (53, 54).

Microbes associated with risk of CRC

Fusobacterium nucleatum

According to two separate investigations, tumor specimens had more *Fusobacterium* DNA and RNA sequences than non-tumor ones (55, 56). Similar relationships have been discovered in several studies, including numerous cohorts of CRC patients worldwide (57, 58). *Fusobacterium nucleatum* (*F. nucleatum*) has been associated with more advanced stages of disease, an increased likelihood of recurrence, and shorter periods of survival for patients, thus presenting compelling evidence of its potential causal role in CRC (59) (Figure 1). It is found in 10%–15% of tumors. Furthermore, *F. nucleatum* levels in tumor tissue have been linked to reduced T-cell infiltration, corroborating studies that claim *F. nucleatum* inhibits the anti-tumor immune response (43). *F. nucleatum* has been linked to distinct clinical and molecular characteristics in epidemiological investigations involving individuals with CRC or precancerous lesions. The aforementioned characteristics encompass the presence of anatomical positioning on the right side, mutations in the BRAF gene, and heightened levels of hypermutation alongside microsatellite instability (58, 60, 61). The described attributes of serrated neoplasia imply that *F. nucleatum* might have a part to play in developing CRC through the serrated pathway. Research has established a connection between *F. nucleatum* and the consensus molecular subtype 1 of CRC (62). This particular subtype is distinguished by an excessive expression of the immune system and the presence of microsatellite instability (63–65). More recently, in paired main tumors and distant metastases from CRC patients, virtually identical, live *Fusobacterium* strains were discovered in similar relative abundances. *Fusobacterium* thus seems to be a crucial part of the tumor microenvironment (66).

Bacteroides fragilis

Enterotoxigenic *Bacteroides fragilis* (ETBF), a significant pathogen that releases virulence factors to advance CRC, generates *B. fragilis* toxin or fragilysin, thereby inciting an adverse

immune reaction (67) (Figure 1). Colitis characterized by a robust, selective colonic Stat3 activation and a selective Th17 response was seen in mice treated with ETBF (68). Notably, a study by Chung et al. (69) provided more evidence that BFTs focus on the epithelial cells of the colon to instigate an immune response within the mucosal lining. This, in turn, triggers a series of inflammatory reactions that require the activation of IL-17, NF-kB, and Stat3. The molecular pathways of ETBF-induced adaptive immunity modification in CRC were further characterized by other researchers. Geis and colleagues (70) demonstrated that the presence of regulatory T cells in the local microenvironment resulted in a decrease in the quantity of IL2, thereby enabling the proliferation of Th17 cells, which is essential for the promotion of ETBF-induced CRC. Exosome miR-149-3p produced by colon cells after ETBF treatment also promotes Th17 differentiation (67). Consequences of long-term ETBF infection and inflammation include carcinogenesis. According to an animal study, BFT was required for the impact of ETBF infection, which increased colonic inflammation and enhanced AOM/DSS-induced CRC (71). An IL-17-driven monocytic-MDSC-dominant immunological profile was shown to be related to ETBF-triggered CRC by Thiele et al. (72), indicating that ETBF infection encourages MDSC-mediated immune suppression. *B. fragilis* emerged as the sole species consistently exhibiting higher levels in the intestinal microbiomes of individuals diagnosed with CRC across various geographical regions. This finding was established through a comprehensive meta-analysis encompassing four case-control studies that examined the metagenomes of CRC patients (73).

Escherichia coli

There is growing proof that pks+ *Escherichia coli (E. coli)* can produce virulence factors that control the development and progression of CRC (74) (Figure 1). A cancer-related pathogen that often infects CRC patients and expresses the polyketide synthase (pks) gene Colibactin, a hybrid peptide-polyketide cytotoxin that *E. coli* produces, induces DNA double-strand breaks and activates the DNA damage checkpoint mechanism in eukaryotic cells (75, 76). The involvement of colibactin in CRC has been shown by recent research. Anaphase bridge development, G2/M cell cycle stoppage, chromosomal aberration, and instability are all signs of the DNA damage response that even brief exposure to pks+ *E. coli* causes in mammalian epithelial cells (76–78). Cougnoux and colleagues (79), on the other hand, showed that acceleration of AOM-DSS-induced CRC by pks+ *E. coli* is facilitated by the stimulation of growth factor-secreting senescent cells, which is achieved through the alteration of p53 SUMOylation. Consequently, this modification encourages the proliferation of uninfected cells. Colibactin-producing *E. coli* also alters the immunological milieu, decreasing antitumor T-cell response and causing immunotherapy resistance and their effects on DNA damage (80).

Peptostreptococcus anaerobius

An anerobic bacteria called *Peptostreptococcus >anaerobius* (*P. anaerobius*) often lives in the mouth cavity. The bacterium *P. anaerobius*, which has been recently identified, was observed to have a higher occurrence rate among individuals diagnosed with CRC in comparison to those who were in good health (81, 82) (Figure 1). This remarkable finding was unearthed by employing the cutting-edge technique of shotgun metagenomic sequencing on fecal samples coupled with the exact 16S ribosomal RNA sequencing method on mucosal samples (83, 84). Subsequent examinations of functional nature revealed that *P. anaerobius* expedited the progression of AOM-induced CRC by augmenting the synthesis of cholesterol through the activation of TLR2/TLR4 signaling, thereby bolstering the proliferation of CRC cells (85, 86). Moreover, investigations on the profiling of the microbiome in the oral cavity have revealed that there are variations in the quantities of different components of the oral biofilm, including *Parvimonas*, *Haemophilus*, *Alloprevotella*, *Prevotella*, *Lachnoanaerobaculum*, *Streptococcus*, and *Neisseria*, between patients with CRC and control subjects (87, 88). The development of CRC has been associated with varying gene expression patterns in the mucosal surfaces of different bacteria. Notably, inquiries that have examined samples derived from individuals with colonic neoplasia and controls have discovered analogous networks of oral bacteria that exist on both the oral and colonic mucosal surfaces (88).

Modulation of microbiota in CRC

In light of the crucial function that the gut microbiota fulfills in CRC, extensive investigations have been conducted to unravel the secrets of regulating gut dysbiosis to avert or combat this ailment (89). Several tactics have been suggested, such as fecal microbial transplantation (FMT), dietary changes, and antibiotic treatment. At present, FMT has demonstrated efficacy in the management of recurrent *Clostridium difficile* infection. Nevertheless, the utilization of FMT in animals remains limited to the prevention and treatment of CRC (90–94). FMT is only marginally beneficial in a preventative situation, though. A more likely method of controlling the microbiota to prevent CRC is dietary intervention. Recent research has revealed how food and the microbiota interact to cause CRC (95). For instance, high-fat diet-fed mice showed a considerable change in the makeup of their intestinal microbial and reduced gut barrier function, confirming the theory that high fat causes CRC by encouraging microbial dysbiosis (96). Contrarily, dietary fiber can promote the proliferation of advantageous commensals, which produce metabolites such as Short-chain fatty acids (SCFAs) linked to tumor-suppressing properties (97). Intriguingly, ETBF-induced CRC in AOM-DSS mouse models was suppressed by high salt diets, suggesting that the effect of diet may depend on the situation (98, 99).

Effect of microbiota on cancer therapy in CRC

Current research suggests that the makeup of microorganisms in the GI system, referred to as the intestinal microbiome, can impact the body's response to different cancer therapies, including immune checkpoint blockade (ICB) therapy and chemotherapy.

The study involved comparing samples of rectal cancer that were locally advanced, with and without treatment for *F. nucleatum*. The results showed that *F. nucleatum* persistence after neoadjuvant chemoradiotherapy (nCRT) is connected to increased recurrence rates and the inhibition of immune cytotoxicity (100). Time and time again, numerous studies have discovered that *F. nucleatum* was more commonly present in patients with CRC who experienced a recurrence following chemotherapy (101). Additionally, it was observed that *F. nucleatum* targeted microRNAs, as well as the innate immunological signaling pathways TLR4 and MYD88, to stimulate the defense autophagy pathway and counteract the response to chemotherapy (102, 103). These findings imply that pathogenic microorganisms may not only influence colorectal carcinogenesis but also enhance treatment resistance (104). Contrarily, much research has also surfaced, demonstrating that gut commensal bacteria can enhance ICB treatment by activating antitumor T cells (105–107).

The role of the microbiome in gastric cancer

Gastric cancer (GC) holds a prominent position as the fourth leading contributor to cancer-related mortality globally. Furthermore, it also ranks as the fifth most commonly detected form of cancer (108). Male rates are two times higher than female rates. Eastern Asia has the highest incidence rates, approximately 26,000 fresh instances and 11,000 fatalities from GC manifest annually within the US. The overall 5-year survival rate for GC is considerably low, standing at 32.4%. This is probably because, in the United States, up to 62% of instances of GC are diagnosed at late stages, which are linked to lower overall survival rates than localized illness (109). GC arises as a result of a multifaceted interplay involving the genetic composition of the host, various environmental factors (e.g., alcohol consumption, smoking, excessive intake of salt and meat, and inadequate consumption of vegetables and fruits), as well as microbial elements (e.g., the presence of *Helicobacter pylori* (*H. pylori*) infection and the composition of the intestinal microbiota). The persistent activation of the immune system resulting from the intestinal microbiota of the host has been associated with long-term inflammation and altered interactions between the host's epithelium and microorganisms, which have been associated with GC (110).

Microbiota in the healthy, non-neoplastic stomach

The standard oxyntic (corpus) region of the human stomach, characterized by a low pH and an acidic milieu, is a barrier against the proliferation of commensal organisms and potentially detrimental pathogens originating from the upper and lower GI tracts. These regions serve as the primary abode for the vast majority of the microorganisms that comprise the body's microbiota. These microorganisms are primarily found in the large and small intestines, as well as the oral cavity (111). The false conclusion was reached due to several factors: the inadequate achievement in isolating and cultivating gastric microbiota, the absence of rapid and non-invasive diagnostic tests, and the emergence of microarray and next-generation sequencing technology, which have focused on the bacterial 16S ribosomal RNA (16S rRNA) as the primary target for accurate taxonomy and phylogeny identifications (112).

The prime pathogen: H. pylori

In 1982, Marshall and Warren discovered the significant revelation that *H. pylori* was the underlying factor responsible for both peptic ulcers and gastritis, thereby drawing attention to the possibility of stomach infection leading to cancer development (113). This particular microorganism, after recent discovery, has been categorized as a type I carcinogen and is projected to have an impact on over 50% of the global populace. In a tiny proportion of the afflicted population (2%), this infection results in a predictable step-by-step pattern of illness progression that, if discovered in time, can be reversed (114). The CagA protein, one of the cag pathogenicity islands, is a mechanism through which *H. pylori* infection can cause cancer (115, 116). Depending on the modifications after translation, CagA is initially introduced into the cell through the Type IV secretion system. Subsequently, it assumes a pathogenic role by stimulating the activation of SHP2, Abl, or Src kinases within the enclosure of GC (114). The EPIYA motif, which is distinguished by the existence of residues such as proline, isoleucine, glutamate, tyrosine, and alanine, functions as the site for phosphorylation within the CagA protein and may display discrepancies depending on the particular strain of *H. pylori* (117, 118). In addition, *H. pylori* can generate peptidoglycan within the cellular environment of the host, thereby augmenting the synthesis of IL-8 and cox, alongside other pro-inflammatory cytokines (119, 120). Consequently, this leads to the persistence of chronic inflammation and ultimately facilitates the emergence of cancer. It has been additionally established that *H. pylori* releases VacA toxin. This substance can potentially diminish T-cell responses and facilitate the formation of lesions with minimal opposition from the immune system (114, 121). Today, *H. pylori* may be detected via a quick urease test, a polymerase chain reaction test, a histological study of biopsy specimens, and a serological test. Infection with *H. pylori* typically develops in childhood and persists throughout the host's life without antibiotic therapy. The transmission of bacteria can occur through direct contact between individuals, either through oral-oral or fecal-oral pathways (122). *H. pylori* is believed to persistently inhabit approximately half of the global populace, with approximately 15% of individuals afflicted by this pathogen subsequently progressing to the development of gastric ulcers (123). *H. pylori* employs flagella to facilitate its entry into the gastric mucosa, seeking refuge from the stomach's highly acidic milieu. It is essential to acknowledge that a substantial percentage, surpassing 20%, of *H. pylori* variants adhere to the exterior of gastric epithelial cells (122). The ability of *H. pylori* to securely attach to the gastric

epithelial cells is facilitated by the implementation of adhesion molecules, including the outer inflammatory protein A (OipA), sialic acid-binding adhesin (SabA), and adherence-associated lipoproteins (AlpA/B) (124).

Dysbiosis of Non-*H. Pylori* microbiota in gastric cancer

For many years, *H. pylori* has been thought of as the predominant, if not the only, bacteria that can live in the stomach's acid environment and encourage gastric carcinogenesis (111). However, new data from 16SrRNA sequencing showed that non- *H. pylori* strains co-occurred in both *H. pylori* + and *H. pylori* - persons with GC (125). Additionally, accumulating evidence indicates that the term "dysbiosis" best describes how the microbiome gradually changes throughout the development of GC (126, 127).

Microbiota in prevention and therapy of GC: from mice to patients

Probiotics, traditionally limited to their use as food additives (such as in yogurt), have been revolutionized by advanced methods like fecal microbiota transplantation (FMT). These techniques have introduced the concept of therapeutically restoring eubiotics in GI illnesses, thereby transforming the field entirely (128). However, there hasn't been any research done yet on the treatment or prevention of GC in humans. Notably, an international expert council has questioned FMT practices due to inconsistent results and the need for standardization and safety (129, 130).

Probiotics: prevention of GC

Proton pump inhibitors (PPIs) are widely used, which has increased interest in how they may interact with the stomach flora. PPI use over an extended period decreases stomach acid output, encouraging bacterial proliferation because of increased pH brought on by *H. pylori*. PPIs thus have a significant influence on the variety and abundance of bacteria (131–133). For instance, significant levels of *Bifidobacteriaceae* from the oral cavity (*Bifidobacterium dentium*, *Scardovia inopinata*, and *Parascardovia denticolens*) were found in human stomachs with hypochlorhydria in gastritis patients on omeprazole (134, 135). PPIs also boost the number of organisms that may colonize the mouth, such as *Clostridiales*, *Streptophyta*, *Veillonella*, *Fusobacterium*, *Leptotrichia*, *Oribacterium*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*, *Granulicatella*, *Campylobacter*, and *Bulleidia* (133, 136). Nonsteroidal anti-inflammatory drugs (NSAIDs) and PPIs can have adverse side effects on the GI mucosa, although taking probiotic strains along with them might lessen these effects. *Bifidobacterium*, as an exemplification, safeguards mice from the occurrence of stomach ulcers caused by aspirin, while *Lactobacillus plantarum*, derived from green tea, has curative properties against gastric ulcers induced by alcohol (137–139). In mice given the PPIs rabeprazole or vonoprazan, oral *Lactobacillus* johnsonii supplementation reduced indomethacin-induced minor intestine damage (140).

Antibiotics: eradication of *H. pylori*

Large-scale clinical trials and field investigations have provided substantial evidence in favor of the cancer-preventive effects of *H. pylori* eradication. However, the emergence of antibiotic resistance poses a significant challenge, as does the disturbance of the gut microbiota and the impact of *H. pylori* on various other disease states, including asthma and esophageal cancer (141). Various microorganisms linked to stomach illness were found in a recent randomized controlled clinical investigation one year after *H. pylori* elimination (142). Probiotic Roseburia, *Faecalibacterium prausnitzii*, and *Sphingomonas* were decreased, whereas *Streptococcus anginosus*, *Acinetobacter lwoffii*, and Ralstonia were enriched. Also linked to chronic gastritis were oral bacteria (*Streptococcus*, *Peptostreptococcus*, *Prevotella*, *Rothia*, Parvimonas, *Granulicatella*). Other researchers who conducted endoscopic ablation of early GC in individuals experiencing a deficiency of beneficial microorganisms, such as *Ralstonia*, *Faecalibacterium*, *Blautia*, *Methylobacterium*, and Megamonas, observed a prolonged presence of dysbiosis in patients after the eradication of *H. pylori* (143, 144). The restoration of beneficial GI microbiota, such as *Bifidobacterium*, *Lactobacillus*, *Lachnoclostridium*, and *Blautia*, has been observed in young asymptomatic individuals following the eradication of *H. pylori*. Additionally, the presence of pathogenic Alistipes has been found to decrease (145, 146).

The role of the microbiome in liver cancer

Liver cancer is the primary reason for cancer deaths, and its prevalence is rising yearly (147). About 90% of initial liver malignancies are hepatocellular carcinomas (HCC), a significant worldwide health issue (148). Several factors increase the risk of HCC, including chronic hepatitis B and C infections, alcoholism, metabolic liver disease (particularly nonalcoholic fatty liver disease), and exposure to food toxins like aristolochic acid and aflatoxins (149). The World Health Organisation predicts that by 2030, this illness will claim the lives of more than a million individuals (150). Sorafenib, an inhibitor of multiple kinases that has received approval for the management of hepatic carcinoma, occupies the position of primary therapeutic modality for advanced HCC. It has been shown to improve overall survival significantly, but it cannot stop the progression of the disease because of the emergence of resistance to antiproliferative therapies (151). The early detection and treatment of HCC contribute to the enhancement of its prognosis, which is also observed in the majority of disease processes. The most optimal opportunity to detect a medical condition at an early stage is by closely monitoring individuals with a heightened likelihood of developing the disease. This group

includes both those who have cirrhosis of any kind and those who carry the hepatitis B virus (152). According to the 2012 NCCN recommendations, individuals at a heightened risk should undergo hepatic ultrasonography and AFP testing on a semiannual to annual basis. Per the 2012 recommendations of the National Comprehensive Cancer Network (NCCN), individuals classified as high-risk should undergo hepatic ultrasonography and AFP testing every six to twelve months (152, 153). The correlation between the presence of microorganisms causing infection and the onset of cancer has been recognized for a significant duration. Among the various mechanisms that contribute to this association, the chronic inflammation induced by infection is considered to be an important causative factor. Emerging evidence indicates that the resulting gut dysbiosis, characterized by an imbalanced state of intestinal microbial composition linked to illness, is responsible for the carcinogenic implications of these microbial stimuli. Consequently, this dysbiosis triggers chronic inflammation and, ultimately, the development of cancer (154). However, it is imperative to acknowledge that not all microorganisms are harmful. A plethora of commensal bacteria have a crucial function in fostering the development of the host's immune system (155, 156). The host's state of health is influenced by the constituent member types (pathogenic or commensal) and abundance arrangement (dysbiotic or eubiotic) of the intestinal microbial. Numerous investigations have effectively highlighted the crucial functions that gut bacteria play in the development of HCC (154, 157). The bidirectional interplay between the GI tract and the hepatic organ transpires via the portal vein, a conduit that expedites the passage of diverse entities originating from the gastrointestinal system, including nourishing compounds, metabolic byproducts of microorganisms, and constituents of said microorganisms, to the hepatic entity (158, 159). Once in the bile duct, these substances go from the liver to the gut. As a result of this enterohepatic circulation, the liver is constantly exposed to substances that originate from the gut (160). Furthermore, the association linking the gut and the liver, commonly called the "gut-liver axis," has garnered increasing attention from researchers due to its pivotal role in preserving liver homeostasis and averting the onset of ailments (161, 162). One common finding in several liver illnesses is that tight connections between adjacent intestinal epithelial cells are impaired with increasing intestinal permeability, indicating that substances coming from the gut have an impact on liver function (160, 163). Furthermore, microbial dysbiosis in the lower GI tract and small intestinal bacterial overgrowth (SIBO) are linked to liver injury (164, 165). This finding implies that the bile produced by a healthy liver, along with other liver-derived compounds, contributes to the probiotic status of the gut microbiota (165). The liver is commonly perceived as an organ devoid of immunological function, instead playing a pivotal role in various metabolic processes, energy source storage, and detoxification (166, 167). The organ can also be perceived as a highly responsive component of the immune system, serving as a dwelling place for various immune cells such as Kupfer cells, natural killer (NK)/NKT cells, and T and B lymphocytes. Additionally, it harbors stromal cells such as liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs), which possess the ability to release cytokines and various other substances that can interact with immune cells (160).

Hepatitis viral infection and gut microbiome

Hepatitis is a liver inflammation that can either go away independently or progress into a dangerous illness that causes cirrhosis or HCC. Hepatitis B and C virus infections typically result in chronic hepatitis, and viral infections are the primary cause of hepatitis worldwide (168). Both viruses cause host immune responses for clearance after infecting hepatocytes. To stop viral replication, nucleoside (or nucleotide) analogs (NAs) are frequently used to treat viral hepatitis. In addition, the mature gut microbiota is necessary for quick HBV clearance via efficient host immune boosting (154). The gut microbiota of individuals with cirrhosis caused by HBV exhibited notable distinctions compared to the gut microbiota of healthy control subjects, as indicated by a study conducted using advanced next-generation sequencing technology (169, 170). Specifically, certain bacterial species such as *Clostridium*, *Prevotella*, *Veillonella*, and *Streptococcus* displayed higher prevalence levels, whereas *Alistipes* and *Eubacterium* were found to be less frequently observed (171). The presence of oral microorganisms in higher quantities indicates that the transfer of microbes from the mouth to the gut is a prevailing occurrence among individuals with cirrhosis (171). The diversity of microorganisms in the GI tract, assessed using the Shannon and Simpson indices, declined in individuals with cirrhosis and recovered to a level comparable to that of healthy individuals in patients with HBV-related HCC (172, 173). In addition, the diversity decreased more in early hepatitis B patients than in intermediate cases, but not considerably, and both were lower than in healthy controls (174). Collectively, the variety of gut bacteria appears to decline during the early stages of HBV infection and then recover to a level comparable to that of healthy individuals as the liver disease advances. The aberrant bile acid production and composition, which compromises the bile acids' antimicrobial defenses and enables the transfer of oral species, are thought to contribute to these gut microbial changes linked to HBV infection (154, 175).

Microbiome and alcoholic liver disease

Alcohol liver disease (ALD), characterized by alcoholic hepatitis, alcoholic fatty liver disease, and alcoholic cirrhosis, represents a significant contributing factor to various liver ailments (176, 177). Alcohol's metabolic byproducts are held responsible for the adverse effects of alcohol abuse. Alcohol undergoes oxidation within the hepatocyte, primarily resulting in the formation of acetaldehyde through the activity of alcohol dehydrogenase. Meanwhile, there is a limited production of reactive oxygen species (ROS). Prolonged exposure to acetaldehyde and ROS can lead to hepatotoxicity and carcinogenicity within the liver (178). Although the liver is the

primary site of alcohol metabolism, intestinal enzymes and microorganisms are also capable of doing so (179, 180). Therefore, drinking too much alcohol increases luminal acetaldehyde and ROS, which disturbs the gut ecology by affecting gut barrier function and promoting gut dysbiosis, which alter the makeup of the gut's microbial population (181–183). As substantiation, the presence of alcoholic hepatitis resulted in a decrease in *Akkermansia* in individuals with severe illness in comparison to individuals who were in good health, and this decrease was even more pronounced (184, 185). Mice that underwent an FMT from individuals suffering from acute hepatitis and alcoholism exhibited increased levels of *Bacteroides*, *Butyricimonas*, *Alistipes*, *Bilophila*, and *Clostridium* XIVa compared to mice that did not receive an FMT (186, 187). Additionally, the presence of alcoholic hepatitis resulted in an elevation of bacterial DNA levels in the bloodstream when compared to individuals who do not consume alcohol. This increase was characterized by a decrease in DNA from *Bacteroidetes* and an increase in DNA from *Fusobacteria* (188).

Non-alcoholic fatty liver disease and microbiome

It is estimated that approximately 80-100 million individuals in the United States, constituting around 25% of the adult population, are believed to be affected by non-alcoholic fatty liver disease (NAFLD). The primary etiology of chronic hepatic ailment presently observed worldwide is NAFLD (189–191). Hepatic steatosis, a condition characterized by the accumulation of fat in the liver exceeding 5% of its overall weight, can be attributed to an excessive intake of alcohol. Abdominal imaging data suggests that the global prevalence of NAFLD is expected to reach 25%, with the African continent experiencing the lowest majority at 13.5% and the Middle East observing the highest at 31.8%. Non-alcoholic steatohepatitis (NASH) emerges in approximately 30% of individuals diagnosed with NAFLD (192). NASH can potentially progress from a state of simple steatosis to the more severe conditions of cirrhosis or HCC, or it may experience a decline in its condition (192, 193). It has been shown that NAFLD causes lipid metabolism to be dysregulated, resulting in the loss of CD4+ T cells and subsequently encouraging the development of hepatocarcinogenesis (194, 195). Similar to this, IgA+ cells that have accumulated in the livers of NASH patients with fibrosis help to promote hepatocarcinogenesis by inhibiting CD8+ T cell activation (196, 197). The importance of the intestinal metagenome in the etiopathogenesis of NAFLD has also been emphasized by recent findings (192). The initial step in establishing the etiological link between gut bacteria and NAFLD was replicating the hepatic changes associated with the disease in mice utilizing co-housing and FMT trials (198, 199). Dysbiosis, in turn, can lead to the development of metabolic disorders, including metabolic syndrome, obesity, Type 2 Diabetes Mellitus (T2DM), and NAFLD (200, 201). Increased *Enterobacteriaceae* was shown to be one of the characteristics that predicted NAFLD-cirrhosis, reflecting its significant involvement in the development of NAFLD (169). Two strains of *Enterobacteriaceae*, which belong to the *Klebsiella pneumonia* family, were fortuitously discovered to produce ethanol among Chinese individuals affected by NAFLD internally. This finding offers valuable knowledge regarding the development of NAFLD in individuals who do not consume alcohol (202). When compared to NASH cirrhosis, individuals with NAFLD-related HCC had lower levels of *Akkermansia* and *Bifidobacterium* species, indicating that gut dysbiosis may worsen the development of NAFLD to hepatocarcinogenesis (203, 204).

The role of the microbiome in esophageal cancer

Esophageal cancer, acknowledged as a highly prevalent form of malignancy worldwide, is projected to have approximately 604,100 novel occurrences in 2020 (205). Nearly 80% of occurrences of this malignant tumor are found in less developed areas, which bear a disproportionately heavy burden. A discrepancy exists in the occurrence and mortality rates between males and females, with males representing approximately 70% of reported cases, resulting in a 2 to 5-fold difference (206). Moreover, the likelihood of developing esophageal cancer increases as individuals grow older, particularly in middle-aged and older demographics (207). In conjunction with the worldwide phenomenon of population growth and aging, the escalating prevalence of risk factors such as alcohol and tobacco consumption, inadequate dietary habits, sedentary lifestyles, and obesity is contributing to the rapid escalation of esophageal cancer globally (208, 209). esophageal cancer comes in two forms: esophageal squamous cell carcinoma (ESCC, also known as SCC) and esophageal adenocarcinoma (AC). AC is more typical in affluent nations, while ESCC is more widespread in East Asia, Southern Africa, East Africa, and Southern Europe (205). Based on the kind of cell from which cancer arises, SCC and AC exhibit significant differences in carcinogenesis (210). In terms of incidence during the previous forty years, AC has been shown to surpass SCC by a wide margin (108). SCC primarily impacts the upper and middle regions of the thoracic esophagus, arising from the squamous cells present within the mucosal lining of the esophagus. AC commences in the epithelial cells, most in the inferior thoracic esophagus (210). Although little is known about the esophageal microbiome, it is recognized that it is not a sterile portion of the digestive system (211). In the esophagus, food passes through quickly, likely limiting the prevalence of microorganisms. However, the pH in healthy people is very steady (about 7), which is ideal for various microbes. The esophagus is home to certain microbes, according to microbiome analysis (212, 213). It is worth mentioning that the composition of microorganisms inhabiting the lower, middle, and upper regions of the esophagus is indistinguishable (214). Most of the esophageal microbiome comprises six phyla, namely *Bacteroides*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Actinobacteria*, and TM7 (215, 216). There is a varied microbial community seen among the Gram-positive bacteria. Particularly, the esophagus of healthy people has the highest concentration of the Streptococcus genus (217). In addition, the esophagus also harbors *Prevotella* and *Veillonella* (211). The microbiome is changed by esophageal disorders. It is

possible to identify esophageal illnesses by the unbalanced changes in the esophageal microbiome (218, 219). Recent research has expanded our understanding of the connection between changes in the gut microbiota and the development of esophageal cancer. It has been proposed that this connection may be essential for the creation and growth of tumors (220). Blackett et al. (221) showed that individuals with Barrett's esophagus and gastroesophageal reflux disease (GERD) had significantly higher concentrations of *Campylobacter*. It is believed that *campylobacter* causes the esophageal mucosa to become inflamed, followed by epithelial metaplasia, which finally results in malignant transformation (222). Elliott and colleagues (223) discovered that certain strains of *Lactobacillus* are concentrated within tumors in roughly 50% of AC patients and that microbial diversity diminishes in AC while relative *Lactobacillus fermentum* abundance rises. Zaidi et al. (224) found that AC contains large amounts of *E. coli*. Additionally, there was a significant increase in the expression of several Toll-like receptors (TLR1, 3, 6, 7, and 9) within the neoplastic tissue of a rat model mimicking AC. Etiological investigations have elucidated that *H. pylori* has the potential to mitigate the occurrence of AC through the suppression of gastric acid secretion, thereby reducing the likelihood of reflux esophagitis while also modulating the quantity of T cells (225). *H. pylori*, on the other hand, has been shown to cause GERD to manifest. Several studies have established a correlation between *Tannerella forsythia* and an increased likelihood of AC. Conversely, symbiotic *Streptococcus pneumoniae* and *Neisseria* have been associated with a decreased risk of AC. Notably, the enrichment of *Porphyromonas gingivalis* (*P. gingivalis*) has been identified as a significant risk factor for ESCC, as highlighted by various investigations (226–230). *P. gingivalis* induces the process of epithelial-mesenchymal transformation (EMT) using the transforming-growth-factor (TGF)-dependent Smad/YAP/TAZ signaling pathway, and also triggers the activation of the nuclear factor (NF)-B signaling pathway, thereby stimulating the proliferation and metastasis of ESCC cells (231, 232). It has been proven that the microbiome of the esophagus contains viral DNA from the Gammapapillomavirus, Betaherpesvirus, and Gammaherpesvirus. With the discovery of Papillomavirus (HPV) DNA from esophageal neoplasia, the probability of developing ESCC was increased in the presence of EBV and HPV infections (233). Fungi infections with inflammation are common in esophageal cancer patients, which may suggest a possible link with the development of esophageal cancer (233). In research by Deng et al. (234) comprising 23 esophageal cancer patients and 23 matched healthy persons, the gut microbiome was examined. By 16S rRNA gene sequencing, the gut microbiota was examined from fresh stool samples. When the strain was considered, it was shown that esophageal cancer patients had much larger amounts of *Actinobacteria* and *Firmicutes* and lower levels of *Bacteroidetes* than healthy people. According to scientists, individuals with esophageal cancer had lower levels of bacteria that produce SCFAs while having higher levels of bacteria that produce lipopolysaccharides (LPS) (234, 235). The significance of the pool of SCFAs ought to be underscored as it possesses various benefits, including its ability to mitigate inflammation and enhance the structural integrity of the intestinal barrier. It is of utmost importance to acknowledge that anaerobic microorganisms located in the distal regions of the GI tract synthesize butyrate, thereby suggesting that it may exert a substantial influence on the pathogenesis of neoplastic growths within this system, encompassing esophageal carcinoma (236). In a study conducted on patients with ESCC, it was discovered that the presence of *Fusobacteria*, *Bacteroidetes*, and *Spirochaetes* was notably reduced (n=18) (176). The high-fat diets (HFD) negatively impact the bile acids composition and the gut flora. According to research in mice, the modifications in bile acid composition brought on by HFD may aid in the emergence of Barrett's esophagus and esophageal cancer (205).

Non-bacteria microbiome (virus, fungi, and archaea) in GI cancer

Bacteria are the predominant microorganisms found throughout the GI tract (237). The impact of specific species or the combined bacteriome on GI cancers has been extensively researched (238). However, in recent times, the presence of viruses, fungi, archaea, and microscopic eukaryotes in the GI tract has been confirmed due to the progress made in sequencing technology and biotechnology (233) (Figure 2).

Viruses in GI cancers

Viruses exhibit a comparatively reduced presence in the gut when compared to bacteria, yet they have been identified as constituents of the enduring commensal microbial consortium within the GI tract (230, 239–241). Viruses have a notable impact on GI cancers. The human virome, encompassing the entirety of viruses found within the human body, is a vital component of the human microbiota and aids in the preservation of tissue equilibrium (242). Bacteriophages have been predominantly recognized within the microbiome, where they are ascribed to various functions. The functions encompassed within this domain encompass the regulation of bacterial populations through the cyclic processes of phages, namely lysogenic and lytic. The proportions of lytic and lysogenic phages are said to have a relationship with the bacteriome and are linked to the overall health condition of an individual (241, 243). Lysogenic bacteriophages might also play a role in the targeted establishment of bacteria and improving the fitness of host bacteria through the exchange of genetic material within the GI tract (243, 244). The involvement of viruses in the development of GI cancers is evident as they impact the abundance of these viruses, infect the cells of the epithelium, or alter the composition of the bacterium (245). A multi-cohort study was conducted in which fecal samples were analyzed using shotgun metagenomics to investigate the virome shift in patients with CRC compared to healthy individuals. Additional examination revealed that there was a fluctuation observed in the colon bacteriophages, with variations evident in both the early and late stages of CRC (246, 247). The analysis conducted subsequently revealed that there was a variation in the displacement of the colon bacteriophages between the initial and advanced stages of CRC (248, 249). *Epsilon15likevirus*, *Betabaculus virus*, *Punalikevirus*, and *Mulikevirus* exhibited a noteworthy augmentation in CRC

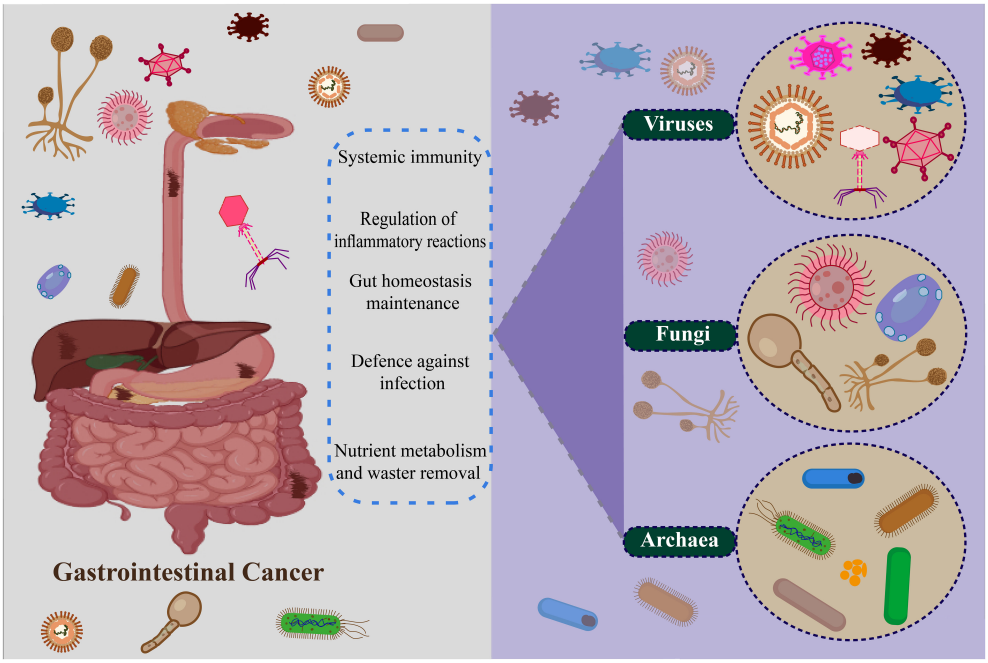


FIGURE 2
Non-bacteria microbiome (virus, fungi, and archaea) in gastrointestinal cancer.

individuals, thereby being linked to escalated intensity and fatality rates. It has been suggested that viruses belonging to the eukaryotic colon could potentially disrupt the balance of the immune system and trigger modifications in the DNA via mechanisms that are dependent on the presence of the virus (250). Indeed, there is a growing body of evidence suggesting that infections caused by eukaryotic viruses are linked to an elevated risk of CRC (251). In cancerous tissues of CRC patients, there was a presence of viral infections including HPV, human herpesviruses, human polyomaviruses, human bocavirus, and Inoue-Melnick virus in comparison to the surrounding normal tissues (252, 253). In the same manner that tumor tissues of CRC patients exhibited the presence of viral DNAs, similar findings were observed in the GC tissues. The well-documented role of Epstein-Barr virus (EBV) as an etiological agent in the development of GC further supports this observation. EBV-positive gastric carcinoma is distinguished by distinct genomic abnormalities and clinicopathological characteristics (254, 255). After being infected, the EBV incorporates its DNA into the host organism. Subsequently, it manifests latent protein and disrupts DNA methylation through the influence of miRNA under the presence of the latent protein. This process ultimately leads to the development of EBV-positive GC (255, 256). The prevalence of human cytomegalovirus (HCMV) is extensive within human populations, encompassing a range of infectious microorganisms. HCMV has been documented to endure within the host for extended durations after the initial infection (257). Recent research has placed greater emphasis on the connections between HCMV and several types of malignancies, such as glioblastoma, breast cancer, GC, and CRC (258). HCMV was observed to exhibit a greater presence in GC tissue compared to the surrounding normal tissues, thus suggesting its potential involvement in the development of carcinogenesis and potentially facilitating the lymphatic metastasis process in GC (259). Furthermore, HCMV has also been documented to elicit disturbances in the GI tract, such as inflammatory bowel disease, ulceration, erosion of the cell wall, and hemorrhage in the mucosal lining (258). Reports have also been documented regarding the identification of viruses, particularly bacteriophages, within the esophageal microbiome. DNA viruses including betaherpesvirus, gammaherpesvirus, and gammapapillomavirus were also found (20, 260). Due to their primary focus on bacteria, it is conceivable that the correlation between the virome of the esophagus and adenocarcinoma could be elucidated in investigations involving larger groups of subjects. In addition, it has been reported that infections caused by EBV and HPV are associated with a heightened susceptibility to ESCC (261). Latent gammaherpesvirus 68 infection in a mouse model exhibited the capacity to induce persistent immune system stimulation, thereby safeguarding against pathogenic infection caused by *Listeria monocytogenes* (262, 263).

Fungi in the GI cancer

Fungi have established their presence as inhabitants of the GI tract of individuals in good health, although their composition is primarily influenced by lifestyle factors, particularly dietary choices (264). These microorganisms have been observed to exist within the gastric compartment, colon, pancreas, and esophagus, albeit in a significantly smaller ratio when compared to bacteria (265, 266). Recent research is commencing to unveil the significance of fungi in the GI tract. A variety of fungi such as *Candida*, *Cryptococcus*, *Saccharomyces*, *Malassezia*, *Debaryomyces*, *Cladosporium*, *Trichosporon*, and *Galactomyces* have been documented as inhabiting the gastrointestinal tract of healthy individuals (264). Fungi play a crucial role in sustaining the

equilibrium of the GI tract. Furthermore, they have been demonstrated to possess functions in systemic immunity, regulation of inflammatory reactions, and protection against infectious agents (267). Fungi are purported to stimulate the activation of T helper 17 cells, which play a crucial role in safeguarding the host against infections. Moreover, these cells contribute to the development of secondary lymphoid organs and the fine-tuning of the host's immune and inflammatory responses (268, 269). Fungal species, namely *Candida albicans*, *Saccharomyces cerevisiae*, and *Candida glabrata*, have been detected in the esophagus of individuals in a non-pathogenic state (270). Candida and Phialemonium demonstrate the capacity to endure the harsh acidic conditions prevalent within the ecosystem, specifically within the gastric fluids (271, 272). The ratio between Basidiomycota and Ascomycota was found to be imbalanced in patients with CRC, similar to other diseases affecting the intestines. In individuals with CRC, an elevation in the population of *Malasseziomycetes* fungi and a decrease in the abundance of *Saccharomycetes* fungi were noted (273, 274). The composition of fungal genera including *Aspergillus*, *Rhodotorula*, *Kwoniella*, *Pseudogymnoascus*, *Malassezia*, *Talaromyces*, *Moniliophthora*, *Debaryomyces*, *Pneumocystis*, and *Nosemia* experienced changes in CRC cases, a finding that was confirmed in separate cohorts of Chinese and European populations (275). In an experimental mouse model investigating esophageal cancer, the administration of oral fungi *Cladosporium cladosporiodes* was found to enhance the severity of ESCC. Interestingly, this detrimental effect was effectively counteracted by treatment with antifungal agents (276). Additionally, infections caused by the *C. albicans* fungus were documented in patients suffering from ESCC (277). The presence of an imbalance in gastric fungi was observed in individuals with GC. The profile of the fungal community (mycobiome) in GC patients exhibited notable differences, including a decrease in diversity, compared to the control group. *Candida* and *Alternaria* exhibited an increased concentration in the GC, whereas *Thermomyces* and *Saitozyma* experienced a decrease in abundance within the GC (278, 279). Similar to other GI microbiome members, alterations in the resident mycobiome that impair their functioning, manipulation of the whole microbiome, or infection by specific pathogenic fungus species may all influence GI malignancies (280, 281).

Archaea in GI cancer

The progress in the field of sequencing and the analytical methods used in bioinformatics have facilitated the examination of archaea, a group that has received less attention in comparison to bacteria, viruses, and fungi within the intestinal ecosystem (282). Archaea represent a distinctive assemblage of prokaryotic organisms characterized by their lack of D-glycerol, esters, fatty acids, and peptidoglycan (283, 284). Owing to their cellular composition, these organisms were recognized for their ability to colonize harsh habitats such as those characterized by high temperatures, alkaline conditions, acidic conditions, and high salinity levels (285). Archaea were commonly presumed to inhabit environments characterized by severe ecological conditions, nevertheless, recent investigations have ascertained their presence in mesophilic conditions comprising human skin, oral cavity, nasal passages, vaginal region, and the GI tract (286). In the GI tract, there have been documented occurrences of both methanogenic archaea and haloarchaea, with their respective proportions being subject to variation dependent on the individual (287). Methanogenic archaea facilitate the reduction of carbon dioxide through the process of methanogenesis, occurring in the GI tract during nutrient digestion. This metabolic activity effectively assists in the elimination of hydrogen from the gut (288, 289). Colonic archaea have also been found to play a role in the elimination of trimethylamine (TMA) from the GI tract. TMA is generated as a byproduct during the degradation of choline mediated by colon microorganisms, and its presence has been linked to elevated probabilities of atherogenesis and the development of cardiovascular ailments (290, 291). The activation of antigen-specific adaptive immune responses by Archaea is a phenomenon that should not be overlooked, as it has the potential to play a crucial role in maintaining immune homeostasis within the GI tract (292). Distinct groups of archaea in the colon were observed in individuals with CRC and colorectal adenoma in comparison to those who were in good health, thus suggesting a modification during the various phases of the development of cancer (293). The presence of Archaea has also been linked to the emergence of IBD, anorexia, and anaerobic abscesses (294, 295) (Table 1).

Microbiomes and therapies for gastrointestinal cancers

Chemotherapy

It is widely established that systemic chemotherapies impact both healthy GI tract cells and cancer cells. The microbiome will undoubtedly experience a disturbance, thus resulting in dysbiosis, which refers to an interruption in the typical makeup of the microbiome. Chemotherapy has been demonstrated to possess a wide-ranging impact on the microbiota, leading to a reduction in the variety of microorganisms. This reduction is accompanied by an elevation in the abundance of Firmicutes and a decline in Bacteroidetes (324). Moreover, gram-negative bacteria tend to increase while gram-positive bacteria decrease due to chemotherapy

TABLE 1 A summary of studies relating the gut microbiome to GI cancers.

Type of cancer	Methods Used	Conclusion	References
CRC	The study analyzed the microbial communities in the colon and the genetic variability of Fusobacterium in 43 Vietnamese patients with CRC and 25 individuals with non-cancerous colorectal polyps. This was achieved through the	- *F. nucleatum* consistently demonstrates an association with CRC. - The diagnostic and therapeutic options can utilize the genomic diversity present in *Fusobacterium*.	Tran et al. (296)

(Continued)

TABLE 1 Continued

Type of cancer	Methods Used	Conclusion	References
	utilization of 16S rRNA gene profiling, anaerobic microbiology, and comprehensive genome analysis		
	The analysis of 18 surgical specimens of human CRC was conducted using 16S rRNA gene sequencing. - Differential examination of microbiomes in tissues and mucus	-*Enterobacteriaceae* and *Sutterella* exhibit a higher presence in the mucus layer that envelops the mucosa. - *Rikenellaceae* exhibits a higher concentration within the mucosal layer that overlays cancerous tissues.	Tajima et al. (297)
	-Using a reverse microbiomics (RM) strategy. -Comparative genomics analysis using Vaxign	- The utilization of the RM methodology was implemented in order to predict the presence of 18 autoantigens and 76 potential virulence factors. - Proposed new model of CRC pathogenesis involving riboflavin synthase	Wang et al. (298)
	- Culture-independent methods for identifying bacterial populations - Sequencing V1-V3 or V3-V5 variable regions of bacterial 16S ribosomal RNA	- The presence of probiotic strains has the potential to impact the treatment of CRC. - Additional investigation is required to ascertain the most effective treatment.	Kim et al. (299)
	N/A	- The intestinal microbiota plays a crucial role in the advancement of colorectal cancer. - The potential of the intestinal microbiota to function as a biomarker in the prompt identification of CRC is considerable.	Ren et al. (300)
	- A systematic search find clinical studies published in the last two decades.	-Bacterial metabolism exhibits a robust correlation with the	Fratila et al. (301)

(Continued)

TABLE 1 Continued

Type of cancer	Methods Used	Conclusion	References
	- A comprehensive analysis was conducted on the following subjects: dietary interventions, potential biomarkers for CRC, probiotic administration in non-surgical patients, and probiotic administration in surgical patients.	development of colonic carcinogenesis and is subject to dietary influences. - Probiotics and prebiotics function as agents that can modify the microbiota by inhibiting the proliferation of epithelial cells and counteracting DNA damage. - As supplementary treatments to surgery or chemotherapy, *Bifidobacteria* and *Lactobacilli* reduce complications.	
	- A comprehensive search of the literature was conducted on March 3rd and 4th, 2023.	-Research indicates that biomarkers based on oral microbiota show potential as a non-invasive means of identifying CRC. However, additional studies are required in order to comprehend the mechanisms of oral dysbiosis.	Negrut et al. (302)
	- To facilitate the screening process, pertinent articles were extracted from different databases by utilizing specific keywords and phrases.	-CRC is linked to imbalances in the GI microbiome.	Eastmond et al. (303)
	- A systematic review of 2009. - Patients diagnosed with any stage of CRC were enrolled in the study.	-Microbiome composition could potentially influence the outcomes of surgery for CRC, although the available evidence is currently limited.	Lauka et al. (304)
	- A Mendelian randomization (MR) study was conducted using a two-sample approach in order to elucidate the causal relationship between the CRC and gut	- The inquiry confirmed the causal correlation between the gut microbiota and CRC, positing a possible linkage between genes and	Xiang et al. (305)

(Continued)

TABLE 1 Continued

Type of cancer	Methods Used	Conclusion	References
	microbiota. - A thorough examination was performed on a total of 166 bacterial characteristics spanning four hierarchical levels: species, genus, family, and order.	pathogenic microbiota in CRC. - The examination of the GI microbiome and its comprehensive analysis involving multiple omics techniques are of utmost importance in the endeavor to impede and manage CRC.	
	- The study evaluated the effectiveness of microbiome-derived biomarkers using noninvasive samples. - A study of 28 studies found that only two explored the co-metabolome as a potential biomarker for colorectal cancer and advanced adenoma patients.	- Based on the current evidence, it is not yet appropriate for routine clinical implementation to utilize the potential of the fecal and oral gut microbiome in order to improve CRC screening tools.	Zwezerijnen et al. (306)
	-A meta-analysis of fecal metagenomics sequencing data from 11 studies involving 692 patients with CRC and 602 healthy controls evaluated features associated with CRC.	- In this investigation, significant correlations were found between CRC status and colibactin, fadA, and *F. nucleatum* compared to control subjects. - Several distinct microbial species were found to be selectively enriched in young patients diagnosed with CRC.	Kharofa et al. (307)
Gastric	- The cutoff value for *H. pylori* infection is determined using pyrosequencing. - Extragastric microbiome is investigated using animal models.	- Relationship between GC and gastric microbiome. - There has been limited advancement in comprehending the non-*H. pylori* function.	Yang et al. (308)
	- Five patients were diagnosed with GC, non-atrophic gastritis, and intestinal metaplasia of the intestinal type.	-The diversity of bacteria declined progressively from non-atrophic gastritis to intestinal metaplasia to GC. - There was a noticeable disparity in microbiota	Aviles et al. (309)

(Continued)

TABLE 1 Continued

Type of cancer	Methods Used	Conclusion	References
		between non-atrophic gastritis and GC	
	- New methods for identifying microbes in the stomach using molecular techniques have been developed. - Studies conducted on the INS-GAS transgenic mouse model	- The development of GC is influenced by the presence of gastric microbiota. - Microorganisms are linked to individuals diagnosed with GC.	Stewart et al. (310)
	N/A	- The significance of the gastric microbiome in the development of cancer is not substantial. - *H. pylori* and inflammation play significant roles in the development of GC.	Engstrand et al. (311)
	- Nucleotide sequencing techniques - Biocomputational tools	- Chronic inflammation is linked to GC. - *H. pylori* and other bacteria contribute to the development of GC.	Schulz et al. (312)
	-The study consisted of 48 individuals diagnosed with GC and 120 individuals without cancer. This group comprised of 20 individuals with normal gastric mucosa, 40 individuals with atrophy, 20 individuals with gastritis, and 40 individuals with intestinal metaplasia	- The group that was under control exhibited the most significant overall bacterial alpha diversity measurements, with the groups with intestinal metaplasia and cancer following closely behind. - The groups with atrophy and gastritis exhibited the lowest level of diversity.	Gantuya et al. (313)
	-The study included 60 individuals diagnosed with chronic gastritis, 30 individuals with early GC, and 30 individuals with advanced GC.	- The inquiry revealed significant variations in the microbial profile and composition when contrasting the initial and progressed stages of GC.	Wang et al. (314)
	- A total of 1630 individuals who were infected with asymptomatic *H.*	- The elimination of *H. pylori* has the potential to offer extended defense	Yan et al. (315)

(Continued)

TABLE 1 Continued

Type of cancer	Methods Used	Conclusion	References
	pylori. -The individuals assigned to undergo *H. pylori* eradication therapy numbered 817, whereas the placebo group consisted of 813 individuals	against GC in populations at high risk, especially for those individuals who are initially infected with the bacteria but do not possess precancerous gastric lesions.	
Liver	- Characterization of intestinal microbial composition in mice and humans - Using bacteriotherapy and antibiotics as potential therapeutic choices is being explored.	- The impact of modifications in the gut microbiota on the progression of hepatic malignancy is of considerable importance. - Bacteriotherapy possesses the capacity to modify the composition of microbiota and decrease inflammation.	Moreno et al. (316)
	- This review analyzes existing evidence and examines potential mechanisms. - Possible therapeutic applications are being discussed	- The microbiota of the GI tract contributes to the development of liver cancer. - Potential therapeutic uses consist of probiotics and FMT.	Zhou et al. (317)
	- Between mice that were free from germs and mice that were. - Alternatives such as gnotobiotic or humanized models were employed.	- The dysbiosis of the GI microbiota exerts a substantial influence on the progression of hepatic disorders. - Gnotobiotic models are applicable for microbiome research.	Hartmann et al. (318)
	N/A	- The connection between an imbalance in liver diseases and gut microbiota. - Therapeutic strategies may be developed by manipulating microbiota.	Abe et al. (319)
Esophageal	N/A	- Microbiota diversity and uniformity decline in cases of esophageal cancer.	Moreira et al. (320)
		- The prevalence of Gram-negative bacteria is elevated in esophageal cancer.	
	- Comparative metagenomic approaches - Sequencing of the 16S rRNA gene	- The imbalance of the microbiota can lead to esophageal tumorigenesis. - The identification of microbiota has the potential to enhance the methods of EC treatment.	Zhou et al. (220)
	- Analysis of bacteria at genus level in gut - Principal coordinate analysis (PCoA) and analysis of similarities (ANSOIM)	- The gut microbiota could potentially play a role in the pathogenesis and progression of esophageal squamous cell carcinoma. - Certain gut bacteria may serve as biomarkers for the screening of these types of cancer.	Shen et al. (321)
	Next-generation sequencing techniques.	- *Streptococcus* is the predominant bacterial group found in the normal esophagus. - Gram-negative bacteria are more prevalent in the diseased esophagus.	Park et al. (322)
	- 16S rRNA gene sequencing - Bioinformatics analysis	- ESCC patients exhibit unique microbial features in comparison to individuals who are in good health. - The development of ESCC may be influenced by the microbiome present in the esophagus.	Lv et al. (323)

(Continued)

TABLE 1 Continued

N/A, No Answer.

treatment. Even though various chemotherapy regimens may have distinct effects on the composition of the GI microbiota, this assertion remains valid (324, 325). The gut microbiota controls toxicity, anticancer effects, and medication effectiveness to control host reactions to chemotherapy medicines (326). The TIMER mechanistic paradigm presents an opportunity to alter the connection between the GI microbiota and chemotherapeutic medications using immunomodulation, translocation, enzyme

degradation, metabolism, and ecological variation (327). Yamamura and colleagues (328) have discovered a significant association between the intratumoral DNA of *F. nucleatum* and the response of patients' ESCC to neoadjuvant treatment. Liu et al. (384) have found that through autophagy, the intracellular bacterium *F. nucleatum* provides chemoresistance to ESCC cells. By inducing the activation of autophagy and enhancing the expression of autophagy-associated genes, *F. nucleatum* specifically acts upon the TLR4/MyD88 signaling pathway, leading to a reduction in the levels of miRNA-4802 and miRNA-18a. Consequently, this molecular modulation results in developing resistance to chemotherapy in CRC. In patients with CRC who are undergoing adjuvant chemotherapy, it has been observed that the presence of *F. nucleatum* infection leads to a reduction in the effectiveness of 5-Fluorouracil (5-FU) treatment by regulating the baculoviral IAP repeat containing 3 (BIRC3) through the TLR4/NF-B signaling pathway (329, 385). In a mouse CRC model, *Mycoplasm hyorhinis* may metabolize gemcitabine into inactive 2', 2'-difluoro deoxyuridine via the CDDL gene (330). Furthermore, it has been established that the majority of the bacteria in PDAC are *Gammaproteobacteria*, which possess the CDDL gene necessary for the metabolization of gemcitabine. Ciprofloxacin can counteract this impact (331). Different commensal microbiota can alter the tumor microenvironment, impacting how well conventional chemotherapy works. By lowering the generation of ROS, the lack of *Lactobacillus* reduces the cytotoxicity of oxaliplatin (332). In one study, Chinese patients receiving FOLFOX treatment for low-lying rectal tumors had their gut microbiomes examined by fecal samples. It has been demonstrated that FOLFOX reduces the variety of the whole microbiome, and intriguingly, this diversity was reduced in patients who reacted to the FOLFOX rather than in nonresponders (333–335). *Lactobacillus rhamnosus*, a probiotic, improved the effectiveness of capecitabine against mouse GC growth (336). Cyclophosphamide (CTX) inhibits several immunological signaling cascades to produce its anticancer action (337). Preclinical research has revealed that some bacterial species, such as *Enterococcus hirae (E. hirae)*, are necessary for CTX-induced immunological activation. To activate the host immune response, CTX causes the bacteria to relocate to the spleen and lymph nodes. The anti-tumor action of CTX is likewise dependent on *E. hirae* and *Barnesiella intestinihominis*, according to further investigations (338, 339). By controlling antitumor cytotoxic CD8+ T cell responses and stimulating the IL-12 signaling pathway, butyrate may increase the effectiveness of oxaliplatin (340). According to prospective research, individuals with locally advanced rectal cancer (LARC) may benefit from using the gut microbiota as possible biomarkers to gauge how well they respond to neoadjuvant chemotherapy and radiation (341).

Immunotherapy

The identification of immunotherapy, a therapeutic approach that harnesses the immune system of the body to tackle cancer, has emerged as a highly promising domain in the realm of cancer therapy (342). In the treatment of GI cancers, especially those that exhibit microsatellite instability (MSI-H), the utilization of immune checkpoint inhibitors (ICIs) like anticytotoxic T lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death 1 (PD1) antibodies is being implemented (343–345). While dysbiosis can establish a connection between the microbiome and carcinogenesis, it is also plausible that a microbiome in good health possesses substantial potential to bolster antitumor immunity (346, 347). It has been proven that the therapeutic agents pembrolizumab and nivolumab, which function as inhibitors of PD-1, exhibit enhanced clinical efficacy in the prevalence of *Akkermansia muciniphila* and *Bifidobacterium* (348, 349). *B. fragilis* and *Bacteroides thetaiotaomicron* are also linked to the effectiveness of anti-CTLA-4 antibodies like ipilimumab (350). As the FMT from individuals who responded to ICI and those who did not into mice was carried out, it is noteworthy to observe that the microbiome of ICI responders exhibited a sustained augmentation of the anti-PD1 effects compared to the nonresponders. This observation highlights the intrinsic capability of the microbiome to stimulate the immune response against tumors (351). The interactions between ICI and microbiome highlight the crucial role that the host microbiome and tumor microenvironment may have in forecasting the response to treatment (351, 352). The potential impact of the microbiome on the effectiveness of ICIs suggests that it could also have a substantial role in regulating immune-related adverse events (iRAEs) associated with ICIs (353, 354). In the context of a practical inquiry, individuals afflicted with severe iRAEs exhibited heightened incidences of *Streptococcus*, *Faecalibacterium*, and *Stenotrophomonas* (355, 356). The concept of harnessing the GI microbiota to enhance the production of the anti-inflammatory compound known as butyrate by the gut microbiota to prevent colitis induced by ICI has already been discussed (357). The utilization of antibiotics to eliminate microbiota appears to diminish the efficacy of immunotherapy (358). In fibrosarcoma, melanoma, and CRC mice models, a combination of ampicillin, colistin, and streptomycin was demonstrated to impede the inhibition of CTLA-4 and subsequently revive the growth of tumors (359). A recent investigation discovered that in mice subjected to anti-CTLA-4 treatment, the administration of Bifidobacterium potentially diminishes autoimmune adversities. However, the absence of vancomycin exacerbates immunotherapy-induced colitis (360). The significant microorganisms that serve as predictive biomarkers for immunotherapy response were identified thanks to these studies. A study conducted on a rat colon adenocarcinoma model has identified a group of 11 bacterial strains that could potentially enhance the efficacy of immunotherapy (361). It's interesting to note that probiotics have been examined as adjuvants in cancer therapies. In a murine model of CRC, the administration of cell lysates derived from Lactobacillus acidophilus in conjunction with a monoclonal antibody targeting CTLA-4 induced a substantial augmentation in CD8+ T lymphocytes, specifically the effector memory subset, along with a noteworthy reduction in regulatory T cells (Tregs). Additionally, the synergistic combination recovered animals with CRC-induced dysbiosis and reduced the aberrant abundance of

Proteobacteria in the tumor microenvironment (362). Therefore, using immunotherapy in concert with probiotics may considerably aid the development of innovative therapeutic methods against CRC (363, 364) (Figure 3).

Radiotherapy

Uncertainty persists over how gut microbiota controls the effectiveness of radiotherapy. Radiotherapy can augment the overall immune response regulated by the immune system in addition to the cytotoxicity of tumors (365) (Figure 3). In addition to causing tumor cell death, local irradiation can boost systemic immunity and inflammation. The therapeutic utility, however, was limited due to adverse outcomes, including bystander effects on adjacent cells, genomic instability, and alterations to commensal microorganisms (366). Research indicates that the microbiota residing in the GI tract could potentially exert a notable influence on the efficacy of radiation therapy (367, 368). The inhibition of apoptosis in cancer cells and the prevention of local immunocyte infiltration were observed when comparing germ-free mice to conventional mice with radiation. The implications of these findings suggest that the commensal microbiota could potentially have a positive impact on the regulation of the body's reaction to radiotherapy treatment (369, 370). In experimental mice and humans getting radiotherapy, the gut flora is destroyed, which may lead to colitis and diarrhea partially mediated by IL-1β (371). Intestinal cell apoptosis and intestinal barrier function degradation are further side effects of radiotherapy that might result in intestinal inflammation (372). Further investigation revealed that angiopoietin-like 4 (ANGPTL4), a protein lipoprotein lipase inhibitor, plays a crucial role in radiotherapy damage resistance (373). *Streptococcus*, *Lactobacillus*, and *Bifidobacterium spp* stimulated the expression of ANGPTL4 to shield germ-free mice and regular mice from the harmful effects of irradiation (369). Additionally, butyrate, a widely recognized advantageous microbial byproduct, was demonstrated to enhance the efficacy of radiation in preclinical patient-derived CRC organoid models, suggesting the potential utilization of butyrate in combination with other therapies for cancer management (374). Additionally, a clinical investigation showed that formulations including *Lactobacillus casei*, *L. acidophilus*, and *B. bifidum* might reduce the intestinal adverse effects of radiation exposure (375, 376). *Lactobacillus rhamnosus*, although it possesses the ability to facilitate the recovery of radiation-induced damage to the intestinal mucosa, induce mesenchymal stem cell pre-migration via the TLR2 pathway, and protect the regular intestinal cavity, its effect on the preservation of transplanted tumor tissue is minimal (387). Additional research has revealed that the radioprotective properties of the microflora are mediated by SCFAs, particularly propionate, and specific tryptophan metabolites generated by the microbiota (377). These results offer a possible therapeutic target for reducing radiotherapy-related side effects and alleviating radiation-induced harm (Table 2).

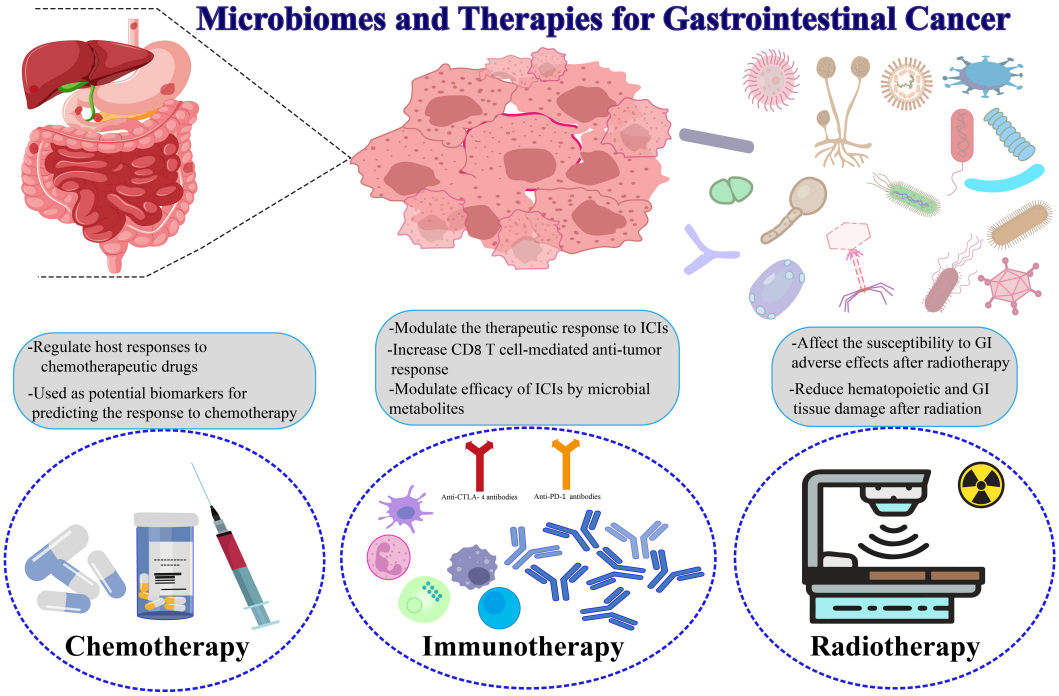


FIGURE 3
Microbiomes and therapies for gastrointestinal cancers.

TABLE 2 A summary of microbiomes and their role in the treatment of GI cancers.

Type of treatment	Methods Used	Conclusion	References
Immunotherapy	N/A	- The success of cancer immunotherapy is influenced by microbiota. - The harnessing of microbiota has the potential to enhance the body's immune response against tumors, thereby promoting antitumor immunity.	Goc et al. (378)
	-High-throughput sequencing technology -Regulation of gut microbiota	- The intestinal microbiota plays a crucial role in the progression and control of GI cancer. - The regulation of gut microbiota is suggested as a novel approach for treating GI issues.	Liu et al. (379)
	N/A	- The dysbiosis of the gut microbiome has an impact on both the prognosis and treatment of tumors. - The microbiota can enhance the anti-cancer immune response.	Wan et al. (380)
	- Meta-analysis was conducted on 16S rRNA gene sequencing data. - A multivariate selbal analysis is employed in order to identify bacterial genera.	- Gut microbiome features may predict immunotherapy response. - The application of machine learning algorithms has the potential to enhance the prognosis of cancer patients.	Liang et al. (381)
	-recruited 74 patients with advanced gastrointestinal cancer receiving anti-PD-1/PD-L1 therapy and collected stool samples before and during immunotherapy, along with clinical evaluations. -16S rRNA taxonomy survey	- The potential of the microbiome as a marker for immune-checkpoint blockade responses is indicated by the impact of gut microbiomes on anti-PD-1/PD-L1 outcomes, particularly in a subset of GI cancer patients.	Peng et al. (382)
Chemotherapy/ immunotherapy/ radiotherapy	- The identification of particular gut microorganisms for use as biomarkers is being investigated through screening processes. -Fine-tuning the gut microbiota for cancer prevention	- Gut microbiota's role in cancer development is crucial. - Improving cancer treatment outcomes can be achieved by adjusting gut microbiota through fine-tuning.	Zhou et al. (383)
Chemotherapy	-The association between *F. nucleatum* and chemotherapy response was investigated in 120 ESCC resected specimens and 30 pre-treatment biopsy specimens.	- *F. nucleatum* induces chemoresistance in ESCC cells through the regulation of autophagy. - Targeting *F. nucleatum* during chemotherapy could lead to different therapeutic results for patients with ESCC.	Liu et al. (384)
	- Genes that are differentially expressed in colorectal cancer cell lines due to infection by *F. nucleatum* were examined using a comprehensive analysis of the entire genome via microarray. - examined the clinical significance of *F. nucleatum* infection, BIRC3 protein expression, and resistance to 5-Fu treatment in patients with CRC.	- *F. nucleatum* and BIRC3 have the potential to be effective therapeutic targets in combating chemoresistance to 5-Fu treatment in advanced CRC.	Zhang et al. (385)
Radiotherapy	Three cohorts of patients (n = 134) were recruited	- The microbiota offers potential for the	Reis et al. (386)

(Continued)

TABLE 2 Continued

Type of treatment	Methods Used	Conclusion	References
	The early cohort (n = 32) The late cohort (n = 87) The colonoscopy cohort compared the intestinal mucosa microenvironment in patients with radiation enteropathy (cases, n = 9) with healthy controls (controls, n = 6)	anticipation, avoidance, or management of radiation enteropathy.	
	- Intestinal radioprotection was simulated through the use of cell lines and enteroids *in vitro*, and through the assessment of clinical outcomes and crypt survival *in vivo*. - The study utilized fractionated abdominal radiation and a single dose of radiation, in combination with syngeneic CT26 colon tumor grafts, to evaluate the efficacy of tumor radioprotection.	-*Lactobacillus rhamnosus* GG (LGG) functions as a controlled-release vehicle, delivering lipoteichoic acid with radioprotective properties. - lipoteichoic acid initiates a multi-step immune response involving macrophages and PGE2-secreting MSCs to safeguard epithelial stem cells within the stem cell niche.	Riehl et al. (387)

N/A, No Answer.

Conclusion

Gastrointestinal (GI) cancer constitutes one of the new cancer cases worldwide and imposes a significant burden on public health, thus presenting a major threat to human population health. Disturbances in the gastrointestinal microbiota may have a significant impact on the development of gastrointestinal cancers. Some bacteria have been found to support the development of cancer, while others appear to protect against it. Studies have shown that changes in the composition and abundance of microbiomes can be associated with the development of various gastrointestinal cancers, such as colon, stomach, liver, and esophageal cancers. In this study, we examine the importance of gut microbiomes in gastrointestinal cancers and their impact on various gastrointestinal cancer treatments, including chemotherapy, immunotherapy, and radiotherapy. The information in this article paves the way for researchers in the field of cancer and microbiome.

Author contributions

AY: Data curation, Writing – original draft. HA: Methodology, Project administration, 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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Host-gut microbiota derived secondary metabolite mediated regulation of Wnt/β-catenin pathway: a potential therapeutic axis in IBD and CRC

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The intestinal tract encompasses one of the largest mucosal surfaces with a well-structured layer of intestinal epithelial cells supported by a network of underlying lamina propria immune cells maintaining barrier integrity. The commensal microflora in this environment is a major contributor to such functional outcomes due to its prominent role in the production of secondary metabolites. Of the several known metabolites of gut microbial origin, such as Short Chain Fatty Acids (SCFAs), amino acid derivatives, etc., secondary bile acids (BAs) are also shown to exhibit pleiotropic effects maintaining gut homeostasis in addition to their canonical role in dietary lipid digestion. However, dysbiosis in the intestine causes an imbalance in microbial diversity, resulting in alterations in the functionally effective concentration of these secondary metabolites, including BAs. This often leads to aberrant activation of the underlying lamina propria immune cells and associated signaling pathways, causing intestinal inflammation. Sustained activation of these signaling pathways drives unregulated cell proliferation and, when coupled with genotoxic stress, promotes tumorigenesis. Here, we aimed to discuss the role of secondary metabolites along with BAs in maintaining immune-gut homeostasis and regulation of inflammation-driven tumorigenesis with emphasis on the classical Wnt/β-Catenin signaling pathway in colon cancer.

KEYWORDS

gut microbiota, secondary metabolites, bile acids, inflammation, Wnt/β-catenin signaling, CRC

1 Introduction

Inflammatory Bowel Diseases (IBD) are a collection of chronic inflammatory disorders associated with the gastrointestinal tract consisting of Crohn's Disease (CD) and Ulcerative Colitis (UC) (1). The presence of IBD also increases the risk of development of colon cancer by 20% at later stages of life (2). Prolonged inflammation, along with other epigenetic factors and a dysregulated immune system, can contribute to the development of Colorectal Cancer (CRC) (2). According to WHO, CRC is the third most prevalent cancer worldwide after breast and lung cancer until 2020 (3). More than 50% of new cases of CRC were reported in Asia, followed by Europe and North America. It is predicted that if the situation persists, then the estimated number of cases will increase from 1.88 million in 2020 to 2.94 million in 2040 globally (4).

Current CRC treatment regimens include chemotherapy, T-cell boosting therapeutics, oncolytic viral treatments, and non-coding RNA therapy. However, these improved treatments did have long-term side effects that can affect quality of life. Up to 85% of survivors treated with Oxaliplatin develop some degree of sensory neuropathy (5). Another survey found that around 20% of patients undergoing chemotherapy experienced grade 3/4 severe toxicities. A smaller percentage (<1%) suffers fatal toxicity, resulting in severe diarrhea, neutropenia, thrombocytopenia, or cardiac symptoms (6). The increased resistance to non-coding RNA therapy over time also poses a major challenge (7). Therefore, despite the developments in biologics, surgery remains one of the major treatment strategies for CRC patients, implying the need for alternative therapies with minimal to no side effects.

2 The gut microbiome as a regulator of intestinal health: a quick overview

The intestinal microbial composition is closely associated with human health and disease. The human gut contains two compartments, the intestinal lumen and lamina propria, separated by the intestinal epithelial barrier. The luminal cavity is colonized by over 1000 species of microbes belonging to the domains *Archaea*, *Bacteria*, and *Eukarya*, which share a commensal relationship with cells of the host. The gut microbiome aids in various biological functions of the host system, such as fermentation of food, vitamin production, secondary metabolite synthesis, and regulation of immune responses (8). It has been reported that certain gut microbiota-derived secondary metabolites influence innate immune cells and non-hematopoietic components of the gut to maintain barrier integrity (9).

Prolonged disease conditions, a change in lifestyle and diet, and imprudent consumption of antibiotics result in gut microbial dysbiosis, subsequently disrupting intestinal homeostasis (9). The modern diet includes calorie-dense and nutritionally deficit options. Additionally, the increased consumption of ultra-processed foods (UPF) is also one of the leading factors contributing to the onset of IBD by reducing gut microbial diversity. Patients suffering from IBD, or gastrointestinal illness, along with medications, are often suggested a strict diet and healthy lifestyle. An appropriate dietary intervention can help in enhancing the effectiveness of the medication. Some dietary strategies have been found effective in improving disease activity and supporting clinical remission; however, some need further prospective evidence (10). For example, in a clinical study conducted in 2015, children suffering from Crohn's disease were subject to a specific carbohydrate diet for 12-52 weeks. They showed reduced severity of the disease with respect to the Harvey-Bradshaw Index from 3.3 +/- 2.0 to 0.6 +/- 1.2 post-treatment (11).

In a healthy individual, the intestinal epithelial cell barrier can prevent the transmission of pathogens, proinflammatory substances, and antigens from the lumen to the internal environment (8). However, an imbalance in intestinal microbiota alters the tight intercellular junctions that allow pathogens and toxins (bacterial lipopolysaccharides, LPS) to cross the intestinal barrier, contributing to the activation of Pattern Recognition Receptors (PRRs) on lamina propria immune cells (12, 13). The intracellular signaling cascades triggered by these PRRs, which include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs), upregulates the expression of inflammatory modulators. These modulators orchestrate the elimination of pathogens and affected cells. However, aberrant activation of this system also leads to the overproduction of immuno-oncogenic signals initiating tumorigenesis (13).

Cumulative studies illustrate that NLRs can negatively regulate cell differentiation and proliferation via the Wnt pathway in various cancers, including CRC (14). Similarly, TLR activation negatively regulates mesenchymal stem cell proliferation by disrupting canonical Wnt signaling by interrupting the expression of Wnt2, Wnt3, Wnt3a, and Wnt8 along with Frizzled Receptors (10). Wnt signaling is involved in the modulation of immune responses during inflammation, providing us with a potential drug target for CRC (12, 15). Therefore, through this review, we aim to shed light on the possible non-invasive methods to treat chronic intestinal inflammation and modulate the Wnt pathway using naturally occurring secondary metabolites of host gut-microbial origin.

3 Wnt signaling cascade and its role in intestinal cancer progression

CRC may result from one or more mechanisms such as chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and microsatellite instability (MSI). The most studied mode of mechanistic progression is chromosomal instability, initiated by adenomatous polyposis coli (APC) mutations. Approximately 80% of CRC cases are a result of APC mutation. This mutation activates Wnt signaling mechanisms, increasing the transcription of several oncogenes (16) An interesting study conducted in 2020 revealed APC is also imperative for controlling Wnt-induced beta-catenin destruction complex recruitment in colonocytes to prevent aberrant cell proliferation and tumorigenesis (17), suggesting the involvement of Wnt signaling in CRC progression.

The Wnt/β-catenin pathway, Wnt/Ca2+ pathway, Wnt planar cell polarization pathway, and intracellular pathway that regulates spindle direction and asymmetric cell division are four major Wnt signaling pathways (18). The Wnt/β-catenin pathway displays a duality while modulating inflammation, possessing anti- and proinflammatory potential (14). In IBD's pathophysiology, Wnt ligands secreted by activated immune cells bind to the Frizzled (Fzd), a G-protein coupled receptor, producing a proinflammatory tumor microenvironment (14, 19). Once Wnt ligands bind to membrane receptor Fzd and Lipoprotein-receptor related protein 5/6 (LRP5/6), they destabilize the β-Catenin degradation complex (GSK-3-β-APC-AXIN- β-Catenin). Accumulated β-Catenin translocates to the nucleus and triggers the TCF-4/LEF-1 (T cell factor/lymphoid enhancer factor) transcription factors to induce the expression of genes involved in cell cycle function and promote cell growth, differentiation, and metastasis (19).

A higher concentration of the Wnt ligands causes greater activation of the Wnt/β-Catenin signaling pathway, leading to increased cell proliferation. This uncontrolled cell proliferation or hypertrophy is followed by hyperplasia, causing an Epithelial-to-Mesenchymal (20) transition and increased cell motility, metastasis, and other related properties of cancer cells (21). Studies show that Wnt3a is the primary ligand involved in oral carcinogenesis, Wnt5a in breast tissue carcinogenesis, and Wnt3 is responsible for colon cancer proliferation (15, 21–23). Additionally, multiple types of cancer are known to be driven by uncontrolled expression of β-Catenin. β-Catenin expression is directly proportional to the depth of tumor infiltration (20). A swelling body of evidence suggests that β-Catenin inhibition suppresses tumor progression and recurrence.

During the clinical treatment of CRC, Wnt inhibitors are a common mode of therapy (24). The transcription factor SP1 (Specificity protein 1) is a crucial factor expressed in cell proliferation pathways (25). The direct interaction of SP1 with β-Catenin prevents the association of SP1 with degrading factors, thereby contributing to its stabilization (25). Interestingly, a study found suppression of the transcription factor SP1 by siRNAs truncated the growth of colon cancer stem cells (CCSCs) (21). Another transcription factor that promotes the proliferation of Wnt-driven colon cancer cells is SOX9. The regulation of gene expression by the Wnt/β-Catenin pathway results from the formation of a β-Catenin complex with the transcription factor TCF7 (T cell factor). TCF7 and SOX9 interact through nonDNA-contacting residues to produce a synergistic effect that encourages cancer cell proliferation (26). Inhibition of such factors can be a potential means of CRC therapeutics. These studies indicate that uncovering molecular targets within the Wnt/β-Catenin pathway will be capable of down-regulating CRC and related predisposing conditions such as IBD.

4 Gut microbiota derived secondary metabolites and their therapeutic potential in CRC

Culture-based studies show the dominance of *Bacteroidetes* and *Firmicutes* in the healthy gut, while *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* are found in minor constituents. Reduction in diversity within the *Firmicutes* phylum is a major contributor to gut microbial dysbiosis causing IBD (8). Molecular cues of gut microbial origin regulating intestinal cell function are attributed to diversified small molecule metabolites (24). These metabolites are the intermediate or end products of host-gut bacterial metabolic processes. They are known to play a significant role in maintaining intestinal barrier integrity and intestinal immune homeostasis. Gut microbiota is widely involved in the metabolism of carbohydrates to generate SCFAs (27). Other majorly explored metabolites include tryptophan and indole derivatives, followed by primary and secondary BAs (27, 28).

Drastic imbalances in the composition of these metabolites have been observed in IBD and CRC patients. IBD patient fecal samples have a lower proportion of SCFA-producing bacteria, whereas mucolytic and pathogenic bacteria are found in abundance. Similarly, an increase in the population of sulfate-reducing bacteria, such as *Desulfovibrio*, is also found in IBD patient's fecal samples. This increases the production of hydrogen sulfate, induces mucosal inflammation, and causes damage to the intestinal epithelial barrier (10). Moreover, IBD and IBD-associated cancers are known to cause malabsorption and reduction in the conversion of primary BAs to secondary BAs, thereby disrupting BA pool composition. Such changes pose a higher risk of infection as the mucosal integrity gets compromised (29).

Thus, their ability to behave as biomarkers and regulate metabolism and other homeostatic mechanisms makes them potential non-invasive therapeutic targets. (Supplementary Table 1).

4.1 Short chain fatty acids

Short Chain Fatty Acids (SCFAs) are crucial in maintaining intestinal barrier integrity, gut homeostasis, and colon health (30). These microbiota-derived SCFAs are the primary energy source for intestinal epithelial cells (IEC) in the digestive tract. The imbalance in SCFAs is known to contribute to intestinal inflammation and associated diseases (30). These SCFAs include butyrate, propionate, and acetate.

One of the significant SCFAs, butyrate, is produced by *Faecalibacterium prausnitzii*, *Clostridium leptum, and Eubacterium rectaleand*, among others, displays superior inhibitory efficacy against CRC proliferation (30). It is essential for human health as it is the primary energy source for colonocytes (31). Additionally, butyrate regulates CRC by inhibiting HDAC 1 and 3 in colon cancer cells and suppressing intestinal inflammation and ROS production (32). Butyrate activates GPR109A and inhibits Protein Kinase B and NF-κB signaling pathways to reverse intestinal epithelium barrier dysfunction (33). Furthermore, evidence shows that butyrate plays a vital role in controlling intestinal inflammation by stimulating the differentiation of Treg cells (34) and promoting an anti-tumor effect (35). It was also reported that *C. butyricum* species indirectly upregulates butyrate production, reduces the levels of β-Catenin, and regulates the Wnt pathway (36).

A study reported that butyrate facilitates M2 macrophage polarization. It was shown that ERK1/2 activation or blockade of Wnt secretion suppressed the beneficial effect of butyrate-primed macrophages on goblet cell function. Adoptive transfer of butyrate-induced M2 macrophages in a dextran sulfate sodium (DSS)-induced mice model of colitis showcased a significant improvement in mucosal layer integrity, mucus secretion, and goblet cell regeneration (37). It is also known that Butyrate stimulates bone formation via T Regulatory cell-mediated regulation of WNT10B expression (38).

A study by Beatrice et al. showed that butyrate inhibits CRC proliferation by autophagy-mediated degradation of β-Catenin. Apart from modulating cancer cell proliferation, the treatment with butyrate plays a significant role in autophagy. Interestingly, the study showed that butyrate promoted the binding between LC3 and β-Catenin, causing its sequestration. The ability of butyrate to inhibit the Wnt/β-Catenin pathway represents a new frontier of targeted cancer therapies (39).

Similarly, propionate, produced by *Veillonella parvula, Bacteroides eggerthii, and Bacteroides fragilis* in the gut, regulates intestinal homeostasis by promoting turnover of the epithelial cells and promoting barrier integrity (40). As a result, stem cells in intestinal crypts differentiate through the Wnt signaling pathway to replenish lost cells. The absence of propionate results in intestinal disbalance, triggering the unregulated proliferation of IECs (30). Valproic acid (VPA) was able to stimulate the differentiation of neuronal stem cells by activating Wnt3a and β-Catenin (18, 41). The fatty acid acetate aids in the acetylation of β-catenin, reducing the Wnt inhibitor SOX-1 and potentially increasing cell proliferation (14). Recently, another study showed β-hydroxybutyrate is capable of suppressing cancer, by inhibiting EMT via the Wnt/β-Catenin pathway (42). (Figure 1A).

4.2 Amino acid metabolites

Numerous amino acid metabolites, including hydrogen sulfide (H_2S) and indole metabolites, are produced due to the fermentation of proteins by the gut microbiota (28). Several studies have shown that tryptophan (Trp), mainly produced by *E. coli*, can downregulate cell proliferation by suppressing the Wnt signaling pathway, implying targeting tryptophan metabolism is a method of CRC treatment (42, 43). A clinical study was conducted on 117 participants comprising 79 CRC patients and 38 age- sex-and body mass index (BMI) matched healthy controls. It was observed that the indole/tryptophan ratio in fecal matter positively correlated to the mRNA expression of tight junction proteins like Zona Occuladins-1 in colon tissue samples collected from the respective participants, suggesting the involvement of Trp metabolites in the tumorigenesis of CRC in humans (43). Several studies have highlighted the role of wnt signaling in shaping immune cell functions. One of the key mechanisms by which Wnt-β-catenin signaling in DCs promotes immune suppression is through the induction of an immunoregulatory enzyme, IDO, thereby causing the degradation of the essential amino acid tryptophan into kynurenines (44). A study found that 1-Methyl-D-tryptophan Reduces Tumor $CD133^+$ cells, Wnt/β-catenin and NF-κβp65 in Murine Pancreatic Adenocarcinoma.1-Methyl-D-tryptophan

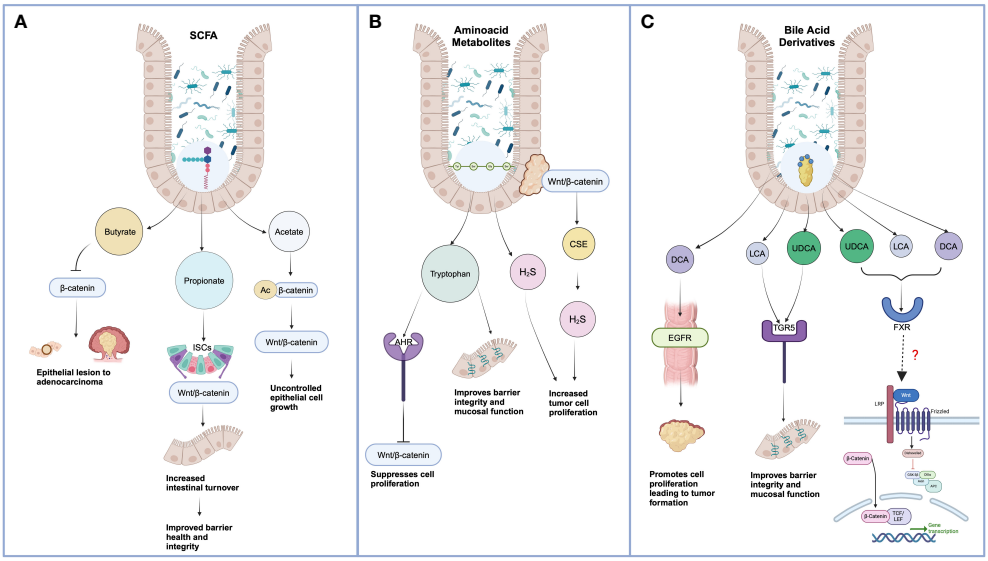

FIGURE 1
Effect of gut microbiota-derived metabolites on Wnt-mediated cancer progression. **(A)** Short-chain Chain Fatty Acids include Butyrate, Propionate, and Acetate. Excess butyrate treatment can promote proteasomal degradation of Wnt ligand β-Catenin mediated by autophagy marker LC3. When propionate synthesis reduces, cell differentiation and polarization diminish, leading to a lower cell turnover and increased aberrant intestinal cell growth. Acetate-mediated acetylation of β-Catenin facilitates a decrease of SOX-1, allowing unchecked Wnt-mediated cell proliferation. **(B)** Lower tryptophan metabolism impairs the integrity of tight junctions and regulates Wnt metabolism by targeting Wnt 3 and β-Catenin. H2S nourishes aberrant cell proliferation via the Wnt pathway. **(C)** Bile Acids bind to the FXR receptor, resulting in the inhibition of the Wnt/β-Catenin pathway. However, few direct co-relations exist between BAs and Wnt signaling ligands and receptors. *Created in BioRender.com).

significantly modulates the regulatory cytokines in the tumor microenvironment, which significantly inhibited tumor growth and tumor immune escaping potency (45).

Studies indicate another predominant amino acid metabolite, H_2S, an energy source for the metabolism of the colonic epithelium (46). H_2S is produced by the action of *Desulfovibrio*, *Escherichia*, *Bilophila*, *Porhyromonas*, *Prevotella*, *Corynebacterium*, *Veillonella*, *Helicobacter*, and *Clostridium* on amino acids (27). Cell culture studies in HT 29 cells discovered the cytotoxic and genotoxic effects of H_2S produced by sulfate-reducing bacteria. However, there has been conflicting data on the inhibitory and stimulatory effects of H_2S on the proliferation and inflammation of CRC cells (47). Upon further analysis in Human Colon Cancer cell line SW480, the study found that the Wnt/β-Catenin pathway regulates Cystathionine-γ-lyase (CSE) on a transcriptional level, upon secretion responsible for increasing H_2S liberation. Furthermore, when tumors were xenografted into nude mice models with a CSE/H_2S knockdown, their tumor growth was reduced, implying that H_2S plays a role in increasing colon cancer (48) (Figure 1B).

4.3 Bile acids

Another well-known host-gut microbiota-derived metabolite of interest are secondary BAs with numerous unknown functions other than role in dietary lipid digestion. BAs are the end-product of cholesterol metabolism generated in the liver by a chain of enzymatic reactions organized in two main metabolic pathways, known as "classic" and "alternative" (49). These liver pathways generate mainly two primary BAs, i.e., cholic acid and chenodeoxycholic acid (CA and CDCA). In hepatocytes, these primary BAs are conjugated with glycine (G) or taurine (T), giving rise to the bile salts. Conjugated BAs are secreted in the intestine, becoming the substrate of an array of bacterial enzymes (49). 7α-dehydroxylation of the OH in the C7 position, a reaction mediated by 7α-hydroxylase expressing bacteria such as *Clostridium* and *Eubacterium*, gives rise to two secondary BAs, i.e., mono-hydroxylated BAs like LCA from CDCA, and 3α-12α-di-hydroxylated BAs like DCA from CA. Additionally, the C7 β-epimerization of CDCA by *Bacteroides, Clostridium, Escherichia, Eubacterium*, and others originates the 7β epimer of CDCA, i.e., the 3α,7β-dihydroxy-5β-cholanoic acid, known as ursodeoxycholic acid (UDCA) (47, 48). The large majority of BA species that reach the terminal ileum are reabsorbed by the intestinal epithelial cells (IEC) and transported back to the liver through the portal vein, completing a cycle in the so-called "entero-hepatic circulation" (49).

4.3.1 Therapeutic potential of BAs

BAs regulate mucosal homeostasis and inflammation by interacting directly with a family of receptors known as bile acid-activated receptors or bile acid receptors (BAR), which include Takeda G protein-coupled receptor 5 (TGR5) and nuclear receptors that include the Farnesoid X Receptor (FXR) and Vitamin D Receptor (VDR) (50). BA signaling is known to suppress the proinflammatory phenotype of intestinal cells by the reduced release of TNF-α, IL-1β, IL-6, or IL-12. Studies have also reported that BA stimulates the production of anti-inflammatory cytokines, promoting epithelial barrier renewal (28).

A study reported that secondary BAs, such as LCA's derivatives, regulate the differentiation of Treg cells, contributing to the suppression of inflammation, maintaining immune homeostasis, and hence, predisposing stages of cancers like CRC (51). LCA is reported to activate VDR on CaCo-2 cells and significantly reduce IL-1β -induced IL-8 secretion by blocking NF-κB inflammatory signaling (52). Kubota et al., in their studies, found VDR mediated the attenuation of Dextran Sulfate Sodium (DSS) induced Colitis in mice fed with LCA (53). Oral administration of LCA suppressed histological injury in an early phase of DSS-induced Colitis in Vdr +/- mice, whereas no significant impact was observed on Vdr-/- mice, suggesting the physiological role of the LCA–VDR axis in intestinal homeostasis (53). Additionally, LCA-dependent PXR activation in epithelial cells promotes TGFβ expression and reduces TLR4-dependent proinflammatory cytokines production by diminishing TLR4 mRNA stability (54). TGR5 is one of the receptors activated by multiple BAs, with LCA being its most potent natural agonist (55). A study found that LCA-induced activation of TGR5 reduces adaptive immune response as there is increased recruitment of NK cells. Another study found that LCA stimulated intestinal epithelial growth in an organoid, as indicated by the increased expression of an intestinal stem cell marker. However, this improved barrier regeneration was lost when LCA was administered to a *Tgr5*-/- organoid, indicating that LCA-associated TGR5 activation is crucial for barrier integrity (55).

Multiple studies have reported the therapeutic role of another secondary BA, UDCA, in Colitis and colitis-associated cancer. UDCA exerts anti-inflammatory and cytoprotective effects in the AOM-DSS-induced colitis mouse model (55). UDCA has also been shown to prevent colon inflammation in rats treated with 2,4,6-trinitrobenzene sulfonic acid (56). Interestingly, deficiency or absence of the TGR5 receptor significantly reduces the modulatory effect of UDCA, both *in vitro* and *in vivo*. He et al. and other studies highlight that UDCA treatment can contribute to intestinal homeostasis by enhancing the intestinal mucosal layer, maintaining epithelial cell integrity, modulating the gut microenvironment, and attenuating intestinal inflammation (55). The collective observation suggests that elucidating the relationship between UDCA and the gut microbiome can be a novel therapeutic strategy for inflammation and inflammation-driven cancer.

DCA has been known to play a significant role in the induction of CRC development. *In vivo* experiments with $APC^{min/+}$ mice suggested that DCA contributes to CRC tumorigenesis by activating EGFR to promote a hyperproliferative effect on colorectal mucosa in DCA-fed mice (57). Ji-Yao et al. reported that oral administration of DCA to germ-free mice increased colonic Rspo3 mRNA levels, which function as ligands for LGR4 and LGR5 and potentiate the activation of the Wnt pathway. In primary myofibroblasts, DCA increases Rspo3 mRNA via TGR5 and mediates high-fat diet-induced intestinal epithelial proliferation (58). However, the impact of therapeutic BAs like UDCA and LCA and its derivatives on wnt regulation are largely unexplored. (Figure 1C).

4.3.2 The cross-talk between Wnt/β-catenin and bile acids

Recent evidence indicates that the Wnt/β-catenin pathway regulates bile homeostasis, including bile synthesis, modification, and transport. Cholesterol synthesis occurs predominantly in periportal hepatocytes (59). CYP7A1 and CYP27, crucial rate-limiting enzymes of BA synthesis, are localized in the perivenous zone of the liver lobule and coincident with β-catenin activation. The close relationship between the two processes was seen in β-catenin KO mice subjected to a methionine and choline-deficient diet, identified by macro vesicular steatosis and fibrosis. Liver-specific β-catenin deletion resulted in increased steatosis, higher hepatic cholesterol accumulation, and jaundice, likely due to defects in cholesterol to bile conversion mechanism and the bile export system. Additionally, conditional β-catenin KO had higher hepatic total BA levels on methionine and choline-deficient and control diets, indicative of basal abnormalities in bile metabolism without β-catenin (60).

Chromatin immunoprecipitation (ChIP) assays showed that CYP27 is a transcriptional target of β-catenin. Similarly, β-catenin KO and LRP5/6 KO models had significantly suppressed expression of CYP7A1, suggesting the involvement of β-catenin in BA metabolism. Interestingly, further studies have found that the β-catenin interacts with FXR, a nuclear receptor that regulates the expression of CYP7A1 and BA efflux transporters. FXR deficiency increases epithelial permeability to luminal bacteria, thereby promoting Wnt/β-catenin signaling, and increasing intestinal inflammation (61).

The crosstalk between Wnt/β-catenin ligands and members of the nuclear receptor (NR) family has been considered a clinically and developmentally important research area of cancer biology. Mao J. et al., in their study, demonstrated that FXR knockdown promotes β-catenin/TCF4 complex formation and, subsequently, its binding ability to the corresponding promoter. Their data indicates a novel mechanism through which FXR expression is mediated during tumor progression, involving the Wnt pathway. Additionally, hepatic bile acid synthesis is downregulated by the activation of the FXR-FGF15/19 signaling pathway (62). Thus, FXR represents a novel Wnt signaling pathway modulator and a potential Wnt signaling cascade molecular target that may be exploited to achieve anti-tumor effects (63).

5 Conclusion/discussion

The role of Wnt signaling in tumorigenesis is predominantly studied in colorectal cancer, where several studies suggest targeting Wnt/β catenin to regulate tumor progression. However, a therapeutic treatment targeting the canonical Wnt pathway achieving efficacy and safety remains a major challenge. Considering the role of FXR in Wnt regulation and the ability of some BAs to activate FXR, understanding the downstream mechanism opens doors to promising hypotheses exploring the impact of BAs via the BAR in regulating pathogenic Wnt signaling and immune modulation in the intestinal inflammation and associated cancers. As new studies describing such processes and our understanding of signaling mechanisms deepen, we must screen for direct interactions between BAs and Wnt pathways with the goal of maintaining intestinal homeostasis. Overall, the development of novel combinatorial therapeutics of natural origin capable of reducing the risk of side effects and improving the treatment outcome in CRC and predisposing IBD is an essential stride.

Author contributions

SSK: Writing – review & editing, Writing – original draft, Conceptualization. AF: Writing – review & editing, Writing – original draft, Conceptualization. PS: Writing – review & editing. TJ: Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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/fonc.2024.1392565/full#supplementary-material

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Untargeted metabolomics in gastric and colorectal cancer patients – preliminary results

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Introduction: Recent years, microbiota-associated aspects have been analysed in multiple disorders regarding cancers. Existing evidence pints that gut microorganisms might take part in tumour origin and therapy efficacy. Nevertheless, to date, data on faecal metabolomics in cancer patients is still strongly limited. Therefore, we aimed to analyse gut untargeted metabolome in gastrointestinal cancer patients (i.e., gastric and colorectal cancer).

Patients and methods: There were 12 patients with either gastric (n=4) or colorectal cancer (n=8) enrolled and 8 analysed (n=4 each). Stool samples were collected prior to anti-cancer treatments. Untargeted metabolomics analyses were conducted by means of mass spectrometry.

Results: A plethora of metabolites in cancer patients we analysed were noted, with higher homogenity in case of gastric cancer patients. We found that the level of Deoxyguanosine,m/z 266.091,[M-H]-, Uridine,m/z 245.075,[M+H]+, Deoxyguanosine,m/z 268.104,[M]+, 3-Indoleacetic acid,m/z 176.07,[M+H]+, Indoxyl,m/z 132.031,[M-H]-, L-Phenylalanine,m/z 164.073,[M-H]-, L-Methionine, m/z 150.058,[M+NH4]+, was significantly higher in colorectal cancer patients and Ethyl hydrogen malonate,m/z 133.031,[M+H]+ in gastric cancer.

Conclusion: The overall insights into untargeted metabolomics showed that most often higher levels of analysed metabolites were detected in colorectal cancer patients compared to gastric cancer patients. The link between gut metabolome and both local and distal metastasis might exist, however it requires confirmation in further multi-centre studies regarding larger sample size.

KEYWORDS

colorectal cancer, gastric cancer, gut microbiome, microbiota-derived metabolites, untargeted metabolomics

1 Introduction

Microbiome and metabolome-related aspects have become objects of interest in oncology (Kaźmierczak-Siedlecka et al., 2023). The reasons are as follows: [1] Currently, it is known that some microbes are involved in development of tumour by creating dysbiotic environment and activating biochemical pathways (Rajagopala et al., 2017). There are therapeutic methods (such as prebiotics, probiotics, synbiotics, postbiotics, next-generation probiotics) which modify the composition of gut microbiome and the activity of microorganisms through for instance affecting production of metabolites and consequently leading to eubiosis restoration. However, it is still under investigation, and it requires further analysis to strengthen the possibility of usage. [2] According to some data, there is a bidirectional link between gut microbiome and drugs (also anti-cancer agents). These interactions are described as pharmacomicrobiomics (Ting et al., 2022). Basis on this bidirectional communications may provide personalized and more effective anti-cancer management. [3] Microbiome profile and metabolomic signature may be considered as biomarkers (Wong and Yu, 2023), which can select subjects with higher risk of tumour development or to detect cancer in early stages. Therefore, it seems that there can be found many benefits from routinely analysis of gut microbiome in cancer patients and include it to screening program.

In contrast to targeted metabolomics, untargeted metabolomics is characterized by wide range of discovery, mainly hypothesis generating, comprehensive analysis, qualitative identifications and relative quantitation of small molecules in sample (Schrimpe-Rutledge et al., 2016). In the level of metabolomics, small molecules are characterized from many types of samples, such as stool, urine, serum, cell extracts, and others. Considering metabolomics it should be emphasized that there are different methods of both separation and detection. Notably, it seems that metabolomics analysis based on mass spectrometry is one of the most significant technology allowing to detect and identify small molecules which are produced by gut microbiota (Bauermeister et al., 2022).

As it was previously mentioned, the imbalance of gut microbiota composition and changes of microbiota-derived metabolites are observed in gastrointestinal cancer patients (Kaźmierczak-Siedlecka et al., 2023; Ohigashi et al., 2013; Tong et al., 2021; Yang et al., 2022; Dai et al., 2021). Recently, in Kaźmierczak-Siedlecka et al. study it was shown that microbiota-derived metabolites based on the proportion between acetate, proprionate, and butyrate is changed in colorectal cancer patients in preoperative period (Kaźmierczak-Siedlecka et al., 2023). Untargeted metabolomics seems to be extremely significant in oncology due to the fact that it allows to collect data without pre-existing knowledge (Schrimpe-Rutledge et al., 2016). It is noteworthy that anti-cancer treatment (such as surgery, chemotherapy, radiotherapy) affects gut microbiome and metabolome-related aspects and vice-versa. Therefore, the aim of this study was to analyse untargeted metabolomics in patients with gastrointestinal cancers (i.e. gastric cancer and colorectal cancer) prior to the introduction of anti-cancer treatment. It allows to obtain more precise data without the potential influence of above mentioned treatment. Moreover, the comparison of untargeted metabolomics in case of gastric and colorectal cancer has been investigated.

2 Patients and methods

Participants (n=12) were recruited in Department of Surgical Oncology (Medical University of Gdansk) and Unit of Surgery with Unit of Surgery with Unit of Oncological Surgery, Specialist Hospital in Koscierzyna, Poland. Inclusion criteria were age ≥18 yr., patients with diagnosed gastric/colorectal cancer prior to the introduction of anti-cancer treatment, written consent to take part in this study. Exclusion criteria included age <18 yr., patients with gastric/colorectal cancer who were under anti-cancer treatment. The stool samples (at least 4 g) were collected after confirming of diagnosis and before introduction of anti-cancer treatment. The stool samples were taken by own patients, placed in sterile tube, and then provided to researchers as soon as possible. Next, they were stored in -80°C in the Fahrenheit Biobank BBMRI.pl, Medical University of Gdansk, until conduction of untargeted metabolomics analysis according to the well-established protocol at Sanprobi Sp. z o. o. The study protocol has been approved by the Independent Bioethics Committee for Scientific Research at the

Medical University of Gdansk (identifiers: NKBBN/129/2021, NKBBN/428/2022, KB/428-314/2023).

2.1 Preparation of material for analysis

Briefly, 500 µl of a mixture of methanol, water and acetonitrile in the proportions of 50:25:25 v/v/v with the addition of deuterated internal standards was added to 60 mg of feces. Then, the samples were shaken at 2000 rpm at 4°C for 30 min. to dissolve the metabolites in the solution and precipitate the proteins. In the next step, the samples were centrifuged for 4 minutes at a speed of 4000 rpm and at a temperature of 4°C. After the samples were centrifuged, the supernatant was decanted to the chromatography tubes through a 0.22 µm syringe filter. The samples were subsequently analysed on the same day by a liquid chromatography–mass spectrometry. QC samples were prepared by mixing test samples in equal proportions and prepared in the same way as the test samples.

2.2 Liquid chromatography-mass spectrometry analysis

The analysis was carried out on an ExionLC liquid chromatograph equipped with a binary pump, autosampler, and column thermostat coupled with a Triple TOF 6600+ mass spectrometer (Sciex, Framingham, MA, USA). The separation was carried out on a Phenomenex Luna® Omega 1.6µm polar C18 150 x 2.1mm column for 45 min in gradient separation. The mobile phases were: Phase A – Water with 10mM ammonium acetate, Phase B - acetonitrile with 0.1% formic acid. The column injection was 2µl and the column temperature was 20°C. The phase flow was 0.2 ml/min. Spectral analysis was performed in the positive ion mode with a capillary voltage of 5500 V, Curtain gas (CUR) was 25 psi, Ion source gas 1 (GS1) 45 psi, Ion source gas 2 (GS2) 60 psi and the ion source temperature was 400°C and the mode negative ions at a capillary voltage of 4500 V, Curtain gas (CUR) was 25 psi, Ion source gas 1 (GS1) 45 psi, Ion source gas 2 (GS2) 60 psi and the ion source temperature was 350°C. Spectrometer collected spectral data in SWATH mode.

2.3 Analysis of the results and statistical analysis

The obtained spectral spectra were analysed and matched to reference spectra contained in the SCIEX All-In-One HR-MS/MS, NIST and own databases using SCIEX OS software. In the next step, based on the results obtained and the identification of metabolites present in the tested samples, a file was created in Microsoft Excel 2019 PL (Poland) for statistical analysis and data visualization on the Metaboanalyst platform (https://www.metaboanalyst.ca/). The t-test and fold change >2 were used to determine differences between the study groups. The statistical analysis was conducted using above mentioned Microsoft Excel 2019 PL (Poland) and STATISTICA version 13.0.

TABLE 1 Characteristics of patients according to the tumour types.

Sample_1G	Stomach cancer T3N0M0
Sample_N2G	Stomach cancer NET, G1
Sample_3G	Cancer of the prepyloric part of the stomach T3N1M0
Sample_4G	Stomach cancer T2N1
Sample_5G	Cancer of the sigmoid-rectal flexure pT3N2a
Sample_9G	Sigmoid colon cancer adenocarcinoma G2 cT4NxM1b
Sample_13AG	Rectal cancer – adenocarcinoma G2 pT2N0M0
Sample_20AG	Ascending colon cancer pT2N0M0

3 Results

This study included 12 patients (n=8 – colorectal cancer, n=4 – gastric cancer). The basic characteristics of these participants is as follows: the median age – 61.78 ± 11.50 years, the median Body Mass Index (BMI) – 29 ± 1.41 kg/m^2, the most commonly co-existing disease – hypertension. Among these patients, 4 were excluded due to incomplete data regarding tumour characteristics. Therefore, the analysis is based on 2 groups: first including gastric cancer patients (n=4) and second regarding colorectal cancer patients (n=4) (Table 1).

The analysis of stool samples revealed the occurrence of wide range of metabolites in gastric and colorectal cancer patients (Table 2).

The metabolic profile of analysed stool samples varies, especially in case of colorectal cancer patients (Figure 1). These differences can be caused by variability of either types of tumours or tumours anatomical localisation. There is higher grouping in case of gastric cancer, which confirms more homogeneous metabolic profile comparing to the analysed group of colorectal cancer. Moreover, in Figure 1 there are subgroups (in gastric cancer) created by Sample_1G and Sample_3G, Sample_4G and Sample_N2G, which show similar characteristics in these subgroups.

The occurrence of metabolites, which varied in both analysed groups, is presented in Figure 2. The metabolites, which significantly varied colorectal cancer and gastric cancer are placed in Figure 2 with blue and red colours and next they are precisely analysed and presented in Figure 3.

The comparison of the levels of particular metabolites detected in colorectal cancer patients and gastric cancer has been presented in Figure 3. Considering 25 metabolites (Figure 3), it is observed that higher level of them are mostly noted in colorectal cancer patients compared to the gastric cancer (21 metabolites *vs.* 4

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TABLE 2 Metabolites identified in analysed stool samples of gastric and colorectal cancer patients.

Compound	Precursor Mass	Adduct	Retention time
Enterolactone	297.115	[M-H]-	23.5
(2-Oxo-2,3-dihydro-1H-indol-3-yl)acetic acid	192.064	[M+H]+	16.8
(2-Oxo-2,3-dihydro-1H-indol-3-yl)acetic acid	190.051	[M-H]-	16.8
Gamma-Undecalactone	185.152	[M+H]+	23.3
1,1-Dimethylbiguanide	130.109	[M+H]+	5.2
1,3,7-Trimethyluric acid	211.082	[M+H]+	16.8
1,3,7-Trimethyluric Acid	209.069	[M-H]-	16.8
1,7-Dimethyluric Acid	197.066	[M+H]+	16.0
1,7-Dimethyluric Acid	195.053	[M-H]-	16.0
1,9-Nonanedicarboxylic acid	215.13	[M-H]-	22.5
12-Hydroxystearic Acid	301.273	[M+H]+	30.4
17.alpha.-Ethyl-5.beta.-estrane-3.alpha.,17.beta.-diol	289.252	[M+H]+	29.1
17a-Ethynylestradiol	295.167	[M-H]-	16.3
1-Aminocyclohexanecarboxylic acid	144.101	[M+H]+	3.4
1-Methyl-1H-purine-2,6(3H,7H)-dione	167.055	[M+H]+	15.5
1-Methyl-1H-purine-2,6(3H,7H)-dione	165.042	[M-H]-	15.6
1-Methyl-4-imidazoleacetic Acid	141.065	[M+H]+	2.4
1-Methyluric Acid	183.05	[M+H]+	9.2
2,2'-Methylene-bis(6-tert-butyl-4 methylphenol)	339.234	[M-H]-	32.3
2,8-Quinolinediol	160.041	[M-H]-	18.9
2,8-Quinolinediol	162.054	[M+H]+	18.9
2-Hydroxy Stearic Acid	299.261	[M-H]-	30.4
2-Hydroxy-3-methoxybenzaldehyde	151.027	[M-H]-	10.1
2-Hydroxyhexadecanoic Acid	271.228	[M-H]-	31.6
2-Methoxymethcathinone	194.117	[M+H]+	20.4
2-Methyl-3-ketovaleric acid	129.056	[M-H]-	11.8
2-Oxindole	134.06	[M+H]+	19.8
2-Phenylbutyric acid	165.09	[M+H]+	9.0
2-Phenylglycine	150.043	[M-H]-	14.9
2-Piperidinone	100.076	[M+H]+	14.9
3b-Hydroxy-5-cholenoic acid	373.276	[M-H]-	31.8
3b-Hydroxy-5-cholenoic acid	419.282	[M +FA-H]-	31.8

(Continued)

TABLE 2 Continued

Compound	Precursor Mass	Adduct	Retention time
3-Hydroxydodecanoic acid	215.166	[M-H]-	25.3
3-Indoleacetic acid	176.07	[M+H]+	20.2
3-Nitrotyrosine	227.081	[M+H]+	18.5
3β-Ursodeoxycholic Acid	391.287	[M-H]-	25.3
4-Methyl-5-thiazoleethanol	144.046	[M+H]+	17.4
5-Aminovaleric acid	116.073	[M-H]-	2.4
7(S),17(S)-Dihydroxy-8(E),10(Z),13(Z),15(E),19(Z)-docosapentaenoic acid	345.237	[M+H]+	30.4
7-Methylguanine	166.072	[M+H]+	15.0
9E,11E-Octadecadienoic acid	281.247	[M+H]+	31.0
Adenine	136.061	[M+H]+	15.2
Aminocaproic acid	130.088	[M-H]-	4.5
Arachidonic Acid	303.234	[M-H]-	32.4
Argininosuccinic acid	291.145	[M+H]+	18.5
Azelaic acid	187.099	[M-H]-	9.2
Benzoic acid	121.03	[M-H]-	19.7
Beta-N-Acetylglucosamine	222.097	[M+H]+	2.4
Biocytin	371.191	[M-H]-	22.0
Biotin	245.095	[M+H]+	17.3
Butyric acid	87.046	[M-H]-	3.2
Cholesterol sulfate	465.306	[M-H]-	31.8
cis-4,10,13,16-Docosatetraenoic acid	331.266	[M-H]-	33.7
cis-4,7,10,13,16,19-Docosahexaenoic acid	327.234	[M-H]-	32.1
cis-5,8,11-Eicosatrienoic acid	305.25	[M-H]-	33.2
Citrulline	176.102	[M+H]+	2.2
Citrulline	174.089	[M-H]-	2.2
Curcumin	369.133	[M+H]+	26.3
Delta-Hexanolactone	115.074	[M+H]+	4.7
Deoxyguanosine	268.104	[M]+	15.2
Deoxyguanosine	266.091	[M-H]-	15.2
Deoxyinosine	253.092	[M+H]+	15.0
Deoxyinosine	251.08	[M-H]-	15.0
D-Glutamine	145.063	[M-H]-	2.0
Dimethylglycine	102.057	[M-H]-	1.8
D-Mannose	179.057	[M-H]-	2.1
Dodecanedioic acid	229.146	[M-H]-	23.3
Dodecanedioic acid	251.128	[M +Na-2H]-	23.3

(Continued)

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TABLE 2 Continued

Compound	Precursor Mass	Adduct	Retention time
Dodecanedioic acid	248.185	[M +NH4]+	23.3
Dodecanoic acid	199.171	[M-H]-	29.9
D-Xylitol	151.062	[M-H]-	2.1
Ethyl hydrogen malonate	133.031	[M+H]+	3.4
Geranyl caprylate	303.231	[M+H]+	28.8
Glutaric acid	131.035	[M-H]-	1.8
Glycodeoxycholic Acid	448.308	[M-H]-	24.0
Glycolithocholic Acid	432.313	[M-H]-	27.0
Guanidinosuccinic acid	174.041	[M-H]-	1.8
Guanosine	284.099	[M+H]+	14.9
Hippuric acid	178.056	[M-H]-	15.3
Hydrocinnamic acid	149.062	[M-H]-	21.7
Hyocholic Acid	409.314	[M+H]+	26.2
Hyodeoxycholic acid	391.287	[M-H]-	27.1
Hyodeoxycholic acid	410.326	[M +NH4]+	25.8
Ile-Ile	245.185	[M+H]+	15.3
Indole-6-carboxaldehyde	146.059	[M+H]+	21.3
Indoxyl	132.031	[M-H]-	1.8
Inosine	269.087	[M+H]+	14.8
Inosine	267.075	[M-H]-	14.8
Isoleukotoxin Diol	313.24	[M-H]-	26.2
Kaempferol	285.056	[M-H]-	14.5
L-Alanine	88.041	[M-H]-	2.0
L-Arginine	173.105	[M-H]-	2.2
L-Glutamic acid	148.06	[M+H]+	1.8
L-Glutamic acid	146.047	[M-H]-	1.8
Linoleic acid	279.234	[M-H]-	30.1
L-Isoleucine	132.101	[M+H]+	3.5
Lithocholic acid	375.292	[M-H]-	30.7
L-Leucine	132.101	[M+H]+	4.6
L-Leucine	132.101	[M+H]+	4.6
L-Lysine	145.099	[M-H]-	1.9
L-Methionine	150.058	[M+H]+	3.4
L-Phenylalanine	166.086	[M+H]+	12.1
L-Phenylalanine	164.073	[M-H]-	11.9
L-Proline	116.07	[M+H]+	2.3
L-Proline	114.057	[M-H]-	2.3

(Continued)

TABLE 2 Continued

Compound	Precursor Mass	Adduct	Retention time
L-Tryptophan	203.083	[M-H]-	16.1
L-Tyrosine	182.08	[M+H]+	7.1
L-Tyrosine	180.067	[M-H]-	6.5
L-Valine	118.086	[M+H]+	2.3
Mandelic acid	151.027	[M-H]-	9.4
Methylcysteine	134.048	[M-H]-	15.1
Myristic acid	227.203	[M-H]-	32.0
N-Acetylglutamic acid	190.07	[M+H]+	1.8
N-Acetylglutamic acid	188.057	[M-H]-	1.8
N-Acetyl-L-phenylalanine	208.096	[M+H]+	16.8
N-Alpha-acetyllysine	187.109	[M-H]-	2.4
Nicotinic acid	124.038	[M+H]+	4.4
Nutriacholic Acid	391.284	[M+H]+	25.5
Nutriacholic Acid	389.271	[M-H]-	25.5
Nutriacholic Acid	413.266	[M+Na]+	25.5
Nutriacholic Acid	408.311	[M +NH4]+	25.5
Nα-Acetyl-L-lysine	189.122	[M+H]+	2.4
Oleic acid	281.25	[M-H]-	34.2
Oleic acid	327.255	[M +FA-H]-	34.2
Ornithine	131.083	[M-H]-	2.2
Palmitoylethanolamide	300.289	[M+H]+	30.4
Pantothenic acid	218.104	[M-H]-	7.5
Phenylacetic acid	135.046	[M-H]-	14.4
Phosphocreatine	212.054	[M+H]+	1.7
Pipecolic acid	130.085	[M+H]+	3.2
Piperine	286.143	[M+H]+	26.2
Pregnenolone	395.172	[M-H]-	18.3
Propane-1,2,3-tricarboxylic acid	175.026	[M-H]-	1.6
Propane-1,2,3-tricarboxylic acid	157.015	[M-H2O-H]-	1.6
Propane-1,2,3-tricarboxylic acid	177.038	[M+H]+	1.7
Pseudouridine	243.063	[M-H]-	4.7
Pyrrolidonecarboxylic acid	130.049	[M+H]+	1.8
Quinolin-2-ol	144.046	[M-H]-	21.3
Sebacic acid	201.114	[M-H]-	20.9
Sodium glycochenodeoxycholate	450.32	[M+H]+	24.1

(Continued)

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TABLE 2 Continued

Compound	Precursor Mass	Adduct	Retention time
Sphinganine	302.305	[M+H]+	27.6
Suberic acid	173.083	[M-H]-	5.7
Tetradecanedioic acid	257.177	[M-H]-	25.1
Tetraethylene glycol	195.122	[M+H]+	15.5
Theobromine	181.071	[M+H]+	16.1
Thymidine	243.096	[M+H]+	15.5
Thymidine	241.084	[M-H]-	15.5
Thymine	127.049	[M+H]+	11.6
Thymine	125.036	[M-H]-	11.4
Aconitic acid	172.995	[M-H]-	1.9
Tyramine	138.091	[M+H]+	12.8
Uracil	113.034	[M+H]+	4.8
Uracil	111.02	[M-H]-	4.8
Uridine	245.075	[M+H]+	10.3
Urocanic acid	139.05	[M+H]+	3.2
Ursodeoxycholic acid	410.327	[M+H]+	27.2
Linoleic acid	325.239	[M +FA-H]-	32.8
2-Hydroxyadenosine	282.086	[M-H]-	14.9
Hederagenin	471.349	[M-H]-	27.8
Kaurenoic acid	301.203	[M-H]-	23.0
6-Hydroxypurine	135.032	[M-H]-	9.5
Betulinic acid	455.354	[M-H]-	33.1
Asiatic acid	487.344	[M-H]-	24.9
Adenosine	268.104	[M+H]+	15.8
Theophylline	181.071	[M+H]+	16.8
Peiminine	430.331	[M+H]+	21.2
Peimine	432.347	[M+H]+	22.5
Ginkgolic Acid	345.244	[M-H]-	34.2
Indirubin	261.078	[M-H]-	21.2

metabolites, respectively). For instance, the levels of L- Leucine, L-tryptophan, L-Phenylalanine are higher in colorectal cancer than in gastric cancer. Moreover, considering extremely precise statistical condition, the statistically significant difference ($p<0.05$ and FC – 2) between analysed groups were found in case of Deoxyguanosine,m/z 266.091,[M-H]-, Uridine,m/z 245.075,[M+H]+, Deoxyguanosine, m/z 268.104,[M]+, 3-Indoleacetic acid,m/z 176.07,[M+H]+, Indoxyl,m/z 132.031,[M-H]-, L-Phenylalanine,m/z 164.073,[M-H]-, L-Methionine,m/z 150.058,[M+NH4]+, Ethyl hydrogen malonate,m/z 133.031,[M+H]+.

4 Discussion

Molecular diagnosis of cancer based on metabolomics can be promising in near future (Cheung et al., 2019). Metabolomic data may be used as biomarkers allowing to detect several cancers, such as oesophageal, gastric, pancreatic, bladder, lung, thyroid, and others (Wang et al., 2022; Yang et al., 2022). Different metabolites/metabolic pathways/metabolism may provide a signature which is specific for diseases/conditions. For instance, in a study by Yang et al., it was noted that glycophospholipid metabolism is related to both tumorigenesis and progression of oesophageal squamous cell carcinoma (ESCC) and that may be therapeutic target in ESCC progression (Yang et al., 2022). Hang et al. reported that untargeted plasma metabolomics can serve as a potential risk prediction of hepatocellular carcinoma (Hang et al., 2022). The aspects of untargeted metabolomics can be also useful in case of other digestive cancers. Plasma metabolomic signatures in precancerous gastric lesions progressing to cancer were identified in a study by Huang et al. (2021). Notably, six plasma metabolites were related to the both overall risk of gastric cancer and early gastric cancer whereas three of these metabolites, such as α-linolenic acid, linoleic acid, palmitic acid were associated with the prediction of risk of gastric lesion progression and early gastric cancer. In another study untargeted metabolome was also analysed in case of gastric cancer (Yu et al., 2021). Serum samples were taken from patients with chronic gastritis/gastric cancer. It was shown that lipid metabolism may affect the development of chronic gastritis to gastric cancer; moreover, hexadecasphinganine, linoleamide, and N-Hydroxy arachidonoyl amine were assessed as diagnostic markers for both chronic gastritis and gastric cancer (Yu et al., 2021). In the current study, we also investigated gut metabolome in cancer patients, but from stool samples. The overall insights showed that higher level of analysed metabolites was mostly noted in colorectal cancer patients compared to gastric cancer patients. For instance, in case of indole-3-acetic acid and tryptophan, the levels are higher in colorectal cancer than in gastric cancer. Indole-3-acetic acid is a tryptophan metabolite produced by gut microbiota according to the following pathway in intestinal epithelial cells: (1) ingested dietary protein, (2) tryptophan, (3) intestinal microbiota, (4) indole-3-acetic acid (Seo and Wargo, 2023; Tomii et al., 2023). This result can be associated with different overall characteristics of gut microbiota in particular types of cancer, i.e. gastric and colorectal cancer. In the current study, it was observed that the level of L-phenylalanine was also higher in colorectal cancer compared to gastric cancer. In previously published data it was reported that some amino-acids including phenylalanine may be considered as a biomarkers in colorectal cancer patients (Hashim et al., 2019). Recently, Chen et al. (2022) presented that gut microbiome-associated serum metabolites can be used to detect colorectal cancer (Chen et al., 2022).

In the current study, it was observed higher grouping in case of gastric cancer in comparison to colorectal cancer, which confirms more homogeneous metabolic profile in gastric cancer patients. Moreover, on Scores Plot analysis there were created two subgroups in case of gastric cancer, such as Sample_1G and Sample_3G,

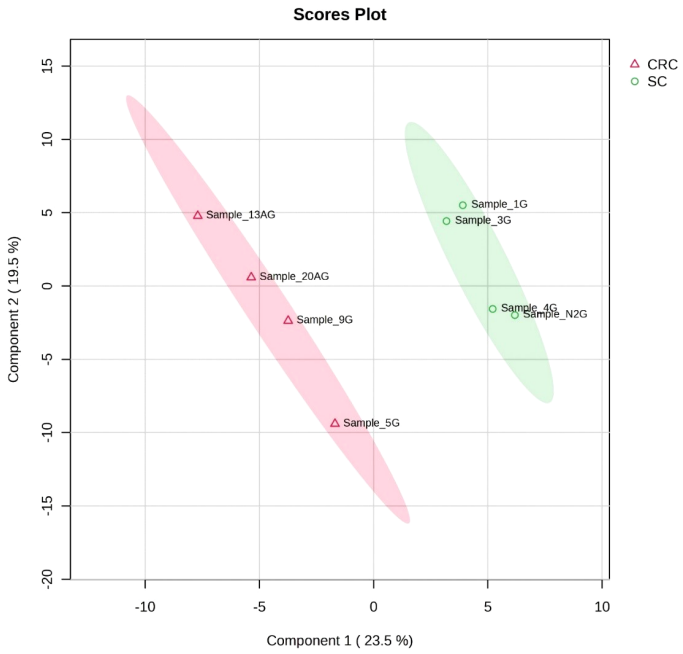

FIGURE 1
Figure PLS_DA – the comparing analysed groups – gastric cancer [SC] and colorectal cancer [CRC]. SC, stomach cancer; CRC, colorectal cancer.

Sample_4G and Sample_N2G. It may suggest similar characteristics in these two subgroups. Notably, the first subgroup created by Sample_1G and Sample_3G regards gastric cancer with similar TNM assessment (i.e. Sample_1G: T3N0M0, Sample_3G: T3N1M0). TNM tool is used to assess as follows: T – tumour, N – nodes (involvement of lymph nodes), and M – metastasis (Piñeros et al., 2019). The link between untargeted metabolomics in gastric cancer and both local and distal metastasis may exist, however it requires confirmation with larger sample size. In the current study, it was demonstrated that the metabolic profile of colorectal cancer patients is varied. It can be associated with different localisation of tumours – there are four cases analysed, i.e. sigmoid-rectal cancer, sigmoid colon cancer, rectal cancer, and ascending colon cancer.

5 Limitations and future directions

This study has been some limitations. First of all, the study was conducted with relatively small sample size. However, it is treated as preliminary results to present basic characteristics of untargeted metabolomics in gastric and colorectal cancer patients as well as to find the directions prior to the next study (KB/428-526/2023, Medical

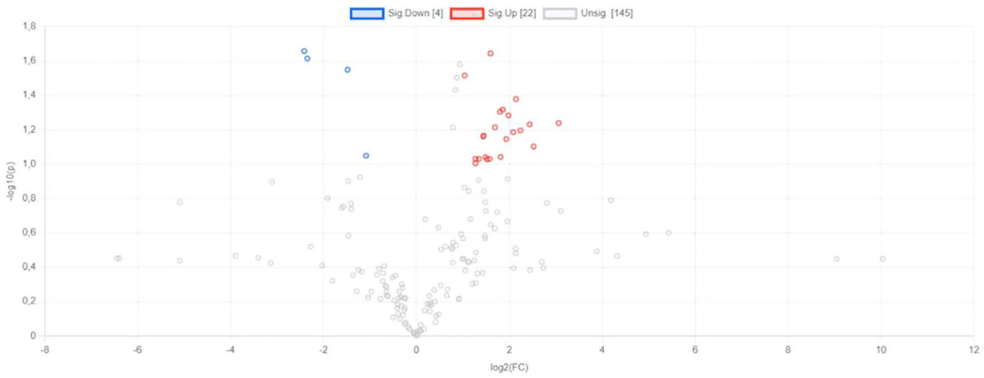

FIGURE 2
Volcano plot presenting metabolites varied groups of gastric cancer [SC] and colorectal cancer [CRC]. Significance parameters: p<0.1; FC > 2.

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FIGURE 3
The levels of metabolites among SC and CRC.

University of Gdansk, Gdansk, Poland) in which we analyse the impact of anti-cancer treatment regarding chemotherapy and radiotherapy on untargeted metabolomics aspects. This project is currently ongoing in cooperation with multi-disciplinary team of both oncologists and oncological surgeons. It is also recommended to investigate untargeted metabolomics among patients with similar both stage and grade of the cancers, nevertheless it might be challenging to collect stool samples with larger sample size. However, it would provide promising strategy to be included in clinical aspects. Metabolomics-based biomarkers might provide earlier detection of cancers allowing to complete resection of tumour. Moreover, metabolomics-related techniques can be attractive due to the fact that they are non-invasive and relatively low cost.

6 Conclusions

The aspect of untargeted metabolomics is a new area, which can be considered in oncology. Notably, the results presented in the current study were obtained prior to the introduction of anti-cancer management, such as surgical treatment. The overall insights into untargeted metabolomics showed that most often higher levels of

analysed metabolites were detected in colorectal cancer patients compared to gastric cancer patients. It can be related to the different activity of gut microbiome in particular types of gastrointestinal cancer. Additionally, it was observed a higher grouping in case of gastric cancer comparing to colorectal cancer, which confirms more homogeneous metabolic profile in this cancer. The link between untargeted metabolomics in gastric cancer and both local and distal metastasis may exist, but it requires confirmation in further multi-centre studies regarding larger sample size.

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD051921.

Ethics statement

The studies involving humans were approved by Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KK-S: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. DM: Writing – original draft. DS: Writing – review & editing, Software, Formal analysis. JM: Writing – review & editing. BS: Writing – review & editing, Data curation. PU: Writing – review & editing, Data curation. KP: Writing – review & editing. ES: Writing – review & editing, Supervision. KS-Z: Writing – review & editing, Supervision. LK: Writing – review & editing, Supervision, Conceptualization.

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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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Amyloid, Crohn's disease, and Alzheimer's disease - are they linked?

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Crohn's disease (CD) is a chronic inflammatory disease that most frequently affects part of the distal ileum, but it may affect any part of the gastrointestinal tract. CD may also be related to systemic inflammation and extraintestinal manifestations. Alzheimer's disease (AD) is the most common neurodegenerative disease, gradually worsening behavioral and cognitive functions. Despite the meaningful progress, both diseases are still incurable and have a not fully explained, heterogeneous pathomechanism that includes immunological, microbiological, genetic, and environmental factors. Recently, emerging evidence indicates that chronic inflammatory condition corresponds to an increased risk of neurodegenerative diseases, and intestinal inflammation, including CD, increases the risk of AD. Even though it is now known that CD increases the risk of AD, the exact pathways connecting these two seemingly unrelated diseases remain still unclear. One of the key postulates is the gut-brain axis. There is increasing evidence that the gut microbiota with its proteins, DNA, and metabolites influence several processes related to the etiology of AD, including β-amyloid abnormality, Tau phosphorylation, and neuroinflammation. Considering the role of microbiota in both CD and AD pathology, in this review, we want to shed light on bacterial amyloids and their potential to influence cerebral amyloid aggregation and neuroinflammation and provide an overview of the current literature on amyloids as a potential linker between AD and CD.

KEYWORDS

inflammatory bowel disease, Crohn's disease, neurodegenerative disease, Alzheimer's disease, β-amyloid (Aβ), curli, microbiota, gut-microbial-brain axis

1 Crohn's disease

1.1 Crohn's disease – general information

Inflammatory bowel disease (IBD) includes ulcerative colitis (UC) and Crohn's disease (CD). The two disease differ in their symptoms, radiographic appearance and histological changes. In this review, we focused on CD, as the histological changes in its course involve the entire thickness of the intestinal wall, characterized by localized lymphocytic

infiltration, granulomas and fibrosis. In contrast to UC, in which lesions are limited to superficial inflammation with the presence of crypt abscesses (Le Berre et al., 2020).

CD is characterized by chronic, transmural, and mostly granulomatous inflammation of the gastrointestinal tract. CD usually affects the distal ileum, cecum, or colon but can affect any part of the gastrointestinal tract. Typically, the CD has an intermittent course with periods of acute flares and remissions. Clinical symptoms vary depending on the severity and section of the gastrointestinal tract involved, ranging from mild to severe. The main symptoms are abdominal pain, diarrhea, low-grade fever, fatigue, unintended weight loss, and malnutrition. Rectal bleeding during CD is less common but can occur when the distal colon is involved. As the disease progresses, the chronic inflammatory process of the intestines disturbs their function, intestinal complications appear, and then – also extraintestinal symptoms (Guan, 2019; Petagna et al., 2020).

Despite comprehensive studies, the exact cause of CD is still not fully understood. Current consensus considers a multifactorial and heterogeneous pathogenesis of CD. It is believed that a complex interaction between genetic, environmental, and microbial factors may lead to dysregulated and enhanced immune response (Sobieszczańska et al., 2019; Ranasinghe; and Hsu, 2023).

1.2 Immunological factors in the pathogenesis of Crohn's disease

An unrestrainable immune response against luminal antigens, leading to tissue inflammation, is an indisputable factor in the pathogenesis of CD. During chronic inflammation, immune cells, including $CD4^+$ and $CD8^+$ T helper (Th) cells, infiltrate and accumulate in the gastrointestinal tract of CD patients. Therefore, dysregulation of various components of the immune system is invariably found in the mucosa of CD patients (Petagna et al., 2020). One of the most expressed alterations that mediate abnormal immune response and subsequent inflammation in the intestinal mucosa include increasing migration, proliferation, and activation of Th cells, especially Th1 and Th17 cells. As a result, there is an upregulation of the synthesis and release of various proinflammatory mediators. Numerous studies have shown increased amounts of mRNA for TNF-α, IL-2, IL-6, IL-8, IL-12, IL-17, IL-21, IL-22, IL-23, CCL20, and chemerin, and increased concentrations of these markers in serum and intestinal mucosa biopsies from CD patients (Guan, 2019).

The role of Th17 cell subpopulation in the pathogenesis of CD is increasingly emphasized. Th17 cells are controlled by Treg cells, which inhibit the former's excessive immune response, they must remain in dynamic balance. When it is lost and shifts towards the proinflammatory Th17 cells, which constantly accumulate, proinflammatory cytokines are continuously synthesized and released. This exceeds the immune tolerance of Treg and leads to persistent mucosal inflammation (Szandruk-Bender et al., 2022a; Chen et al., 2023; Szandruk-Bender et al., 2023). Intestinal Tregs have T-cell receptors (TCRs) specific for intestinal antigens. They are essential for suppressing the immune response against the gut microbiota (Choi et al., 2022). In addition, Treg stimulate the development of intestinal stem cells (ISCs), ensuring the integrity of the intestinal epithelium and maintaining intestinal homeostasis (Harada et al., 2022). Treg can enter the central nervous system via three routes, through: (i) the blood-brain barrier (BBB) (into the perivascular space); (ii) the subarachnoid space in the meninges, and (iii) the choroid plexus (into the cerebrospinal fluid). Treg accumulating in damaged areas infiltrate the brain and, being able to interact with microglia, exacerbate inflammation within the nervous system contributing to the development of neurodegenerative diseases (Ma et al., 2024).

Both Th17 and Treg cells are differentiated from naive CD4+ T cells under the influence of relevant transcription factors and microenvironmental cues. Differentiation of Th17 cells is driven by retinoic acid related orphan receptor γt (RORγt) and signal transducer and activator of transcription 3 (STAT3) in the presence of proinflammatory cytokines, especially IL-6 and IL-23, while Treg cells – by forkhead box protein 3 (Foxp3) transcription factor (Szandruk-Bender et al., 2022a; Szandruk-bender et al., 2023). Importantlythe gut microbiota directly or through metabolites indirectly can regulate Th17 and Treg cell differentiation and, thus, the progression of CD (Chen et al., 2023).

Intestine inflammatory response is also determined through the remodeling of the extracellular matrix by the action of upregulated metalloproteins, e.g., MMP1, MMP3, MMP9, and the overexpression of such adhesion. Its overexpression enables increased migration of lymphocytes to the healthy gastrointestinal tract and sites of inflammation (Petagna et al., 2020). Disturbances in the apoptosis process also contribute to CD pathogenesis. Excessively expressed apoptosis of epithelial cells leads to their increased elimination and damage to the intestinal barrier, that said reduced programmed death of inflammatory cellsresults in their accumulation in the wall of the gastrointestinal tract and maintenance of inflammatory process (Guan, 2019).

1.3 Genetic factors in the pathogenesis of Crohn's disease

There is a growing body of evidence that genetic factors influence the risk of developing CD increasingly confirmed susceptibility loci for CD (Graham and Xavier, 2020). The first gene whose mutations were associated with this disease is nucleotide-binding oligomerization domain 2 (NOD2). NOD2 mutations occur in around one-third of the CD patients. The 1007fs mutation in this gene manifests itself in a more severe course of the disease, and the R702W and G908R mutations lead to an intensified response from proinflammatory cytokines and the induction of inflammation (Guan, 2019). In addition to *NOD2*, genes associated with the risk of developing CD are related to i) the innate pattern recognition receptors, e.g., caspase activating recruitment domain 15 (CARD15), organic cation transporters novel (OCTN), toll-like receptors (TLRs); ii) the integrity of the intestinal barrier, e.g., (DLG5, IBD5); iii) autophagy, microbial detection, and effector pathways, e.g., autophagy-related gene 16L1 (ATG16L1), immunity-related GTPase M (IRGM), leucine-

rich repeat kinase 2 (LRRK2); and iv) lymphocyte differentiation, e.g., interleukin-23 receptor (IL23R), STAT3, ROR, TNFSF15, Janus kinase 2 (JAK2), chemokine receptor 6 (CCR6) (Tsianos et al., 2012; Graham and Xavier, 2020). Moreover, many genes appear to be not only susceptibility genes but also influence the prognosis (NOD2, IL23R, ATG16L1, DLG5, IRGM), disease activity (NOD2), location (ATG16L1, NOD2, IL-10, STAT3, TLRs) of CD, as well as the presence of intestinal and extraintestinal manifestations in the course of CD (TLRs, CARD15, NOD2, IL-6, IL-10, IRGM, STAT3) (Tsianos et al., 2012). Even though many people carry loci that increase the risk of CD, only a small proportion of the population develops CD. The occurrence of the disease requires exposure to environmental factors and disruption of the interaction between the intestinal microbiota and the immune system of the intestinal mucosa (Graham and Xavier, 2020).

1.4 Environmental factors in the pathogenesis of Crohn's disease

Prenatal life: Development of CD in children is influenced by the mother's age (>35 years old) and smoking during pregnancy (Roberts et al., 2011). Increased risk for IBD, including CD, also occurs after exposure to antibiotics during the 3rd trimester of pregnancy (Örtqvist et al., 2019).

Perinatal factors: The studies on these factors looked at prematurity, month of birth, birth weight, and Apgar score obtained. It was shown that only an Apgar score of.7 at one minute was associated with a higher probability of CD (Canova et al., 2020).

Neonatal and infancy period: Many case-control studies have shown an association of breastfeeding with later incidence of CD (Gruber et al., 1996; Basson et al., 2014). One study reported that CD patients lived in smaller households and had lower numbers of siblings (Bernstein et al., 2006). In addition, the study performed by Hampe et al. additionally showed that lower birth position is a possible indicator of increased exposure to infections, resulting in a higher risk of CD (Hampe et al., 2003). The place of living is also important. Numerous studies have shown that people who spent their childhood in the countryside have a much lower probability of developing CD in adulthood (Radon et al., 2007; Benchimol et al., 2017). Other factors related to childhood include the level of hygiene. Indeed, it has been proven a directly proportional relationship that the higher the level of hygiene, the greater the likelihood of CD (Amre et al., 2006; Lashner and Loftus, 2006).

Specialist risk factors in adult life: *1) Smoking*. Studies have shown that active smokers and ex-smokers have a significantly increased risk of CD compared to people who have never smoked (Lakatos et al., 2007; Berkowitz et al., 2018). It has been suggested that they are contributed to by i) the T-cell-nicotine connection (released immune messengers lead to intestinal inflammation) (Razani-Boroujerdi et al., 2007); ii) modifications of mucus production by the gastric mucosa (Li et al., 2014) and intestines (Allais et al., 2016); iii) disorders of the intestinal mucosal repair (Li et al., 2014) and iv) disruption of blood flow to the mucosa of the gastrointestinal tract (Hunsballe et al., 2001). *2) Supplementation of chemical substances*. Many studies have confirmed the impact of using antibiotics (Card et al., 2004; Hildebrand et al., 2008), and nonsteroidal anti-inflammatory drugs (Felder et al., 2000) on the development of CD. These compounds probably cause damage to the mucosa of the gastrointestinal tract, consequently disrupting the formation of the its microbiome. Moreover, the use of oral hormone therapy has been shown to be positively associated with the risk of CD, it has been proven that it is independent of the dose of estrogen used (Cornish et al., 2008). *3) Diet*. A high consumption of animal protein and long-chain omega-6 polyunsaturated fatty acids has been associated with an increased risk of CD (Shoda et al., 1996). It is due to the fact that omega-6 fatty acids are indirectly involved in the production leukotrienes and prostaglandins. *4) Other*. Exacerbation of symptoms in CD is also influenced by strong stress and sleep disturbances, which have been observed during periods of recurrence (Anthony Sofia et al., 2020; de Dios-Duarte et al., 2022).

1.5 Microbial factors in the pathogenesis of Crohn's disease

In fact, many studies have shown that one of the most likely factors in CD is an imbalance in the gut microbiota. These changes contribute to the impairment of intestinal innate immunity carried out by neutrophils, monocytes, macrophages, dendritic cells, innate lymphoid cells, and natural killer (NK) cells, representatives of non-specific first-line defense. Furthermore, studies have shown that in the situation of impaired intestinal microbiota, intestinal CX3C chemokine receptor 1 high ($CX3CR1^{high}$) macrophages differentiate into pro-inflammatory effector cells, acquiring the ability to present antigens to lymphocytes and becoming a critical predisposing factor in the development of IBD, including CD (Zigmond et al., 2014).

Explicit experimental evidence was provided by the studies of Schaubeck et al. (2016). In their study, they used a transplant of CD-associated microbiota that transferred features of colitis into the recipient's body. This confirmed the direct causal role of intestinal bacterial dysbiosis in the development of chronic enterocolitis.

One theory regarding the etiology of CD points to the involvement of completely different microorganisms in the initiation of the disease than in its development. Types that represent a small percentage of the gastrointestinal microflora appear to be involved in the initiation. These include: *Proteobacteria* (e.g., *E. coli*, *Helicobacter* spp.) (Arumugam et al., 2011; Carrière et al., 2014), *Actinobacteria (e.g., Mycobacterium avium* subsp. *paratuberculosis)* (McNees et al., 2015; Elmagzoub et al., 2022), and also viruses (e.g., norovirus, polyomavirus, anellovirus, herpesvirus, adenovirus, sapovirus, rotavirus) (Haag et al., 2015; Lecuit and Eloit, 2017; Cao et al., 2022; Matsuzawa-Ishimoto et al., 2022; Dehghani et al., 2023; Ding et al., 2023) and fungi (e.g., *Candida* spp.) (Šašala et al., 2020; Di Martino et al., 2022). In contrast, the role in maintaining inflammation is mostly attributed to species of the genus *Firmicutes* and *Bacteroides*, which account for >90% of the total human intestinal microbiota (Frank et al., 2007; Sokol et al., 2008; Mondot et al., 2011). Studies have shown that sustained inflammation is associated with a reduction in the amount of *Faecalibacterium prausnitzii* and *Bacteroides fragilis* and an increase in *E. coli* and mucolytic bacteria: i.e., *Ruminococcus*

gnavus and *Ruminococcus torques* (Darfeuille-Michaud et al., 2004; Sokol et al., 2008; Png et al., 2010). Due to the fact that no microorganism has been isolated that is present in all patients with CD, the direct role of microorganisms in the progression of the disease has not been determined. Therefore, the contribution of dysbiosis is highly probable. The conducted studies confirm that dysbiosis is a cause and also an effect of CD. In the early stages of the disease, a significantly reduced diversity of bacteria belonging to the intestinal microbiota is observed The aggressive groups (i.e., *Proteobacteria* spp., *Fusobacterium* spp. and *R. qnavus*) are dominant, developing at the expense of protective groups (i.e., *Lachnospiraceae* spp., *Bifidobacterium* spp., *Roseburia* spp. and *Sutterella* spp.) (Sartor and Wu, 2017).

Confirmed decreased numbers of bacteria from the *Bacteroidales* family contribute to lower control of our immune system during infection. This is because this family is the main producer of mucins, the glycoproteins that make up mucus, which plays a protective and uptake role against pathogens (Szewczyk et al., 2019). As a result, the gut becomes more susceptible to infection.

Furthermore, disorders in the intestinal microbiota lead to increased levels of zonulin, a protein responsible for controlling the permeability of the intestinal barrier (Sturgeon and Fasano, 2016; Ohlsson et al., 2017). Increased amounts of this protein lead to disturbances in the integrity of the tight junctions between enterocytes, consequently contributing to the leaky gut syndrome. Bacteria of the *Ruminococcaceae* family, whose increasing amounts have been confirmed in the progression of CD, are also involved in this process. These bacteria produce secondary bile acids, promoting the overproduction of reactive oxygen species (ROS), thereby reducing the integrity of the intestinal barrier (Hang et al., 2022).

An increase in the number of *Ruminococcus* spp. (i.e., *R. gnavus, R. torques*) may also contribute to an abnormal response from the immune system. Indeed, these bacteria produce short-chain fatty acid (SCFA), thus playing a huge role in the body's immune system response, including regulating the production of cytokines (Igudesman et al., 2022).

On the other hand, *B. fragilis* whose decline has been documented in the progression of CD, secretes lipopolysaccharide (LPS), which activates the transcription nuclear factor kappa light chain enhancer of activated B cells (NF-κB). This factor plays a significant role in immune and inflammatory processes, as it regulates the expression of many genes, including those associated with the production of cytokines, acute-phase proteins, collagenases, stromilysins, and matrix-degrading enzymes. Moreover, it demonstrates the ability to inhibit apoptosis, induce proliferation, and enhance the angiogenesis process, suggesting its involvement in the processes of oncogenesis and tumor progression (Kou et al., 2020). Furthermore, NK-κB is responsible for inducing the transcription of microRNA (i.e., miRNA-9, miRNA-34, miRNA-125b, miRNA-146a, miRNA-155) with proinflammatory effects. Additionally, it activates miRNA-34a, which inhibits the expression of the triggering receptor expressed in myeloid/microglial cells (TREM) (Bhattacharjee et al., 2016; Lukiw et al., 2021). Accordingly, this contributes to the disruption of the microglia's anti-phagocytic abilities, promoting neuroinflammatory diseases. Therefore, the reduction in the abundance of *B. fragilis*, and consequently a decrease in the LPS produced by these bacteria, contributes to the development of inflammatory and neoplastic diseases.

Moreover, bacteria belonging to the gastrointestinal microbiome adapt to participate in diseases with coexisting genetic, environmental, and immunological conditions. They do this not only by influencing mucus components and tight junctions but also by producing adhesins. One of these are curli fimbriae, which exhibit the biochemical and structural properties of β-amyloid (Aβ) (Sobieszczańska et al., 2019). This protein is a constituent of various healthy tissues, including the heart, muscle, liver, kidney, and brain. However, under favorable conditions, it can also become pathogenic in these tissues (Sonthalia et al., 2016; Martínez-Naharro et al., 2018; Pinto et al., 2021; Deng et al., 2022; Gurung and Li, 2022). Furthermore, previous studies have confirmed that human amyloid and bacterial amyloid share many common features, undoubtedly warranting a more in-depth analysis (Das et al., 2022; Bessho et al., 2023).

It is evident that dysbiosis within the gastrointestinal tract opens the gates of the intestines to toxins and proinflammatory cytokines, thereby likely increasing the probability of inflammatory diseases (including CD), and, consequently, the development of neurodegenerative diseases, i.e., Alzheimer's disease (AD).

However, it is a known fact that the functions attributed to the intestine, i.e., immune activation, intestinal permeability, its reflexes, and enteroendocrine transmission, are controlled by the gut-brain axis (GBA) (Carabotti et al., 2015). GBA plays a significant role in shaping both the structure and development of the central nervous system (CNS) (Foster et al., 2017). This communication between the gastrointestinal tract and the CNS occurs bidirectionally: through indirect and direct pathways, involving neuronal, humoral, and immunologic paths (Montiel-Castro et al., 2013). Numerous studies conducted in recent years have shown that the role of gut microbiota goes far beyond functions related to the digestive system. It influences, among other things, the immune system, carbohydrate metabolism, bone health, and also plays a crucial role in connection with GBA (Anglin et al., 2015). Experiments performed on animals have provided valuable insights into this topic. Indeed, it has been demonstrated that intestinal colonization is essential for the proper development of the CNS. On the other hand, the absence of gut microbiota in the studied animals clearly affected the disturbance of neurotransmitter expression and activity, thereby disrupting the functioning of the CNS. This manifested as memory problems, the development of anxiety, and depressive disorders (Carabotti et al., 2015). The enteric nervous system and the brain are in constant communication, and their interaction is made possible through the gut-microbiota-brain axis (GMBA).

2 Properties of amyloid protein

Amyloids are a diverse group containing many different proteins, which have in common β-sheet structures that aggregate into fibers. Their assembly starts from a monomer that oligomerizes and assembles into fibrils, which then organize into sheets (Bissig et al., 2016), are shown on Figure 1.

The AmyloGraph is comprehensive database highlighting interactions between 46 amyloid proteins or peptides (Burdukiewicz et al., 2023). According to the collected data, Aβ can alter fibrillization speed (faster/slower aggregation) on both the same or another amyloid which can also result in the heterogenous fibers. This can have positive and negative effects on the human body. Aβ contributes in various disease and important pathways, and understanding these interactions may be helpful in the prevention and management of them. Atrial natriuretic peptide (ANP) through a process called 'cross-seeding' inhibits Aβ aggregation (Tang et al., 2023). Gelsolin responsible for Finnish type of familial amyloidosis just like ANP inhibits the fibrilization of Aβ (Maury, 1991; Ray et al., 2000). In Table 1 are presented several protein capable of amyloid formation, diseases caused by them and its functions. Some of them are neurodegenerative diseases, e.g., Parkinson's disease (PD), AD and prion disease, e.g., Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI) thus the concept that tumors are prion like disease is reflected: S100A9, p53, Beta-2-microglobulin (B2M) amyloids are involved in numerous tumors (Li et al., 2022). Moreover, Aβ can be both positive and negative for our health, yet there is still vast advantage for harmful aspects.

3 Amyloids in neurodegenerative disorders

Neurodegenerative disorders (ND) affect millions of people in the world and seem to become one of the greatest global health problem (Van Schependom and D'haeseleer, 2023). Classification of ND can be based on anatomical, cellular ground or according to type of amyloid involved (Kovacs, 2016; Kovacs, 2018) (Figure 2). Anatomical classification emphasizes the affected regions in the neural system and, as a result of this localization, the clinical

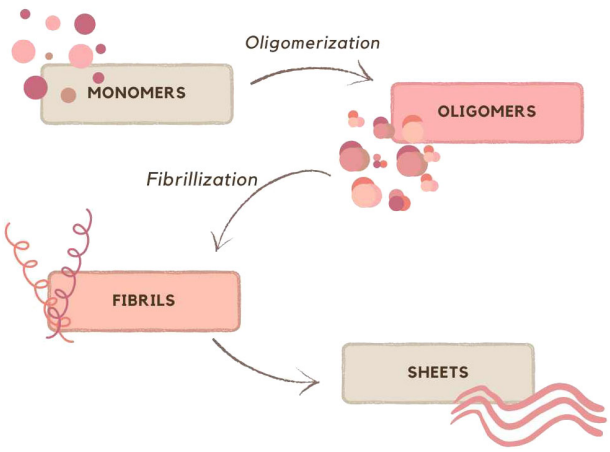

FIGURE 1
Schematic representation of formation sheets; based on (Bissig et al., 2016).

TABLE 1 Contribution of amyloid protein to disease pathogenesis.

Protein	Disease	Function	Source
α-synuclein	Parkinson's disease	intracellular and synaptic vesicle trafficking	(Villar-Piqué et al., 2016; Mehra et al., 2019)
Aβ	Alzheimer's disease	neurite growth, neuronal adhesion	(Baumkötter et al., 2014; Trejo-Lopez et al., 2019)
Apolipoprotein A-I	Hereditary AApoAI	formation of HDL, lipid transport	(Traynor et al., 2013; Frankel et al., 2022)
Cystatin C	HCCAA	inhibitor of cysteine proteinases	(Osk Snorradottir et al., 2015; Ding et al., 2022)
IAPP	Diabetes type II	regulator of energy metabolism	(Wiltzius et al., 2009; Hay et al., 2015)
Insulin	Insulin amyloidosis	lowers glucose level, anabolic hormone	(Tokarz et al., 2018; Kano, 2022)
Lysozyme	OTA	bacteriolytic function	(Pepys et al., 1993; Wu et al., 2019)
Pmel 17	NN	melanosome morphogenesis, pigmentation	(Bissig et al., 2016)
proSP-C	chILD	lowers surface tension in alveolars	(Griese et al., 2016; Barriga et al., 2021)
PrP	CJD, FFI, GSD, HDL1	neuronal development and synaptic plasticity	(Taylor et al., 2009)
S100A9	tumor development	Ca^{2+};Zn^{2+} binding protein	(Marinković et al., 2020)
Serum amyloid A	PCOS	acute-phase response	(Sun and Ye, 2016; Liu et al., 2022)
p53	tumors, cancer, LFS	tumor suppressor in many tumor types	(Li et al., 2022)
B2M	breast cancer, RCC	tumor-promoting and tumor-suppressing	(Wang et al., 2023)
Tau	FTD	promotes microtubule assembly and stability	(Yoshida and Goedert, 2012)
TDP-43	ALS	RNA-binding protein	(Bhardwaj et al., 2013; Yu et al., 2020)
Transthyretin	ATTR-CM	thyroid hormone-binding protein.	(Ruberg et al., 2019)

Aβ, β-amyloid; IAPP, islet amyloid polypeptide; Pmel 17, premelanosome protein 17; proSP-C, prosurfactant protein C; PrP, prion protein; S100A9, S100 calcium-binding; protein A9; p53, regulatory protein; B2M, beta-2- microglobulin; TDP-43, TAR; DNA-binding protein 43; AApoAI, Apolipoprotein AI-derived amyloidosis; HCCAA, Hereditary Cystatin C Amyloid Angiopathy; OTA, Ostertag-type amyloidosis; NN, not named; chILD, children's interstitial lung disease; CJD, Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; GSD, Gerstmann-Straussler disease; HDL1, Huntington disease-like type 1; PCOS, polycystic ovary syndrome; LFS, Li-Fraumeni syndrome; RCC, renal cell carcinoma; FTD, Frontotemporal dementia; ALS, Amyotrophic lateral sclerosis; ATTR-CM, Transthyretin Amyloid Cardiomyopathy; HDL, high-density lipoprotein

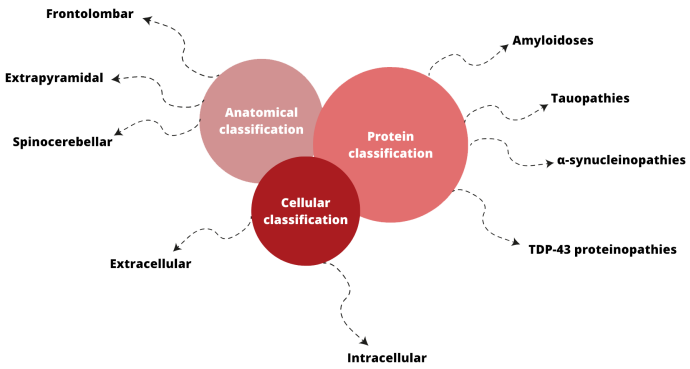

FIGURE 2
Classifications of neurodegenerative disorders; based on (Kovacs, 2016; Kovacs, 2018; Sahoo et al., 2022).

manifestations. On the other hand, the cellular classification focuses on molecular pathology and distinguishes whether amyloid deposits accumulate intracellularly or extracellularly (Kovacs, 2016).

Although exact causes of these diseases are still unknown, their pathomechanism is associated with misprocessing of proteins that aggregate and accumulate in neural tissue (Wolfe and Cyr, 2011; Wells et al., 2021). The factor that is responsible for this proteostasis dysfunction is unidentified, yet there are theories about possible processes which can lead to abnormal protein aggregation, for example oxidative stress, mitochondrial disfunction or neuroinflammation (Bonafede and Mariotti, 2017; Alexander, 2004). Although the pathomechanism of many of these diseases has not yet been fully clarified, some of them have been the subject of many studies aimed at explaining them. These include PD, amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), the pathomechanism of which is briefly discussed below.

Parkinson's disease, second most common neurodegenerative disorder, develops when α-synuclein forms intracellular aggregates, Lewy's bodies, in dopaminergic neurons of the *substantia nigra* (Sveinbjornsdottir, 2016; Kouli et al., 2018; Alexander, 2004). It leads to loss of neurons and decreased levels of dopamine that is responsible for clinical symptoms like bradykinesia, rigidity, tremor, and balance problems (Sveinbjornsdottir, 2016; Kouli et al., 2018).

Among neurodegenerative diseases there are also motor neuron diseases that affect motoneurons and cause muscle paralysis (Motor neuron diseases; Bonafede and Mariotti, 2017). Amyotrophic lateral sclerosis is the most common out of these diseases though new evidence reveal that ALS is a multisystem disorder (Mishra et al., 2020; Matrone, 2023). Not only it attacks both upper and lower motor neurons but also non-motor structures what results in fronto-temporal dementia (FTD) (Mishra et al., 2020; Mahoney et al., 2021). In conclusion, ALS cause progressive muscle paralysis and behavioral, language, cognition changes (Mishra et al., 2020; Mahoney et al., 2021). The factor that is considered to play a role in the pathogenesis of ALS is mutated TAR DNA binding protein 43 (TDP-43) (Yu et al., 2020) (Figure 3).

However, TDP–43 may not be the only one amyloid protein that is involved in ALS pathogenesis. According to Bryson et al. onset of ALS symptoms coincide with increase of Aβ and amyloid precursor

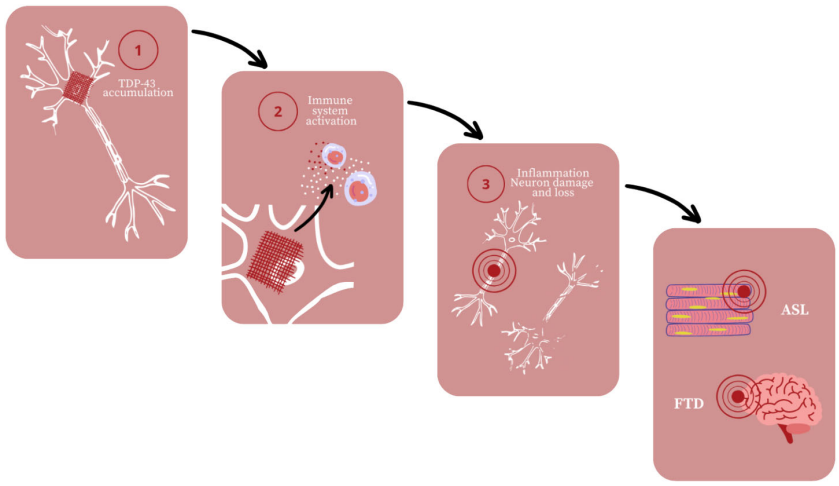

FIGURE 3
Amyotrophic lateral sclerosis (ALS) and fronto-temporal dementia (FTD) development; based on (Yu et al., 2020).

protein (APP) in muscles (Zhang and Shi YD, 2022). Although there are no evidences that these amyloids aggregation causes ALS the altered levels of them were observed in ALS patients, so it may be worth considering in future studies (Nishikawa et al., 2021).

Huntington's disease is an autosomal dominantly inherited, late-onset, polyglutamine, neurodegenerative disorder (McGowan et al., 2000; Churkina et al., 2022). It is a result of the expansion of trinucleotide cytosine–adenine–guanine (CAG) in *HTT* gene that causes formation of mutated protein huntingtin (Churkina et al., 2022). Because of its deformed structure, huntingtin aggregates inside the neurons and causes dysregulation in cell's processes such as protein degradation, mitosis or signaling pathways (Matlahov and van der Wel, 2019; Churkina et al., 2022). It leads to neurons death and manifest as uncontrolled movement, abnormal behaviour, changes in personality and emotions (Huntington's disease).

The great breakthrough has been made in the field of amyloid diseases as scientists from the Stowers Institute for Medical Research have uncovered the structure of the first step in Aβ formation for Huntington's disease (Stowers Institute; Kandola et al., 2023). It may give new prospects for HD treatment and reveal some secrets of amyloids.

All things considered, NDs form diverse group of diseases that may vary in pathomechanism or location of leasions, but have common thread – amyloids. Aβ, among all of amyloid proteins, seems to play a significant role in many of these disorders and is noteworthy in future research.

4 Contribution of amyloid protein to the pathomechanism of Alzheimer's disease

Neurodegenerative diseases, including AD, can be called proteinopathy. Aggregated extracellular Aβ plaques and intracellular Tau protein (Tau) tangles are well-known protein pathologies of AD. Increasing evidence suggests that the development of AD characteristic pathological features, i.e., β-amyloid plaques and Tau tangles, can be associated with microorganisms (Dow, 2021).

Alzheimer's disease, a neurodegenerative disorder, is most common dementia, possibly contributes to 60-70% of cases worldwide which is over 30 million people with AD according to the World Health Organization (Zhang et al., 2021).

4.1 AD's pathology

Pathophysiology of AD is very complex, main contributing factors are: genetics, epigenetics, microbiota, immunology and environment. It is based on many known mechanisms of neurodegeneration, including dysregulation of calcium homeostasis, abnormal accumulation of Aβ and dysfunctional Tau, imbalance of neurotransmitters, necrotic and apoptotic neuronal death, disappearance of synapses, and neuroinflammation with pathological microglia and astrocyte activation in the brain, white matter changes and finally brain atrophy (Pluta et al., 2020). AD is divided into 2 subtype: early-onset Alzheimer's disease (EOAD), defined as Alzheimer's disease occurring before age 65 and late-onset Alzheimer's disease (LOAD). LOAD is more frequent, thus well studied and usually is more mild progressive (Tellechea et al., 2018; Perkovic et al., 2021).

4.2 Genetic factors

The amyloid cascade hypothesis is based on Aβ accumulation resulting in the initiation of a cascade leading to neurodegeneration. The integral genes contributing to this process are *APP*, presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes (Reitz, 2015). These genes affect amyloid production or cleavage and are primarily involved in the EOAD. The development of LOAD is more complex and those mutations are not mandatory. The basic principle of overproduction and/or impaired clearance of Aβ stays the same for both EOAD and LOAD yet the pathways are different (Robinson et al., 2017). The apolipoprotein E (APOE) was first genetic risk factor for LOAD. Its presence determines increased risk of AD, accelerating symptoms and lowers the age at onset by 6-7 years (Reitz, 2015; Robinson et al., 2017). Also, *APOE4* and *TREM2* genes are involved in AD, being responsible for cholesterol metabolism and immune response, respectively (Karch and Goate, 2015).

4.3 Epigenetics

Epigenetics play a major role in the development, diagnosis and therapy of AD (Perkovic et al., 2021). The epigenetic alterations in AD include: DNA methylation/hydroxymethylation, mitochondrial DNA (mtDNA) methylation, histone modifications, the microRNAs. Results collected from other work by Perkovic et al. suggest involvement of 5mC (5-methyl cytosine) and 5hmC (5-hydroxymethyl cytosine) in AD pathology and progression. These compounds are products of cytosine methylation leading to weaker binding of transcriptional factors. Authors point out its difficult to compare results from experiments using different methods (methylation array technology, next generation sequencing, and pyrosequencing and immunochemistry) and different brain regions tissues. miRNAs significant in pathology of AD have biomarker potential as easily monitored in body fluids, their level can be used as distinction from other dementias as AD does not have common detecting test. Lastly mtDNA also may be potential marker (Perkovic et al., 2021). The dysregulation of DNA methylation dynamics, encompassing both hypermethylation and hypomethylation events, contributes to the disruption of transcriptional programs underlying synaptic plasticity, neuroinflammation, and Aβ deposition, thereby exacerbating the neurodegenerative cascade characteristic of AD (Maity et al., 2021; Sommerer et al., 2023). Histone modifications, encompassing an array of reversible post-translational alterations to histone tails, exert fine-tuned control over chromatin accessibility and gene expression. Perturbations in histone acetylation, methylation, and phosphorylation have been implicated in AD pathophysiology, modulating the expression of genes central to neuronal survival, synaptic integrity, and cognitive function (Anderson and Turko,

2015; Santana et al., 2023). Notably, the dysregulation of epigenetic enzymes, including DNA methyltransferases and histone-modifying enzymes, underscores the intricate interplay between genetic and epigenetic factors in AD susceptibility and progression. Targeting epigenetic modifiers presents a tantalizing avenue for therapeutic intervention, with epigenetic-based therapies poised to mitigate the progression of AD pathology and ameliorate cognitive decline.

4.4 Microbiota

The growth of microbiota starts even before birth of the child, crucial for development is the first year of age, nevertheless its state is dynamic until a person dies with it (Gomaa, 2020; Vandenplas et al., 2020). That gives us opportunity to maintain it during life time. Microbiota is element of the GMBA capable of altering mood, behavior and other processes through immune, neuroendocrine and direct nerve mechanisms. Its role is multi-level, over the last years scientists discovered many connections between some illnesses and microbiota, e.g., HIV, obesity, allergies and many other (Desai and Landay, 2018; Vandenplas et al., 2020). Changes in microbiota can cause anxiety, memory impairment, cognitive and neurodegenerative disorders (Pluta et al., 2020). As indicated above the intestinal dysbiosis is the source of Aβ, LPS and other toxins, which contribute to systemic inflammation and disruption of physiological barriers, e.g., intestinal wall (Megur et al., 2020). These products can transfer, through X cranial nerve, to CNS over years, triggering inflammation and microglia activation. Neuroinflammation is the reason of neuron loss in the brain. Combined with bacterial amyloid it promotes misfolding and aggregation of human amyloids (Megur et al., 2020; Pluta et al., 2020). There is hypothesis of antimicrobial protection in AD. According to theory Aβ deposition is an early immune response to mistakenly perceived immunostymuli. Aβ fibrillization helps to combat the infection, in AD, chronic activation of this pathway leads to sustained inflammation and neurodegeneration (Moir et al., 2018). Therefore the regulation of microbiota shines like a prominent opportunity to manage AD. It is possible mostly through healthy diet rich in fibers, yet also probiotics and antibiotics have its role. The effect of fructants on reducing AD incidence in the elderly, among other things, has been demonstrated (Nishikawa et al., 2021). By appropriate distribution of some antibiotics: amoxicillin, minocycline, rapamycin D-cycloserin, doxycycline it is possible to improve cognition, reduce Tau, Aβ, inflammation and microglia activation. However antibiotics: streptomycin, ampicillin, cefepime have negative impact on the animals and humans with AD (Angelucci et al., 2019). In the AD rat model administration of *Lactobacillus plantarum MTCC 1325*, *Lactobacillus* spp. *and Bifidobacterium*, *Bifidobacterium breve strain A1* have had positive impact on Aβ formation or its effects on cognitive functions (Megur et al., 2020). The pathways to the development of Alzheimer's disease are shown in Figure 4.

As there is connection between gut microbiota and ND development and progression, alterations in the intestinal microbial flora may be promising treatment option. Akbari E et al. investigated effect of probiotic supplementation in patients with AD (Akbari et al., 2016). During randomized, double-blind, and controlled clinical trial, treatment group of patients supplemented probiotic milk containing *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum* and *Lactobacillus fermentum* for 12 weeks (Maity et al., 2021). Similar trial was conducted by Tamtaji et al., as patients were aministered probiotic containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Bifidobacterium longum* with selenium for 12 weeks (Tamtaji et al., 2019). Both trials showed positive effect of probiotics on cognitive function and some metabolic profiles in AD (Maity et al., 2021; Santana et al., 2023). Beneficial effect on AD appears to be treatment involving fecal microbiota transplantation (FMT) (Xiang et al., 2023). *In vivo* studies conducted by Soriano et al. in the C57BL/6 mouse model confirmed the roles of the intestinal microbiota in the pathogenesis of AD. Healthy mice treated with fecal microflora from mice with AD revealed larger areas of brain damage, increased numbers of activated microglia cells and reduced motor regeneration (Soriano et al., 2022). These studies provide the basis for the hypothesis that it is the microbiota that can improve

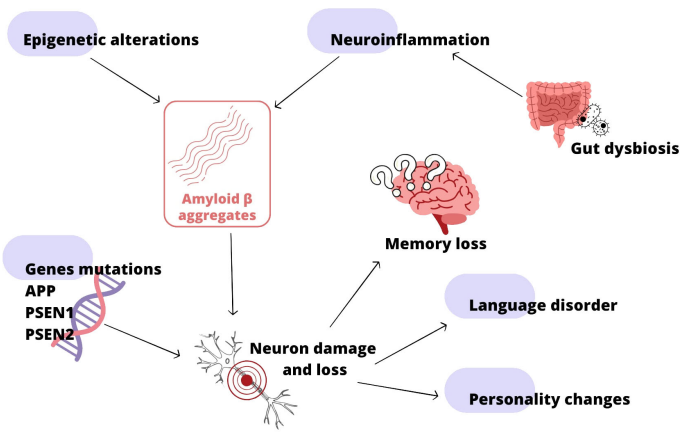

FIGURE 4
Alzheimer's disease (AD) development; based on (Reitz, 2015; Moir et al., 2018; Megur et al., 2020; Pluta et al., 2020; Perkovic et al., 2021).

cognitive function in NDs and improve recovery. These optimistic results create basics for further studies in this area.

5 Crohn's disease association with Alzheimer's disease

Extensive research in recent years has provided incontrovertible conclusions that the gut microbiota is closely linked to neurodegenerative diseases. Increasing evidence suggests that the gastrointestinal tract plays a meaningful role in AD. Moreover, patients with CD are at an increased risk of developing AD (Wang et al., 2022). Previous studies confirm that the pathophysiological mechanisms leading to gastrointestinal disorders terminally lead to neurodegeneration. A meta-analysis concerning residents of Taiwan, conducted by Liu et al., showed that the likelihood of dementia occurrence in people with IBD, including CD was twice as high. Furthermore, the risk of developing AD was the greatest among all types of neurodegenerative diseases (six times higher in people with vs without IBD). These conclusions are supported by a meta-analysis conducted by Szandruk-Bender et al., based on the search of Pubmed and Embase databases (Szandruk-Bender et al., 2022b).

5.1 Chronic inflammatory bowel disease is associated with increased inflammation of the nervous system

In recent years, numerous meta-analyses have been conducted to achieve consensus on the relationship between IBD and neurodegenerative diseases. Research by Zhang et al. provided evidence of potential dementia indicators in the course of IBD. They demonstrated that the risk of developing AD in patients with CD was twice as high [risk ratio (RR) of 2.79] compared to the general population [(RR) = 1.35] (Zhang and Shi YD, 2022). Furthermore, significant evidence was provided by Kim et al. in studies conducted among the Korean population. It was shown that in patients with IBD aged ≥ 65 years, the risk of AD was increased compared to the control group [adjusted hazard ratio (HR) = 1.14] (Kim et al., 2022).

Research by Heston et al. suggests that inflammatory bowel disease is linked to brain inflammation even in the early stages of the disease. These authors demonstrated that inflammation of the intestines may exacerbate the progression of AD. Their study involved measuring calprotectin, a marker of intestinal inflammation, in the feces of people with confirmed AD. The obtained results were subjected to multiple regression analysis with maximum likelihood estimation and Satorra-Bentler correlations, using 11C-Pittsburgh compound B positron emission tomography (PiB-PET) imaging, and synchronized with cognitive test results. It was shown that in patients diagnosed with AD, the level of calprotectin was higher. Furthermore, it also exhibited a higher level in those with impaired verbal memory functions but normal cognitive functions, thus indicating a very early stage of AD (Heston et al., 2023). Elevated levels of calprotectin observed in neurodegenerative diseases are also associated with its elevation in the course of CD (Bourgonje et al., 2018; Kennedy et al., 2019).

Additionally, studies performed by Liu et al. suggested that chronic inflammation combined with an abnormal gut microbiome may degrade cognitive functions proportionally to the duration of this condition. Indeed, the longer the period of IBD, the greater the risk of dementia development (Liu et al., 2022).

Research conducted by Kaneko et al. using *in vivo* studies in wild-type mouse models and the AD mouse model, $App^{NL\text{-}G\text{-}F}$, demonstrated the involvement of the immune system in AD and CD. Upon inducing intestinal inflammation (using 2% dextran sodium sulfate, DSS), an increase in Aβ accumulation was observed in the brains of mice exhibiting AD-like symptoms. Through detailed single-cell RNA sequencing analysis (scRNA-req), a significant presence of neutrophils in the brains of these animals was identified. Furthermore, the administration of antibodies inhibited the polymerization reaction. These studies clearly indicate that neutrophil infiltration in the AD-altered brain is associated with the progression of intestinal inflammation (Kaneko et al., 2023). This occurs because neutrophils activated by microglia can cross the BBB and positively respond to Aβ aggregation, leading to the production of inflammatory cytokines (Park et al., 2019).

5.2 Dysbiosis of the intestinal microbiota

The above data point to the critical role of the gut microbiota in CD, implicating it in the development of AD. It is very interesting that studies conducted to date have shown that bacteria belonging to the same families and even species are involved in both CD and AD. Patients with AD have been shown to have significant changes in the intestinal microbiota composition for many types of bacteria (Ferreiro et al., 2023). The most significant changes involve genera: *Firmicutes*, *Bifidobacterium, Actinobacteria, Eubacteria*, and *Bacteroidetes*, as well as *E. coli, Shigella* spp., and *Salmonella* spp (Vogt et al., 2017). In addition, meta-analyses by Hung et al. involving patients with AD *vs* a control group showed increased amounts of *Proteobacteria, Firmicutes, Clostridiaceae, Lachnospiraceae* and *Rikenellaceae* in the AD spectrum group (Hung et al., 2022). Research on the gut microbiome in the course of CD and AD has revealed a decrease in the levels of *Prevotellaceae*, *Firmicutes*, *Actinobacteria*, and *Eubacterium*. This situation leads to the disruption of mucin synthesis and tight junctions between enterocytes. Consequently, it contributes to an increased permeability of the intestinal mucosal membrane (Paray et al., 2020; Socała et al., 2021). As a result, there is the occurrence of leaky gut syndrome, which is characterized not only by disruptions in the integrity of tight junctions but also by a decrease in the level of immunoglobulin A. Together, they constitute the first line of defense of the gastrointestinal tract against pathogens, a defense that is clearly compromised in the course of neurodegenerative diseases (Maruya et al., 2013). On the other hand, an increase in the quantity of bacteria from the *Ruminococcus* genus is associated with the production of secondary bile acids. This leads to DNA damage and an overproduction of ROS (Hang et al., 2022). It is known that

ROS are a key harmful factor influencing the pathogenesis of neurodegenerative diseases, including AD. Simultaneously, oxidative stress leads to persistent damage to brain cells and disrupts the conduction of nerve impulses (Manoharan et al., 2016; Bhatt et al., 2021). As the involvement of the same bacteria has been confirmed in CD, the results of this research have directed scientists to make an effect-causal connection between diseases involving the gut and neurological diseases.

5.3 Decreased production of anti-inflammatory metabolites and increased bacterial neutotoxic and neuromodulatory molecules

Numerous studies conducted in recent years have provided interesting insights. They have demonstrated the direct involvement of bacterial products (such as proteins and metabolites) in the pathogenicity of the GMB-amyloid-AD connection, resulting from endothelial dysfunction (Marizzoni et al., 2020). Due to increased permeability of the gut-blood barrier, BBB, and GBA in progressing neurodegenerative diseases, the penetration of small particles such as amyloids, cytokines induced by LPS, or other small pro-inflammatory molecules is observed (Zhu et al., 2022). Confirmation of this phenomenon comes from studies conducted by Gonzalez Cordero et al., who analyzed eight observational experiments involving patients diagnosed with AD or PD. The results of their analysis clearly point to disruptions in the GBA, indicating a link between gut microbiota and cognitive dysfunction (Cordero et al., 2022). Furthermore, the direct involvement of bacterial factors in neurodegenerative diseases has been confirmed by researchers who have shown the impact of extracellular bacterial DNA (including its presence in the bloodstream) on the misfolding of Tau and the aggregation of Aβ (Tetz et al., 2020; Tetz and Tetz, 2021; Giacconi et al., 2023).

Other evidence linking the development of CD to changes occurring in patients with AD is the reduction in the quantity of *Bifidobacterium* spp. This genus actively participates in brain metabolic processes, including the production of the neurotransmitter gamma-aminobutyric acid (GABA). The level of this neurotransmitter in the gut nervous system correlates with its level in the central nervous system. Therefore, a decrease in the number of *Bifidobacterium* spp. species may be a factor contributing to changes characteristic of AD, such as the development of depression or abnormal cognitive functions (Chen et al., 2021).

Intestinal dysbiosis leads to a reduction in the production of beneficial anti-inflammatory metabolites by the intestinal microbiome, such as SCFAs, certain bile acids (e.g., tauroursodeoxycholic acid), and ligands for the aryl hydrocarbon receptor. These substances have the ability to traverse the BBB. On the other hand, chronic inflammation in IBD promotes the production of neurotoxic metabolites that contribute to inflammation within the nervous system. These neurotoxic metabolites include kynurenine, certain bile acids, LPS, and enterotoxins. They damage the lining of the large intestine, increasing its permeability. "Leaky, damaged intestines" serve as gateways for the migration of these metabolites from the intestinal lumen to the central nervous system, crossing through the GBA pathway (Jia et al., 2020; Mulak, 2021; Wang et al., 2022).

5.4 Association of amyloid (including curli fimbriae) with the development of neurodegenerative diseases

One link between neurodegenerative diseases and gastrointestinal diseases is the fact that some proinflammatory bacteria, such as *E. coli, Shigella* spp., *Salmonella* spp., and those of the genus *Bacteroidetes*, which growth has been demonstrated in both CD and AD, have the ability to produce amyloid peptides. These contribute directly to the development of AD. Furthermore, studies have shown that bacterial amyloid peptides (curli) structurally similar to amyloid fibers deposited in the brain in the form of plaques during AD (Miller et al., 2021). Previous studies have shown that curli fibers are an important factor in facilitating the bacteria that produce them to successfully colonize the colonic epithelium in people with IBD (including CD) (Sobieszczańska et al., 2019). Therefore, bacterial amyloids potentially affect amyloid aggregation in the brain and inflammation of the nervous system (Friedland and Chapman, 2017). Additionally, curli fibers have a very specific protective role for bacteria against external factors. They form "nets" preventing an effective response from the immune system. Moreover, the accumulation of Aβ is associated with the formation of neurofibrillary tangles composed of hyperphosphorylated Tau. This phenomenon is exactly one of the most characteristic changes in the course of AD. Indeed, it has been shown that there can be two *vs.* eight or more phosphoryl groups per molecule of Tau, for a healthy and an AD patients, respectively (Mandelkow and Mandelkow, 2011).

Moreover, *in vitro* and *in vivo* studies have demonstrated a connection between bacterial endotoxins and the development of AD. *Bacteroidetes*, which have been shown to increase in both CD and AD patients, are undoubtedly implicated. It has been proven that LPS from Gram-negative bacteria enhances amyloid fibrillogenesis, facilitates the deposition of amyloid in larger quantities, and promotes the formation of Tau (Kitazawa et al., 2005; Kahn et al., 2012; Asti and Gioglio, 2014). The direct involvement of Gram-negative bacteria, and the LPS and curli fimbriae they produce, in the pathomechanism of AD was proven by the study of Zhan et al. These authors provided unequivocal evidence for the co-localization of *E. coli*, and various compounds produced by them, including the curli fibers with amyloid plaques in postmortem brain tissue obtained from a patient with AD (Zhan, 2017). The CD-Aβ-AD association has been further confirmed by studies conducted by Sun et al. These authors demonstrated that after injection into the stomach wall of mice, Aβ1-42 oligomers were detected in the small intestine, vagus nerve, and brain after one year. Therefore, amyloid induced changes in the functioning of the gastrointestinal organs, ultimately contributing to amyloidosis in the central nervous system and AD-like dementia (Sun et al., 2020). These studies strongly suggest that Aβ oligomers from the gastrointestinal tract may cross into the brain, thereby participating in the pathogenesis of neurodegenerative diseases.

Cognitive impairment following retrograde transport of intra-GI administration of Aβ oligomers were demonstrated *in vivo* studies (Homolak et al., 2023).

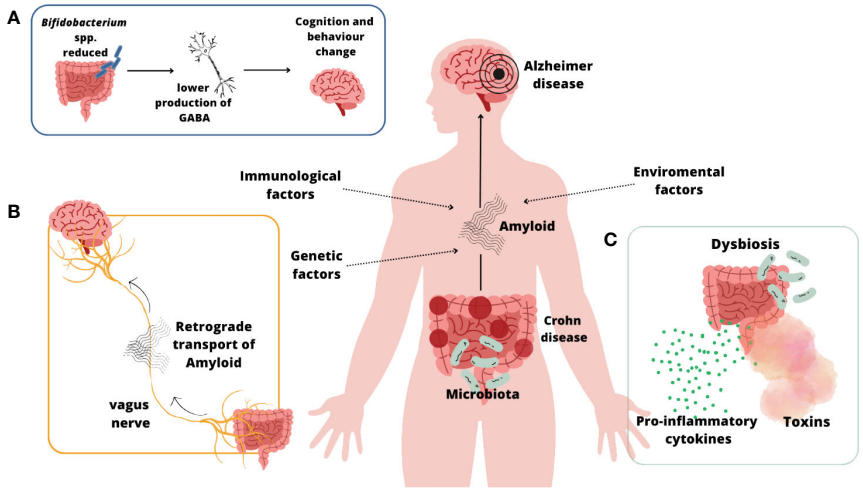

FIGURE 5
CD and AD connection (Mandelkow and Mandelkow, 2011; Villar-Piqué et al., 2016; Friedland and Chapman, 2017; Mehra et al., 2019; Li and Wen, 2022; Zeng et al., 2022; Xing et al., 2023)][**(A)**; The link between reduction in the quantity of *Bifidobacterium* spp., neurotransmitter GABA and developing of AD symptoms (Li and Wen, 2022); **(B)** Retrograde transport of amyloid fibers through vagus nerve (Zeng et al., 2022; Xing et al., 2023); **(C)** Leaky gut syndrome (Villar-Piqué et al., 2016; Mehra et al., 2019).

Figure 5 shows some of mechanism mentioned above that take part in AD development on the base of IBD – CD.

Moreover, genetic and environmental risk factors, as described in the introduction of this Review, may contribute not only to the development of CD, but also to neurodegeneration. However, this is only speculation, as genetic meta-analyses conducted to date have not shown a link between AD and CD (Li and Wen, 2022; Zeng et al., 2022; Xing et al., 2023; Liao et al., 2024).

6 Conclusions

Based on the above evidence, AD can be added to the growing list of gastrointestinal microbiological diseases associated with disruptions in their microbiota. Given the constantly increasing amount of evidence, both from experimental and clinical studies, we now know that CD increases the risk of AD, and Aβ seems to be one of the links between these pathological conditions. The question that requires an answer and further research is whether the increased risk of developing AD in the course of CD is an implication or rather a co-occurrence, the cause of which lies somewhere deeper.

Although research on amyloid proteins produced by representatives of the microbiota and their impact on health and disease is still in the 'crawl' stage and requires significant time and scientific solutions. However, this topic is worth attention as possible interference with the microbiota or the products produced by it (including Aβ) could prove to be an effective therapeutic solution in the fight against neurodegenerative diseases, which effectively seem to have taken over humanity. One such direction is therapies that inhibit Aβ accumulation to prevent increased risk of AD due to colitis. In addition, therapeutic options aimed at inhibiting neutrophil infiltration offer great hope for the future. Also promising are studies of fecal calprotectin levels and Th1- and Th17-related cytokines in serum. Appropriate early detection of these biomarkers would allow the determination of CD disease activity and the implementation of effective treatment, thereby preventing complications, including the development of AD. Based on the studies described above, a future strategy for combating NDs seems to be the transplantation of synthetic intestinal microbiota. This concept would focus on producing such a preparation that would be enriched with probiotics beneficial to NDs patients. This method would be a more effective alternative to FMT in this group of patients.

Author contributions

AD: Conceptualization, Data curation, Investigation, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. JS: Investigation, Resources, Visualization, Writing – original draft. NS: Investigation, Resources, Software, Visualization, Writing – original draft. AM: Resources, Visualization, Writing – original draft. MS: Data curation, Investigation, Resources, Supervision, Visualization, 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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Glossary

AApoAl	apolipoprotein Al-derived amyloidosis
Aβ	β-amyloid
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANP	atrial natriuretic peptide
APOE	apolipoprotein E
APP	amyloid precursor protein
ATG	autophagy related gene
ATTR-CM	transthyretin amyloid cardiomyopathy
B2M	β2-microglobulin
BBB	blood-brain barrier
CAG	cytosine–adenine–guanine
CARD	caspase activating recruitment domain
CCR6	chemokine receptor 6
CD	Crohn's disease
chILD	children's interstitial lung disease
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
$CX3CR1^{high}$	intestinal CX3C chemokine receptor (high)
DLG5	discs large MAGUK (membrane associated guanylate kinases) scaffold protein 5
DSS	dextran sodium sulfate
EOAD	early-onset Alzheimer's disease
FFI	fatal familial insomnia
FMT	fecal microbiota transplantation
Foxp3	forkhead box protein 3
FTD	fronto-temporal dementia
GABA	gamma-aminobutyric acid
GBA	gut-brain axis
GMBA	gut-microbiota-brain axis
GSD	Gerstmann-Straussler disease
HCCAA	hereditary cystatin C amyloid angiopathy
HD	Huntington's disease
HDL	high-density lipoprotein
HDL1	Huntington disease-like type 1
HIV	human immunodeficiency virus
HR	hazard ratio
IAPP	islet amyloid polypeptide (amylin)
IBD	inflammatory bowel disease
IBD5	inflammatory bowel disease 5
IL	interleukin
IL23R	interleukin-23 receptor
IRGM	immunity related GTPase M
JAK2	Janus kinase 2
LFS	Li-Fraumeni syndrome
LOAD	late-onset Alzheimer's disease
LPS	lipopolysaccharide
LRRK2	leucine-rich repeat kinase 2
miRNA	microRNA
MMP	metalloproteins
mtDNA	mitochondrial DNA
ND	neurodegenerative disorders
NK	natural killer
NFkB	nuclear factor kappa light chain enhancer of activated B cells
NOD	nucleotide-binding oligomerisation domain
OCTN	organic cation transporters novel
OTA	ostertag-type amyloidosis
p53	regulatory protein important protein in the cell cycle, DNA repair and apoptosis initiation, mutated in human cancers
PCOS	polycystic ovary syndrome
PD	Parkinson's disease
PiB-PET	11C-Pittsburgh compound B positron emission tomography
PMEL	pre-melanosomal protein
proSP-C	prosurfactant protein C
PrP	prion protein
PSEN	presenilin
RCC	renal cell carcinoma
ROR	retinoic acid related orphan receptor
RORγt	retinoic acid related orphan receptor γt
ROS	reactive oxygen species
RR	risk ratio
S100A9	S100 calcium-binding protein A9
SCFA	short-chain fatty acid
STAT3	signal transducer and activator of transcription 3
Tau	Tau protein
TDP-43	TAR DNA binding protein 43
Th	T helper
TNF-α	tumor necrosis factor alpha
TNFSF15	TNF superfamily member 15

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Continued

TLR	toll-like receptor
Treg	regulatory T cells
TREM	triggering receptor expressed in myeloid/microglial cells
UC	ulcerative colitis
5hmC	5-hydroxymethyl cytosine
5mC	5-methyl cytosine.

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The impact of pre-, pro- and synbiotics supplementation in colorectal cancer treatment: a systematic review

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Introduction: The effectiveness of the supplementation of prebiotics, probiotics and synbiotics as a therapeutic approach in colorectal cancer (CRC) remains unclear. The aim of this systematic review is to critically examine the current scientific evidence on the impact of modulating the microbiota, through the use of prebiotics, probiotics and synbiotics, in patients diagnosed with CRC undergoing treatment, to determine the potential therapeutic use of this approach.

Methods: This systematic review was made according to the PRISMA 2020 guidelines. Inclusion criteria were randomized controlled trials (RCT) comparing the impact of pre-, pro-, or synbiotic supplementation with placebo or standard care in patients with CRC undergoing treatment. Exclusion criteria were non-human studies, non-RCTs, and studies in languages other than English or Portuguese. Six databases were consulted, namely, Cochrane Library, Pubmed, Scopus, Cinahl, MedicLatina and Web of Science until May of 2023. RAYYAN software was used to manage the search results and risk of bias was assessed according to the guidelines of the Cochrane Collaboration using the Rob 2.0 tool.

Results: Twenty-four RCTs met the inclusion criteria and were included in this review. Administration of pre-, pro-, or synbiotics improved surgical outcomes such as the incidence of infectious and non-infectious postoperative complications, return to normal gut function, hospital length of stay, and antibiotic usage. The supplementation of these microorganisms also alleviated some symptoms from chemotherapy and radiotherapy, mainly diarrhea.

Evidence on the best approach in terms of types of strains, dosage and duration of intervention is still scarce.

Conclusions: Pre-, pro-, and synbiotics supplementation appears to be a beneficial therapeutic approach in CRC treatment to improve surgical outcomes and to alleviate side-effects such as treatment toxicity. More RCTs with larger sample sizes and less heterogeneity are needed to confirm these potential benefits and to determine the best strains, dosage, and duration of administration in each situation.

Systematic review registration: https://www.crd.york.ac.uk/prospero, identifier CRD42023413958.

KEYWORDS

probiotics, synbiotics, prebiotics, microbiota, colorectal neoplasms, systematic review

1 Introduction

According to GLOBOCAN, in 2022, colorectal cancer (CRC) ranked as the third most diagnosed cancer, with over 1.9 million new cases, and the second most deadly malignancy causing roughly 904,000 deaths. This accounted for 9.3% of cancer-related deaths (1). GLOBOCAN also estimates that by 2040 the burden of CRC will rise to 3.2 million new cases and 1.6 million deaths with most cases predicted to occur in developing countries with the numbers increasing along with the increase of the Human Development Index. Conversely, in highly developed countries, where the screening is now a routine, numbers are expected to stabilize or even decline (2, 3).

Surgery stands as the primary treatment for CRC, but chemotherapy and radiotherapy are also commonly used as neoadjuvant or adjuvant treatments. These approaches often lead to several side-effects such as postoperative infectious complications, diarrhea, vomiting, nausea, etc. (4–6). More recently, immunotherapy and targeted therapy have emerged as viable options in select cases (5, 7).

The etiology of CRC is multifactorial, involving genetic factors, epigenetic alterations, and environmental factors such as being overweight, smoker, heavy drinker and following an unhealthy diet (8–10). More recently, the development of CRC has also been associated with chronic inflammation, immune system dysfunction, and dysbiosis. Dysbiosis is the compositional and functional alteration caused by an imbalance between symbiotic and opportunistic microbiomes. It can be categorized in three types: loss of beneficial microbes, expansion of pathogenic microbes, and loss of microbial diversity (11, 12).

Over the past decade, the relationship between the gut microbiota and CRC has gained significant attention with studies showing that patients with CRC harbor a distinct microbiota composition compared to healthy control subjects (3, 13). These studies show that CRC patients' microbiota has lower bacterial diversity, lower abundance of commensal bacteria such as *Akkermansia muciniphila*, *Lactobacillus rhamnosus* and *Bifidobacterium breve*, and higher abundance of pro-carcinogenic bacteria such as *Fusobacterium nucleatum, Escherichia coli, Bacteroides fragilis, Streptococcus gallolyticus* and *Peptostreptococcus anaerobius* (3, 10, 13, 14). Studies have also shown that butyrate-producing bacteria are less represented in CRC patients. Butyrate is a short-chain fatty acid with very important health-promoting and antineoplastic properties such as being the main energy source for colonocytes, maintaining the mucosal barrier integrity, reducing pro-inflammatory cytokines, and inducing apoptosis (9, 15, 16).

The microbiota has been studied not only as a potential risk factor for CRC but also as a therapeutic approach in the treatment of this malignancy through its modulation with pre-, pro-, and synbiotics (17, 18). Prebiotics are defined as a non-digestible food ingredient that promote changes in the composition and/or activity of the microbiota conferring health benefits to the host (3). On the other hand, according to the most accepted definition and the one proposed by the expert panel convened by the International Scientific Association of Probiotics and Prebiotics in 2014, probiotics are "live microorganisms which, when administered in an adequate amount, confer a health benefit to the host" (19). The most commonly used strains are from the *Lactobacillus, Bifidobacterium, Streptococcus* and *Enterococcus* genera (10, 14). When prebiotics and probiotics are administered together, in a way that prebiotics promote the growth and survival of probiotics, it's called a synbiotic (20, 21).

Recent studies indicate that modulating the microbiota, through the supplementation of pre-, pro-, or synbiotics, appears to have an impact on CRC treatment. This can be due to the reduction of postoperative infectious and non-infectious complications and side-effects of chemotherapy and radiotherapy or even directly on the efficacy of the drugs used in chemotherapy or, more recently, on the sensitivity to immunotherapy (12, 22, 23).

While some systematic reviews have delved into this area (24–31), the majority focused on only one treatment for CRC, or one outcome and some results appear to be contradictory. To overcome these previous limitations, the aim of this systematic review is to critically examine the present scientific evidence, including more recent findings, on the impact of modulating the microbiota, through the use of pre-, pro-, and synbiotics, in CRC patients undergoing treatment. This is also performed to determine the potential therapeutic use of this approach.

2 Materials and methods

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (32) and was registered in the PROSPERO database (registration number CRD42023413958).

2.1 Literature search

This systematic review was conducted in six databases, namely, Cochrane Library, Pubmed, Scopus, Cinahl, MedicLatina and Web of Science, until May 2023. The MESH terms or equivalents and search terms for title and abstract were selected according to the Population, Intervention, Comparison, Outcome and Study (PICOS) model. The MESH terms used for the Pubmed database are shown in Table 1. The search strategy for the databases is shown in detail in Appendix 1. The results were filtered to identify studies in the English or Portuguese language. The references of the selected studies were also scanned to identify additional studies missed in the initial search.

2.2 Inclusion and exclusion criteria

The inclusion criteria were based on the PICOS model. The selected population comprised patients diagnosed with CRC (colon cancer or rectal cancer), the intervention was the supplementation with pre-, pro-, or synbiotics. The considered control treatment was placebo or standard care, the primary outcomes were the impact of this intervention on the efficacy, toxicity, or side-effects of treatments such as chemotherapy, radiotherapy, surgery or immunotherapy and the studies selected were randomized controlled studies.

The exclusion criteria were studies in languages other than English or Portuguese, studies where the population were patients with other types of cancer and studies where the intervention wasn't exclusively the supplementation of prebiotics, probiotics or synbiotics.

TABLE 1 Mesh terms used for research in Pubmed database.

	Mesh Terms
Population	**Colonic Neoplasms OR Colorectal Neoplasms OR Rectal Neoplasms OR Anus Neoplasms OR Colorectal Neoplasms, Hereditary Nonpolyposis OR Sigmoid Neoplasms OR Colitis-Associated Neoplasms**
Intervention	**Prebiotics OR Probiotics OR Synbiotics OR *Lactobacillus* OR *Bifidobacterium***
Control	–
Outcome	**Radiotherapy OR Immunotherapy OR Immune Checkpoint Inhibitors OR Antineoplastic Agents OR Colorectal Surgery OR Postoperative Complications OR Surgical Wound Infection OR Diarrhea OR Nausea OR Postoperative Nausea and Vomiting OR Vomiting OR Signs and Symptoms, Digestive OR Mucositis OR Quality of Life OR Biomarkers OR Biomarkers, Tumor**

2.3 Study selection

The studies obtained from the initial search were uploaded to the RAYYAN software and were analyzed and selected by two independent reviewers (MM and MC). The articles were screened by title and abstract and then full text of relevant studies were retrieved and assessed based on the inclusion and exclusion criteria. Disagreements in study selections were resolved by discussion between the two reviewers.

2.4 Data extraction

Data such as author, publication year, participant, placebo, and intervention details and outcomes were extracted from articles considered eligible and compiled in a summary table.

2.5 Risk of bias assessment

The risk of bias of all included studies was assessed through the RoB 2.0 tool, according to the guidelines of the Cochrane Collaboration (33), using Review Manager Software (Revman Web 5.5 - online). Risk of bias was assessed in the following domains: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias) and other bias. The risk of bias was then classified as high, low, or unclear risk.

3 Results

3.1 Study selection

A total of 2308 studies were obtained from the initial search through six databases, namely, Cochrane Library, Pubmed, Scopus, CINAHL, MedicLatina and Web of Science. These results were uploaded to the RAYYAN software, and the duplicates were identified (n = 784). After the duplicates were removed, 1524 articles remained and were screened by title and abstract. Afterwards, 1480 articles were excluded for reasons such as being

non-human studies, being reviews or case reports or not being relevant to this review. The remaining 44 studies were full text screened for eligibility and 24 studies were included in this review. The other 20 studies were excluded because they did not meet the inclusion criteria or were unavailable in full-text format. The studies that did not meet the inclusion criteria are shown in Appendix 2. The PRISMA flow diagram, shown in Figure 1, summarizes the selection process.

3.2 Description of the selected studies

The 24 studies included in this review were all randomized controlled studies. The year of publication ranged from 2007 to 2023, where approximately 70% of the studies were published in the last 10 years. In terms of geographic localization the included studies are from China (n=6) (34–39), Turkey (n=2) (40, 41), Slovenia (n=2) (42, 43), Japan (n=2) (44, 45), Greece (n=2) (46, 47), Brazil (n=2) (48, 49), Iran (n=2) (50, 51), Malaysia (n=1) (52), Finland (n=1) (53), Sweden (n=1) (54), Slovakia (n=1) (55), Republic of Korea (n=1) (56) and Bosnia and Herzegovina (n=1) (57). There were a total of 2204 participants, 1139 in the intervention groups and 1065 in the control groups. Of these, 1220 participants were male, 984 were female, and their age ranged from 19 to 92 years. Considering the intervention, 11 out of the 24 studies used a mixture of probiotics (34–39, 46, 52, 55–57), 8 used synbiotics (42, 43, 45, 47–51), 3 used a single probiotic (44, 53, 54) and 2 studies used Kefir (40, 41). Species of the *Lactobacillus, Bifidobacterium* and *Enterococcus* genera were the most commonly used for the probiotics or synbiotics intervention. Placebo was used for the control group in 18 of the studies (34–42, 46–50, 52, 54–56) while the other 6 used standard care treatment (43–45, 51, 53, 57). Characterization of these studies is shown in Table 2.

3.3 Risk of bias assessment

The summary results of the risk-of-bias assessment are shown in Figure 2. All studies included in this review were randomized but 7 studies failed to mention how the randomization was done (35, 38–40, 43, 54, 57). Therefore, there is an unclear risk of bias in this parameter and in 1 study randomizations were performed by one of the authors leading to a high risk of bias (44). In terms of allocation concealment, 7 studies had an unclear risk of bias as there was no clear description if allocation was concealed until the beginning of the intervention (38–41, 43, 44, 57). Ten studies presented a high

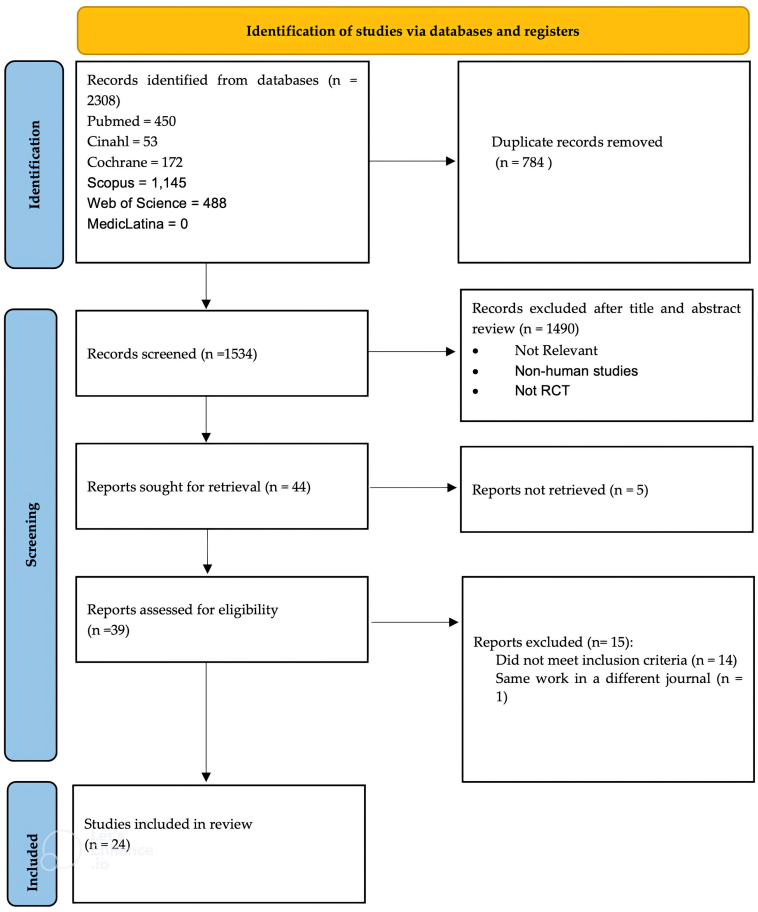

FIGURE 1
PRISMA flowchart that summarizes the screening and selection process.

TABLE 2 Characteristics of the 24 included studies.

Year	Author	Country	Study Design	Reference
2007	Österlund, P.	Finland	RCT	(53)
2008	Topuz, E.	Turkey	RCT	(40)
2009	Can, G.	Turkey	RCT	(41)
2010	Horvat, M.	Slovenia	RCT/double blind	(42)
2011	Liu, Z.	China	RCT/double blind	(34)
2012	Mangell, P.	Sweden	RCT	(54)
2012	Zhang, J.	China	RCT	(35)
2013	Liu, Z.	China	RCT/double blind	(36)
2014	Sadahiro, S.	Japan	RCT	(44)
2015	Kotzampassi, K.	Greece	RCT/double blind	(46)
2015	Mego, M.	Slovakia	RCT/double blind	(55)
2016	Komatsu, S.	Japan	RCT	(45)
2016	Tan, C.	Malaysia	RCT/double blind	(52)
2016	Krebs, B.	Slovenia	RCT/double blind	(43)
2016	Theodoropoulos, G.	Greece	RCT	(47)
2016	Yang, Y.	China	RCT	(37)
2017	Flesch, A.	Brazil	RCT/double blind	(48)
2019	Polakowski, C.	Brazil	RCT/double blind	(49)
2019	Xu, Q.	China	RCT	(38)
2019	Bajramagic, S.	Bosnia and Herzegovina	RCT	(57)
2020	Radvar, F.	Iran	RCT/double blind	(50)
2020	Park, I.	Korea	RCT/double blind	(56)
2023	Mohebian, F.	Iran	RCT	(51)
2023	Huang, F.	China	RCT	(39)

risk of bias in the performance domain because participants and/or personnel weren't blinded during the intervention (38–41, 43–45, 51, 53, 57). Blinding of outcome assessment risk was unclear in 8 studies (38–41, 43, 44, 51, 57) and high in 2 studies (45, 53) while incomplete data outcome risk was unclear in only 2 of the studies (38, 43). Nineteen of the studies were considered to have an unclear risk in terms of selective reporting (35, 37–45, 48–52, 54–57) and 2 studies were considered to have a high risk of other bias because they were ended prematurely before full recruitment of participants was completed (46, 55).

3.4 Outcomes

Out of the 24 included studies, 17 evaluated the impact of probiotics or synbiotics supplementation in colorectal cancer surgery (34–38, 42–49, 52, 54, 56, 57) while the remaining 7 studied the impact on treatment with chemotherapy (39–41, 50, 51, 53, 55). One study selected patients undergoing chemoradiotherapy so outcomes on the impact of this approach in treatment with radiotherapy was also assessed (50). None of the studies evaluated the impact of probiotics or synbiotics supplementation in CRC patients undergoing immunotherapy.

3.4.1 Surgery

The impact of probiotics or synbiotics supplementation in CRC patients undergoing surgery treatment was assessed in 17 studies (34–38, 42–49, 52, 54, 56, 57). Details from each study are shown in Table 3. Six studies used synbiotics for the intervention (42, 43, 45, 47–49), 9 used a mixture of probiotics strains (34–38, 46, 52, 56, 57) and 2 studies used a single strain in the intervention (44, 54). The administration of the probiotic/synbiotic was done pre-operatively in 6 studies (35, 38, 42, 43, 49, 52), pre- and post-operatively in 8 studies (34, 36, 37, 44, 45, 48, 54, 56) and 3 studies focused on the post-operative period (46, 47, 57). Considering control groups, 4 studies used standard care (43–45, 57) while the remaining 13 used placebo (34–38, 42, 46–49, 52, 54, 56).

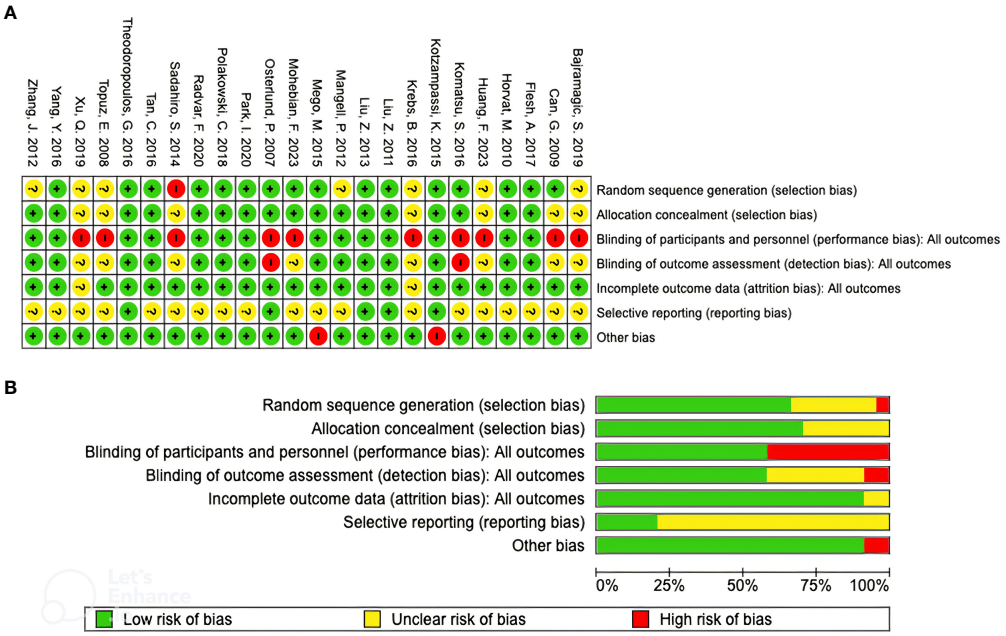

FIGURE 2
Risk of bias summary **(A)** and risk of bias assessment graph **(B)**.

3.4.1.1 Post-operative complications

Fourteen studies (34–37, 43–46, 48, 49, 52, 54, 56, 57) assessed the incidence of post-operative infectious complications such as wound infection, septicemia, and pneumonia. Among them, 9 studies (34–37, 46, 48, 49, 56, 57) reported that the supplementation of probiotics or synbiotics could decrease the incidence of post-operative infectious complications, with significant results being observed in all but 2 of the studies (37, 57). Additionally, 5 studies (43–45, 52, 54) found no differences between the intervention and the control group.

In a study by Flesch et al., where the intervention group took synbiotics for 5 days before surgical procedure and for 14 days after surgery, it was observed that only one patient in the synbiotics group presented surgical wound infection, while 9 such cases were diagnosed in the control group (p=0.002). Furthermore, there was a significant difference between groups in relation to other infectious complications such as intra-abdominal abscess (n=3) and pneumonia (n=4) in the control group and no cases in the synbiotics group (p=0.001) (48).

A study by Liu et al., where patients received a mixture of probiotics for 6 days preoperatively and 10 days post-operatively, also reported a significant difference in postoperative infectious complications between the intervention and the control group, being the incidence of post-operative septicemia 73% in the control group and 55% in the probiotics group (p= 0.017) (36). In contrast, Komatsu et al. reported no statistical differences in postoperative infectious complications between the intervention and control group where synbiotics were administered from day 7 to day 11 before surgery and reintroduced from day 2 to day 7 postoperative (45). Mangell et al. also noted a higher number of complications in the placebo compared with the intervention group, where an administration of a single strain for 8 days preoperatively and 5 days postoperative was performed, although, this difference did not reach statistical significance (54). Four studies (34, 36, 38, 56) demonstrated a lower bacterial translocation and intestinal permeability in the intervention group and 2 of the studies reported lower zonulin levels which is used as a biomarker of impaired gut function barrier (36, 56).

Regarding non-infectious postoperative complications such as diarrhea, ileus, and anatomic leakage, 8 studies (34, 36–38, 46, 49, 56, 57) indicated that the supplementation of probiotics and synbiotics could decrease their incidence, with all studies showing statistically significant results but one (49). Additionally, 1 study (48) found no statistical difference between the intervention and the control group when considering non-infectious complications.

A study by Yang et al. reported a lower incidence of diarrhea in the intervention group (26.7%) compared to the placebo (53.3%) (p=0.035), after administration of probiotics for 12 days (5 prior to surgery and 7 postoperatively). The results concerning anastomotic leakage and abdominal distension were essentially quite comparable between the 2 groups (37).

Similarly, Bajramagic et al. also reported a lower number of non-infectious complications in the intervention group after administration of probiotics for 30 days starting on day 3 postoperative, but this difference was only statistically significant for ileus development (57).

Another study, where the intervention group received a mixture of probiotics for 6 days preoperatively and 10 days post-operatively, also observed a significant lower incidence of non-infectious complications, compared with the placebo group: diarrhea (10% vs. 30%, p< 0.05), abdominal cramping (26% vs. 38%, p< 0.05) and distension (22% vs. 36%, p< 0.05), and a shorter duration of pyrexia (>38.5°C) (5.9 days vs. 7.2 days, p< 0.05) (34).

TABLE 3 Details of the included studies that assessed the impact on surgery outcomes.

Reference	Intervention Group			Control Group				Cancer Stage	Intervention		Outcome
	N°	Mean age (min-máx)	Male/ Female	Type	N°	Mean age (min-máx)	Male/ Female		Type	Dose/ Duration	
Horvat, M. 2010 (42)	48	62 (29-86)	19/29	Placebo	20	65 (52-78)	11/9	–	Synbiotic group = mixture of four *lactobacilli (Pediacoccus pentosaceus Leuconostoc mesenteroides Lactobacillus paracasei Lactobacillus plantarum)* + betaglucan, inulin, pectin and resistant starch Prebiotic group = betaglucan, inulin, pectin and resistant starch	40 billion *lactobacilli* plus 10 g plant fibers, twice a day for three days before the scheduled operation date.	Preoperative prebiotic administration had the same protective effect in preventing the postoperative inflammatory response (leukocytes and differential counts (lymphocyte/granulocyte ratio), fibrinogen and C-reactive protein) as mechanical bowel cleaning. Further prospective studies are needed to verify the effects of synbiotics.
Liu, Z. 2011 (34)	50	65	28/22	Placebo	50	66	31/19	I-III	*Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium longum*	2 g/day, in a total daily dose of 2.6x1014 CFU, 6 days preoperatively and 10 days post-operatively	Probiotics reduced the infection risk ($p<0.01$), improved gut barrier function ($p<0.01$) and reduced post-operative infectious complications ($p<0.05$).
Mangell, P. 2012 (54)	32	74 (70-80)	16/16	Placebo	32	70 (64-79)	20/12	–	*Lactobacillus plantarum 299v*	10^9 CFU per ml, 100ml 8 days preoperatively and 5 days post-operatively	Lactobacillus plantarum299v was detected in the intestine, but no inhibitory effect on enteric bacteria, bacterial translocation, or postoperative complications was found.
Zhang, J. 2012 (35)	30	68 (45-87)	10/20	Placebo	30	62 (46-82)	14/16	I-III	*Enterococcus faecalis, Lactobacillus acidophilus and Bifidobacterium longum*	3 oral bifid triple viable capsules, each of which contained 0.21 g (108cfu/g), 3 times a day for 3 days before surgery (days -5 to -3)	Probiotics reduced the postoperative occurrence of infectious complications ($p<0.05$). Moreover, the probiotics maintained microbial colonization resistance and restricted bacterial translocation from the intestine.
Liu, Z. 2013 (36)	75	66	38/37	Placebo	75	62	40/35	I-III	*Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium longum*	2 g/day, in a total daily dose of $2.6x10^{14}$ CFU, 6 days preoperatively and 10 days post-operatively	Probiotics group had significantly lower bacterial translocation ($p=0.027$) and intestinal permeability ($p=0.001$=. Probiotics also reduced the rate of postoperative septicemia ($p=0.017$) and were associated with reduced serum zonulin concentrations ($p=0.001$).
Sadahiro, S. 2014 (44)	100	67	49/51	Standard Care	95	66	51/44	I-III	*Bifidobacterium bifidum*	Three tablets orally after each meal three times a day for 7 days before the operation and from	The preventive effect of oral administration of probiotics on postoperative infection could not be confirmed.

(Continued)

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TABLE 3 Continued

Reference	Intervention Group			Control Group				Cancer Stage	Intervention		Outcome
	N°	Mean age (min-máx)	Male/ Female	Type	N°	Mean age (min-máx)	Male/ Female		Type	Dose/ Duration	
										postoperative D-5 to D-15.	
Kotzampassi, K. 2015 (46)	84	66	57/27	Placebo	80	66	58/22	–	*Lactobacillus plantarum, Lactobacillus acidophilus, Bifidobacterium lactis and Saccharomyces boulardii*	1 capsule twice a day for 14 days post-operatively.	Administration of probiotics significantly decreased the rate of all postoperative major complications (p=0.010). Major benefit was found in the reduction of the rate of postoperative pneumonia (p=0.029), of surgical site infections (p=0.020) and of anastomotic leakage (0.031). The time until hospital discharge was shortened as well (p<0.0001).
Komatsu, S. 2016 (45)	168	69 (29-92)	92/76	Standard Care	194	69 (30-89)	118/76	I-IV	*Lactobacillus casei and Bifidobacterium breve* + galactoligosaccharides	$4x10^{10}$ CFU L.casei + $1x10^{10}$ B.breve + 2,5g galactoligosaccharide, orally administered daily for 7–11 days before surgery and reintroduced at 2–7 postoperative days.	The preventive effect of oral administration of probiotics on postoperative infection could not be confirmed.
Tan, C. 2016 (52)	20	64	11/9	Placebo	20	68	13/7	0-III	*Lactobacillus lactis, Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium infantis, Bifidobacterium bifidum and Bifidobacterium longum*	30 billion CFU administered orally twice daily (1 sachet in the morning and 1 in the evening) 7 days prior to surgery	The treatment group demonstrated significantly faster return of normal gut function (48 h earlier than the placebo group) (p=0.022) The duration of hospital stay in the treatment group was also reduced (p=0.012).
Krebs, B. 2016 (43)	38	63 (43-87)	24/14	Standard Care	16	67 (52-78)	9/7	–	Synbiotic group = mixture of four *lactobacilli (Pediacoccus pentosaceus Leuconostoc mesenteroides Lactobacillus paracasei Lactobacillus plantarum)* + betaglucan, inulin, pectin and resistant starch Prebiotic group = betaglucan, inulin, pectin and resistant starch	1 sachet twice a day, 3 days prior to surgery.	No statistical differences in systemic inflammatory response measured by upper factors and no differences in postoperative course and complications rate were found.

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TABLE 3 Continued

Reference	Intervention Group			Control Group				Cancer Stage	Intervention		Outcome
	N°	Mean age (min-máx)	Male/ Female	Type	N°	Mean age (min-máx)	Male/ Female		Type	Dose/ Duration	
Theodoropoulos, G. 2016 (47)	38	67	20/18	Placebo	37	69	23/14	0-IV	Mixture of four *lactobacilli (Pediacoccus pentosaceus Leuconostoc mesenteroides Lactobacillus paracasei Lactobacillus plantarum)* + betaglucan, inulin, pectin and resistant starch	2 g in 250 mL of water once a day for 15 days post-operatively	Synbiotic group had a better global score in the Gastrointestinal Quality of Life Index (p=0.01). The scores on the domain "diarrhea" were better in the synbiotic group after 3 (p=0.04) and 6 months (p=0.003). No significant effect was observed in the "constipation" domain.
Yang, Y. 2016 (37)	30	64	15/15	Placebo	30	62	12/18	0-III	*Enterococcus faecalis, Lactobacillus acidophilus and Bifidobacterium longum*	$1x10^7$ CFU of each strain once a day for 12 days (5 prior to surgery and 7 post-operatively.	The days to first flatus (p=0.03) and the days to first defecation (p=0.03) were significantly improved in the probiotic treated patients. The incidence of diarrhea was significantly lower in probiotics group (p=0.04). There were no statistical differences in other infectious and non-infectious complication rates.
Flesch, A. 2017 (48)	49	65	18/31	Placebo	41	61	19/23	I-IV	*Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus paracasei and Bifidobacterium lactis* + fructoligosaccharides	2 sachets twice a day for 5 days prior to surgery and 14 days post-operatively	The perioperative administration of synbiotics significantly reduced postoperative infection rates (p=0.002). The incidence of noninfectious postoperative complications wasn't different between the study groups. There were no significant differences between the groups regarding mortality rates and re-hospitalization.
Polakowski, C. 2019 (49)	36	61	20/16	Placebo	37	59	19/18	I-III	*Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium lactis* + fructoligosaccharides	1 sachet twice a day for 7 days prior to the surgery	There were significant reductions in IL-6 and CRP levels in the synbiotic group (p<0.001). Postoperative infectious complications were reduced in the synbiotic group (p=0.02). Administration of synbiotics was also associated with reduced length of hospital stay (p<0.001) and use of antibiotics (p<0.001).
Xu, Q. 2019 (38)	30	61	20/10	Placebo	30	62	18/12	–	Bfiidus-Triple Viable Preparation (Inner Mongolia Shuangqi Pharmaceutical Co., Ltd., Inner Mongolia, China)	Once a day for 7 consecutive days prior to surgery.	Probiotics group had significantly lower bacterial translocation and intestinal permeability (p<0.05). The duration of post-operative fever, average heart rate at 7 days after surgery and first exhaust time were shorter and lower (p<0.05).
Bajramagic, S. 2019 (57)	39	–	–	Standard Care	38	–	–	III	*Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus rhamnosus,*	2x1 capsules from the third postoperative day during the next thirty	There was a significant difference in the duration of postoperative hospitalization in the group of patients treated with probiotic (p<0.05). All complications

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TABLE 3 Continued

Reference	Intervention Group			Control Group			Cancer Stage	Intervention		Outcome
	N°	Mean age (min-max)	Male/ Female	Type	N°	Mean age (min-max)	Male/ Female		Type	Dose/ Duration	
									Lactobacillus casei, Bifidobacterium lactis, Bifidobacterium breve, Bifidobacterium bifidum, and Streptococcus thermopilus	days, and then 1x1 for two weeks each next month to a total of one year.	were more present in the group of patients untreated with probiotic, with statistical significance shown only in the case of ileus (p<0.05).
Park, I. 2020 (56)	29	60	19/10	Placebo	31	61	13/18	I-IV	*Lactobacillus plantarum, Lactobacillus casei and Bifidobacterium animalis*	Twice a day for 1 week prior to surgery and 21 days post-operatively.	The probiotic group's "Anterior Resection Syndrome" Score showed an improving trend (p=0.063) particularly for flatus control (p=0.03). Serum zonulin levels significantly decreased with probiotics (p=0.035).

Polakowski et al. also evaluated the incidence of postoperative non-infectious complications after synbiotic supplementation, for 7 days prior to surgery. Even though the incidence was higher in the control group, it did not reach statistical significance (p=0.42) (49).

Conversely, in the study by Flesch et al. the incidence of non-infectious postoperative complications such as nausea, vomiting, abdominal distension, ileus, diarrhea or constipation was not different between the study groups (p=0.161) (48).

3.4.1.2 Return to normal gut function

Nine studies (34, 37, 42, 43, 46, 47, 52, 54, 56) evaluated the time to return to normal gut function. Six studies (34, 37, 46, 47, 52, 56) found that the supplementation of probiotics or synbiotics could significantly improve the return to normal gut function, and other 3 studies (42, 43, 54) found some improvements in the intervention group, but the results did not reach statistical difference.

A study by Tan et al., where the intervention group received a mixture of probiotics for 7 days prior to surgery, demonstrated a significantly earlier return of normal gut function compared to the placebo group (108.5 h vs. 156.5 h respectively, p=0.022) (52).

Another study reported a significant improvement in the days to first flatus (3.63 ± 0.67 days in the placebo group versus 3.27 ± 0.58 days in the probiotics group, p = 0.0274) and the days to first defecation (4.53 ± 1.11 days in the placebo group versus 3.87 ± 1.17 days in the probiotics group, p = 0.0268), after probiotics administration for 12 days (5 prior to surgery and 7 postoperative) (37).

In a study by Liu et al. a shorter time to first defecation when comparing the supplementation of a mixture of probiotics for 6 days preoperatively and 10 days post-operatively to placebo (3.3 days vs. 4.2 days, p< 0.05) was reported (34).

Horvat et al. demonstrated that patients receiving synbiotics and prebiotics twice a day for 3 days prior to surgery, passed flatus and stool after the operation earlier than the control. However, this difference did not reach statistical difference (2.3 days with synbiotics, 2.2 with prebiotics, and 2.5 days in the control, p=0.41) (42).

3.4.1.3 Hospital length of stay

Eleven studies (34–37, 42, 43, 46, 48, 49, 52, 57) assessed the length of hospital stay after surgery. While 7 studies (36, 42, 43, 46, 49, 52, 57) found that the supplementation of probiotics or synbiotics could decrease the duration of hospital stay [significantly except for 2 studies (42, 43)], another 4 studies (34, 35, 37, 48) found no statistical difference between the intervention and the control group.

One study, where patients received probiotics for 7 days prior to surgery, the length of hospital stay was shorter for the intervention group in comparison to the placebo group (6.5 vs. 13 days, p=0.012) (52). Another study, where synbiotic supplementation was also administered for 7 days prior to surgery, reported a shorter length of hospitalization in the synbiotic group compared with the placebo group [3.0 (3-5) days, vs. 4.0 (3-21) days (p <0.001)] (49).

In contrast the study by Zhang et al. did not find differences in length of stay after probiotics administration for 3 days prior to surgery (35) contrary to studies by Krebs et al. (43) and Flesch et al. (48) that reported a shorter length of stay in the intervention groups, although they did not reach statistical significance.

3.4.1.4 Usage of antibiotics

In terms of use of antibiotics, 2 studies (36, 49) found that the supplementation of probiotics or synbiotics could decrease the duration of antibiotic usage, and another 2 studies (34, 37) found no statistical difference between the intervention and the control group. Specifically, the study by Liu et al. reported a shorter duration of antibiotic therapy in the probiotics group compared with the control (5.69 vs. 7.29 days, p=0.001) (36). In agreement to Liu et al., Polakowski et al. also reported a shorter duration of antibiotic usage in the synbiotic group compared to the placebo group (1.42 vs. 3.74, p<0.001) (49). Contrary to these results, studies by Liu et al. and Yang et al. did not find significant differences in length of antibiotic therapy between the intervention and control groups (34, 37).

3.4.2 Chemotherapy

Seven studies (39–41, 50, 51, 53, 55) assessed the effect of probiotics or synbiotics supplementation in CRC patients undergoing chemotherapy treatment. Details from each study are shown in Table 4. One study used synbiotics for the intervention group (50), 3 used a mixture of probiotics strains (39, 51, 55), 2 studies used kefir and one study used a single strain in the intervention group (53). For the control group, 2 studies used standard of care (51, 53), while the remaining 5 used some type of placebo (39–41, 50, 55).

The impact of probiotics or synbiotics supplementation on the incidence of diarrhea was assessed in all studies but one (40). Significant improvement of this side-effect was reported in 3 of the following studies: (1) Österlund et al. reported a lower incidence of diarrhea grade 3 – 4 in patients who received *Lactobacillus rhamnosus* twice a day for the 24 weeks of adjuvant chemotherapy (22% vs 37%, p=0.027) (53), comparing to the placebo group; (2) Mohebian et al. noted a lower severity of diarrhea and improved stool consistency in patients who took a mixture of probiotics twice a day for one week (51); and (3) Huang et al. also demonstrated a lower incidence of diarrhea in patients who received probiotics from day 3 postoperative to the end of the first neoadjuvant chemotherapy cycle, in a total of 6 weeks (16% vs. 40%, p=0,008) (39). Mego et al. also observed a lower incidence of overall diarrhea and lower incidence of grade 3 – 4 diarrhea when probiotics were administered 3 times a day for 12 weeks, however, these results did not reach statistical difference when compared to the placebo (0% vs. 17.4% p=0.11 and 39.1% vs. 60.9% p=0.24 respectively) (55). In contrast, a study by Can et al., where 250ml of kefir was consumed during the first 5 days of each chemotherapy cycle, reported that this intervention did not prevent diarrhea but increased constipation (41).

Besides lower incidence of diarrhea, the study by Huang et al. also reported, when compared to placebo, lower incidences of abdominal pain (6% vs 24%, p=0.025), abdominal distension (10% vs 28%, p=0.041) and constipation (8% vs 28%, p=0.019) in the group who took probiotics (39). Furthermore, Österlund et al. also demonstrated a lower abdominal discomfort resulting from flatulence and less abdominal distension in patients who took *Lactobacillus rhamnosus* (2% vs. 12%, p=0,025). This study also showed statistical significance for less chemotherapy-dose reductions due to bowel toxicity (21% vs 47%, p=0,008) in the intervention group (53). Accordingly, Farshi Radvar et al. demonstrated that synbiotic administration for 6 weeks, starting one week before chemoradiotherapy, decreased the incidence of symptoms such as nausea, vomiting, appetite loss and diarrhea, even though these results weren't statistically significant, the placebo group has significant increases in these symptoms (50).

One study assessed the impact of kefir supplementation on mucositis development and reported no preventive effect of supplementation during the first 5 days of each chemotherapy cycle (40).

3.4.3 Radiotherapy

Farshi Radvar et al. assessed the impact of synbiotics supplementation during radiotherapy, in rectal cancer patients undergoing neoadjuvant chemoradiotherapy. All of the participants received pelvic radiotherapy 5 times a week for 5 to 6 weeks and an intravenous dose of chemotherapy daily for 5 days in the beginning and at the end of radiotherapy. Participants in the intervention group took synbiotics for 6 weeks starting 1 week before beginning chemoradiotherapy. Quality of life was assessed through the European Organization for Cancer Research and Treatment of Cancer's 30-item quality of life questionnaire version 3.0 which is composed of 3 scales: global health status, functional scale, and symptom scale (50, 58).

The results showed that, in terms of global health status, the synbiotic group had a higher improvement (69.73 to 74.12; p=0.39) compared to the control group (68.42 to 68.85; p=0.96) but the results weren't of statistical significance (p=0.60). No improvements were observed in the functional scale but the synbiotic group had a decrease in the overall mean of the symptom scale (18.45 to 16.95; p=0.56) while the control group had an increase (21.37 to 24.88; p=0.29), although the results showed no statistical significance between the 2 groups (p=0.22). Particularly, nausea and vomiting (4.38 to 3.50; p=0.71) and diarrhea (33.33 to 26.31; p=0.49) decreased slightly in the synbiotic group and increased [(10.52 to 17.54; p=0.17), and (45.51 to 57.89; p=0.27) respectively] in the placebo group. This study also evaluated the quality of life in both groups, showing that the synbiotic group had a bigger increase in this parameter compared to the placebo group, but no statistically significant difference was reached (69.73 to 74.12 vs. 68.42 to 68.85, p=0.60) (50).

4 Discussion

CRC treatment has evolved in recent years, yet the accompanying side-effects still significantly impact patients' quality of life and prognosis (6, 59). For this reason, it remains imperative to explore solutions that may decrease the occurrence of associated toxicity and complications in order to achieve a more successful outcome (4). In the present systematic review, we aimed to critically examine the current scientific evidence on the impact of pre-, pro-, and synbiotics used for modulating the microbiota, in

TABLE 4 Details of the included studies that assessed the impact on chemotherapy and radiotherapy outcomes.

Reference	Intervention Group			Control Group				Cancer Stage	Intervention		Outcome
	N°	Mean age (min-máx)	Male/ Female	Type	N°	Mean age (min-máx)	Male/ Female		Type	Dose/ Duration	
Österlund, P. 2007 (53)	98	61 (35-74)	51/47	Standard Care	52	57 (31-75)	25/27	II-IV	*Lactobacillus rhamnosus GG*	1-2x10^{10} per day, twice a day during the 24 weeks of adjuvant cancer CT*.	Participants had less grade 3 or 4 diarrhea (p=0.027), reported less abdominal discomfort (p=0.025), needed less hospital care (p=0.021) and had fewer CT dose reductions due to bowel toxicity (p=0.0008).
Topuz, E. 2008 (40)	17	51 (19-75)	12/5	Placebo	20	58 (34-72)	12/8	II-IV	Kefir	250 ml of kefir twice a day after meals on the first 5 days of each CT cycle.	Kefir consumption made no statistically significant effect on serum proinflammatory cytokine levels and on the incidence of mucositis development.
Can, G. 2009 (41)	17	–	12/5	Placebo	20	–	12/8	II-IV	Kefir	250 ml of kefir twice a day after meals on the first 5 days of each CT cycle.	Kefir does not prevent or decrease gastrointestinal complaints in patients undergoing CT for colorectal cancer. No difference was found between the two groups for quality of life.
Mego, M. 2015 (55)	23	62 (45-75)	14/9	Placebo	23	64 (42-81)	12/11	–	*Lactobacillus plantarum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus brevis, Bifidobacterium infantis, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium longum and Streptococcus thermopilus*	10×10^{9} CFU per capsule. 3×1 capsule per day orally for 12 weeks.	Administration of probiotics reduced the incidence of enterocolitis (p=0.49), of diarrhea (p=0.24) and of severe diarrhea (grade 3 or 4) (p=0.11). Usage of antidiarrheal drugs was also reduced (p=0.45). There was no infection caused by probiotics recorded. Results did not reach statistical significance.
Farshi Radvar F. 2020 (50)	19	58	13/6	Placebo	19	63	12/7	II-III	*Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium longum, Bifidobacterium breve and Streptococcus thermopilus* + fructoligosaccharides	1x10^{8} CFU twice a day before meals for 6 weeks starting 1 week before treatment to the end of radiotherapy.	Synbiotic supplementation caused improvement in global health status (p=0.60), symptom scale scores (p=0.75) and scores of functional scales (p=0.57). Nausea and vomiting (p=0.16), insomnia (p=0.25) and diarrhea (p=0.20) decreased slightly in the synbiotic group but increased significantly in the placebo group.
Mohebian, F. 2023 (51)	19	–	13/6	Standard Care	25	–	18/7	–	*Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium longum, Bifidobacterium breve and*	1 capsule (500mg) twice a day for 1 week.	The number of defecations in the yogurt group with probiotics and yogurt was significantly lower than the control group p<0.05). The severity of diarrhea in the group with probiotics decreased more rapidly (p<0.05). Stool consistency in the group with probiotics was significantly better than the control (p<0.05).

(Continued)

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TABLE 4 Continued

Reference	Intervention Group			Control Group				Cancer Stage	Intervention		Outcome
	N°	Mean age (min-máx)	Male/ Female	Type	N°	Mean age (min-máx)	Male/ Female		Type	Dose/ Duration	
									Streptococcus thermopilus + fructoligosaccharides		
Huang, F. 2023 (39)	50	58	24/26	Placebo	50	62	29/21	I-III	*Enterococcus faecalis, Lactobacillus acidophilus, Bacillus cereus and Bifidobacterium infantis*	1×3 tablets (1 capsule 3 times per day) from the 3rd post-operative day to the end of the 1st CT cycle.	Results showed that probiotics administration could effectively reduce CT-induced gastrointestinal complications, particularly in diarrhea ($p<0.01$).

CRC patients undergoing treatment, and to determine the potential therapeutic use of such approach.

Gut microbiota, specifically dysbiosis, has been associated with the development and progression of CRC (60). Specific bacteria such as *Fusobacterium nucleatum, Escherichia coli, Bacteroides fragilis, Streptococcus gallolyticus* and *Peptostreptococcus anaerobius* are frequent in CRC patients and have been linked with its development in various studies. Studies show that dysbiosis and the presence of these bacteria may alter the inflammatory, genomic, and metabolic processes in the host in a way that promotes carcinogenesis through different mechanisms such as the ability to induce DNA damage, interference with the DNA damage repair, impact on signaling pathways and immune suppression (60–63). Dysbiosis has also been observed in cancer patients undergoing immunotherapy and chemotherapy and has been associated with the efficacy of these treatments and their gastrointestinal toxicity and side-effects (62, 64). Consequently, gut microbiota modulation, in order to restore gut microbiota balance, through pre-, pro- and synbiotics has been studied as a potential therapeutic agent, potentiating cancer treatment effect or preventing and managing treatment-related toxicity or complications (65, 66).

Surgery remains the primary treatment for nearly all CRC patients. Although effective, surgery can lead to postoperative infectious or non-infectious complications that may impact prognosis (67, 68). In this systematic review, studies showed that the probiotics and synbiotics supplementation can have a role in reducing the incidence of postoperative complications. However, some evidence remains contradictory, and no conclusions can be drawn regarding the optimal formulation, duration and dosage of the intervention. Studies with shorter intervention duration and those using only one strain of probiotics appeared to yield less significant results (43, 44, 54), suggesting that a mixture of probiotic strains for a longer period of time may be more effective to reduce the incidence of postoperative complications. Accordingly, the following three systematic reviews and meta-analyses reported that the supplementation of probiotics and synbiotics can reduce the incidence of postoperative complications in CRC patients: (1) Chen et al. reported that probiotic or synbiotic administration significantly reduced the risk of developing postoperative infectious complications by 37% (RR = 0.63; 95%CI: 0.54–0.74) (28); (2) Veziant et al. reported that there were significantly fewer infectious complications in the probiotic or synbiotic group (RR = 0.59; 95%CI: 0.47–0.75) (29); (3) Araújo et al. reported that probiotic supplementation reduced the incidence of surgical site infection (OR = 0.53; 95%CI: 0.36 - 0.78) (31). These systematic reviews and meta-analyses also highlight that more evidence is needed regarding which strains of probiotics to use and what is the ideal intervention duration.

Probiotics and synbiotics may also facilitate a faster return to normal gut function, reduce hospital length of stay and decrease antibiotics usage after surgery, as evidenced by the majority of studies (34, 36, 37, 46, 47, 49, 52, 56, 57). However, three studies showed no significant results and (42, 43, 48) other two showed no difference between the control and intervention groups (35, 37). The heterogeneity between studies, considering sample size and type, dosage and duration of the intervention may explain the differences between the results. A systematic review and meta-

analysis by Amitay et al. reported that perioperative probiotics/synbiotics administration was associated with faster return to normal gut function, shorter postoperative antibiotics use, and shorter length of hospital stay (30). Zeng et al. also reported a shorter duration of antibiotic therapy but found no statistical differences in hospital length of stay (26) whereas An et al. concluded that probiotics may result in little to no difference in hospital length of stay after colorectal cancer surgery (69).

Both chemotherapy and radiotherapy can lead to several toxicity-related side-effects. Their dose or intensity reductions may thus be necessary, which in turn can result in less efficient outcome. In the past years, studies and systematic reviews have reported that gut modulation interventions can reduce the incidence of cancer treatment-related side-effects such as diarrhea and mucositis. However, most studies have included several types of cancers and not only CRC patients, which weakens conclusions, as results cannot be extrapolated (70–72).

This systematic review includes all studies that assessed the impact of probiotic or synbiotic supplementation in chemotherapy or radiotherapy exclusively in CRC patients. It was found that these interventions may have a potential role in alleviating gastrointestinal symptoms and overall quality of life of these patients (39, 50, 51, 53). Two studies using kefir in the intervention group, reported no improvements, as so kefir may not be an effective approach (40, 41). One study found no statistical significance when comparing probiotics to placebo, but this study ended prematurely due to slow accrual with only 46 out of the planned 220 patients, which may have compromised statistical power (55). Mahdavi et al. (27) reported that probiotics were not related to diarrhea incidence in patients undergoing chemotherapy, but, this systematic review only included three studies, compared to six studies included in the present review, which may explain the difference between the findings. Even though the evidence is promising, more studies with lower heterogeneity and exclusive for CRC patients are necessary to allow strong conclusions about the impact of these interventions in chemotherapy and radiotherapy toxicity and side-effects.

As previously mentioned, studies included in this review exhibit high heterogeneity between them in terms of used strains, dose, and duration of the intervention. Nonetheless, it can be observed that among studies where probiotics or synbiotics supplementation had a beneficial effect, some strains were present in the intervention across most of them. This is the case of species such as *Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum and Bifidobacterium lactis*. These are well known beneficial bacteria that help maintain a functional and structured gut barrier with preclinical studies showing that *Lactobacillus* spp. and *Bifidobacterium* spp. have anticancer functions such as inhibition of cell proliferation, induction of cancer cell apoptosis, modulation of host immunity and reduction of inflammation (62, 66). Furthermore, these strains are butyrate-producing bacteria which can repair and enhance gut barrier function and appears to inhibit proliferation of CRC cells (61). Taking this into account, administration of a combination of *Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum, and Bifidobacterium lactis* can be recommended for these types on interventions.

In terms of efficacy of probiotics versus synbiotics in CRC treatment, more robust evidence is needed in order to make stronger conclusions. The prebiotics contained in the synbiotics used in the studies included in the present review varied from fructoligosaccharides and galactoligosaccharides to betaglucan, inulin, pectin, and resistant starch with fructoligosaccharides being the one present in most of the studies with results of statistical significance that used synbiotics. The consumption of these types of prebiotics has been associated with increased counts of *Lactobacillus* spp. and *Bifidobacterium* spp. and as a result, higher levels of short-chain fatty acids including butyrate which, as previously mentioned, have anticancer properties (73, 74). Yet, in the present review, interventions with synbiotics do not appear to be more efficient than interventions with probiotics. Again, randomized controlled studies with less heterogeneity and larger sample sizes are needed in order to determine which gut modulation intervention is more adequate to improve CRC treatment.

Some studies have showed that gut microbiota composition differs across CRC progression. Notably patients with early-stage CRC (stage I-II) exhibit a distinct microbiota compared to those with late-stage CRC (stage III-IV) (9, 75). This raises questions about whether the efficacy of pre-, pro-, and synbiotic supplementation differs based on CRC stage. The majority of the studies encompassed in this systematic review included patients with different stages of CRC but didn't divide them accordingly. A systematic review from Dikeocha et al. (24) reported that probiotics supplementation has beneficial effects regardless of CRC stage. Nonetheless, future studies should take this information into consideration and compare the effectiveness of these types of interventions in different stages of CRC. Similarly, it has been noted that gut microbiota composition varies depending on the location of the CRC tumor with tumors on the left-side of the colon presenting a different microbiota than those on the right-side (76, 77). To our knowledge, no study accounted for this distinction by dividing the intervention group based on the tumor location. However, it would be interesting to future studies to investigate whether these factors influence the effectiveness of the intervention.

Although this systematic review also aimed to study the impact of microbiota modulation in immunotherapy, no studies meeting our inclusion criteria, specifically focusing on CRC patients undergoing immunotherapy were found. This treatment is a relatively new approach for CRC patients so it's possible that research may still be ongoing, and results will be published upcoming years regarding the role of pre-, pro-, and synbiotic supplementation in CRC patients undergoing immunotherapy (7, 78). Nevertheless, a recent meta-analysis, that included 6 studies, reported that probiotics improved the efficacy of immune checkpoint inhibitors in non-small cell lung cancer patients with the intervention group having better overall survival and higher objective response rate and disease control rate (79).

One point that also has to be considered is the safety of these interventions in CRC patients. Prebiotics, probiotics and synbiotics are in general considered safe, specially the most common studied and used strains such as *Lactobacillus* and *Bifidobacterium* (14, 80). In addition, none of the studies included in this review reported major adverse reactions caused by the intervention. Yet, microbiota modulation may

impact the prognosis, the immune function and toxicity, as so safe strains have to be confirmed and immunocompromised patients should be carefully monitored. More studies to assess the safety of gut modulation in this population are needed (81).

This systematic review has limitations, including high heterogeneity between intervention groups (strains, dose, and duration) and different primary outcomes, which compromise the accuracy of comparisons between studies. Most studies had a small sample size, and some had a short intervention time which decreases the probability of obtaining statistically significant results. Additionally, not all existing databases were searched, and only studies in English or Portuguese were full text screened and included in this review which may have led to a loss of relevant data.

Nonetheless, this review has its strengths. A comprehensive search was performed in several electronic databases and all current available evidence, including more recent studies, was analyzed. The search was not limited in terms of interval of years of publication, and it studied the impact of pre-, pro-, and synbiotic supplementation in different types of CRC treatment and not just one specific treatment.

5 Conclusion

In conclusion, the comprehensive analysis conducted in this systematic review suggests that supplementation with prebiotics, probiotics, and synbiotics may be beneficial for patients undergoing treatment for CRC. There is moderate evidence that this type of intervention in CRC patients may potentially facilitate return to normal gut function and decrease the occurrence of both infectious and non-infectious postoperative complications, reduce hospital length of stay, and mitigate antibiotic usage. Furthermore, there is also some evidence suggesting that probiotic and synbiotic administration may help lessen some side effects, mainly diarrhea, associated with chemo- and radiotherapy. Interventions with more than one strain type, and longer duration, appear to be more effective. Randomized controlled studies with less heterogeneity and larger sample sizes are needed in order to determine the best approach regarding strain selection, dosage, and duration of the intervention in gut modulation interventions in CRC patients.

Data availability statement

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

Author contributions

MM: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. MC: Data curation, Investigation, Writing – review & editing. MC: Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. NP: Writing – review & editing. TS: Writing – review & editing. SG: Writing – review & editing. AM: Writing – review & editing. PR: Formal analysis, Supervision, Validation, Writing – review & editing.

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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/fonc.2024.1395966/full#supplementary-material

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Mendelian randomization study and mediation analysis about the relation of inflammatory bowel disease and diabetic retinopathy: the further exploration of gut-retina axis

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Background: The concept of the gut-retinal axis proposed by previous scholars primarily focused on the relationship between intestinal microbiota and retinal diseases, and few further expanded the relationship between intestinal diseases and retinal diseases. To further substantiate the concept of the gut-retinal axis, we analyzed inflammatory bowel disease (IBD) and diabetic retinopathy (DR) using Mendelian randomization (MR), and use mediation analysis to further explore the potential substances that influence this causal relationship.

Methods: The genome-wide association study's (GWAS) summary statistics for genetic variations were utilized in a Mendelian randomization (MR) investigation. GWAS data on IBD (including ulcerative colitis (UC), Crohn's disease (CD), and IBD) for non-Finnish Europeans (NFE) were sourced from published articles. In contrast, data on DR (including DR and diabetic maculopathy (DMP)) were obtained from FinnGen R9. The causal relationship has been investigated using inverse variance weighted (IVW), MR-Egger, and weighted median and sensitivity analysis was applied to verify the stability of the results. In addition, we applied mediation analysis to investigate whether circulating inflammatory proteins and plasma lipids played a mediating role, and calculated its effect ratio.

Results: The causal relationship between IBD and DR was discovered by employing the inverse variance weighted (IVW) method and weighted median method. In forward MR, UC was significantly associated with lower risk of DR (IVW: OR=0.874; 95%CI= 0.835–0.916; P value= 1.28E-08) (Weighted median: OR=0.893; 95%CI= 0.837–0.954; P value= 7.40E-04). In reverse MR, it was shown that DR (IVW: OR=0.870; 95%CI= 0.828–0.914; P value= 2.79E-08) (Weighted median: OR=0.857; 95%CI= 0.801–0.916; P value= 6.40E-06) and DMP (IVW: OR=0.900; 95%CI= 0.865–0.937; P value= 3.34E-07)(Weighted median: OR=0.882; 95%CI= 0.841–0.924; P value= 1.82E-07) could reduce the risk of CD. What's more, DR is associated with a lower risk of IBD according to

genetic prediction (IVW: OR=0.922; 95%CI= 0.873–0.972; P value= 0.002) (Weighted median: OR=0.924; 95%CI= 0.861–0.992; P value= 0.029). Fibroblast growth factor 21 (FGF21), phosphatidylcholine (PC), and triacylglycerol (TG) serve as mediators in these relationships.

Conclusions: Our research offers novel insights and sources for investigating the gut-retina axis in the genetic relationship between IBD and DR. We discover four mediators and more about the association between the intestine and retinal disorders and provide more evidence for the gut-retinal axis theory.

KEYWORDS

gut-retina axis, inflammatory bowel disease, diabetic retinopathy, Mendelian randomization, mediation analysis

1 Introduction

During the past few years, there has been a significant global surge in the incidence of inflammatory bowel disease (IBD), which consists of Crohn's disease (CD), ulcerative colitis (UC), and indeterminate colitis. IBD is a chronic condition with a predilection for children and young adults, and it is linked to a range of adverse outcomes such as cancer, hospitalizations, surgical interventions, and infections (1). The pathogenesis of IBD is intricate and multifactorial, involving intricate interactions between genetic susceptibility, environmental triggers, immune-mediated mechanisms, and microbial influences. Extensive research efforts have been dedicated to unraveling the complexities of this disease. The management of IBD aims to achieve clinical remission through a combination of dietary interventions and pharmacotherapy targeting inflammation. This comprehensive approach enhances the overall quality of life for individuals afflicted by IBD (2).

Diabetic retinopathy (DR) is a noticeable ocular manifestation of diabetes mellitus (DM), affecting approximately 30 to 40% of individuals with diabetes (3). Hyperglycemia can cause capillary occlusion and an increase in vascular permeability, which can result in nonproliferative DR (NPDR). A proliferative phase of DR (PDR), typified by the creation of new blood vessels, may occur after this phase (4). Among the various complications of DR, diabetic maculopathy (DMP) is a prominent cause of legal blindness, primarily characterized by diabetic macular edema (DME). DME occurs when fluid builds up in the macula, leading to visual impairment. Adequate assessment, screening, and imaging techniques can aid in preventing vision loss associated with DR. However, despite these measures, DR still imposes a substantial societal burden, as individuals cope with increased life pressures and potentially modify their dietary habits (5, 6).

Currently, numerous studies have demonstrated a connection between the metabolism of intestinal microorganisms and retinal diseases, so the concept of the gut-retinal axis has been proposed by scholars (7). The gut microbiota has been found to influence the expansion of various retinal disorders, including diabetic retinopathy, optic neuritis, age-related macular degeneration, and retinopathy of prematurity. As a result, controlling the gut microbiota has grown into a potential approach for treating or preventing such eye disorders (8). However, most studies investigating the gut-retinal axis have primarily focused on the field of intestinal microorganisms, whereas the relationship between intestinal diseases and retinal diseases remains largely unclear. The connection between intestinal and retinal diseases has been studied through observational research, but the findings have been limited. Uveitis associated with IBD is well-documented, but there have been few studies investigating the link between IBD and DR. Both IBD and DR involve barrier dysfunction. In IBD, large amounts of tissue inhibitors of metalloproteases (TIMP)-free matrix metalloproteases (MMPs) are produced by neutrophils, while in DR, mononuclear leukocytes synthesize MMP-9 in a balanced manner with TIMP-1. Therefore, MMP-9 may be a therapeutic target for both conditions (9). Additionally, APE1/Ref-1, a multifunctional signal transduction enzyme, is implicated in the pathogenesis of both IBD and DR (10). To further explore the gut-retina axis, we have chosen IBD and DR as research subjects due to their close association with metabolism and inflammatory factors.

In randomized controlled trials, there are always issues with the viability, logic, and safety of investigation. The conclusions obtained from individual randomized controlled trials are potentially biased by uncontrolled confounding factors. A statistical technique known as Mendelian randomization (MR) can be employed to overcome these limitations. MR prevents reverse causation and facilitates the derivation of valid conclusions regarding the causal relationship between exposure and outcome variables. This approach utilizes single-nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to assess the causal association between two phenotypes (11). The alleles of the genetic variant associated with the exposure of target phenotype are randomly assigned and not susceptible to reverse causation (12, 13). In our study, we utilized genotype-

phenotype associations from publicly available Genome-wide association studies (GWASs) in a two-sample bidirectional Mendelian randomization investigation to examine whether causal link exists between IBD and DR.

2 Materials and methods

2.1 Study design

A brief description of this bidirectional MR design between IBD and DR is displayed in Figure 1. The data about the IBD could be extracted from the study of non-Finnish European (NFE) published in *Nature genetics* by Katrina (14, 15). The data about DR could be divided into DR and diabetic maculopathy, an important subcategory. All data come from published articles or publicly available GWAS statistics. With bidirectional MR, IBD is viewed as the exposure and DR as the result in forward MR analysis. In contrast, DR is considered as the exposure and IBD as the result in reverse MR analyses (16). Due to the limited size of SNPs of DMP, we could only use this data in reverse MR analysis. After conducting a bidirectional Mendelian randomization analysis, we proceeded with a mediation analysis to further investigate the mechanisms underlying the interaction between IBD and DR. The whole study is based on three basics instrumental variable (IV) assumptions: I The chosen instrumental variables should have a substantial correlation with exposure. II The chosen instrumental variables should be separated from confounding factors. III The chosen instrumental variables must affect the outcome only through exposure. Research may only be conducted following those as mentioned above three fundamental principles.

2.2 Data source of exposures, outcomes and mediators

The GWAS statistics on IBD and its two subtypes, UC and CD, come from the GWAS study published in *Nature genetics* (14). The study subjects were European populations not included in the whole-genome meta-analysis, referred to as non-Finnish European (NFE) (15). The study involved 12,366 UC cases, 12,194 CD cases, and 25,042 IBD cases. This data is available from the GWAS catalog (https://www.ebi.ac.uk/gwas/publications/28067908) and the IEU open GWAS database (https://gwas.mrcieu.ac.uk/). The GWAS statistics of DR were collected from the FinnGen R9 research consortium (https://r9.finngen.fi/pheno/DM_RETINOPATHY_EXMORE). This data included 10413 DR cases and 308633 controls of Finnish ancestry. The definition of DR in our statistics is a chronic, pathological complication associated with diabetes mellitus (DM), where retinal damages are incurred due to microaneurysms in the vasculature of the retina. In addition, the GWAS of DMP was used in our analysis because it's an important sub-disease of DR. This GWAS was also extracted from the FinnGen R9 research consortium (https://r9.finngen.fi/pheno/DM_MACULOPATHY_EXMORE) which encompassed 3572 cases and 308547 controls. DMP is recognized as a loss of vision in the central portion of the retina (macula) caused by DM.

The relationship between IBD and DR and their association with the body's inflammatory state and lipid composition is currently recognized (17). We conducted a study to investigate if circulating inflammatory proteins and plasma lipids play a role in mediating the relationship between IBD and DR. *Zhao* conducted a genome-wide study on 91 plasma proteins (GCST90274758 - GCST90274848) (18), and we utilized this data for our research.

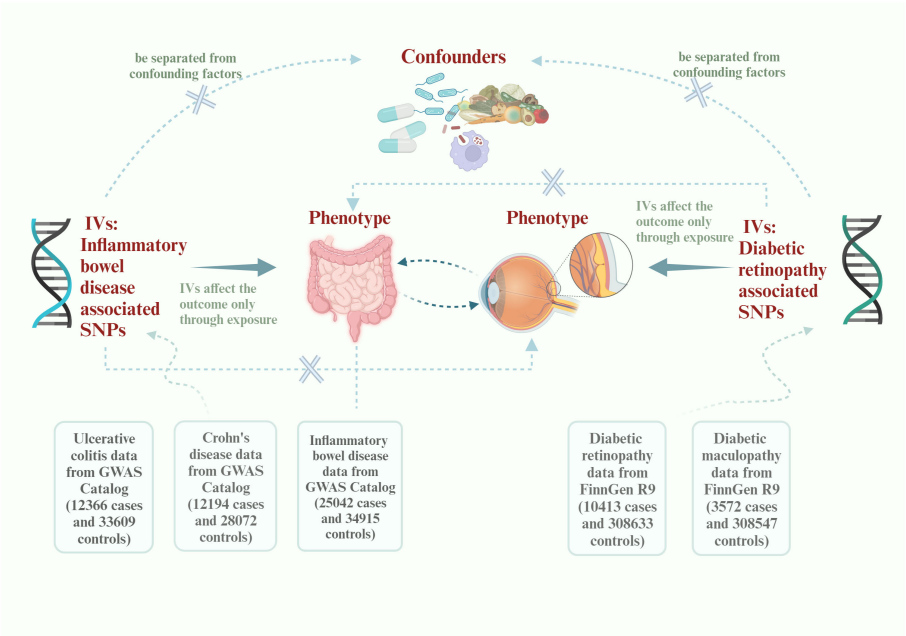

FIGURE 1
Flowchart of this Mendelian randomization study. IVs, instrumental variables; SNPs, single-nucleotide polymorphisms; GWAS, Genome-wide association study.

Additionally, *Ottensmann* carried out a genome-wide analysis on 179 lipids from 13 lipid classes (GCST90277238 - GCST90277416) (19). The data is available from the GWAS catalog (https://www.ebi.ac.uk/gwas/home).

2.3 Ethics statement

The data used in this study to analyze the relationship between IBD and DR can be obtained from public literature or databases. The institutional ethical committees of each GWASs utilized in this study confirmed their approval. No further ethical approval is required in this situation.

2.4 Selection of genetic instrumental variables

At first, we performed forward MR analysis, and UC, CD and IBD were chosen for the exposure variable. Firstly, a subset of SNPs was chosen as IVs based on their significance level below the genome-wide threshold of 5×10^{-8}, respectively. Secondly, we implemented a linkage disequilibrium (LD) analysis based on European-based 1,000 Genome Projects (R2 <0.001, clumping distance = 10,000 kb) to meet the MR core assumptions. The SNPs that failed to satisfy the requirements were deleted. Thirdly, Each SNP's efficacy as an IV was determined using the F-statistic: a value above ten suggested a strong instrument. Finally, we removed all palindromic sequences to ensure the selected SNPs were referred to the same allele when harmonizing the effects of the SNPs on exposure and outcome. We corrected the strand for SNPs with different effect alleles, guaranteeing the effect allele was the same in both databases.

In reverse MR analysis, the DR and DMP were selected for the exposure variable. Considering the limited size of IVs obtained from the GWAS of DMP, we selected the wide significance ($P<5\times10^{-6}$) as the threshold to get more SNPs. The settings for the parameters and leftover flow were the same as in the forward MR.

In the mediation analysis, 91 circulating inflammatory proteins and 171 plasma lipid components were considered as potential mediators. Due to the limited number of IVs, we also relaxed the selection threshold ($P<5\times10^{-6}$) to include more SNPs.

2.5 Mendelian randomization analysis

The primary approach for estimating the causal effect values unbiasedly was the inverse variance weighted (IVW) test, which prevented confounding factors in the absence of a horizontal pleiotropy. IVW is the most widely used method combining the Wald ratio in fixed-effect meta-analysis to infer the presence and the strength of the causal effect between an exposure and an outcome (20). At the same time, we highlighted the significance of the weighted median method because it can maintain consistency where invalid instrumental variables account for as much as 50% of the data (21). By combining the results obtained by these two methods, we can improve the solidity of the final results. Furthermore, MR-Egger regression was performed to estimate the causal effect adjusted for directional pleiotropy. It has the lowest capability so its results should be cautiously treated. When only one SNP passed quality control, causal associations were evaluated using the Wald ratio (WR) method.

2.6 Sensitivity analysis

Several sensitivity analyses were applied to detect the horizontal pleiotropy and heterogeneity, which may improve the reliability of results. Cochran's Q test, heterogeneity, the leave-one-out method, RadialMR and MR PRESSO each play a special role in a sensitivity analysis. Finding the heterogeneity is essential since it helps decide when it's suitable to combine all individual outcomes into a single summary measure (22). Cochran's Q test is the main method to evaluate heterogeneity between different samples. Since we consider the outcomes of our study to be stable and reliable in the absence of heterogeneity, the test's P value ought to be greater than 0.05. We are more concerned about horizontal pleiotropy since it defies fundamental MR analysis assumptions (the chosen instrumental variables must impact the outcome only through exposure). MR-Egger intercept was applied to assess the presence of horizontal pleiotropy between the IVs and the result, while MR PRESSO global test also detected horizontal pleiotropy. According to statistical presumptions, we cannot conclude that the study shows horizontal pleiotropy when the P value>0.05. The leave-one-out method detected whether results change due to a certain SNP. This is another efficient way to guarantee the outcome's stability. RadialMR was applied to exclude the outlier SNPs because methods for removing outliers can successfully lessen bias in MR estimates.

The Bonferroni method was adopted to correct all P values. Calculating Bonferroni's adjustment involves dividing the total number of tests by the alpha value. In forward MR analysis, if the corrected P value <0.016, we have more grounds for believing that exposure and result are strongly correlated. If the adjusted P value is less than 0.05 but greater than 0.016, we may consider the possibility of an exposure-outcome relationship into account. As with forward MR, we may be more certain that the exposure is highly correlated with the result in reverse MR when the P value is smaller than 0.0083. P values between 0.0083 and 0.05 indicate a possible association between the two factors.

2.7 Mediation analysis linking IBD with DR via circulating inflammatory proteins and plasma lipids

We conducted a mediation analysis to connect IBD with diabetic retinopathy DR through circulating inflammatory proteins and plasma lipids. After performing a bidirectional Mendelian randomization analysis, we found positive associations between exposure factor A1 and outcome factor B1. Next, we investigated the correlations between 91 circulating inflammatory proteins and 179 plasma lipids with the outcome factor B1. We selected the substances that showed positive associations for further analysis. These selected substances were then analyzed to determine

their correlation with exposure factor A1 when considered as outcome factors B2. When intermediary substances were found to be correlated with both exposure and outcome factors, we calculated the proportion of the mediating effect attributable to each intermediary substance using delta method (23).

We use RStudio (2024.04.1 + 748) to perform the above process, and with the help of TwoSampleMR R packages (version 0.5.7).

3 Results

3.1 Causal effect in forward MR analysis

A forward MR analysis was conducted to examine the IBD and its two subtypes, whether about DR. The figure shows that IBD did not show a genetic relationship with DR. However, the results of two subtypes are not fully aligned with IBD (IVW: OR=0.999; 95% CI= 0.974–1.024; P value= 0.948) (Weighted median: OR=0.981; 95%CI= 0.939–1.023; P value= 0.366). UC was negatively correlated with DR (IVW: OR=0.874; 95%CI= 0.835–0.916; P value= 1.28E-08) (Weighted median: OR=0.893; 95%CI= 0.837–0.954; P value= 7.40E-04), CD did not show a causal relationship with DR as well (IVW: OR=1.022; 95%CI= 0.994–1.049; P value= 0.118) (Weighted median: OR=1.012; 95%CI= 0.974–1.051; P value= 0.538). Supplementary Material 1 shows the complete results and Table 1 shows the sensitivity analysis results. Figure 2 shows the forest plot of the analysis results.

We rigorously conduct sensitivity analysis to guarantee the integrity of the findings. The results of UC had passed various tests. The p-value in the heterogeneity test was 0.383, and the p-value in the Egger-intercept test was 0.101. scatter plots and Leave-one-out plots are presented in Figures 3, 4. After applying the Bonferroni correction, the relationship between UC and DR was still statistically significant.

3.2 Causal effect in reverse MR analysis

In reverse MR analysis, we respectively investigated the possibility that DR and DMP are genetically related to IBD and its subtypes. To find possible correlations between DMP and IBD, we used a lenient P-value (P<5E-06). As shown in Figure 2, the results indicated that DR (IVW: OR=0.870; 95%CI= 0.828–0.914; P value= 2.79E-08)(Weighted median: OR=0.857; 95%CI= 0.801–0.916; P value= 6.40E-06) and DMP (IVW: OR=0.900; 95%CI= 0.865–0.937; P value= 3.34E-07)(Weighted median: OR=0.882; 95% CI= 0.841–0.924; P value= 1.82E-07) had causal relationships with the risk of CD. It is genetically predicted that DR is about the risk IBD (IVW: OR=0.922; 95%CI= 0.873–0.972; P value= 0.002) (Weighted median: OR=0.924; 95%CI= 0.861–0.992; P value=

TABLE 1 Heterogeneity and pleiotropy analysis of inflammatory bowel disease with diabetic retinopathy.

Exposure	Outcome	Methods	Q	P (heterogeneity)	P (Egger intercept)	MR-PRESSO global test P value
UC	DR	IVW	27.52	0.383		
		MR Egger	24.61	0.484	0.101	0.393
CD	DR	IVW	69.23	0.170		
		MR Egger	69.23	0.148	0.986	0.227
IBD	DR	IVW	79.59	0.523		
		MR Egger	79.41	0.497	0.673	0.567
DR	UC	IVW	10.03	0.528		
		MR Egger	8.43	0.587	0.235	0.648
DMP	UC	IVW	9.76	0.462		
		MR Egger	9.76	0.552	0.975	0.616
DR	CD	IVW	13.55	0.632		
		MR Egger	9.92	0.825	0.076	0.652
DMP	CD	IVW	15.78	0.397		
		MR Egger	13.62	0.478	0.164	0.370
DR	IBD	IVW	8.01	0.843		
		MR Egger	7.44	0.827	0.462	0.920
DMP	IBD	IVW	13.82	0.312		
		MR Egger	11.18	0.428	0.136	0.299

MR, Mendelian Randomization; UC, Ulcerative colitis; CD, Crohn's disease; IBD, Inflammatory bowel disease; DR, Diabetic retinopathy; DMP, Diabetic maculopathy; IVW, Inverse variance weighted.

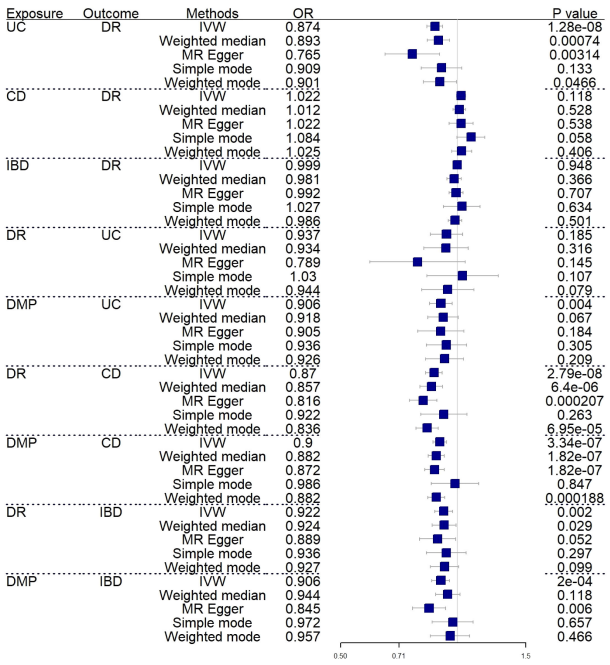

FIGURE 2
Forest plot of MR analysis results between inflammatory bowel disease and diabetic retinopathy. UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; DR, diabetic retinopathy; DMP, diabetic maculopathy; IVW, inverse variance weighted; OR, odds ratio.

0.029). What's more, the IVW results indicated DMP is about the UC (IVW: OR=0.906; 95%CI= 0.848–0.969; P value= 0.004) and the IBD (IVW: OR=0.906; 95%CI= 0.859–0.955; P value= 0.0002). Only one analysis, IVW, showed a positive relationship between DMP and UC/IBD, so we have reservations about the positive results. Sensitivity analysis revealed no notable heterogeneity and horizontal pleiotropy.

3.3 Result of mediation analysis

Based on the positive results mentioned earlier, we initially used the MR method to analyze the connection between 91 circulating inflammatory proteins and 171 plasma lipids with CD (for more information, please refer to Supplementary Materials S2, S6). We identified that a total of 13 circulating inflammatory proteins were associated with CD, with 9 proteins positively correlated and 4 proteins negatively correlated. Additionally, 25 plasma lipids were found to be associated with CD, with 10 lipids showing a positive correlation and 15 lipids showing a negative correlation. We then analyzed the correlation between these 13 circulating inflammatory proteins and 25 plasma lipids as the outcome factor with DR and DME as the exposure factor. We also calculated the mediating effect ratios. Similarly, we analyzed the association between the 91 circulating inflammatory proteins and 171 plasma lipids with IBD and DR, respectively (for more information, please refer to Supplementary Materials S3–S5, S7). Relevant results are shown in Figure 5; Table 2. After completing these steps, we identified Fibroblast Growth Factor 21, Phosphatidylcholine (20:4_0:0), and Phosphatidylcholine (O-18:0_20:4) as potential mediators that reduce the likelihood of CD. Additionally, Triacylglycerol (48:0) was identified as a potential mediator that reduces the likelihood of IBD.

4 Discussion

Numerous activities of the intestines are provided by the large amount of intestinal flora and its survival metabolism. Previous proposals have been proposed for the gut-kidney and gut-brain axes, and research on the relationship between the intestines and other tissues and organs has been conducted piecemeal. Based on our research, it was possible to link inflammation and metabolism to DR and IBD. Though these two illnesses appear to have different causes and manifestations in the human body, one must consider the significance of inflammatory variables and the integrity of human metabolism when considering the broader context. As there are no direct observational studies on DR and IBD, we cannot compare our results with those of real-world epidemiological surveys. We are investigating whether alternative mechanisms exist to bolster the notion of the gut-retina axis.

4.1 Causal effect of IBD on DR

Forward MR analysis has revealed a substantial negative correlation between UC and the risk of DR. Although our results indicated that circulating inflammatory proteins and plasma lipids did not mediate the causal relationship from IBD to DR in the mediation analysis, this relationship remained detectable. An increasing amount of research conclusively links iron to the pathological progression of retina disease, such as age-related macular degeneration (AMD) and DR. The inner blood-retina barrier (iBRB) protects retinal neural tissue from potentially hazardous compounds in circulation (24). Abnormal iron deposition disrupts iron homeostasis in retinal vascular endothelial cells (ECs), causing impairment of the blood-retinal barrier. Prior research has indicated that iron plays a pivotal role in the pathophysiology of DR by acting as a catalyst and facilitating reactive oxygen species (ROS)-induced dysfunction or death of ECs (25). Oxidative stress, characterized by a rise in hydroxyl radicals via the Fenton/Haber-Weiss mechanism, contributes significantly to retinal damage, affecting neurons, retinal pigment epithelial cells (RPEC), and retinal endothelial cells (REC) (26, 27). Iron also triggers canonical Wnt/β-catenin signaling in RPEC, which has been correlated with fibrosis, inflammation, and angiogenesis (28). The maintenance of retinal iron homeostasis is closely interconnected with systemic iron metabolism, relying on efficient iron absorption, transport, and utilization. Regardless of the structural integrity of the blood-retinal barrier, elevated serum iron levels can surpass regional processes that regulate retinal iron, potentially increasing the risk of age-related retinal diseases (29). In IBD, anemia, particularly iron deficiency anemia (IDA), is a common extraintestinal symptom. Increased intestinal mucosal

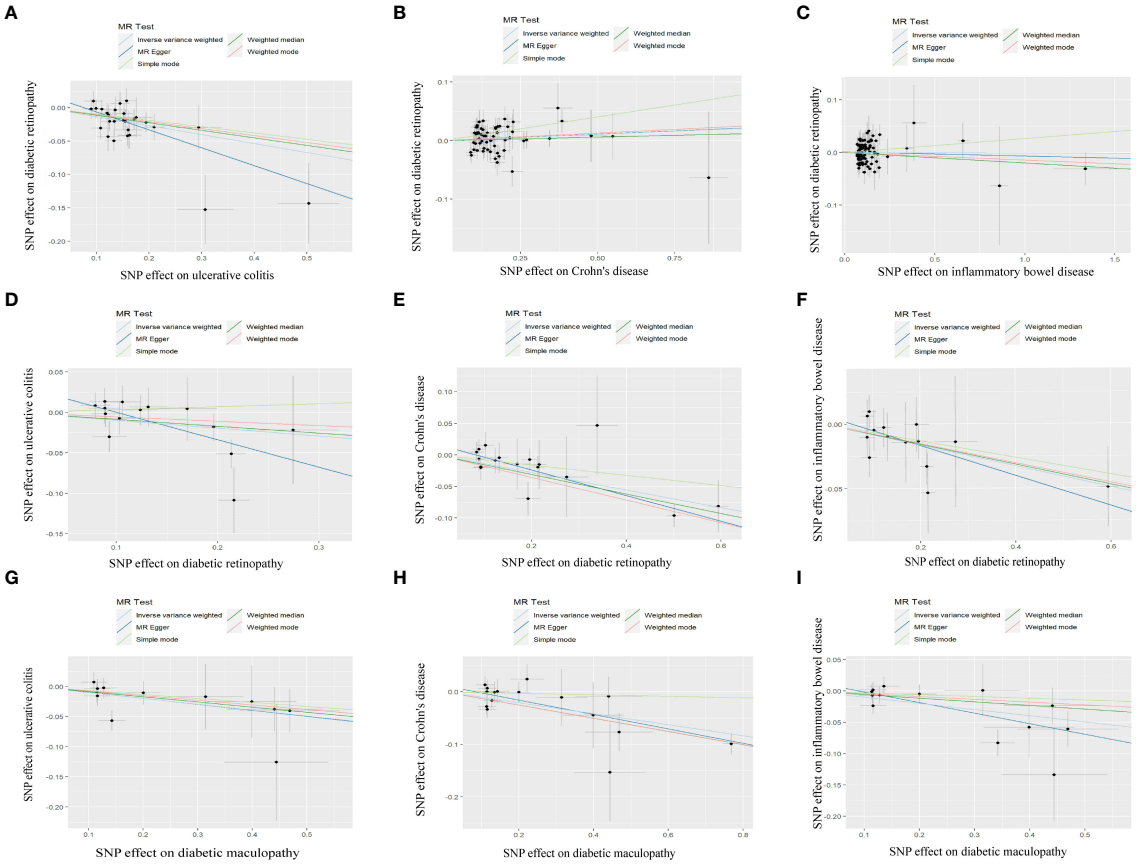

FIGURE 3
Scatter plot of MR analysis results: **(A)** scatter plot of MR analysis results between UC and DR; **(B)** scatter plot of MR analysis results between CD and DR; **(C)** scatter plot of MR analysis results between IBD and DR; **(D)** scatter plot of MR analysis results between DR and UC; **(E)** scatter plot of MR analysis results between DR and CD; **(F)** scatter plot of MR analysis results between DR and IBD; **(G)** scatter plot of MR analysis results between DMP and UC; **(H)** scatter plot of MR analysis results between DMP and CD; **(I)** scatter plot of MR analysis results between DMP and IBD. UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; DR, diabetic retinopathy; DMP, diabetic maculopathy.

inflammation in IBD leads to iron loss through gastrointestinal bleeding and impaired iron absorption, contributing to iron deficiency (30). The severity of intestinal inflammation has been shown to fluctuate with the extent of blood loss, which is more pronounced in UC than CD (31). Excessive blood loss exceeding dietary iron absorption can disrupt iron metabolism. Besides, hepcidin, a hepatocyte peptide hormone, regulates ferroportin activity on duodenal enterocytes. During inflammatory processes, cytokines like Interleukin-6 (IL-6) may induce hepcidin production, producing decreased iron transfer to plasma by altering ferroportin conformation (32). Systemic iron metabolism and storage are intricately linked to local iron homeostasis in the retina, necessitating further research to balance these factors effectively.

Furthermore, there's an interesting substance called adiponectin worth thinking about. White adipose tissue secretes the secretory protein adiponectin, which has pro- and anti-inflammatory characteristics. It is important in insulin sensitivity, anti-inflammatory/anti-fibrotic processes, and anti-apoptotic mechanisms (33). Adiponectin has been proven to elevate insulin sensitivity and exert antidiabetic effects in mouse models. Research has demonstrated that adiponectin protects against pathological retinal microvessel formation by down-regulating TNF-α-mediated inflammatory responses (34). TNF-α is a cytokine involved in inflammation, insulin resistance, and diabetic vasculopathy. Adiponectin suppresses TNF-α production by inhibiting nuclear factor κB activity in macrophages and promotes macrophage clearance of apoptotic bodies, leading to decreased TNF-α levels (35, 36). Studies have shown that adiponectin levels tend to be greater in male and female UC patients compared to healthy controls, and elevated levels of adiponectin are observed in the serum of patients with CD (37, 38). Adiponectin levels vary in autoimmune and chronic inflammatory diseases such as rheumatoid arthritis (RA), chronic kidney disease (CKD), and IBD, exhibiting both pro- and anti-inflammatory properties relying on the specific tissue and signaling pathways involved (39).

Intestinal barrier dysfunction in UC can lead to decreased absorption of fluid, electrolyte, amino acid, fat, and carbohydrate, and disrupted intestinal motility. Abnormal results in tests assessing D-Xylose absorption, and reduced absorption of fat, folic acid, and amino acids, have been observed in individuals with UC (40). Systemic inflammation in UC and CD patients has been connected with lower levels of total and low-density lipoprotein (LDL) cholesterol compared with healthy individuals (41). Lowering cholesterol is considered an important aspect of

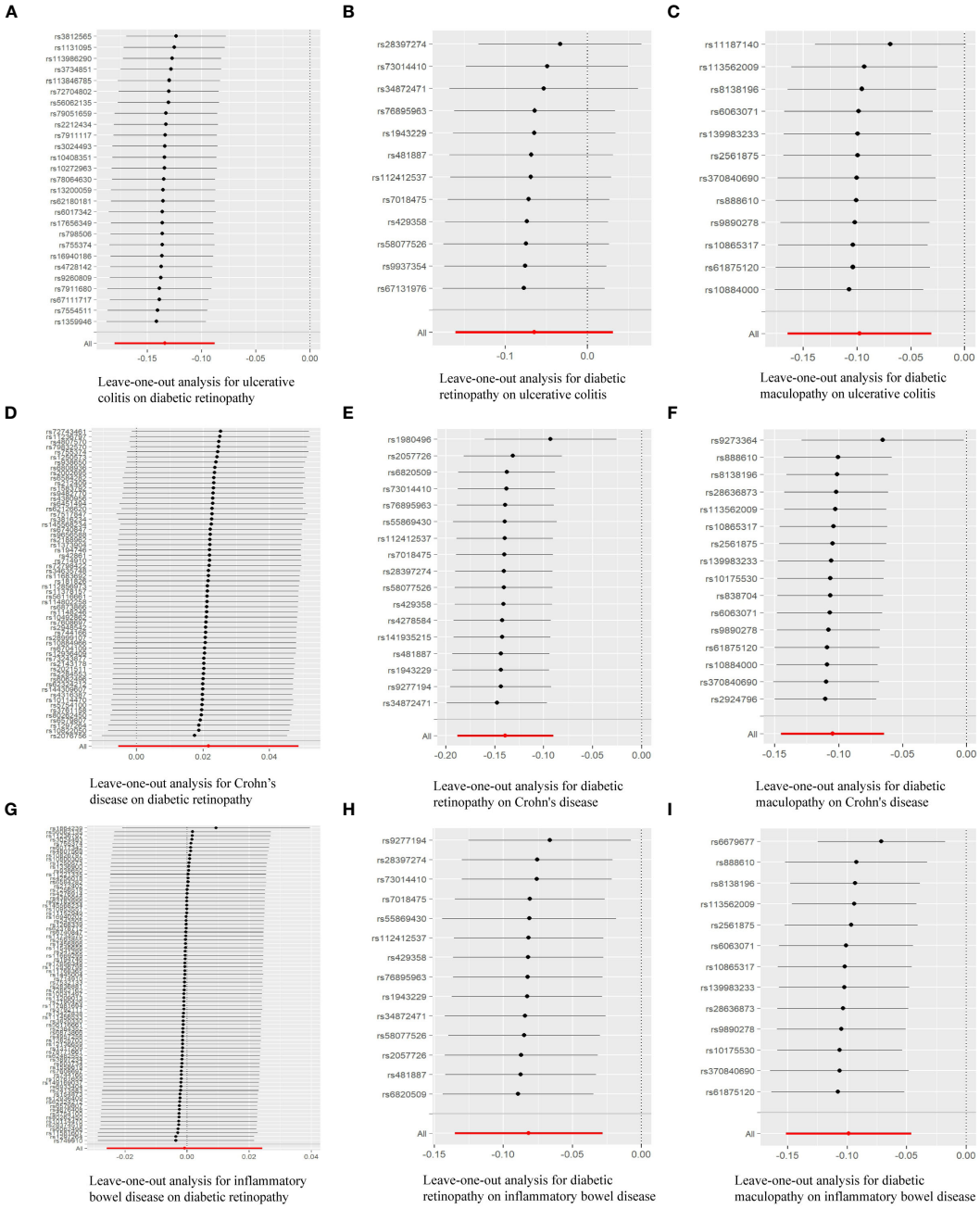

FIGURE 4
Leave-one-out analysis of the effect of individual SNPs of MR analysis results: **(A)** Leave-one-out analysis for ulcerative colitis on diabetic retinopathy; **(B)** Leave-one-out analysis for diabetic retinopathy on ulcerative colitis; **(C)** Leave-one-out analysis for diabetic maculopathy on ulcerative colitis; **(D)** Leave-one-out analysis for Crohn's disease on diabetic retinopathy; **(E)** Leave-one-out analysis for diabetic retinopathy on Crohn's disease; **(F)** Leave-one-out analysis for diabetic maculopathy on Crohn's disease; **(G)** Leave-one-out analysis for inflammatory bowel disease on diabetic retinopathy; **(H)** Leave-one-out analysis for diabetic retinopathy on inflammatory bowel disease; **(I)** Leave-one-out analysis for diabetic maculopathy on inflammatory bowel disease.

diabetes treatment, and statins, lipid-lowering drugs, have been shown to bring down the risk of vision loss and the occurrence of hard exudates and microaneurysms in DR (42, 43). The formation of cholesterol crystals during the development of DR disrupts retinal lipid metabolism and contributes to persistent inflammation (44). Moreover, high-fat intake can induce hypothalamic inflammation, characterized by increased levels of circulating cytokines and free fatty acids, resulting in neuroinflammation and the release of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (45, 46). This process also causes neurons to release fractalkine (CX3CL1), further intensifying inflammation by attracting peripheral monocytes to the hypothalamus (47). Hyperactivity of the sympathetic nervous system and hypothalamic inflammation are features of type 2 diabetes and metabolic syndrome (48). Hypothalamic inflammation and insulin resistance promote the development of

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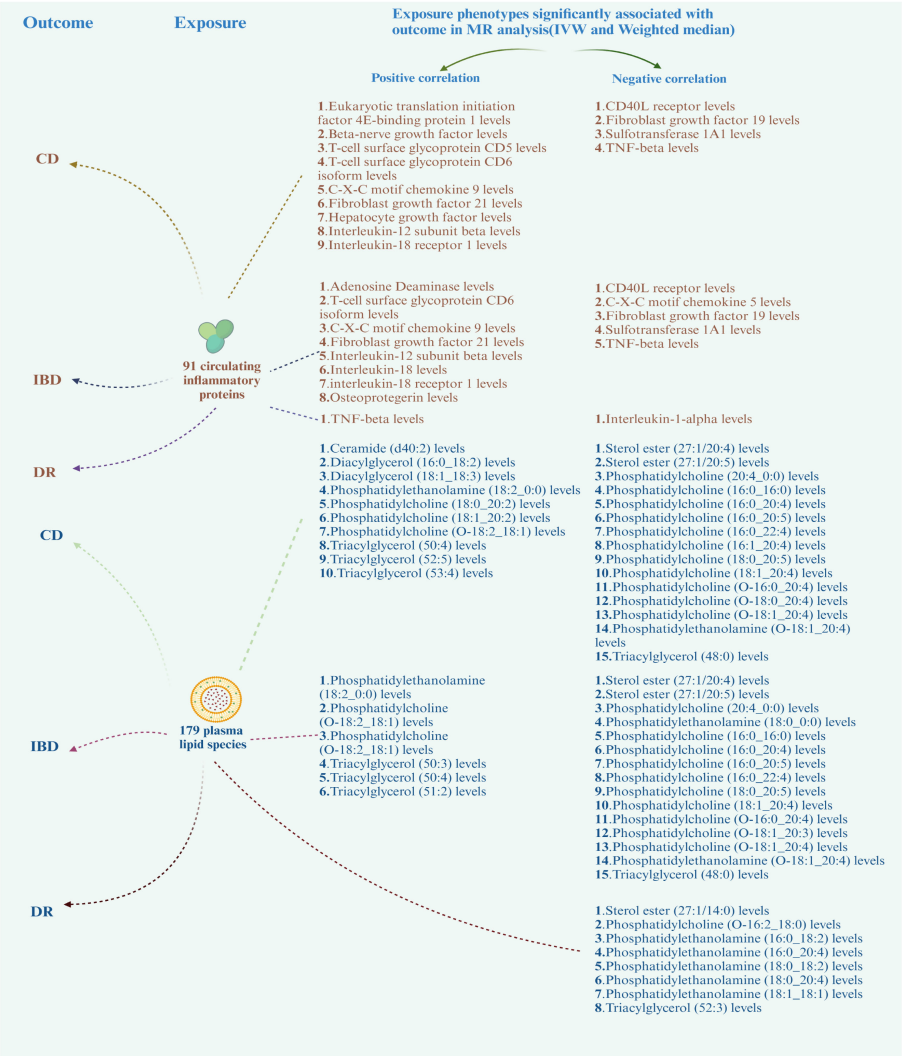

FIGURE 5
Correlation of circulating inflammatory proteins and plasma lipids with CD, IBD, and DR. CD, Crohn's disease; IBD, inflammatory bowel disease; DR, diabetic retinopathy.

TABLE 2 The positive mediation effect of DR on IBD through circulating inflammatory proteins and plasma lipids.

Exposure	Mediator	Outcome	Beta (SE), P value			Mediating effect (95%CI)
			Exposure-Outcome	Mediator-Outcome	Exposure-Mediator	
DMP	Fibroblast growth factor 21	CD	-0.10484 (0.02054) P_value=3.34e-07	0.35156 (0.08875) P_value=7.46e-05	-0.036 (0.01259) P_value=4.26e-03	12.1% (8.6%-23.3%)
DMP	Phosphatidylcholine (20:4_0:0)	CD	-0.10484 (0.02054) P_value=3.34e-07	-0.13264 (0.031489) P_value=2.53e-05	0.04362 (0.01915) P_value=0.022709	5.5% (0–11.3%)
DR	Phosphatidylcholine (O-18:0_20:4)	CD	-0.139 (0.025) P_value=2.79e-08	-0.14466 (0.0656) P_value=0.02765	0.0387 (0.01934) P_value=0.0454	4.0% (0–9.5%)
DR	Triacylglycerol (48:0)	IBD	-0.08169 (0.02732) P_value=0.0027	-0.13789 (0.04293) P_value=0.00132	0.05259 (0.02246) P_value=0.01919	8.8% (0–19.7%)

CD, Crohn's disease; IBD, Inflammatory bowel disease; DR, Diabetic retinopathy; DMP, Diabetic maculopathy; SE, Standard error; CI, Confidence interval.

DR by initiating inflammatory cascades and impairing pancreatic β-cell function and insulin production (49). In conclusion, a lower intake of lipids in UC may reduce the risk of triggering hypothalamic inflammation, thereby protecting against the development of DR.

4.2 Causal effect of DR on IBD

DR is a chronic inflammatory disease closely linked to lipid metabolism. MR studies suggest that phosphatidylcholine (PC) may mediate interactions between DR and CD. PC, a multifunctional phospholipid, is essential for cell membrane structure, fat metabolism, and cell signaling (50). Metabolomic analyses indicate some lower serum PC levels in NPDR and PDR groups compared to type 2 diabetes mellitus (T2DM) patients, except for PC (C34:4 and C36:6) which are lower in PDR than NPDR (51). Conversely, another study found elevated PC levels in DR patients compared to T2DM and non-DR patients (52), making the PC-DR relationship controversial and unclear. DR disrupts retinal lipid metabolism, which differs from plasma metabolism. The retina can synthesize docosahexaenoic acid (DHA, 22:6n3) from α-linolenic acid (18:3n3) and eicosapentaenoic acid (EPA, 20:5n3) (53). The relationship between DR and lipid compounds is influenced by medication and compensatory mechanisms, complicating cross-sectional studies. Similarly, IBD patients exhibit altered lipid profiles. In animal models, PC supplementation reduces DSS-induced colonic lesions and pro-inflammatory cytokines. PC is involved in tryptophan, arginine, proline, and purine metabolism, bile secretion, and vitamin absorption (54, 55). It helps treat IBD by regulating the intestinal barrier, reshaping gut microbiota, modulating macrophage polarization, and reducing inflammation. Dietary PC supplementation can alleviate intestinal inflammation and is emerging as a novel approach in clinical treatment (56, 57).

In studies of type 2 diabetes and insulin resistance, elevated FGF21 levels are considered a compensatory response to metabolic stress and are associated with both microvascular and macrovascular complications. However, the connection between FGF21 and DR is debated. Some researchers propose FGF21 as a biomarker for DR, noting higher blood levels in proliferative and non-proliferative DR patients compared to controls (58). On the other hand, other studies find no positive correlation, with some proposing a U-shaped relationship (59). They argue that individual conditions and medication history can influence the measurement of FGF21 in DR patients. Pemafibrate increases plasma and liver FGF21, which reduces retinal neovascularization, restoring retinal function, and lowering inflammatory markers (60). FGF21 also reduces hypoxia-induced neovascularization in proliferative DR (36). In animal models, FGF21 reduction exacerbates intestinal inflammation, while increased IL-22-mediated STAT3 activation maintains intestinal cell homeostasis (61). MR result suggests that reducing FGF21 can indirectly alleviate CD. Due to the controversial nature of FGF21's relationship with DR and the impact of lifestyle and drugs on its levels, further investigation is necessary.

It is well-established that patients with DR and IBD often exhibit dyslipidemia. Cohort and case-control studies have shown that serum total cholesterol (TC), low-density lipoproteins (LDLs), and serum triglycerides (TGs) are significantly higher in patients with DME compared to non-DME patients (62). A predictive nomogram for DR risk in type 2 diabetes patients indicated that elevated TGs promote DR development (63). Cross-sectional studies have identified TGs as independent risk factors for DR, although observational studies only highlight associations between DR and TC without establishing causality (64). Our genetic analysis suggests that DR promotes increased TG levels, elucidating the interrelationship between these conditions. In the context of TGs and IBD, observational studies can determine whether IBD patients develop dyslipidemia but cannot assess whether dyslipidemia influences IBD progression. Data from the Korean National Health Insurance Service (2009–2016) showed an inverse relationship between serum TG levels and the incidence of UC, with lower TG levels linked to higher UC incidence (65). Our mediation analysis corroborates current observational studies and identifies causal relationships.

4.3 Limitations

It is important to consider the many restrictions that apply to our investigation. Firstly, DMP cannot be utilized as an outcome variable in forward MR to investigate the gut-retinal axis relationship because the number of SNPs in it is too limited. Secondly, since only people of European ancestry were included in the GWAS, it's possible that the study's conclusions cannot be applied to other ethnic groups. Thirdly, we selected the wide significance ($P<5\times10^{-6}$) as the threshold to get more SNPs in reverse MR, which could induce bias and false positive variants. Finally, the results of MR analysis and mediation analysis can point us in the right direction, but more clinical practice data are needed to supplement it. We are trying to study the signaling pathways associated with IBD and DR in future.

5 Conclusion

This MR study investigates the relationship between the gut-retinal axis in more detail. Although they manifest in different parts of the human body, IBD and DR share similar molecular processes and signaling pathways. Our MR and mediation analysis results indicate that DR and DME can reduce the incidence of CD. Additionally, DR can lower the incidence of IBD, while UC can reduce the incidence of DR. FGF21, PC and TG serve as mediators in these relationships. This research will help direct the development of specific medications and the management of these disorders.

Data availability statement

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

Author contributions

JYL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. YC: Investigation, Methodology, Project administration, Validation, Visualization, Writing – review & editing. SG: Formal analysis, Investigation, Writing – review & editing. SS: Resources, Visualization, Writing – review & editing. HZ: Data curation, Resources, Writing – review & editing. JBL: Conceptualization, Project administration, Supervision, Writing – review & editing. SL: Conceptualization, Funding acquisition, Project administration, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1382777/full#supplementary-material

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The relationship between small intestinal bacterial overgrowth and constipation in children – a comprehensive review

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Small intestinal bacterial overgrowth (SIBO) is characterized by an increase in the bacterial population of the small intestine due to an imbalance between the amount of bacteria and the intestinal barrier. Pediatric SIBO presents with a wide spectrum of symptoms, ranging from mild gastrointestinal complaints to malabsorption or malnutrition. Breath tests are commonly used as noninvasive diagnostic tools for SIBO, but a standardized methodology is currently unavailable. Intestinal flora produces methane which slows intestinal transit and increases the contractile activity of small intestine. Emerging literature suggests a correlation between overgrowth of methanogenic bacteria in the intestines and constipation. Treatment of SIBO involves administration of antibacterial therapy in addition to management of underlying conditions and optimal dietary adjustments. However, research on antibiotic treatment for pediatric patients with constipation and SIBO is limited and has yielded conflicting results. In the current review, we summarize the state-of-the-art of the field and discuss previous treatment attempts and currently used regimens for SIBO patients with constipation, with a focus on pediatric populations.

KEYWORDS

small intestinal bacterial overgrowth (SIBO), constipation, children, irritable bowel syndrome, functional gastrointestinal disorders

1 Introduction

Small intestinal bacterial overgrowth (SIBO) consists of an increase in the bacterial content of the small intestine of more than 10^5 colony-forming units (CFU)/mL (Bushyhead and Quigley, 2022; Skrzydło-Radomańska and Cukrowska, 2022), which produce gas in the small intestine, causing variable clinical aspects ranging from mild digestive symptoms (bloating, periumbilical pain) to more severe manifestations, such as malabsorption,

malnutrition, nutritional deficiencies, as well as osmotic diarrhea (Hammer et al., 2022). SIBO can also determine irritable bowel syndrome (IBS) with symptoms of constipation-predominant syndrome in 54.6% of children and diarrhea-predominant type in the rest of children, according to Hutyra et al (Hutyra and Iwańczak, 2010).

The most common symptoms of SIBO are abdominal pain, diarrhea, constipation, flatulence, belching, foul-smelling stools with mucus, nausea and stunted growth (Cho et al., 2023). SIBO occurs when the balance between bacteria and the intestinal tract protection barrier is altered (Banaszak et al., 2023). Typically, the bacterial count in the proximal bowel is around 10^2 CFU)/mL, which increases gradually towards the terminal ileum (Rana and Bhardwaj, 2008). The mechanisms that control bacterial proliferation are gastric acid secretion, digestive tract integrity, propulsive peristalsis and IgA immunoglobulins (Hammer et al., 2022). Therefore, numerous conditions in which these mechanisms are altered are associated with SIBO: ileo-cecal valve resection; small bowel diverticulosis; treatment with proton pump inhibitors, atrophic gastritis or gastric bypass which lower gastric pH; treatment with drugs that slow intestinal motility (antidiarrheals, anticholinergics) or abnormal small intestinal motility in different pathologies (celiac disease, inflammatory bowel disease, scleroderma, diabetes, Parkinson's disease) (Sachdev and Pimentel, 2013; Marginean et al., 2017; Quigley et al., 2020; Hammer et al., 2022).

Additionally, an increase in lipopolysaccharide permeability exacerbates the inflammatory response causing chronic inflammation that can lead to SIBO. Inflammation of the small intestine in SIBO was demonstrated by elevated levels of pro-inflammatory cytokines (interleukin-1β -IL1β, interleukin 6 - IL6 and tumor necrosis factor α – TNFα) in the duodenal fluid (Rizos et al., 2022). Moreover, elevated levels of fecal calprotectin, a marker of intestinal inflammation have been reported in SIBO (Donowitz et al., 2016). Increase in ghrelin, leptin, or trimethylamine N-oxide (TMAO) levels, along with a higher gastric pH, could also contribute to the development of SIBO (Cheung and Wu, 2013; Augustyn et al., 2019; Banaszak et al., 2023).

In pediatric patients, the involvement of SIBO in various clinical conditions such as IBS (Chumpitazi et al., 2017), obesity (Esposito et al., 2020), failure to thrive (Collard et al., 2022) constipation, cystic fibrosis (Furnari et al., 2019) and short bowel syndrome has been investigated. Treatment with proton pumps inhibitors (PPI), altered gastrointestinal anatomy and living in impoverished conditions were identified as risk factors for SIBO in children (Leiby et al., 2010; Chumpitazi et al., 2017; Furnari et al., 2019; Esposito et al., 2020; Collard et al., 2022; Caporilli et al., 2023). SIBO prevalence varies between 14,3% in children with IBS (Korterink et al., 2015) and approximately 90% in children with failure to thrive (Collard et al., 2022) and chronic abdominal pain (Collins and Lin, 2011). However, data on the epidemiology of SIBO in children is limited by the small number of studies and varying test methodologies applied.

In the last 30 years, the Roma Foundation carried out the diagnostic framework and formulated the therapeutic recommendations for functional gastrointestinal disorders (Drossman, 2016). IBS, one of the main functional digestive disorders, has been described as a disturbance of the microbiota-gut-brain axis (Drossman, 2016). The main clinical features of IBS (abdominal pain, diarrhea, constipation) overlap those of SIBO, and several studies have shown a frequent association between the two entities (Drossman, 2016). Moreover, functional constipation, an entity with a high incidence in pediatric patients (van den Berg et al., 2006), has been linked with intestinal dysbiosis, related to an increase in the number of methane-producing intestinal bacteria (Leiby et al., 2010).

In this review, we *aimed* to investigate the link between SIBO and constipation in children. The article mainly addresses the constipation subtype of IBS and functional constipation.

2 Search strategy and selection criteria

A thorough literature search was conducted using PubMed, Scopus and Web of Science databases to gather all articles indexed until April 2024, investigating the association between SIBO and constipation in adults and children. Additionally, the "snowball" method was employed involving the examination of reference lists within articles, to identify additional pertinent studies (Wohlin et al., 2002).

Search terms included a combination of "SIBO", "small intestinal bacterial overgrowth", "small bowel bacterial overgrowth", "intestinal methanogenic overgrowth (IMO)", "ĪMO", "methane", "CH_4", "breath test", "methane breath test", "constipation", "transit", "motility", "irritable bowel syndrome", "irritable colon", "child", "pediatric".

Two authors independently conducted an initial screen of titles and abstracts.

The *inclusion criteria* were population-based human studies, literature published in English and research articles examining the relationship between intestinal bacterial overgrowth and constipation. Full-text papers, including randomized controlled trials, prospective cohort studies, retrospective cross-sectional studies, and longitudinal studies, were included. *Exclusion criteria* comprised of studies which did not align with our research objectives, case reports, editorials, review articles, non-English publications, articles lacking free-available abstracts and duplicate entries. Additionally, abstracts and conference proceedings were omitted from the search results due to inadequate detail regarding the characteristics of the study population, diagnostic methodologies, or treatment modalities employed.

3 Results

3.1 Selection outcome

The database search yielded 467 articles. The 393 studies remaining after removing the duplicates and articles with full-text in other languages than English were screened by title or abstract. Only 136 of these studies were relevant to the research question out of which 69 were excluded for various reasons. By screening the reference list of the articles included we identified 12 additional studies. Therefore, we included in the review 79 articles which have

complied with our inclusion and exclusion criteria, as summarized in Figure 1.

3.2 SIBO diagnosis

There have been numerous discussions regarding the interpretation of diagnostic tests for SIBO. In many studies, the gold standard for diagnosis was the presence of $>10^5$ CFU/ml, determined from samples obtained by jejunal aspirate. However, in 2017, the North-American Consensus established the cutoff of $\geq 10^3$ CFU/mL as significant for the diagnosis of SIBO (Khoshini et al., 2008; Rezaie et al., 2017).

Jejunal aspiration is used for diagnosing SIBO, but is invasive and expensive, requiring a qualified gastroenterologist. In pediatric patients, the invasiveness of the procedure further restricts its use. Moreover, the sampling from the middle and distal regions of the small intestine is difficult, while sampling only from the proximal regions may cause false negative results. Also, the contamination of the samples with bacteria from the esophageal and oral flora can influence the culture results (Takakura and Pimentel, 2020). Given these disadvantages of the jejunal aspiration technique, breath tests (BTs) are frequently used to assess microbial overgrowth in the gut. These tests use different carbohydrate substrates, most commonly glucose and lactulose. The intestinal microflora transforms these substrates into hydrogen (H_2) and methane (CH_4), through anaerobic fermentation which are subsequently eliminated through respiration (Bond et al., 1971; Christl et al., 1992). The North American consensus defines a positive result for SIBO as an increase in $H_2 \geq 20$ parts per million (ppm) from baseline within 90 minutes of substrate ingestion and a CH_4 level ≥ 10 ppm at any time of the test (Rezaie et al., 2017). The European guidelines for H_2 and CH_4 breath testing in adults and children (Hammer et al., 2022) published in 2021 refrain from defining a single cutoff for H_2 and CH_4 values in the diagnosis of SIBO, as the diagnostic criteria have not been sufficiently confirmed and uniformly accepted. The results of the breath test should be interpreted considering the pre-test probability of SIBO (the presence of risk factors or associated conditions, abdominal pain, bloating, malabsorption in the absence of another diagnosis on endoscopy and imaging) and serial tests with H_2BT followed by a transit test with scintigraphy can be used in order to distinguish SIBO from rapid intestinal transit (Miller et al., 1997; Rao and Lele, 2002; Bratten et al., 2008; Yu et al., 2011; Hammer et al., 2022). Glucose is absorbed in the duodenum and jejunum, thus false negative BTs may occur (as high as 30–50%) if the bacteria are mainly located in the distal parts of the small intestine (Sellin and Hart, 1992; Saad and Chey, 2014). On the contrary, false positive results may be obtained in approximately 50% of patients with a rapid oro-cecal transit time (OCTT), as the glucose quickly reaches the colon (Lin and Massey, 2016). Lactulose is not absorbed in the small bowel and a BT using this substrate will show the contact with bacteria in the small bowel as well as in the colon (Saad and Chey, 2014). As a consequence, only early rises in the concentration of H_2 during the lactulose BT indicate the presence of small bowel bacteria, although early increases may be secondary to a rapid OCTT (Yu et al., 2011). The specificity of both substrates in diagnosing SIBO is similar (80%-85%), but glucose BT is considered to have a higher sensitivity (62% versus 52%) (Hammer et al., 2022). For both substrates the diagnostic accuracy can be improved by combining a technique to evaluate OCTT (Zhao et al., 2010; Zhao et al., 2014; Lin and Massey, 2016). In the absence of scintigraphy to evaluate OCTT, glucose should be the preferred substrate, especially in non-surgical patients (Hammer et al., 2022). H_2 detection through breath tests has been used since the 70s (Levitt and Donaldson, 1970), and more recently CH_4 detection was introduced in the test protocols (Rezaie et al., 2017). Increase in H_2 above the limit values during BTs have been associated with the diarrheal and the mixed form of IBS (Chen et al.,

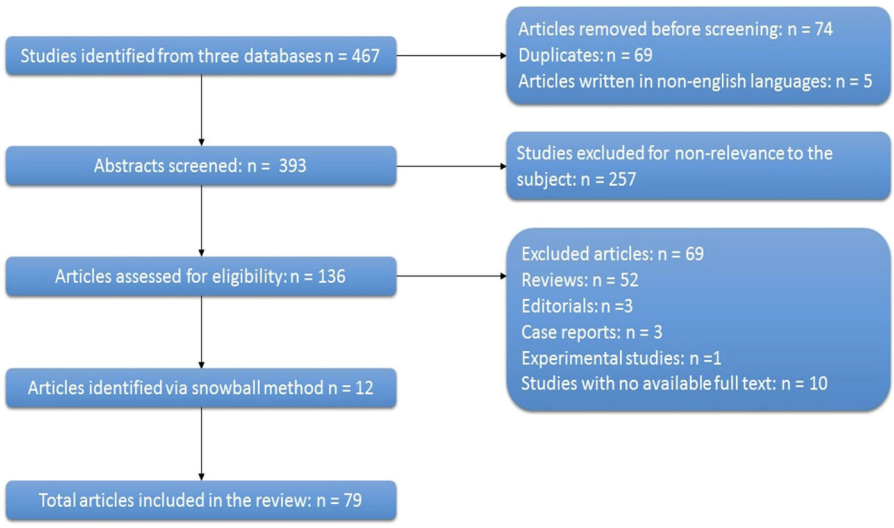

FIGURE 1
Flow diagram of studies assessed and included in the review. (n, number).

2018), while increases of CH_4 have been linked to the constipation form of IBS (Hwang et al., 2010; Ghoshal et al., 2016). An acid pH in the colon, high dietary sulfate intake or increased methane production (as Archea use H_2 to produce CH_4) (Pimentel et al., 2020) can cause low rates of colonic H_2 accumulation, resulting in false-negative results for hydrogen detection during BT. It has been suggested that measurement of breath CH_4 concentrations may help in improving the sensitivity of BTs (Rezaie et al., 2017; Pimentel et al., 2020; Hammer et al., 2022).

Therefore, the limitations of BTs are related to false positive and negative results as well as the lack of a clear standardization of protocols. Also, the correct result of a breath test depends on the patient's adherence to pre-test dietary and therapeutic restrictions, as well as a correct technique during the procedure. Despite these limitations, BTs remain valuable for SIBO diagnosis in pediatric patients, as they are practical and non-invasive. Notably, there haven't been any significant side effects reported with the H_2/CH_4 BT, aside from occasional transient abdominal pain or vomiting during the procedure (Hammer et al., 2022). In pediatric patients, specific technical adjustments are implemented during BTs. For instance, in younger children, a face mask connected to a double bag via a T-valve is frequently employed. When a child cooperates both mentally and physically, adult breath collection techniques are utilized (Hammer et al., 2022). Other changes in BT protocol are the decrease in the minimum fasting period of 8 hours before the BT to 4–6 hours in infants. Moreover, glucose and lactulose substrate doses are calculated according to weight (Hammer et al., 2022).

3.3 SIBO and constipation

Functional gastrointestinal disorders (FGID) are commonly diagnosed conditions and are associated with transit abnormalities (Rao et al., 2011). Constipation, a common symptom in multiple FGIDs (Drossman, 2016) is caused by one of the following mechanisms: impaired rectal evacuation, IBS with constipation or secondary to slow transit caused by abnormalities of the enteric nerves (Pritchard et al., 2017). In pediatric patients, constipation is more commonly caused by changes in diet, toilet training or a painful defecation episode leading to withholding (Afzal et al., 2011; Colombo et al., 2015; Robin et al., 2018; Sharif et al., 2019).

IBS is one of the most commonly evaluated conditions linked to SIBO. It is defined as a functional gastrointestinal disorder, characterized by abdominal pain at least 4 days per month over at least 2 months, related to defecation or to changes in the form or frequency of stools. Importantly, the symptoms cannot be attributed to other medical conditions (Drossman, 2016).The main bowel symptoms determine the IBS-subgroups: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), and IBS with a mixture of constipation and diarrhea (IBS-M). This classification is important, as different subgroups require specific diagnostic tests and treatments (Menees et al., 2012). Prevalence of SIBO in IBS patients varies in different studies depending on the methodology and diagnostic criteria used (Reddymasu et al., 2010; Sachdeva et al., 2011; Ghoshal et al., 2020). A recent meta-analysis (Bratten et al., 2008) including 37 articles and 5379 IBS patients found a 36,7% global prevalence of SIBO in IBS patients, varying between 4,3% and 83,7%. This high variability of SIBO incidence can be explained by differences in the methodology of the included studies. SIBO was more prevalent in patients with IBS compared to controls, when assessed through the glucose hydrogen breath test and upper gut aspirate culture. However, the lactulose breath test (LBT) did not show a higher detection rate of SIBO in IBS patients compared to the control group. This finding suggests that LBT might lack specificity and could often yield false-positive results in healthy individuals. Patients with IBS-D were more likely to have SIBO than patients with other subtypes of IBS (Efremova et al., 2023).

A similar global SIBO prevalence in patients with IBS (31%) was obtained in another meta-analysis that included 25 studies (Shah et al., 2020). SIBO prevalence in IBS patients was 35,5% using BT and only 13.9% using cultures from aspirates. Similar to the previous meta-analysis, LBT led to a much higher prevalence of SIBO in IBS, compared to glucose breath test (GBT) and cultures. Furthermore, SIBO prevalence was greater in patients with IBS-D (35.5%) compared with patients with IBS-C (22.5%) and IBS-M (25.2%).

Studies in children have found a prevalence of SIBO in IBS ranging from 14,3% to 91%. The high variability may be due to different inclusion criteria and diagnostic methodologies. Studies which used glucose as a substrate found a lower prevalence [14,3% (Korterink et al., 2015) – 34% (de Boissieu et al., 1996)], while lactulose yielded a higher prevalence of the same condition [39% (Ojetti et al., 2014) – 91% (Collins and Lin, 2011)].

A large meta-analysis from 2020 (Shah et al., 2020) included 3192 patients with IBS and 3320 controls and found that patients with IBS-C had a three-times higher prevalence of methane positive SIBO (25.3%) compared with patients with IBS-D (8.8%). The OR for methane-positive SIBO in patients with IBS compared with controls was 1.2. SIBO was much more prevalent in patients with IBS versus controls. Moreover, the prevalence of SIBO diagnosed through LBT was higher than the one established though GBT in both patients and controls (Shah et al., 2020). Similarly, in a meta-analysis conducted by Kunkel et al, a significant association was found between methane detected on breath tests and constipation (OR = 3.51) (Kunkel et al., 2011).

However, other studies did not find a correlation between SIBO and diarrhea or constipation, nor between the prevalence of methane-positive SIBO in chronic constipation compared to controls (Reddymasu et al., 2010).

Another meta-analysis revealed that the incidence of methane-positive SIBO in patients with IBS was 25%, which was not substantially different from the control group (Chuah et al., 2022). Nevertheless, methane-positive SIBO was more prevalent in the constipation subtype compared to IBS-D (OR = 3.1). LBT yielded positive results for methane-positive SIBO nearly three times as often as GBT (29.0% vs 11.5%) (Chuah et al., 2022).

Different tests have been used to determine the impact of SIBO on transit times in the small intestine and colon. Suri et al (Suri et al., 2018) used scintigraphy to study SBT (small bowel transit) and CT (colonic transit) in patients with hydrogen-positive (H-SIBO) and methane-positive (M-SIBO) LBT, and found that the presence of SIBO does not affect SBT nor CT. However, M-SIBO

exhibited significantly delayed SBT and CT compared to H-SIBO, indicating the presence of delayed motility in patients with elevated methane levels, as found on LBT (Suri et al., 2018). On the contrary, another study (Yu et al., 2011), which employed oro-caecal scintigraphy and LBT in IBS patients, concluded that abnormal increases in hydrogen levels measured during the breath test could be attributed to variations in oro-caecal transit time rather than SIBO. However, it's worth noting that this study did not include measurements of breath methane.

A study utilizing a wireless motility device compared intestinal transit patterns and breath tests among individuals with IBS and discovered no discernible link between SBT and abnormal breath H_2 or CH_4 excretion (DuPont et al., 2014). The study also showed that 76% of IBS patients exhibited prolonged gastric emptying times, with IBS-C being associated with increased gut transit times (DuPont et al., 2014).

Lastly, a study using a barostat (Grover et al., 2008) has shown that methane-producing IBS patients have higher urge thresholds and higher baseline levels of colon phasic contractions than SIBO-negative IBS patients, and report an increased consistency of stools.

CH_4, a product of intestinal fermentation, has been shown to directly slow intestinal transit and cause constipation in animal models, as well as humans (Takakura and Pimentel, 2020). Multiple studies have demonstrated an association between positive methane breath test and constipation, as well as between the degree of constipation and breath CH_4 levels in subjects with IBS (Chatterjee et al., 2007; Attaluri et al., 2010; Furnari et al., 2012). Increased methane production was also found in diverticulosis, a condition frequently associated with constipation (Weaver et al., 1986; Yazici et al., 2016). CH_4 appears to amplify neuronal activity in the intestine through the anticholinergic pathway and initiates slowing of peristalsis in the proximal intestinal segment. Contractile activity in the proximal intestinal segments is inhibited through a feedback loop when the distal segments are exposed to excess amounts of methane. Another proposed mechanism is the generation of non-propagating small bowel contractions, leading to delayed transit times (Pimentel et al., 2003b; Park et al., 2017; Suri et al., 2018). In another study methane- producing IBS patients had lower postprandial serotonin levels compared to the hydrogen-producing group. As serotonin is a key mediator of the peristaltic reflex, it may be a cause of delayed intestinal peristalsis in methane-producing patients (Pimentel et al., 2004). *Methanobrevibacter smithii* (*M. smithii*), a member of the Archaea domain, has been linked to constipation-predominant IBS and is the main methanogen responsible of CH_4 production. Because archaea are not bacteria, intestinal methanogenic overgrowth (IMO) and not SIBO is a more appropriate term for *M. smithii* overgrowth (Cho et al., 2023).

3.4 SIBO and constipation in children

In children few studies have investigated the association between SIBO and constipation. Ojetti et al. investigated 18 children with myelomeningocele, a condition frequently associated with constipation, and diagnosed SIBO in 38% of the patients, using LBT (Ojetti et al., 2014). Interestingly, all children who produced CH_4 showed a delayed OCTT with a lower frequency of evacuation.

Similarly, a study using LBT in children with fecal retentive incontinence found a prevalence of SIBO of 42% (Leiby et al., 2010). Moreover, 48% of patients with fecal incontinence showed high CH_4 values compared to only 10% in the control group. Fecal impaction scores were significantly increased in children with encopresis who were methane producers.

Soares et al., investigated the relationship between CT time, determined by radio-opaque markers and CH4 production in children with constipation (Soares et al., 2005). An increase in CH_4 production was found in 73.5% of children with constipation and incontinence, but only in 16.7% of children with constipation and no incontinence. Similarly, another study found increased CH_4 production in 65% of encopretic patients and in only 11% of patients with constipation and no encopresis (Fiedorek et al., 1990). Therefore, pediatric constipated patients with encopresis are more likely to be CH_4 producers than constipated patients without encopresis (Soares et al., 2005). Soares et al. also found that CH_4 producers had a prolonged CTT, which decreased after successful treatment (Soares et al., 2005). Similar results indicated that in children with IBS, CH_4 production correlated positively with whole intestinal transit time and negatively with bowel movement frequency (Chumpitazi et al., 2017).

Other studies in children have failed to demonstrate significant correlations between breath tests and transit changes. Scarpellini et al. used LBT to measure H_2 and CH_4 in 43 children with IBS and 56 controls (Scarpellini et al., 2009). They observed a higher prevalence of abnormal LBT results among patients diagnosed with IBS (65%, 28 out of 43 patients) compared to controls (7%, 4 out of 56 patients). However, no association between CH_4 production and intestinal transit changes was found (Scarpellini et al., 2009). A similar result was obtained in a study on 54 children with IBS, which showed no strong correlation between symptoms (constipation, diarrhea, bloating, abdominal pain, nausea) and H_2 and CH_4 breath test results (Peinado Fabregat et al., 2022). However, there was a small correlation between the presence of diarrhea and nausea and increased H_2 production (Peinado Fabregat et al., 2022). Contrarily, Hutyra et al. found a higher prevalence of SIBO in children with constipation-predominant IBS (54.55%) compared to diarrhea-predominant IBS (2.86%) (Hutyra et al., 2009).

Mello et al. studied the association between CH_4 production and SIBO in two socioeconomically distinct categories of children in Brazil (Mello et al., 2012). One group consisted of children living in poor conditions in a slum, while the second group of children came from socioeconomically advantaged families. The study revealed a high CH_4 production, regardless of SIBO presence in children living in unfavorable environments. However, there wasn't a clear correlation between SIBO and increased CH_4 production (Mello et al., 2012). Among children residing in slum areas, there was no obvious link between CH_4 production and constipation. Conversely, within the private school group, 3 out of 8 children who produced CH_4 complained of constipation (Mello et al., 2012).

SIBO presence was also studied in pediatric patients with Abdominal Pain–Related Functional Gastrointestinal Disorders (AP-FGID). Korterink et al. found that 14.3% of AP-FGID

patients were diagnosed with SIBO, and IBS was significantly more frequent in children with SIBO compared to those without SIBO (Korterink et al., 2015). A similar prevalence of SIBO (20.6%) was identified in another study on 68 children with AP-FGID (Lee et al., 2022). Loose stools were notably more prevalent among patients testing positive for H_2 or CH_4, although no other correlations with bowel symptoms were identified.

Table 1 shows the main characteristics and results of the studies published in pediatric patients with FGID. The prevalence of SIBO in studies including children with FGID is summarized in Figure 2.

3.5 SIBO treatment in adults and children

Treating SIBO in both children and adults involves a complex approach aimed to reduce bacterial overgrowth, relieve symptoms and address any underlying causes. Treatment strategies often involve a combination of antibiotics, dietary modifications and probiotics (Quigley et al., 2020). Antibiotics are the first line of treatment for SIBO, and are often empirically initiated, due to difficulties in obtaining culture aspirates and isolation of bacterial pathogens. Commonly prescribed antibiotics for SIBO include rifaximin, neomycin and metronidazole (Collins and Lin, 2011; Cho et al., 2023).

Neomycin is one of the first antibiotics studied in adult IBS patients. Although it was effective, resulting in a 35% improvement in IBS symptoms composite scores compared to 11% for placebo, its use was limited by the numerous side effects (Pimentel et al., 2003a). Rifaximin, the most studied antibiotic in the treatment of IBS, inhibits bacterial RNA synthesis, thereby disrupting the growth and reproduction of bacteria in the gut (Scarpignato and Pelosini, 2005; Koo et al., 2012). Unlike many other antibiotics absorbed systemically into the bloodstream, the effects of rifaximin are mainly restricted to the gastrointestinal tract after oral administration (Scarpignato and Pelosini, 2005). Because of its safety features (less frequently reported systemic side effects commonly associated with other antibiotics), rifaximin has been approved by the FDA for the treatment of IBS-D (Pimentel and Lembo, 2020). Rifaximin has been shown to eradicate bacterial overgrowth in up to 80% of adult patients diagnosed with SIBO (Scarpellini et al., 2013). A meta-analysis (Gatta and Scarpignato, 2017) that included 32 studies and 1331 adults SIBO patients identified an overall eradication rate of 70% of bacterial overgrowth, with adverse effects occurring in less than 5% of cases. A dose-dependent effect was demonstrated, the most commonly used dose being 1200 mg per day. In addition, rifaximin is more effective in reducing symptoms in IBS patients compared to placebo (Menees et al., 2012). Notably, symptom improvement was seen more frequently in patients treated with rifaximin compared to other antibiotics, such as neomycin, doxycycline, amoxicillin/clavulanate and ciprofloxacin (Yang et al., 2008).

In adult patients with IBS-C, treatment with specific antibiotics decreased CH_4 levels, which correlated with relief of constipation

TABLE 1 SIBO and functional gastrointestinal disorders in children.

Authors	Year	Study population - children	Diagnostic test	Results
de Boissieu et al (de Boissieu et al., 1996)	1996	50 children with chronic diarrhea, abdominal pain, or both, further included into 4 groups: - Group 1 – subjects with positive BT treated with antibiotics - Group 2 – subjects with negative BT - Group 3 – controls - Group 4 – patients with bacteriologically proven SIBO	Glucose H_2 BT Positivity was defined by a change in H_2 value ≥ 10 ppm after ingestion of glucose.	34% SIBO prevalence
Soares et al (Soares et al., 2005)	2005	- 40 children with chronic constipation evaluated before and after 6 weeks of treatment	CH_4 BT Positivity was defined as a methane concentration > 3 ppm	73,5% SIBO prevalence in patients with constipation and encopresis and 16,7% in patients with isolated constipation. CH_4-positive patients had a prolonged colonic transit time
Scarpellini et al (Scarpellini et al., 2009)	2009	- 43 children with IBS (Rome II criteria) and - 56 healthy controls	Lactulose H_2/CH_4 BT. Positivity was defined as an early rise in H_2 or CH_4 excretion of > 20 ppm within the first 90 min.	SIBO prevalence: Cases: 65%/ Controls: 7% No correlation between H_2/CH_4 values and bowel habits
Hutyra et al (Hutyra et al., 2009)	2009	- 136 children with functional dyspepsia, chronic abdominal pain and IBS; - 28 controls, children treated for other pathologies	Lactulose H_2 BT Positivity was defined as an increase in H_2 > 20 ppm within the first hour	54.55% SIBO prevalence in constipation predominant IBS, 2.86% in diarrhea predominant IBS
Collins et al (Collins and Lin, 2011)	2010	- 75 Children with chronic abdominal pain randomized into two groups, one treated with Rifaximin and one with placebo; - 40 healthy controls	Lactulose H_2 BT Positivity was defined as a rise in H_2 >20 ppm in the first 90 min	SIBO prevalence of 91% in cases and 35% in controls Abnormal LBTs persisted after treatment in 80% children who received Rifaximin and 86% children who received placebo

(Continued)

TABLE 1 Continued

Authors	Year	Study population - children	Diagnostic test	Results
Leiby et al (Leiby et al., 2010)	2010	- 50 children with fecal incontinence; - 39 controls with gastrointestinal symptoms but without fecal incontinence	Lactulose H_2/CH_4 BT Positivity was defined as an increase in H_2 > 20 ppm or in CH_4 >10 ppm over baseline at < 60 min. Patients were considered CH_4 producers if their level was >3 ppm at any point in the study	SIBO prevalence 42% in cases, 23% in controls
Jones et al (Jones et al., 2011)	2011	287 children with chronic diarrhea, abdominal pain, bloating or irritability	H_2 and CH_4 levels Positivity was defined as an increase in H_2 >10 ppm over baseline in the initial 45 min of the test. Patients were classified as H_2 or CH_4 producers if they produced >10 ppm of these gases at any time point	87% SIBO prevalence
Scarpellini et al (Scarpellini et al., 2009)	2013	50 children with IBS (Rome II criteria) treated with Rifaximin	Lactulose H_2/CH_4 BT Positivity was defined as an increase in H_2 or CH_4 excretion >20 ppm within the first 90 min	66% SIBO prevalence 64% LBT normalization rate after treatment
Ojetti et al (Ojetti et al., 2014)	2013	18 children with myelomeningocele and constipation	Lactulose H_2/CH_4 breath test. Positivity was defined as an increase in H2 or CH4 excretion >20 ppm within the first 90 min	39% SIBO prevalence
Korterink et al (Korterink et al., 2015)	2014	161 children with abdominal pain related functional gastro-intestinal disorders (Rome III criteria), divided into two groups: SIBO-positive and SIBO-negative	GHBT. Positivity was defined as fasting breath H_2 concentration > 20 ppm or increase in H_2 >12 ppm over baseline value	14.3% SIBO prevalence
Siniewicz-Luzenczyk et al (Siniewicz-Luzeńczyk et al., 2015)	2015	100 children with abdominal pain	Positivity was defined by a baseline concentration of H_2 > 20 ppm or an increase in H_2 > 20 ppm in the first hour of the test.	63% SIBO prevalence 88% HBT normalization rate after Rifaximin treatment and improvement in symptoms
Chumpitazi et al (Chumpitazi et al., 2017)	2017	87 children with IBS (Rome III criteria)	Subjects who excreted ≥ 3 ppm of CH_4 in at least one of the breath samples were characterized as CH_4 producers	LBT CH4 correlated positively with whole intestinal transit time and negatively with bowel movement frequency. 58.6% were CH_4 producers No differences by IBS subtype in H2 or CH_4 production
Garg et al (Garg et al., 2017)	2017	- 62 children with chronic abdominal pain underwent BT - 21 were diagnosed as lactose intolerant and 8 as SIBO-positive	GHBT and LHBT SIBO positivity defined as an increase in H2 < 10 ppm in 30 minutes Lactose intolerance defined as an increase in H2 > 20 ppm	17% SIBO prevalence on LHBT
Lee et al (Lee et al., 2022)	2022	68 children with functional abdominal pain disorders (Rome IV)	Glucose H_2 and CH_4 breath test Positivity was defined by an increase in H_2 > 12 ppm above baseline within 90 minutes or an increase in CH_4 > 10 ppm above baseline within 90 minutes	20.6% SIBO prevalence

BT, Breath test; LBT, lactose breath test; LHBT, lactose hydrogen breath test; GHBT, glucose hydrogen breath test; H_2, Hydrogen; CH_4, Methane; SIBO, Small intestinal bacterial overgrowth; IBS, irritable bowel syndrome; ppm, parts per million.

(Pimentel et al., 2006; Low et al., 2010). Both neomycin and rifaximin have been shown to reduce constipation in IBS-C, but using a combination of the two drugs appears to be more effective (Low et al., 2010). Additionally, reduction of CH_4 to undetectable levels (< 3 ppm) on repeat BT was obtained in 33% of patients treated with neomycin, 28% patients treated with rifaximin and 87% patients treated with both antibiotics (Pimentel et al., 2014).

In the pediatric population, data are limited regarding the use of antibiotics to treat SIBO. The effect of rifaximin in children with IBS was evaluated in a study that included 33 subjects with a positive LBT (Scarpellini et al., 2013). The breath test normalized in 64% of cases treated with rifaximin 200 mg daily for 7 days. Furthermore, visual analogic scale scores for gastrointestinal symptoms improved after successful treatment. Similar results were reported by Siniewicz-Luzenczyk et al, with a normalization of BT in 88% cases after treatment (Siniewicz-Luzeńczyk et al., 2015). Another course of antibiotics was used to treat children with SIBO consisting of the combination of trimethoprim-sulphametoxazole 30 mg/kg daily and

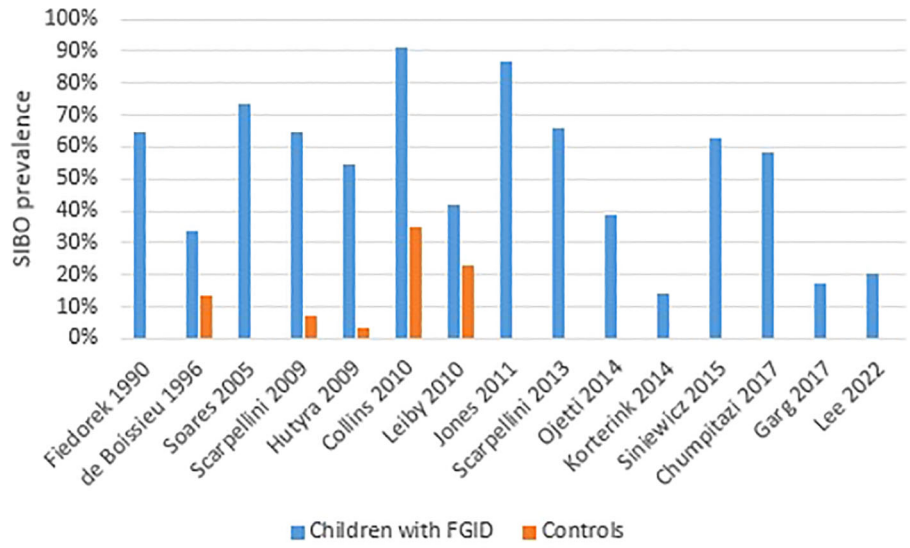

FIGURE 2
SIBO prevalence in children with functional gastro-intestinal disorders. The majority of studies did not include a control group.

metronidazole 20 mg/kg daily for 14 days, which normalized BTs in 95% of cases (Tahan et al., 2013). Furthermore, different antibiotics and probiotics regimens have been studied in children with SIBO and a positive GBT or LBT (Peinado Fabregat et al., 2022). Treatment with the combination of probiotics and antibiotics demonstrated a better resolution of symptoms compared to treatment with antibiotics alone (81% vs. 67.7%). The effects were similar with respect to the antibiotic used. An overview of the current therapeutic options of SIBO has been illustrated in Figure 3.

Non-pharmacological methods were also used in the treatment of SIBO. Dietary approaches for managing SIBO typically involve reducing the intake of fermentable substances such as fiber, sugar alcohols and sweeteners like sucralose (Staudacher and Whelan, 2017; Souza et al., 2022). These strategies are often based on dietary guidelines for IBS, emphasizing low-FODMAP diets, which restrict fermentable oligosaccharides, disaccharides, monosaccharides, and polyols. However, the mechanisms behind clinical improvements resulting from dietary changes remains unclear. It is uncertain whether these changes primarily affect the intestinal microbiota or simply reduce fermentation and gas production.

The role of probiotics in the treatment of SIBO has also been investigated. A recent meta-analysis has found that probiotics appeared to reduce hydrogen production (Zhong et al., 2017). In randomized clinical trials examining probiotic use in SIBO, variations were observed in the strains used and the duration of treatment (Souza et al., 2022). One study investigated the effect of *Bifidobacterium* in 126 patients diagnosed with gastrointestinal cancer and SIBO (Liang et al., 2016). Following the treatment regimen, SIBO was eradicated in 81% of individuals administered probiotics, compared to 25.4% in the placebo group. Furthermore, symptoms were significantly reduced in the probiotic group but not in the placebo group.

Similar results were reported in a study on IBS- D and SIBO (Bustos Fernández et al., 2023). Participants received either *Saccharomyces boulardii CNCM I-74* (Sb) along with dietary advice (DA) or DA alone. The researchers observed a more pronounced reduction in hydrogen excretion in the Sb group compared to the DA group. Additionally, Sb supplementation led to an improvement in digestive symptoms.

Conversely, in a randomized, double-blind trial (Stotzer et al., 1996) involving 17 individuals diagnosed with SIBO, *Lactobacillus fermentum KLD* did not produce significant changes in BT outcomes, clinical symptoms, or stool frequency when compared to the baseline measures.

In children with SIBO we identified only one retrospective report studying the effect of probiotics in a limited group of only 10 patients (Ockeloen and Deckers-Kocken, 2012). Of these, 7 children had an improvement in their abdominal complaints after treatment with *Bifidobacterium* and *Lactobacillus*, but the difference was not statistically significant.

Additionally, most studies exhibited moderate methodological quality, therefore no recommendations for standardized treatment are currently available (Pimentel and Lembo, 2020; Souza et al., 2022).

4 Future research directions and limitations of current data

To the best of our knowledge, this review is the first to explore the association between SIBO and constipation in children. A strong point of this review was the comprehensive literature search of all the studies including pediatric patients with SIBO and constipation.

In the adult population, there are numerous studies investigating the diagnosis and treatment of SIBO. However, in children research related to this topic is very limited, even less regarding the SIBO-constipation relationship. Technical difficulties in performing both digestive endoscopy with the collection of jejunal aspirate, and respiratory tests in children largely explain this paucity of studies.

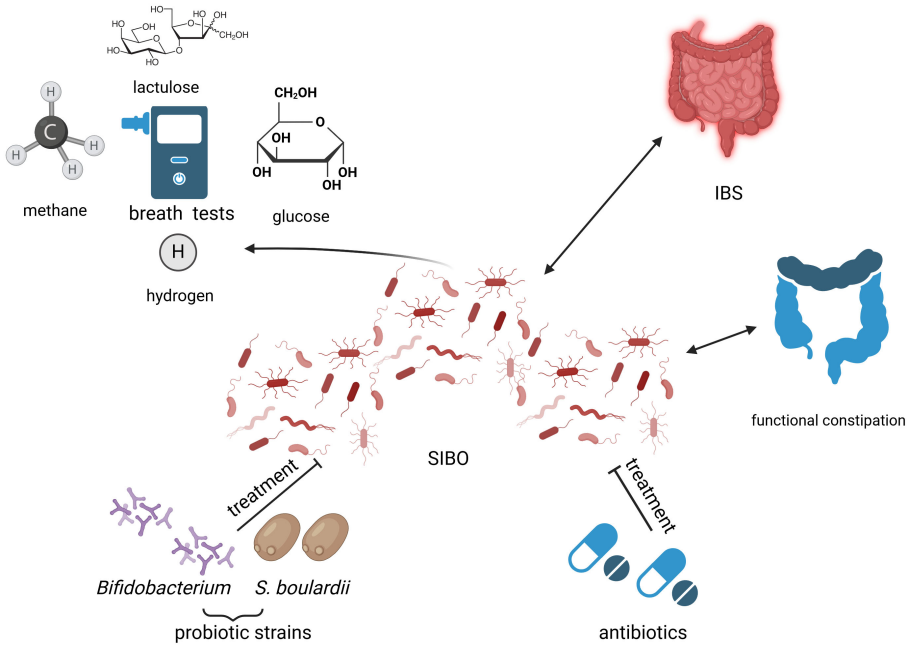

FIGURE 3
Small intestinal bacterial overgrowth (SIBO) in relation to childhood constipation: diagnostic and treatment opportunities. Created with BioRender.com (https://biorender.com/) Small intestinal bacterial overgrowth (SIBO) has been linked to functional constipation and irritable bowel syndrome (IBS). The possibility of SIBO depiction through multiple types of breath tests (BTs), such as lactulose BT, hydrogen BT, glucose BT or methane BT leads to miscellaneous results of currently available studies, due to methodology-related disparities. Current treatment options of SIBO include probiotics belonging to the Bifidobacterium genre and *Saccharomyces boulardii* (*S. boulardii*), as well as antibiotics, but research on this matter is ongoing. IBS, irritable bowel syndrome; *S. boulardii, Saccharomyces boulardii*; SIBO, small intestinal bacterial overgrowth.

The main limitations of our review are generated by the small number of studies available. The majority of these studies featured small sample sizes and lacked control groups. In addition, we noticed a considerable variability between studies regarding diagnostic methods and threshold values for BT, therefore strong conclusions could not be formulated. Most studies have used lactulose as a substrate for BT, although the most recent European consensus recommends the use of glucose because it leads to a higher sensitivity (Hammer et al., 2022). Figure 2 also highlights the miscellaneous methods used for breath test related diagnosis of SIBO.

Cut-off points for SIBO diagnosis using BT have not been adapted to the pediatric population. This is problematic since recent studies have demonstrated variations between the gut microbiota of children and adolescents compared to adults (Hollister et al., 2015; Derrien et al, 2019).

In adult studies, a positive CH_4 BT has been associated with constipation and delayed intestinal motility (Chatterjee et al., 2007; Attaluri et al., 2010; Furnari et al., 2012; Suri et al., 2018). Nevertheless, research on children has produced inconsistent results on this issue. Several studies have suggested that children experiencing constipation and encopresis exhibit elevated CH_4 production and a delayed OCTT (Fiedorek et al., 1990; Soares et al., 2005; Ojetti et al., 2014). Conversely, other studies found no correlation between BT results and bowel movement frequency in children with isolated constipation (Scarpellini et al., 2009; Peinado Fabregat et al., 2022).

Lastly, there is also a paucity of studies focusing on the treatment of SIBO in children. While rifaximin has similar positive outcomes in children as in adults (Scarpellini et al., 2013; Siniewicz-Luzeńczyk et al., 2015), a previous study noted a higher rate of SIBO eradication with trimethoprim-sulphamethoxazole (Tahan et al., 2013), indicating that additional studies are necessary.

5 Conclusions

SIBO is a poorly understood condition with variable clinical manifestations, ranging from mild symptoms to malabsorption and failure to thrive. Identifying SIBO in pediatric patients is crucial to prevent long-term complications and optimizing growth and development.

In adults, numerous studies have demonstrated a significant link between intestinal methanogenesis and constipation. However, as this review points out, research on this correlation in the pediatric population has yielded conflicting results, potentially due to the limited sample size and methodological variations across studies.

Additionally, determining SIBO incidence in pediatric patients remains challenging due to the lack of standardized diagnostic criteria and limited studies focusing on this age group. Rigorous case-control studies on large population samples, using glucose as a substrate and simultaneously measuring intestinal transit time via scintigraphy or other diagnostic methods, could improve the

diagnostic criteria for SIBO, as currently suggested through European consensus. Further investigations are necessary to establish a universally accepted diagnostic criteria and threshold values for pediatric SIBO and these values are difficult to establish without future research enrolling control groups, missing from most available studies. Moreover, exploring the impact of diet, probiotic therapy or the adaptation of effective antibiotics from adult treatments in pediatric patients should be addressed in future studies, as this subject is worth exploring from a therapeutic point of view in children as well.

Author contributions

CRM: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MS: Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CM: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Project administration, Validation, Visualization.

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Conflict of interest

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

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The causal relationship between gut microbiota and diabetic neuropathy: a bi-directional two-sample Mendelian randomization study

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Background: Many studies suggest a strong correlation between gut microbiota (GM) and diabetic neuropathy (DN). However, the precise causal relationship between GM and DN has yet to be fully elucidated. Hence, a bi-directional Mendelian randomization (MR) analysis was used to examine the association between GM and DN.

Methods: Widely known genome-wide association study (GWAS) of GM was collected from the MiBio Gen project. Summary-level datasets for DN were taken from the FinnGen project. Inverse variance weighted approach was used for evaluating the causal relationship between GM and DN. Subsequently, pleiotropy and heterogeneity tests were performed to verify the reliability of the data. Furthermore, a bidirectional two-sample MR analysis was done to investigate the directionality of the causal relationships. Gene Ontology analysis was conducted to identify the associations that could indicate biological functions.

Results: We identified potential causal associations between GM and DN ($p< 0.05$ in all three MR methods). Among them, we found increased levels of Christensenellaceae R-7 (Odds ratio, OR= 1.52; 95% confidence interval, CI = 1.03–2.23; $p = 0.03$), Ruminococcaceae UCG013 (OR =1.35; 95% CI = 1.00–1.85; $p = 0.04$), and *Eggerthella* groups (OR = 1.27; 95% CI = 1.05–1.55; $p = 0.01$), which may be associated with a higher risk of DN, while increased levels of Peptococcaceae (OR = 0.69; 95% CI = 0.54–0.90; $p< 0.01$) and *Eubacterium coprostanoligenes* groups (OR = 0.68; 95% CI = 0.49–0.93; $p = 0.01$) could be associated with a lower risk. Gene Ontology pathway analysis revealed enrichment of genes regulated by the associated single-nucleotide polymorphisms (SNPs) in the apical plasma membrane, glycosyltransferase activity, hexosyltransferase activity and membrane raft. Reverse MR analyses indicated that DN was associated with five microbial taxa in all three MR methods.

Conclusion: The results of our study validate the possible causative relationship between GM and DN. This discovery gives new perspectives into the mechanism on how GM influences DN, and establishes a theoretical foundation for future investigations into targeted preventive measures.

KEYWORDS

gut microbiota, diabetic neuropathy, Mendelian randomization, causal relationship, bi-directional

1 Introduction

Diabetic neuropathy (DN) is a common and burdensome complication of diabetes that is significant but often over-looked. It can markedly impair psychological functions and quality of life (1). Diabetic Peripheral Neuropathy (DPN) is the most frequently observed type of DN that affects the feet and legs. It presents a range of symptoms, including pain, numbness, and severe discomfort. autonomic dysfunction, however, affects the autonomic nervous system that regulates involuntary bodily functions. This dysfunction contributes to various problems, such as cardiovascular dysfunction characterized by blood pressure and heart rate changes, gastrointestinal dysfunction leading to gastroparesis, and urogenital dysfunction affecting bladder control and sexual function (2). DPN alone contributes to more than $10 billion in annual healthcare expenses, exceeding one-fourth of the total direct medical costs of diabetes (3). Managing DN requires a comprehensive approach that includes strict glycemic control to slow neuropathy progression, pain management and treatment of autonomic symptoms to enhance quality of life (4). Early diagnosis and thorough management are key to prevent complications and improve patient outcomes. Due to the limited treatment options for DN, it is crucial to investigate and identify new therapeutic targets (5).

Gut microbiota (GM) generally refers to the bacteria residing in the human gut. It plays an important role in regulating a wide array of physiological functions in the host and providing protection against pathogenic bacteria (6). GM is involved in processes such as digestion, immune system modulation, also influencing mood and behavior through the gut–brain axis (7, 8). The pathogenesis of microbiota dysbiosis significantly contributes to the onset and advancement of diabetes mellitus and its complications, such as cardiovascular disease, nephropathy, and DN, by promoting systemic inflammation and disrupting metabolic functions (9). However, these observational studies did not show a causal relationship between GM with DN, and it is still uncertain whether reverse causality weakens this correlation.

Abbreviations: GM, Gut microbiota; DN, diabetic neuropathy; IVW, inverse variance weighted; SM, simple mode; WMe, weighted median; WMo, weighted mode; MR, mendelian randomization; GO, Gene ontology; SNPs, Single nucleotide polymorphisms; OR, Odds ratio; CI, Confidence interval.

Mendelian randomization (MR) is a method in genetic epidemiology that uses genetic variants as instrumental variables (IVs) to assess the causal relationship between an exposure and outcome (10). Genome-wide association studies (GWASs) offer extensive datasets featuring many single nucleotide polymorphisms (SNPs) and significant sample sizes. MR leverages Mendelian inheritance laws using one or more genetic polymorphisms as the exposure variable. This makes GWAS-based MR a compelling method for determining causality (11, 12).

The two-sample MR technique offers increased statistical power to identify the causal effects between exposure factors and outcomes using published summary estimates from various large-scale GWASs (13). Also, large-scale summary statistics enable the analysis of the relationships between GM and DN by enhancing the statistical power of two-sample MR analysis. Hence, a bidirectional MR methodology was employed to investigate the potential causal association between GM and DN by combining data from the MiBioGen and FinnGen consortiums' GWASs on genetic variations. The adoption of a bidirectional MR strategy enhanced the robustness of our findings against potential confounding variables and reverse causation. Finally, we conducted a gene ontology (GO) analysis using lead SNPs to investigate the GM's biological impact on DN. This GO analysis offered deeper understanding of the physiological mechanisms involved. Our research opens new avenues and provides fresh insights for future DN studies.

2 Methods

2.1 Study design

To establish the potential causal relationships between GM and DN, we employed a bi-directional MR analysis, which provides stronger associations by minimizing the biases present in the traditional epidemiologic observational studies. The flow chart of the study design is shown in Figure 1. To perform the study, it is necessary that three fundamental assumptions are satisfied: (1) A strong correlation between IVs and exposure; (2) No correlation between IVs and confounders; and (3) IVs can only affect the outcomes through exposure (14). IVs that fulfill these three

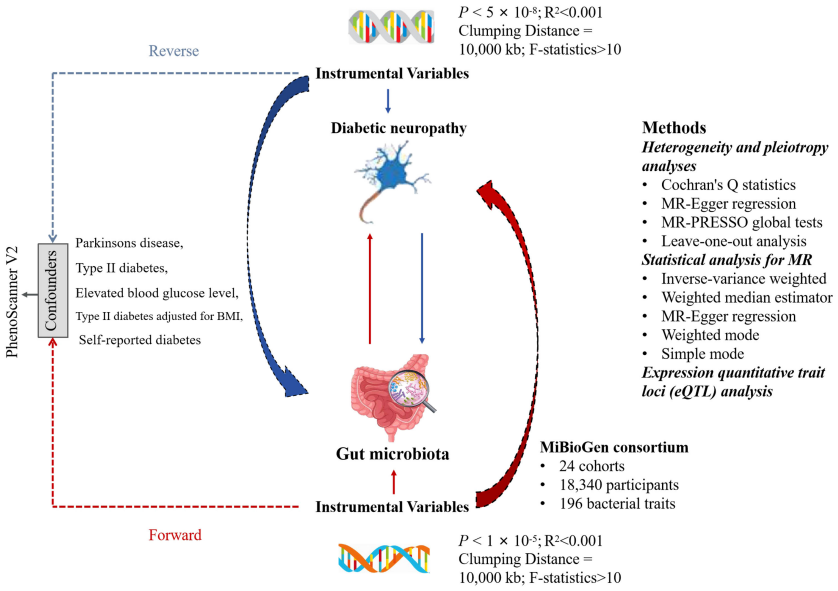

FIGURE 1
The flowchart for the study of the association between gut microbiota and Diabetic neuropathy. LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

assumptions were included in this MR study. Our results were reported according to the STROBE-MR guidelines (15).

2.2 Data source of exposure and outcome

This was a multi-ethnic large-scale GWAS that coordinated 16S ribosomal RNA gene sequencing profiles and genotyping data from 18,340 participants of 24 cohorts from the USA, Canada, Israel, South Korea, Germany, Denmark, Netherlands, Belgium, Sweden, Finland, and UK to explore the association between the autosomal human genetic variants and GM (16). A total of 211 bacterial traits (classified into specific phylum, class, order, family, and genus) were obtained, and the sample size was 14,306. Out of the 211 traits selected, 15 bacterial traits did not have specific species names. Hence, we excluded them and used the remaining 196 traits for analysis. All the original studies were approved ethically and participants' consents were obtained. In this study, the GWASs and associated datasets were shown in Table 1.

2.3 Instrumental variables

IVs were chosen from a GWAS dataset provided by the international consortium MiBio Gen. These IVs are specifically associated with the makeup of the human GM. First, consistent with prior MR studies, we identified significant SNPs for the respective GM using a cut-off value of $p< 1\times10^{-5}$ (17). When conducting a reverse MR analysis with DN as the exposure, we set the threshold at $p< 5\times10^{-8}$ for selecting SNPs. Second, the clump program in PLINK software was adopted to exclude the dependent IVs of $R^2< 0.001$ (clumping window size = 10,000 kb), which were obtained using the 1000 Genome Projects reference panel in Europe (18). Third, an important step in MR is to ensure that the effects of the SNPs on the exposure correspond to the same allele as that on the outcome. To avoid distortion of strand orientation or allele coding, we removed palindromic SNPs (such as, with A/T or G/C alleles). To assess the presence of weak instrument bias, the F-statistic for the IVs was computed using the formula $F = \frac{R^2(N-1-K)}{(1-R^2)K}$, where R^2 is the proportion of variance in the exposure explained by the genetic variants, N is the sample size, and K is the number of instruments (19). A weak instrument, indicated by an F-value below 10, was excluded (20). Additionally, by searching for pleiotropic SNPs of confounders in PhenoScanner V2 (21), we eliminated certain IVs that were significantly associated with potential confounders ($p< 1\times10^{-5}$). When the exposure was GM, potential confounders included Parkinsons disease, type II diabetes mellitus, elevated blood glucose level, type II diabetes adjusted for body mass index (BMI), and self-reported diabetes. In reverse MR analysis with DN as the exposure, no potential confounders were identified. The remaining IVs were then used for subsequent MR analysis.

TABLE 1 The present study used genome-wide association studies (GWAS) and associated datasets to conduct our analysis.

Exposure or outcome	Sample size	Ancestry	Links for data download	PMID
Human gut microbiome	18,340 participants	Mixed	https://mibiogen.gcc.rug.nl	33462485
Diabetic neuropathy	1,415 cases, 162,201 controls	European	https://gwas.mrcieu.ac.uk/datasets/finn-b-DM_NEUROPATHY/	–

2.4 Heterogeneity and pleiotropy analyses

We conducted a heterogeneity test utilizing Cochran's Q statistics. A $p< 0.05$ indicated significant heterogeneity (10). Horizontal pleiotropy, which implies that IVs influence outcomes through paths other than the causal effects, can potentially lead to false-positive results ($p< 0.05$) (22). To evaluate the direct relationship between the chosen IVs and outcome, horizontal pleiotropy was tested using MR-Egger intercept test and MR-PRESSO global tests. Significant outliers identified in the MR-PRESSO analysis were excluded to reduce the influence of horizontal pleiotropy (23). Furthermore, a leave-one-out analysis was conducted to validate the robustness of the results (24).

2.5 Statistical analysis for MR

For the MR analysis, we employed five methods: the inverse-variance weighted (IVW) test, weighted median estimator (WME), MR-Egger regression, weighted mode (WMe) and simple mode (SM). IVW was the primary method, complemented by the other four methods (25). All the statistical analyses were conducted using R programming, version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria). For MR analyses, we utilized the "Two sample MR" (version 0.5.7) and "MR_PRESSO" (version 1.0) R packages (23).

2.6 Gene ontology enrichment analysis

To examine the function of IVs in mediating causality between exposure factors and outcomes, we utilized IV SNPs derived from MR analysis. By integrating these SNPs with the data from the eQTLGen database, we analyzed the genes regulating gene expression from the cis-expression quantitative trait loci (cis-eQTL) standpoint and nearest gene method (26). Using the R package "ClusterProfiler" we conducted a gene ontology (GO) enrichment analysis on these genes to investigate the patterns of gene expression regulation (27).

3 Results

3.1 Selection of IVs

To analyze the effects of GM on DN, we selected 2–12 SNPs for GM species as IVs. Some analyses were unsuccessful due to the absence of SNPs following harmonization. The F statistics for IVs indicated that the estimates were less likely to suffer from weak instrumental bias ($F > 10$, Supplementary Table 1).

3.2 Potential causal associations between the GM and DN

As seen in Figure 2, in both circular heatmaps, the data layers, from inside to out, represent the odds ratios (OR) calculated using the IVW method, followed by –log10 (p values) for IVW, Weighted Median (WMo), WMe, SM, and MR-Egger methods, respectively. The outermost ring illustrates the agreement of effect direction as determined by the five MR methodologies: IVW ($p< 0.05$), MR-Egger, SM, WMe, and WMo. We identified three risk factors (genus Christensenellaceae R-7group, *Eggerthella*, and Ruminococcaceae UCG-013) and two protecting factors (family. Peptococcaceae and *Eubacterium coprostanoligenes* group) related to DN after setting a standard in which the IVW method demonstrated a significant difference ($p< 0.05$), and the five methods indicated consistent directions. Details and statistics are given in Figure 3. Specifically, we observed elevated levels of Christensenellaceae R-7 (OR = 1.52; 95% confidence interval, CI = 1.03–2.23; $p = 0.03$), Ruminococcaceae UCG-013 (OR = 1.35; 95% CI = 1.00–1.85; $p = 0.04$), and *Eggerthella* groups (OR = 1.27; 95% CI = 1.05–1.55; $p = 0.01$), which may be linked to an increased risk of DN. Conversely, higher levels of Peptococcaceae (OR = 0.69; 95% CI = 0.54–0.90; $p< 0.01$) and *Eubacterium coprostanoligenes* groups (OR = 0.68; 95% CI = 0.49–0.93; $p = 0.01$) could indicate a reduced risk of DN (Supplementary Table 2). The leave-one-out investigation revealed that removing any of the SNPs did not affect the overall results, suggesting that this MR analysis is extremely robust (Supplementary Figure 1).

3.3 Sensitivity analyses

The MR-Egger, WMe, SM, and WMo techniques showed comparable causal estimates for size and direction. We discovered no evidence of horizontal pleiotropy for GM in DN with $p > 0.05$ when utilizing the MR-Egger regression intercept method. MR-PRESSO analysis indicated no outliers in the findings. In addition, the findings of the Cochrane's Q statistics indicated no substantial heterogeneity ($p > 0.05$) (Supplementary Table 3). Scatter plots were utilized to assess the MR models and show the intercept of the MR-Egger slope (Supplementary Figure 2).

3.4 Reverse MR analysis

Among the 211 bacterial traits, five exhibited elevated levels that could be associated with an increased risk of DN. Details and statistics are given in Figure 4. These include the genus *Anaerofilum* (OR = 1.07; 95% CI = 1.00–1.13; $p< 0.05$), *Dorea* (OR = 1.05; 95% CI = 1.02–1.08; $p< 0.01$), *Lachnospiraceae* UCG-010 (OR = 1.05; 95% CI = 1.01–1.09; $p = 0.02$), *Ruminococcus 2* (OR = 1.06; 95% CI = 1.01–1.10; $p = 0.01$), and the order. NB1n (OR = 1.08; 95% CI = 1.01–1.14; $p = 0.02$). Forest plots were drawn using IVW, MR-Egger, and WMo (Figure 5). Next, sensitivity analysis of the MR results between DN and the five GMs (Supplementary Table 4) was performed, and the test showed no heterogeneity or horizontal pleiotropy. Details of IVs for reverse MR are listed in Table 2. The intercepts of the MR-Egger regression demonstrated no evidence of horizontal pleiotropy, as shown by p value > 0.05. The MR-PRESSO global test score of $p > 0.05$ indicated that there is no evidence of horizontal pleiotropy (Supplementary Table 5). Scatterplots (Supplementary Figure 3) and leave-one-out plots (Supplementary Figure 4) revealed no outliers.

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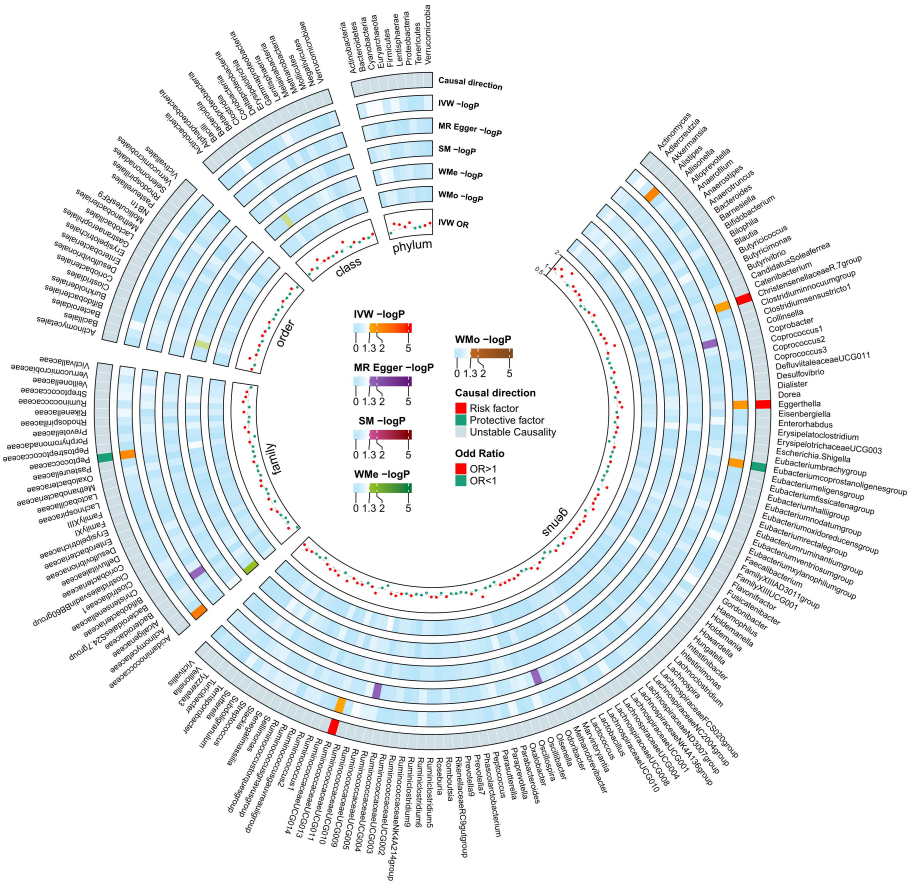

FIGURE 2
Mendelian Randomization analyses illustrating the causal effect of the gut microbiome on diabetic neuropathy. In both circular heatmaps, the data layers, from inside to out, represent the odds ratios calculated using the Inverse Variance Weighted (IVW) method, followed by –log10(p values) for IVW, Weighted Median (WMo), Weighted Mode (WMe), Simple Median (SM), and MR-Egger methods, respectively. The outermost ring illustrates the acceptance of effect direction as determined by the five MR methodologies: IVW ($p < 0.05$), MR-Egger, SM, WMe, and WMo. IVW, inverse variance weighted; SM, simple mode; WMe, weighted median; WMo: weighted mode; MR, mendelian randomization.

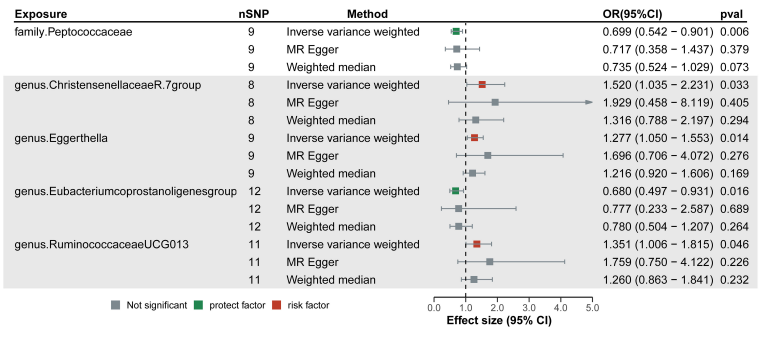

FIGURE 3
Forest plots of the significant causal effect of gut microbiota on diabetic neuropathy were calculated using the inverse variance weighted method, MR-Egger, and weighted median. The forest plots demonstrate that elevated levels of Christensenellaceae R-7, Ruminococcaceae UCG-013, and *Eggerthella* groups were risk factors for of diabetic neuropathy. While higher levels of Peptococcaceae and *Eubacterium coprostanoligenes* groups were protective factors. IVW, Inverse variance weighted.

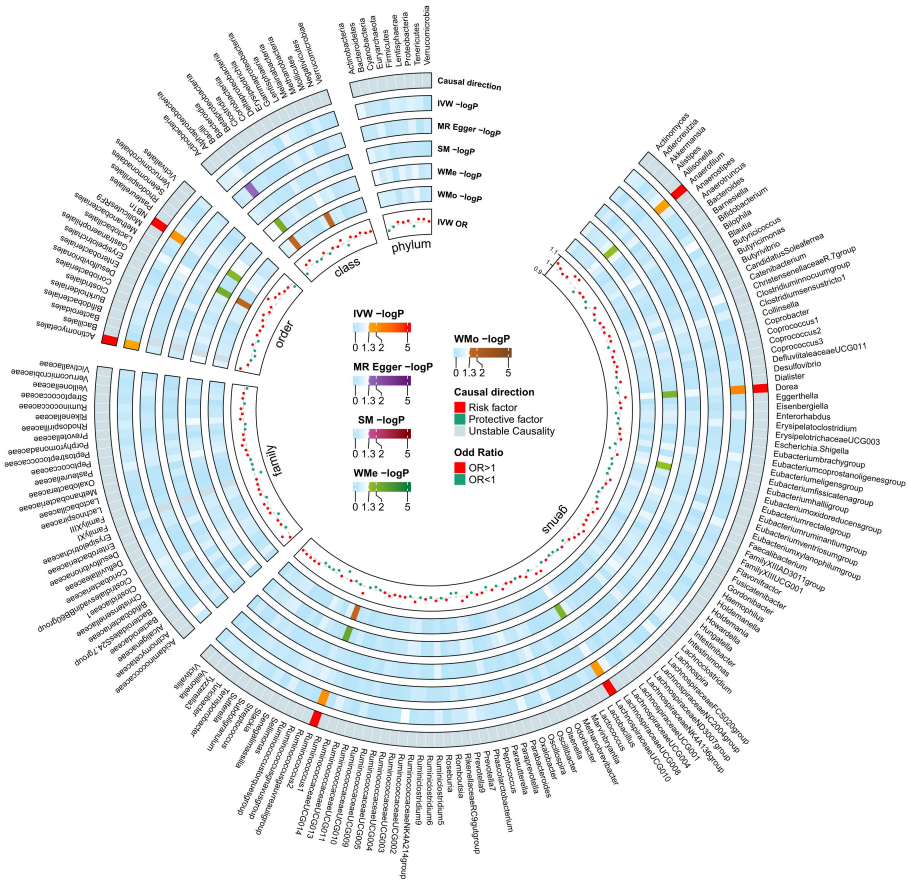

FIGURE 4
Mendelian Randomization analyses illustrating the causal effect of the diabetic neuropathy on gut microbiome. In both circular heatmaps, the data layers, from inside to out, represent the odds ratios calculated using the Inverse Variance Weighted (IVW) method, followed by –log10(*p* values) for IVW, Weighted Median (WMo), Weighted Mode (WMe), Simple Median (SM), and MR-Egger methods, respectively. The outermost ring illustrates the concordance of effect direction as determined by five MR methodologies: IVW ($p < 0.05$), MR-Egger, SM, WMe, and WMo. IVW, inverse variance weighted; SM, simple mode; WMe, weighted median; WMo: weighted mode; MR, mendelian randomization.

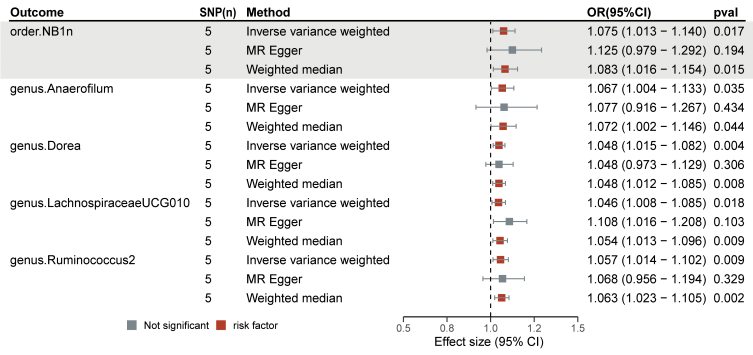

FIGURE 5
Forest plots of the significant causal effect of diabetic neuropathy on gut microbiota were calculated using the IVW method, MR-Egger, and weighted median. The forest plots demonstrate that diabetic neuropathy was a risk factor for the *Anaerofilum* (OR = 1.07; 95% CI = 1.00–1.13; $p < 0.05$), *Dorea* (OR = 1.05; 95% CI = 1.02–1.08; $p < 0.01$), *Lachnospiraceae UCG-010* (OR = 1.05; 95% CI = 1.01–1.09; $p = 0.02$), *Ruminococcus 2* (OR = 1.06; 95% CI = 1.01–1.10; $p = 0.01$), and order NB1n (OR = 1.08; 95% CI = 1.01–1.14; $p = 0.02$).

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TABLE 2 Instrumental variables used in MR analysis of the association between Diabetic neuropathy and gut microbiota.

Exposure	SNP	chr.	pos.	Beta	SE	*p*-value	R^2	F
Diabetic neuropathy	rs13212435	6	32454571	-0.273	0.034	1.21787E-15	2.33e-04	64.0
	rs2476601	1	113834946	-0.274	0.036	5.60919E-14	2.06e-04	56.5
	rs2736428	6	31876147	0.195	0.029	1.78115E-11	1.65e-04	45.2
	rs73410776	6	32822178	0.525	0.04	4.55198E-40	6.39e-04	175.5
	rs9273364	6	32658525	0.593	0.027	5.7148E-105	1.72e-03	473.4

3.5 GO enrichment analysis

In the set of IVs from the forward MR analysis, a total of 13 genes were identified: *ADCYAP1R1, MPDZ, DLG1, SLC9A1, SLC2A5, CD226, B3GNT5, EXTL3, GBE1, TMTC1, NPY2R, ADARB2*, and *GNPDA1*, which exhibited cis-regulatory control over gene expression. The GO enrichment analysis of these 13 genes yielded 12 significant results ($p< 0.05$), such as apical plasma membrane, glycosyltransferase activity, hexosyltransferase activity, and membrane raft (Figure 6).

4 Discussion

In recent times, there has been significant focus on the gut microbiota in metabolic illnesses, specifically type 2 diabetes. This topic has been extensively explored in scientific literature (28). In addition, previous studies have used MR analysis to elucidate potential causal relationship between various biomarkers from different sources and the risk of various diseases (29–31). In this study, we utilized GM data derived from a GWAS meta-analysis conducted by the MiBioGen consortium and DN data from the R8 release of the FinnGen consortium. The causal effects of GM taxa (from phylum to genus level) on DN were investigated. We found increased levels of Christensenellaceae R-7, Ruminococcaceae UCG013 and *Eggerthella* groups, which may be associated with a higher risk of DN, while increased levels of Peptococcaceae and *Eubacterium coprostanoligenes* groups could be linked to a lower risk. Additionally, we performed a reverse MR analysis to demonstrate the causal relationships between DN and GM, and found that the risk of DN may be potentially linked with elevated levels of *Anaerofilum, Dorea, Lachnospiraceae UCG-010, Ruminococcus_2*, and order NB1n. The GO enrichment analysis showed a considerable enrichment of the genes involved in glycosyltransferase and hexosyltransferase activities.

There is an increasing interest in studying the harmful effects of the microbiome on various human diseases. Dysbiosis of GM may disrupt normal gut microbial activity, leading to various neurological defects (32). Similarly, previous studies *in vivo* have shown that transplanting dysbiotic GM from individuals with distal symmetric polyneuropathy, a prevalent neuropathy in people with diabetes mellitus, to *db/db* mice had accelerated the development of peripheral neuropathy (33). *Ruminococcus* has been reclassified as *Blautia*, a genus of anaerobic bacteria that play specific roles in metabolic disorders, inflammatory diseases, and biotransformation (34). Recent investigations have shown that *Ruminococcus torques*

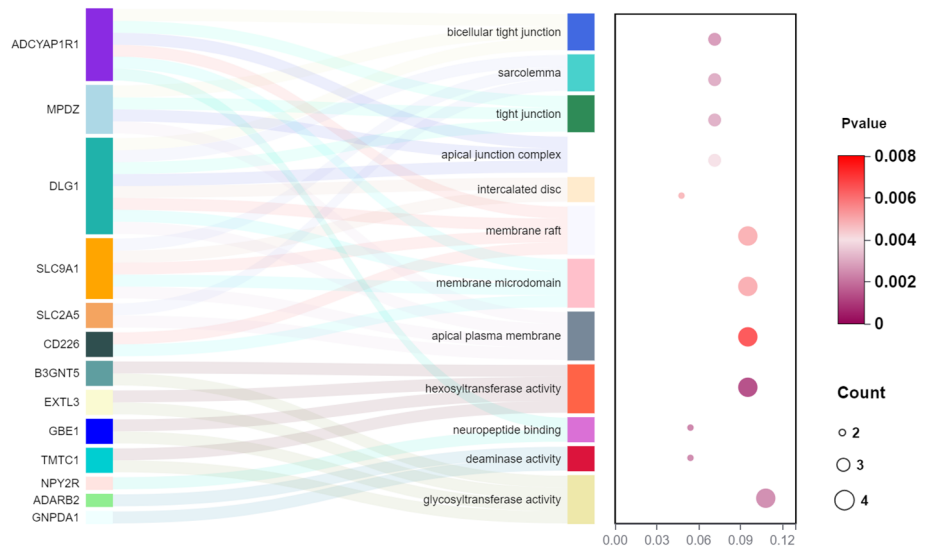

FIGURE 6
The GO enrichment analysis of the 13 genes from a cis-eQTL perspective in the forward MR analysis (gut microbiota as exposure and diabetic neuropathy as outcome).

level was significantly elevated in the clinically diagnosed DPN group, relative to the normal or disease controls (35). Similarly, Ruminococcaceae_UCG013 may be a causative agent of DN in our study. A prospective cohort analysis showed that *Eggerthella* is an important risk factor for diabetic foot ulcers (36), whereas peripheral neuropathy was identified as one of the most prominent variables linked with diabetic foot ulcers (37). implying that *Eggerthella* may be a potential risk factor for DN. These results were consistent with our study. Previous studies have confirmed that Peptococcaceae is a protective factor for diabetic retinopathy (38). However, the role of Peptococcaceae in DN has not been previously investigated. Besides, the reverse MR analysis suggested that DN may have a causal association with the elevated levels of *Ruminococcus_2*. There may be a two-way causal relationship between different genera of GM and the same disease. Therefore, further investigations are needed to clarify the functional significance of specific genera of GM and explore targeted therapies for gut bacterial flora. Previous research has shown a potential genetic relationship between the Christensenellaceae R-7 group and frailty, highlighting the significance of GM in human physiology (39). Our research confirms that Christensenellaceae R-7group has been associated with an increased incidence of DN, offering a novel avenue to explore the impact of GM.

There is less research on the effect of DN on GM. GM is dynamic and mostly stable in healthy people, but it can be influenced by many disorders (40). From the perspective of metabolic and immune processes, the distribution of GM significantly differed between the patients with and without diabetes (41). In our study, increased levels of *Anaerofilum*, *Dorea*, *Lachnospiraceae UCG-010*, *Ruminococcus_2*, and order NB1n may potentially be associated with the risk of DN. Previous studies have shown that delayed neurocognitive recovery was enriched by *Anaerofilum* compared to the non-delayed neurocognitive recovery group (42). The study of the relationship between *Dorea* and insomnia (43), *Lachnospiraceae UCG-010* and chronic kidney disease (44), *Ruminococcus_2* and rheumatoid arthritis (45), order NB1n and gastroduodenal ulcers (46) highlight the potential for GM-focused treatments.

The precise processes through which gut bacteria influence the likelihood of developing metabolic diseases are still unknown. Prior research has shown specific factors contributing to the progression of diabetic issues are elevated levels of reactive oxygen species, chronic hyperglycemia, reduced antioxidant capacity (47) and the anti-inflammatory effects of certain bacteria (such as, *Faecalibacterium* in patients with DN) (48). Moreover, numerous recent studies have recognized the gut–brain axis as a pivotal mechanism for investigating the advancement of diseases. However, there is a lack of research on the connection between the microbiota and various types of pain that lack a clearly identifiable localized cause, such as DN (49, 50). In our study, we performed a GO analysis for the 13 genes to find potential mechanisms of disease pathogenesis. The analysis showed an increase in the apical plasma membrane, glycosyltransferase activity, hexosyltransferase activity, and membrane raft.

This research has several strengths. Causal inference between GM and DN was determined using an MR analysis to exclude the confounding variables and reverse causation. The genetic variations of GM were derived from the most extensive GWAS meta-analysis to ensure that the robustness of the instruments used in the MR analysis. Horizontal pleiotropy was identified and ruled out by the MR-PRESSO and MR-Egger regression intercept term analyses. The leave-one-out analysis confirmed the robustness of the results. A two-sample MR test was used, utilizing non-overlapping exposure and result summary-level data to prevent bias (51). Nevertheless, our research has some drawbacks. We chose SNPs with $p< 1 \times 10^{-5}$ as IVs due to the limited number of SNPs with $p< 5 \times 10^{-8}$. We conducted multiple IV screening processes to ensure the reliability of IVs. This included removing SNPs with an F-value< 10 to prevent bias from weak IVs and scanning all SNPs in PhenoScanner V2 to eliminate any confounding effects. This study examines the relationship between GM and DN without studying the underlying mechanism. This MR analysis can be affected by potential pleiotropy. Each exposure in our study had a minimum of three IVs, which could potentially reduce the impact of pleiotropy given that distinct IVs are unlikely to exhibit the same correlation due to pleiotropy. The genetic IVs showed a slight effect on the variances of certain microbial taxa, possibly limiting the statistical power of the association findings.

The research participants were mostly of European descent, with limited GM data collected from other ethnic groups, who were less influenced by ethnic bias. Hence, this prevents the generalizability of the findings to other groups. Therefore, future research should examine the complex interactions and communications between the host and gut bacteria to enhance our understanding of the relation between GM and illness.

5 Conclusion

In conclusion, by carrying out a two-sample MR analysis using publicly available GWAS summary-level data, we investigated the causal influence of GM on DN neuropathy and found potential flora for DN development. This work may be relevant for screening gut microbial-derived metabolites and indicators for early diagnosis of DN, which could serve as non-invasive diagnostic or therapeutic targets.

Data availability statement

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

Author contributions

LX: Conceptualization, Data curation, Formal analysis, Project administration, Writing – original draft, Writing – review & editing. WG: Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

GC: Formal analysis, Funding acquisition, Project administration, Resources, Validation, 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/fendo.2024.1402014/full#supplementary-material

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Association between gut microbiota and adrenal disease: a two-sample Mendelian randomized study

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Background: Some observational studies and clinical experiments suggest a close association between gut microbiota and metabolic diseases. However, the causal effects of gut microbiota on adrenal diseases, including Adrenocortical insufficiency, Cushing syndrome, and Hyperaldosteronism, remain unclear.

Methods: This study conducted a two-sample Mendelian randomization analysis using summary statistics data of gut microbiota from a large-scale genome-wide association study conducted by the MiBioGen Consortium. Summary statistics data for the three adrenal diseases were obtained from the FinnGen study. The study employed Inverse variance weighting, MR-Egger, and MR-PRESSO methods to assess the causal relationship between gut microbiota and these three adrenal diseases. Additionally, a reverse Mendelian randomization analysis was performed for bacteria found to have a causal relationship with these three adrenal diseases in the forward Mendelian randomization analysis. Cochran's Q statistic was used to test for heterogeneity of instrumental variables.

Results: The IVW test results demonstrate that class Deltaproteobacteria, Family Desulfovibrionaceae, and Order Desulfovibrionales exhibit protective effects against adrenocortical insufficiency. Conversely, Family Porphyromonadaceae, Genus Lachnoclostridium, and Order MollicutesRF9 are associated with an increased risk of adrenocortical insufficiency. Additionally, Family Acidaminococcaceae confers a certain level of protection against Cushing syndrome. In contrast, Class Methanobacteria, Family Lactobacillaceae, Family Methanobacteriaceae, Genus. Lactobacillus and Order Methanobacteriales are protective against Hyperaldosteronism. Conversely, Genus Parasutterella, Genus Peptococcus, and Genus Veillonella are identified as risk factors for Hyperaldosteronism.

Conclusions: This two-sample Mendelian randomization analysis revealed a causal relationship between microbial taxa such as Deltaproteobacteria and Desulfovibrionaceae and Adrenocortical insufficiency, Cushing syndrome, and Hyperaldosteronism. These findings offer new avenues for comprehending the development of adrenal diseases mediated by gut microbiota.

KEYWORDS

gut microbiota, adrenal disease, Mendelian randomized, EPI - epidemiology, risk

Introduction

Adrenal diseases are vital components of endocrine system disorders, chiefly encompassing Adrenocortical insufficiency (AI), Cushing syndrome(CS), and Hyperaldosteronism(HA) (Salman and Cohen, 2021). AI manifests as adrenal cortex dysfunction, resulting in absolute or relative insufficiency of cortisol secretion (Bancos et al., 2015). Primary adrenal insufficiency, such as Addison's disease, is relatively rare and typically arises from direct adrenal failure (Erichsen et al., 2009; Ross and Levitt, 2013; Bornstein et al., 2016). Conversely, secondary adrenal cortex insufficiency, more prevalent, stems mainly from pituitary damage affecting adrenocortical hormone secretion (Grossman, 2010; Hahner et al., 2021). AI frequently progresses to adrenal crisis, significantly heightening patient mortality rates (Dineen et al., 2019). Prolonged elevation of endogenous cortisol levels can result in Cushing's syndrome, leading to numerous organ complications such as hypertension, obesity, dysregulation of glucose and lipid metabolism, and cognitive impairment (Pivonello et al., 2016). These complications arise due to the impact on the nervous and immune systems, ultimately diminishing the patient's quality of life (Hatipoglu, 2012; Ferriere and Tabarin, 2020). Hyperaldosteronism primarily involves primary aldosterone elevation and stands as a frequent cause of hypertension, often inflicting direct damage on target organs (Zennaro et al., 2020). Compared to diabetes, the incidence of adrenal diseases is relatively lower. However, with advancements in medical technology, their global incidence is on the rise. The prevalence of CS is 39.1 per million, with an annual incidence of 2.4 per 100,000. Reports indicate that since 1974, the prevalence of CS has increased almost linearly (Steffensen et al., 2010), while the prevalence of AI has reached 100-140 per million (Hahner et al., 2021). HS most commonly manifests as hypertension, and it is estimated that approximately 6%-10% of hypertensive patients are affected by HS (Monticone et al., 2017). These data suggest that adrenal diseases are impacting an increasing number of patients, gradually becoming a significant focus of global public health efforts.

Abbreviations: AI, Adrenocortical insufficiency; CS, Cushing syndrome; GWAS, Genome-wide association studies; HA, Hyperaldosteronism; IV, instrumental variables; IVW, Inverse variance weighting; MR, Mendelian randomization; SNP, Single nucleotide polymorphisms.

The gut microbiota, defined as the microbial community residing in the human gastrointestinal tract, has garnered increasing attention due to mounting evidence suggesting its close association with various diseases in the body. For example, a case-control study observed a reduction in Bacteroidetes and an increase in Firmicutes and Proteobacteria in patients with CS (Zhang et al., 2022). In a study involving 54 psoriasis patients and 27 healthy controls, genetic material from gut bacteria was detected in the plasma of 16 psoriasis patients, but not in any healthy controls. This discrepancy may be attributed to a reduction in the abundance of potential probiotics in psoriasis patients, resulting in immune system imbalance (Mahmud et al). Moreover, the relationship between obesity and gut microbiota is well established (Gomes et al., 2018). It is widely believed that gut microbiota dysbiosis primarily induces disease by influencing the gut-brain axis and regulating brain function (Anand et al., 2022). Animal studies have shown that microbial colonization in mice affects the development of the hypothalamic-pituitary-adrenal axis postnatally, suggesting that gut microbiota significantly impact adrenal function (Sudo et al., 2004). Another study found that the excessive release of lipopolysaccharides into the blood by Gram-negative bacteria can hyperactivate the hypothalamic-pituitary-adrenal axis, inducing systemic and neuroinflammation, leading to increased cortisol secretion and severely disrupting central nervous system homeostasis (Moylan et al., 2014). The probiotic B. pseudocatenulatum (CECT 7765) has been shown to reverse abnormal stress responses caused by dysregulation of glucocorticoid receptors (Agusti et al., 2018). Treatment with Lactobacillus sp. during early maternal separation stress can normalize hypothalamic-pituitary-adrenal axis activity (Gareau et al., 2007). L. farciminis has also been found to prevent excessive activation of the hypothalamic-pituitary-adrenal axis caused by restraint stress (Ait-Belgnaoui et al., 2012).However, due to challenges in confirming exposure and outcome times in case-control studies, and because similar observational studies draw conclusions based on changes in microbial composition in patients' feces, they are susceptible to various confounding factors such as age and environment (Rinninella et al., 2019). These limitations impede causal inferences between gut microbiota and adrenal diseases, leaving the causal relationship between gut microbiota and adrenal diseases unresolved.

Mendelian randomization(MR) is currently recognized as a method capable of inferring causality between exposure and outcome. It utilizes genetic variants associated with exposure as instrumental variables to assess the association between exposure and outcome (Greenland, 2000; Emdin et al., 2017). According to Mendelian genetic laws, genetic information is randomly allocated at conception, occurring prior to the onset of any disease. This randomization significantly minimizes the influence of environmental and lifestyle confounding factors. MR has been widely utilized to explore causal relationships between gut microbiota and various diseases, including preeclampsia (Li et al., 2022), autoimmune diseases (Xu et al., 2021), and others. However, research on the relationship between gut microbiota and AI, CS, and HA is relatively scarce. Thus, this study aimed to investigate the causal relationship between gut microbiota and various adrenal diseases by conducting a comprehensive two-sample MR analysis on three adrenal diseases, namely AI, CS, and HA.

Method

This Mendelian randomization analysis utilized summary-level MR analysis genome-wide association studies(GWAS) data, all of which were publicly available and did not involve the collection of new data, hence no additional ethical approval was required. The study workflow is depicted in Supplementary Figure 1.

Exposure data

The instrumental variables (IVs) for gut microbiota were derived from a large-scale GWAS on human gut microbiota composition conducted by the MiBioGen Consortium (Kurilshikov et al., 2021). This study pooled 16S rRNA sequencing data from a total of 18,340 participants across 24 cohort studies to explore potential associations between common genetic variants and gut microbiota. A total of 211 taxonomic groups (131 genera, 35 families, 20 orders, 16 classes, and 9 phyla) were included in this study.

Outcome data

GWAS data for the three adrenal diseases were sourced from the FinnGen study, a nationwide GWAS conducted in Finland (Kurki et al., 2023).

Selection of instrumental variables

In this study, the following criteria were used to select IVs: 1. Single nucleotide polymorphisms(SNPs) significantly associated with gut microbiota were chosen as potential IVs, with a P-value less than the genome-wide significance threshold (5×10e-8); 2. SNPs with strong linkage disequilibrium were excluded based on an R2<0.001, with a clumping window size of 10,000 kb; 3. SNPs with a minor allele frequency ≤ 0.01 were eliminated; 4. In the presence of palindromic SNPs, allele frequency information was used to infer the alleles on the forward strand.

MR analysis

This study primarily utilized inverse variance-weighted (IVW), MR-Egger regression, and MR-PRESSO methods to examine the causal relationships and horizontal pleiotropy between gut microbiota and three adrenal diseases, with the IVW method being the main approach. Cochran's IVW Q statistic was used to test the heterogeneity of IVs. Additionally, to identify potential heterogeneity SNPs, a "leave-one-out" analysis was conducted by sequentially excluding each instrumental SNP. Furthermore, reverse MR analysis was performed on gut microbiota identified in forward MR analysis to have causal relationships with the three adrenal diseases, using the same methods as forward MR. To assess the strength of selected SNPs, the following formula was used to calculate the F-statistic for each bacterial taxonomic group:

$$F = \frac{R^2(N-1-K)}{(1-R^2)K}$$

where R^2 represents the proportion of exposure variance explained by genetic variation, N represents the sample size, and K represents the number of IVs. If the corresponding F-statistic is >10, then no significant weak instrument bias is considered (Pierce et al., 2011).

All statistical analyses were conducted using R version 4.3.3 (R Foundation for Statistical Computing). The TwosampleMR (version 0.5.11) packages were used for MR analysis.

Result

SNP selection

According to the criteria for selecting IVs, we respectively used 81, 8, and 99 SNPs as IVs for AI, CS, and HA. The F-statistics for all selected IVs were >10 (detailed in Supplementary Table 1), indicating the exclusion of weak instrument bias as much as possible. The results of Cochran's IVW Q test and MR-PRESSO global test showed no significant heterogeneity among these IVs (Supplementary Table 3, Supplementary Table 5). Furthermore, the MR-Egger regression intercept analysis showed no significant horizontal pleiotropy.

Causal influence of gut microbiota on the development of three adrenal diseases

Adrenocortical insufficiency

Among the 211 taxa of gut microbiota, we identified 6 bacterial taxa associated with AI, namely class Deltaproteobacteria, Family Desulfovibrionaceae, Family Porphyromonadaceae, Genus

Lachnoclostridium, Order Desulfovibrionales, and Order MollicutesRF9. IVW test results showed that class Deltaproteobacteria, Family Desulfovibrionaceae, and Order Desulfovibrionales were protective factors for AI, with odds ratios (ORs) of 0.47 (95% CI: 0.24, 0.90), 0.43 (95% CI: 0.21, 0.87), and 0.39 (95% CI: 0.20, 0.77), respectively, while Family Porphyromonadaceae, Genus Lachnoclostridium, and Order MollicutesRF9 were risk factors for AI, with ORs of 4.12 (95% CI: 1.72, 9.85), 2.25 (95% CI: 1.12, 4.52), and 1.73 (95% CI: 1.03, 2.92), respectively (Figure 1, Supplementary Table 2).

Cushing syndrome

Among the 211 taxa of gut microbiota, we found that only Family Acidaminococcaceae exhibited a certain protective effect against CS, with an odds ratio (OR) of 0.14 (95% CI: 0.04, 0.45) (Figure 1, Supplementary Table 2).

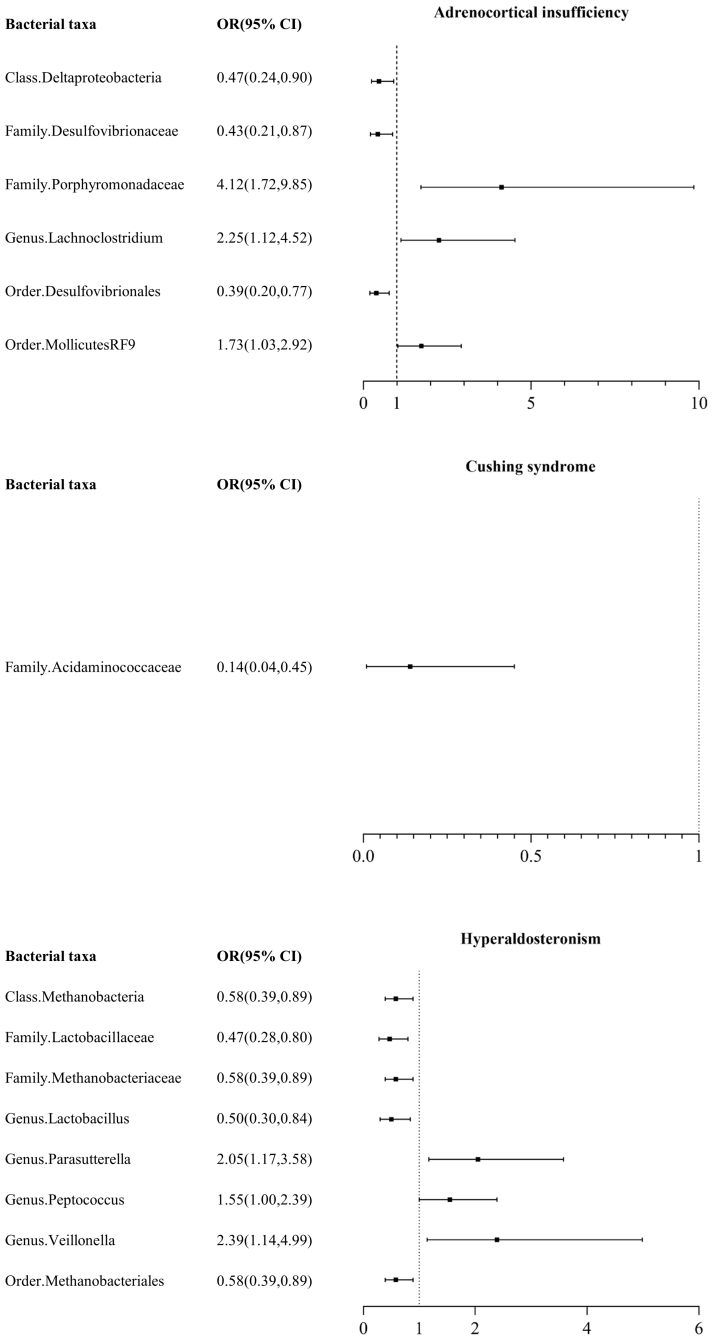

FIGURE 1
Mendelian randomization results of causal effects between gut microbiome and adrenal disease.

Hyperaldosteronism

Among the 211 taxa of gut microbiota, we found that Class Methanobacteria (OR: 0.58, 95% CI: 0.39, 0.89), Family Lactobacillaceae (OR: 0.47, 95% CI: 0.28, 0.80), Family Methanobacteriaceae (OR: 0.58, 95% CI: 0.39, 0.89), Genus Lactobacillus (OR: 0.50, 95% CI: 0.30, 0.84), and Order Methanobacteriales (OR: 0.58, 95% CI: 0.39, 0.89) were protective factors for HA, while Genus Parasutterella (OR: 2.05, 95% CI: 1.17, 3.58), Genus Peptococcus (OR: 1.55, 95% CI: 1.00, 2.39), and Genus Veillonella (OR: 2.39, 95% CI: 1.14, 4.99) were risk factors for HA.

Sensitivity analyses

The magnitude and direction of causal estimates obtained by the MR-Egger method were similar to those of the IVW method (Figure 2 and Supplementary Table 2). In the scatter plot (Figure 2) and leave-one-out plot (Figure 3), potential outliers were visually observed for some bacterial taxa IVs. Still, further, Cochran's IVW Q test results and MR-PRESSO global test results showed no significant heterogeneity (Supplementary Table 3, Supplementary Table 5). Additionally, when using the MR-Egger regression

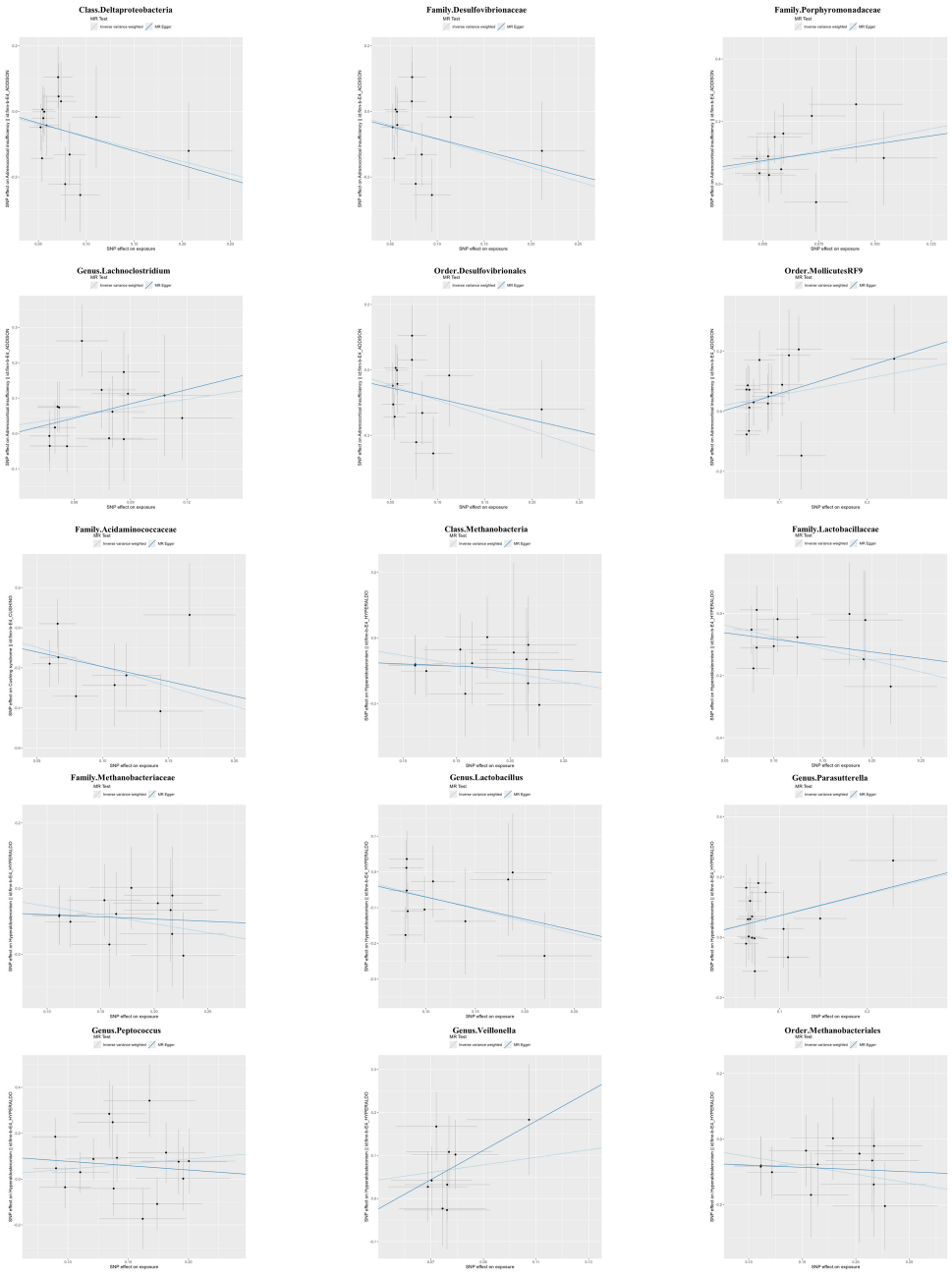

FIGURE 2
Scatterplots of the causal relationship between gut microbiota and adrenal diseases.

FIGURE 3
Leave-one-out plots for the causal association between gut microbiota and adrenal diseases.

intercept method, we found no evidence of horizontal pleiotropy in the causal relationship between the three adrenal diseases and gut microbiota (Supplementary Table 4).

Reverse causality between 3 adrenal diseases and gut microbiota

According to the results of reverse MR analysis, we did not find significant causal relationships between the three adrenal diseases and corresponding gut microbiota, and MR-Egger regression intercept analysis also did not reveal significant horizontal pleiotropy (Supplementary Table 6).

Discussion

In this study, we conducted a two-sample MR analysis using summary statistics data on human gut microbiota from the large-scale GWAS analysis conducted by the MiBioGen consortium and summary statistics data on three adrenal diseases from the FinnGen study. The aim was to evaluate the causal relationships between gut

microbiota and the three adrenal diseases. Our findings revealed that Class Deltaproteobacteria, Family Desulfovibrionaceae, and Order Desulfovibrionales were protective factors for AI, while Family Acidaminococcaceae exhibited a protective effect against CS. Additionally, Class Methanobacteria, Family Lactobacillaceae, Family Methanobacteriaceae, Genus Lactobacillus, and Order Methanobacteriales were identified as protective factors for HA.

Increasing research indicates a causal relationship between the gut microbiota we selected and various diseases. For instance, studies have identified Class Deltaproteobacteria as a major risk factor for Graves' disease and a potential risk factor for chronic kidney disease (Cao et al., 2023; Luo et al., 2023). Meanwhile, Desulfovibrionaceae is recognized as a harmful bacterial genus in the gut, capable of producing toxic effects on the intestinal epithelium by reducing sulfate to H2S under anaerobic conditions, leading to gastrointestinal diseases (Cabrera et al., 2006; Pires et al., 2006). Some research has found a significant correlation between high concentrations of Desulfovibrionaceae and the development of Parkinson's disease (Murros et al., 2021). However, a study from the Guangdong Gut Microbiome Program in China revealed a negative correlation between the relative abundance of Desulfovibrionaceae and BMI, waist circumference, and uric acid levels (Chen et al., 2021). Our study also discovered a protective effect of Desulfovibrionaceae against AI, suggesting that Desulfovibrionaceae is not universally associated with adverse health conditions. This could be attributed to the positive correlation between the relative abundance of Desulfovibrionaceae and microbial diversity, which benefits the stability of the microbiome and host health (Le Chatelier et al., 2013).

Some studies suggest that Porphyromonadaceae may act as regulators of obesity by producing short-chain fatty acids such as acetate and propionate (Lu et al., 2016). However, research by Teresa Tavella (Tavella et al., 2021) and others found that elderly individuals with higher concentrations of Porphyromonadaceae have significantly lower serum levels of branched-chain amino acids, which are associated with insulin deficiency and insulin resistance (Rietman et al., 2016; Holeček, 2018). This may also be a reason for the increased risk of Adrenocortical insufficiency (AI). Lachnoclostridium is one of the core genera in the gut microbiota and is significantly associated with many metabolic diseases. Some studies suggest that a high abundance of Lachnoclostridium may decrease levels of acetate in circulation, leading to increased abdominal fat and negative effects on obesity and type 2 diabetes (Cai et al., 2022).

There is currently limited research on MollicutesRF9, but we found that it increases the risk of AI. We speculate that this may be similar to mycoplasma, which also belongs to Mollicutes, but the specific mechanism needs further exploration.

Acidaminococcaceae mainly produces butyrate in the human body. Clinical evidence suggests that the abundance of bacteria-producing butyrate is associated with blood pressure reduction in obese pregnant women. A recent study found that supplementing fiber and acetate can improve gut dysbiosis, related to the increase of Acidaminococcaceae. Acidaminococcaceae may play a protective role in hypertension and heart failure in hypertensive mice (Xu et al., 2020).

Methanobacteria are mainly located in the human gastrointestinal tract and are responsible for methane production (Hoegenauer et al., 2022). Compared to healthy individuals, patients with inflammatory bowel disease, periodontal disease (Lepp et al., 2004), obesity (Maya-Lucas et al., 2019), and cancer (Cai et al., 2022) have higher concentrations of Methanobacteria, but there is currently no evidence to suggest that they are pathogens (Mafra et al., 2022). Lactobacillaceae, as a well-recognized probiotic, is also the most widely used microbial genus (Gourbeyre et al., 2011). It can protect the body by enhancing the intestinal epithelial barrier (Collado et al., 2005), producing antimicrobial substances (Bierbaum and Sahl, 2009), and playing an immunomodulatory role (Lebeer et al., 2010). Research on Parasutterella is currently limited. Some studies have found that an increase in the abundance of Parasutterella is associated with a decrease in gut microbiota diversity (Chiodini et al., 2015) and gut dysbiosis may be associated with many metabolic diseases (Carding et al., 2015; Chen et al., 2018). This study found that the concentration of Parasutterella is elevated in patients with HA, but the specific mechanism is unclear and requires further exploration through more basic research.

This study has several strengths. It determined the causal relationship between gut microbiota and three adrenal diseases through MR analysis, minimizing the interference of confounding factors, and supplemented by reverse MR analysis. The genetic variation of gut microbiota was obtained from the largest GWAS meta-analysis, ensuring the strength of the instruments in MR analysis. Horizontal pleiotropy was detected and eliminated using MR-PRESSO and MR-Egger regression intercept tests. A two-sample MR design was used, and non-overlapping summary-level data for exposure and outcome were used to avoid bias (Burgess et al., 2016).

However, this study also has some limitations. Firstly, because the data used in this study are summary-level GWAS data, subgroup analysis cannot be performed. Secondly, most patients in this study are of European descent, so the results may not apply to other ethnic groups. Thirdly, due to the strict criteria used in the selection of IVs in this study, a large number of SNPs of gut microbiota were excluded, so false discovery rate (FDR) correction was not applied to the research results, which may introduce some errors. Finally, we must acknowledge the inherent limitations of MR, including trait heterogeneity and developmental compensation, which may impact the accuracy and applicability of our research findings (Haycock et al., 2016).

Conclusions

In conclusion, we comprehensively evaluated the causal relationship between gut microbiota and three adrenal diseases. Our results indicate that microbial taxa such as Deltaproteobacteria and Desulfovibrionaceae may serve as pathways for the diagnosis and treatment of AI, CS, and HA, which require further experimental exploration. This study may provide new directions for understanding the occurrence and development of adrenal diseases mediated by gut microbiota.

Data availability statement

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

Author contributions

Y-YZ: Conceptualization, Formal analysis, Methodology, Writing – original draft. Y-WL: Data curation, Methodology, Writing – original draft. B-XC: Software, Visualization, Writing – original draft. QW: Funding acquisition, 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.2024.1421128/full#supplementary-material

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The beneficial effect of probiotics in the prevention of irinotecan-induced diarrhea in colorectal cancer patients with colostomy: a pooled analysis of two probiotic trials (Probio-SK-003 and Probio-SK-005) led by Slovak Cooperative Oncology Group

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Background: Probiotics could decrease irinotecan-induced diarrhea due to the reduction of intestinal beta-d-glucuronidase activity. This study included a combined analysis of two clinical trials aimed to determine the effectiveness of the probiotics in the prophylaxis of irinotecan-induced diarrhea in metastatic colorectal cancer (CRC) patients.

Methods: This combined analysis included 46 patients with CRC enrolled in the Probio-SK-003 (NCT01410955) and 233 patients from Probio-SK-005 (NCT02819960) starting a new line of irinotecan-based therapy with identical eligibility criteria. Patients were randomized in a ratio 1:1 to probiotic formulas vs. placebo administered for 12 and 6 weeks, respectively. Due to the different durations of study treatments, only the first 6 weeks of therapy were used for analysis.

Results: In total, 279 patients were randomized, including 142 patients in the placebo and 137 participants in the probiotic arm. Administration of probiotics did not significantly reduce the incidence of grade 3/4 diarrhea compared to placebo (placebo 12.7% vs. probiotics 6.6%, p = 0.11). Neither the overall

incidence of diarrhea (placebo 48.6% vs. probiotics 41.6%, p = 0.28) nor the incidence of enterocolitis (placebo 4.2% vs. probiotics 0.7%, p = 0.12) was different in the placebo vs. probiotic arm. However, subgroup analysis revealed that patients with a colostomy who received a placebo had a significantly higher incidence of any diarrhea (placebo 51.2% vs. probiotics 25.7%, p = 0.028) and grade 3/4 diarrhea (placebo 14.6% vs. probiotics 0.0%, p = 0.03) compared to the probiotic arm.

Conclusions: This combined analysis suggests that probiotics could be beneficial in the prevention of irinotecan-induced diarrhea in colorectal cancer patients with colostomy.

KEYWORDS

pooled analysis, irinotecan, diarrhea, probiotics, colorectal cancer, beta-glucuronidase

Introduction

Diarrhea represents a common condition in cancer patients undergoing chemotherapy that can severely impact the quality of life and treatment outcomes. Chemotherapy-associated diarrhea is a complex condition requiring a proper understanding of its underlying mechanisms and effective strategies for prevention and management (1).

Diarrhea in cancer patients is caused by various factors, primarily triggered by the aggressive nature of cancer and the side effects of therapeutic interventions such as chemotherapy. The gastrointestinal mucosa, a critical barrier protecting the digestive system, becomes susceptible to damage by treatments that disrupt normal cellular processes. Chemotherapy-induced diarrhea, a common manifestation, is characterized by the toxic effects of anticancer drugs on rapidly dividing cells within the intestinal lining. Additionally, alterations in the gut microbiota, inflammation, and the release of various signaling molecules further contribute to the disruption of physiological bowel functions (1).

The use of probiotics in preventing and managing diarrhea is based on both theoretical considerations and the outcomes of numerous clinical trials (2–6). Lactic acid bacteria play a pivotal role in addressing dysbiosis by competing for substrates with pathogenic bacteria, producing bacteriocins, and enhancing transepithelial resistance (7). Their enzymatic activity influences the activation or deactivation of metabolites responsible for inducing diarrhea (8). Moreover, the production of short-chain fatty acids, essential for the well-being of intestinal mucosal cells, further contributes to the anti-diarrheal effects of probiotics (9, 10).

Irinotecan, a topoisomerase I inhibitor widely used in the treatment of various cancers, including colorectal cancer, has been associated with a higher incidence of diarrhea compared to other chemotherapeutic agents (11). This side effect not only poses discomfort to patients but may also lead to dose reductions or interruptions, compromising the efficacy of the treatment. The incidence of irinotecan-induced diarrhea ranges widely, encompassing 60-90%, with severe diarrhea affecting 20-40% of patients. This gastrointestinal complication assumes critical significance in the landscape of morbidity and mortality associated with irinotecan-based chemotherapy. Identified predisposing factors include age exceeding 65 years, an Eastern Cooperative Oncology Group performance status (ECOG PS) of ≥1, and a history of abdominopelvic radiation (11, 12).

The mechanism of irinotecan-induced diarrhea is mediated by its metabolite SN-38, which is glucuronidated in the liver and subsequently excreted into the intestine. Within the intestinal lumen, bacterial beta-D-glucuronidase deconjugates SN-38, initiating a cascade of events that inflict direct damage to the intestinal mucosa, resulting in malabsorption of water and electrolytes, ultimately culminating in the onset of diarrhea (12). Understanding the intricate mechanisms of irinotecan-induced diarrhea is imperative for devising targeted interventions to enhance the overall management of this chemotherapy-related side effect (13–16). Certain probiotic bacteria have demonstrated the capability to diminish the activity of intestinal beta-D-glucuronidase (14, 15). This suggests a potential avenue for the application of these bacteria in preventing diarrhea in patients undergoing irinotecan-based therapy (Figure 1).

Previously, we conducted two clinical trials focused on preventing irinotecan-induced diarrhea in metastatic colorectal cancer patients (17, 18). In the pilot study, which included 46 patients who received the probiotic formula Colon Dophilus™ or placebo, we observed a decreased diarrhea incidence in the probiotic arm with no grade 3/4 diarrhea (17). Based on these results, we performed a phase III trial in the same patient population. In this trial, patients received a combination of *Bifidobacterium* BB-12 and *Lactobacillus rhamnosus* GG, LGG (18). The results of this trial did not confirm the effectivity of probiotics in the prevention of irinotecan-induced diarrhea;

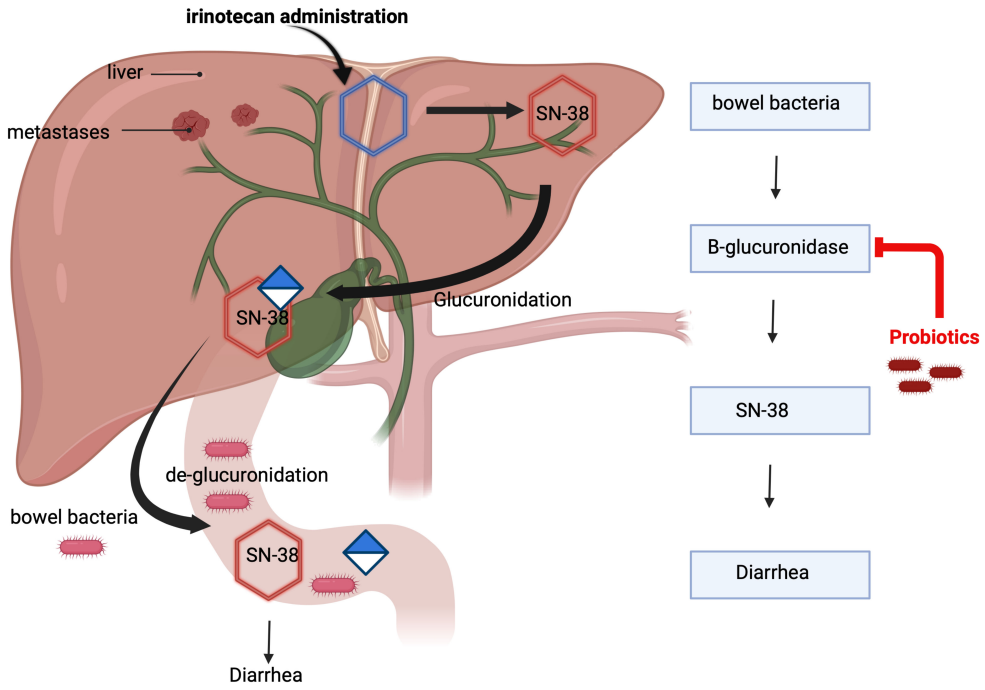

FIGURE 1
Study hypothesis.

however, subgroup analysis suggested their effectivity in patients with colostomy. These trials utilized different probiotic formulas widely available for patients without prescription. The choice of formulas was determined mainly by their availability for investigator-initiated trials from pharmaceutical companies. While Colon Dophilus™ is more complex and contains 10 different probiotic strains, the probiotic formula Probio-Tec® BG-Vcap-6.5 is composed of two strains and has been more widely studied in various clinical scenarios.

The statistical power of subgroup analysis, especially in underrepresented subgroups, is limited in single trials. Taking advantage of identical eligibility criteria and a very similar statistical design of these two clinical trials, we performed pooled analysis aiming to determine the effectiveness of the probiotics in the prophylaxis of irinotecan-induced diarrhea in metastatic colorectal cancer (CRC) patients and identifying specific subgroups that could benefit from preventive administration of probiotics during irinotecan-based chemotherapy. Besides having higher statistical power for the primary endpoint, the dataset of this pooled analysis has increased the number of patients in several specific subgroups compared to individual previous trials, which enables more robust testing, enhances the ability to detect heterogeneity, and improves the generalizability of study results.

Patients and methods

This combined analysis included two studies; 46 patients with CRC enrolled in the Probio-SK-003 (NCT01410955) between January 2011 and December 2013, starting a new line of irinotecan-based therapy (17) and 233 patients of Probio-SK-005 study (NCT02819960) randomized from March 2016 to May 2022 with identical eligibility criteria as previous trial (18).

Eligibility criteria

Both trials had the same eligibility criteria (17, 18). Eligible participants were adult patients with histologically proven colorectal cancer starting a new line of chemotherapy based on irinotecan with ECOG PS 0-1 at study entry. Exclusion criteria comprised impossibility to take oral medication, active infection treated by antibiotic therapy, ileostomy or jejunostomy, hypersensitivity to study drug, and any concurrent malignancy other than non-melanoma skin cancer, no other cancer in the past 5 years.

Trial design

Both trials were multi-centered, double-blinded clinical studies conducted to evaluate the effectiveness of oral probiotic supplements compared to a placebo in preventing severe diarrhea in patients with colorectal cancer who were starting a new round of chemotherapy treatment involving irinotecan. Patients were randomly assigned to receive either the probiotic supplement or the placebo, with an equal number of patients in each group. The randomization process was centralized, where each patient was given a unique identification number and received a corresponding container with the assigned treatment. These containers,

indistinguishable from each other, were labeled with sequential numbers assigned randomly to preserve blinding. All researchers, statisticians, and patients remained unaware of which treatment each patient received until the final result analysis.

Treatment

In Probio-SK-003, the probiotic formula Colon Dophilus™ (produced by Harmoniom International, Inc., Mirabel, Canada) was administered orally at a dose of 3×1cps per day for 12 weeks and each capsule contained 10×10^9 CFU of bacteria. Whereas, in Probio-SK-005, the probiotic formula Probio-Tec® BG-Vcap-6.5 (produced by Chr. Hansen A/S, Hoersholm, Denmark) containing $2.7x10^9$ CFU was administered orally at a dose of 3x1 cps per day for 6 weeks. No premedication or patient monitoring after probiotic supplementation was required in both trials. The probiotic formula might be taken after meals or snacks to reduce stomach upset. The probiotic formula might be taken after meals or snacks to reduce stomach upset. The capsule should be swallowed whole or opened, and the content mixed with a small amount of food in case of problems with swallowing. Probiotic formula Colon Dophilus™ contained *Bifidobacterium breve HA-129* (25%), *Bifidobacterium bifidum* HA-132 (20%), *Bifidobacterium longum* HA-135 (14.5%), *Lactobacillus rhamnosus* HA-111 (8%), *Lactobacillus acidophilus* HA-122 (8%), *Lactobacillus casei* HA-108 (8%), *Lactobacillus plantarum* HA-119 (8%), *Streptococcus thermophilus* HA-110 (6%), *Lactobacillus brevis* HA-112 (2%), *Bifidobacterium infantis* HA-116 (0.5%). Probio-Tec BG-Vcap-6.5® contained *Bifidobacterium* BB-12 (50%) and *Lactobacillus rhamnosus* GG, LGG (50%).

Duration of therapy

In Probio-SK-003, the probiotic formula was administered during irinotecan-based chemotherapy for 12 weeks, while in Probio-SK-005, probiotic supplementation lasted for 6 weeks. Due to the different durations of study treatments, only the first 6 weeks of therapy were used for the analysis.

In both trials, patients might also discontinue protocol therapy in the case of intercurrent illness, affecting the patients' safety in investigator judgment, the ability to deliver treatment or the primary study endpoints, and/or by patient request.

Concomitant therapy

Patients received full supportive care during the study, including transfusion of blood and blood products, antibiotic treatment, anti-emetics, antidiarrheal agents, analgesics, erythropoietin, or bisphosphonates, when appropriate.

Treatment evaluation

The clinical assessment encompassed various factors such as demographic information, birthdate, ethnicity, gender, and medical background. This included a detailed account of cancer-specific history, encompassing the date of diagnosis, primary tumor type along with histology findings, past surgical and/or radiological treatments (including dates and specific organ/anatomic regions targeted), current cancer stage, previous systemic therapies, persistent side effects from prior treatments, any history of additional malignancies, and significant medical events within the last six months. The assessment of adverse effects, including diarrhea and enterocolitis, was conducted according to the NCI Common Terminology Criteria for Adverse Events Version 4.1 (CTCAE) (18). Patients maintained diaries to record daily stool frequency and consistency, as well as the use of antidiarrheal medications throughout the study. However, evaluation of patients' compliance with the prescribed study medications was not performed (17, 18).

Statistical analysis

Data analysis followed the pre-specified plan for statistical analysis. The patients' attributes were summarized by presenting the median (range) for continuous variables and frequency (percentage) for categorical variables. The Kolmogorov-Smirnov test was applied to assess the distribution's normality. If the data followed a normal distribution, sample means were tested using either the Student t-test or analysis of variance (ANOVA), with adjustments like Bonferroni's or Tamhane's based on variance homogeneity. For non-normally distributed data, the nonparametric Mann-Whitney U or Kruskal-Wallis H test was utilized. Fisher's exact test or Chi-square test was employed for categorical data. Event-free survival, specifically concerning diarrhea, was determined utilizing Kaplan-Meier methods, and compared between study arms using the log-rank test. The data were computed from the initiation of probiotic administration (day 1) until the event or the end of the study, at which point the data were censored. All presented p-values are two-sided, with associations considered significant if the p-value was 0.05 or lower. The statistical analyses were conducted using NCSS 2022 statistical software (Hintze J, 2022, Kaysville, UT, USA).

Results

Patient characteristics and chemotherapy protocols can be found in Table 1. There were disparities observed between the groups receiving different treatments. The probiotic arm had a higher proportion of patients with colon cancer compared to rectal cancer, which was in line with previous radiation therapy patterns for rectal cancer. The placebo arm had slightly more patients receiving adjuvant therapy, whereas the probiotic arm had a higher number of patients treated with first-line chemotherapy. Colostomy was slightly more prevalent in the placebo arm. The distribution of irinotecan regimens and other therapies, including 5-FU-based, anti-EGFR, and anti-VEGF therapy, was balanced across both arms.

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TABLE 1 Patients' characteristics.

	Placebo	A	Probiotics	B
	N	%	N	%
All patients	142	100.0	137	100.0
Age. median (range)	65 (36-82)		64 (29-82)	
Gender				
male	83	58.5	81	59.1
female	59	41.5	56	40.9
Tumor localization				
colon	86	60.6	96	70.1
rectum	53	37.3	40	29.2
Surgery of primary tumor				
no	28	19.7	36	26.3
yes	112	78.9	101	73.7
Colostomy				
no	101	71.1	102	74.5
yes	41	28.9	35	25.5
Previous radiotherapy to rectum				
yes	34	23.9	22	16.1
no	108	76.1	115	83.9
Previous therapy				
adjuvant chemotherapy	56	39.4	41	29.9
chemotherapy for metastatic disease	79	55.6	70	51.1
5-Fluorouracil-based including capecitabine	75	52.8	63	46.0
anti-VEGF	33	23.2	35	25.5
anti-EGFR	11	7.7	10	7.3
Current therapy				
Line of therapy				
1st line	63	44.4	67	48.9
2nd line	70	49.3	62	45.3
3rd line	9	6.3	6	4.4
4th line	0	0.0	2	1.5
Chemotherapy				
irinotecan weekly	32	22.5	34	24.8
irinotecan every 2 weeks	88	62.0	83	60.6
irinotecan every 3 weeks	22	15.5	20	14.6
5-Fluorouracil	73	51.4	66	48.2
5-Fluorouracil bolus	34	23.9	71	51.8
5-Fluorouracil continues	47	33.1	51	37.2
Capecitabine	48	33.8	41	29.9
5-Fluorouracil-based chemotherapy	122	85.9	112	81.8
anti-EGFR	19	13.4	16	11.7
anti-VEGF	47	33.1	44	32.1

Totally 279 patients were randomized (placebo 142, probiotics 137). Administration of probiotics did not significantly reduce the incidence of grade 3/4 diarrhea compared to placebo (placebo 12.7% vs. probiotics 6.6%, p = 0.11) (Figure 2A). Neither the overall incidence of diarrhea (placebo 48.6% vs. probiotics 41.6%, p = 0.28) nor the incidence of enterocolitis (placebo 4.2% vs. probiotics 0.7%, p = 0.12) was different in the placebo vs. probiotic arm (Table 2). However, subgroup analysis revealed that patients with a colostomy who received a placebo had a significantly higher incidence of any diarrhea (placebo 51.2% vs. probiotics 25.7%, p = 0.028) and grade 3/4 diarrhea (placebo 14.6% vs. probiotics 0.0%, p = 0.03) compared to the probiotic arm. Moreover, patients with colostomy had no enterocolitis compared to 7.3% of patients in a placebo arm (Table 3; Figure 2B). Patients in the probiotic arm needed numerically less salvage medication (loperamide) in contrast to the placebo arm (placebo 29.3% vs. probiotics 14.3%, p = 0.17). We did not observe any infection caused by probiotic strains in this study.

Discussion

In this pooled analysis, the administration of probiotics did not yield statistically significant reductions in grade 3/4 diarrhea, overall diarrhea incidence, or enterocolitis compared to the placebo group. However, a subgroup analysis identified a benefit for patients with colostomy receiving probiotics, showing significantly lower incidences of any diarrhea and grade 3/4 diarrhea compared to the placebo group. Patients with colostomy in the probiotic arm also had no cases of enterocolitis, in contrast to 7.3% in the placebo arm. Additionally, patients in the probiotic arm required numerically less salvage medication (loperamide) than those in the placebo arm. Importantly, no infections were observed related to the probiotic strains used in the study.

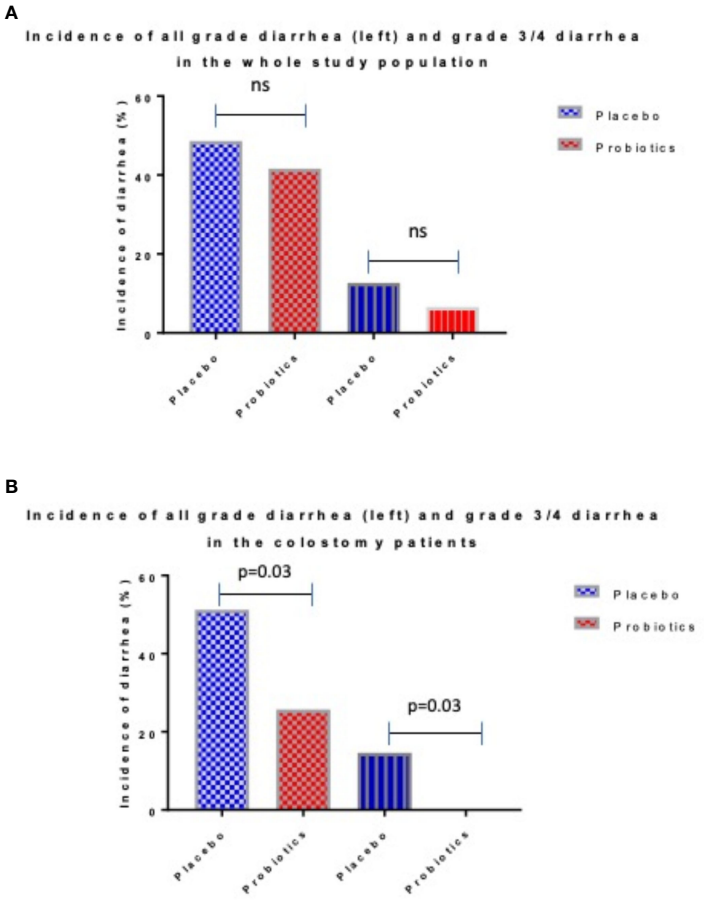

FIGURE 2
Incidence of diarrhea in whole study populations **(A)** and patients with colostomy **(B)**.

Animal models focusing on irinotecan administration have revealed shifts in microbiota composition, marked by increased presence of intestinal *Enterobacteriaceae* spp. and *Clostridium* cluster XL, accompanied by heightened pro-inflammatory cytokines and alterations in mucosa composition leading to reduced adhesion sites (19, 20). These changes contribute to a decrease in symbiotic bacteria and an increase in opportunistic pathogens. While numerous preclinical data suggest the potential benefits of probiotics in mitigating irinotecan-induced gastrointestinal toxicity, clinical evidence remains limited (21–23). A prospective observational trial hints at the ameliorative effects of *Lentilactobacillus kefiri* LKF01 (Fefibios®) on severe irinotecan-induced diarrhea in cancer patients (24). Conversely, a phase II/III, randomized, double-blind, placebo-controlled study failed to meet its primary endpoint of reducing grade 3/4 irinotecan-induced diarrhea using a high-concentration multi-strain probiotic supplement (25). This observation aligns with our trials (17, 18). The disparity underscores the complexity of translating preclinical findings into clinical efficacy and emphasizes the need for further investigation into the optimal probiotic strategies for managing irinotecan-induced diarrhea.

Both these trials had the same eligibility criteria, which enabled data pooling. Due to the different durations of study treatment, only the first 6 weeks of therapy were used for this analysis. Differences in outcome in each trial could be related to the different probiotic formulas used as well as different incidences of diarrhea in control arms, which could be related to better management of irinotecan toxicity in the last years. Despite these differences, both trials consistently showed the most pronounced effect of probiotics in the prevention of diarrhea in patients with a colostomy (17, 18). There was no overlap in any probiotic strain used in these clinical trials. However, both formulas contained *Lactobacillus* and *Bifidobacterium*, which are widely utilized in numerous probiotic products, thus increasing the generalizability of study results. Taking into account the results of a similar trial published in the abstract form (25), we suggest that the efficacy of probiotics in reducing irinotecan-induced diarrhea in the unselected patient population is unlikely. These results can't exclude the potential beneficial effect of gut microbiome modification by other probiotic formulas and/or fecal microbiota transplantation in the study patient population treated with irinotecan-based chemotherapy. Unfortunately, any of the utilized probiotic formulas underwent preclinical testing in animal models of irinotecan-induced diarrhea, which could also affect study results. Future studies assessing any other intervention to modify the gut microbiome composition

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TABLE 2 Study results (n=279).

	Placebo	A	Probiotics	B	P-value
Variables	N	%	N	%	
Diarrhea any grade	69	48.6	57	41.6	0.28
Diarrhea grade 3/4	18	12.7	9	6.6	0.11
Diarrhea (grades)					
0	73	51.4	80	58.4	0.05
1	34	23.9	22	16.1	
2	17	12.0	26	19.0	
3	16	11.3	9	6.6	
4	2	1.4	0	0.0	
Enterocolitis	6	4.2	1	0.7	0.12
Abdominal bloating	13	9.2	9	6.6	0.5
Patients' diaries					
mushy stool	112	78.9	112	81.8	0.55
watery stool	75	52.8	76	55.5	0.72
loperamide	32	22.5	31	22.6	1.00
diphenoxylate	34	23.9	24	17.5	0.24
loperamide or diphenoxylate	51	35.9	40	29.2	0.25

TABLE 3 Study results. Colostomy patients only (n=76).

	Placebo	A	Probiotics	B	
Variables	N	%	N	%	P-value
Diarrhea any grade	21	51.2	9	25.7	0.03
Diarrhea grade 3/4	6	14.6	0	0.0	0.03
Diarrhea (grades)					
0	20	48.8	26	74.3	0.008
1	9	22.0	3	8.6	
2	6	14.6	6	17.1	
3	6	14.6	0	0.0	
4	0	0.0	0	0.0	
Enterocolitis	3	7.3	0	0.0	0.24
Abdominal bloating	7	17.1	3	8.6	0.33
Patients' diaries					
mushy stool	33	80.5	28	80.0	1.00
watery stool	26	63.4	19	54.3	0.49
loperamide	12	29.3	5	14.3	0.17
diphenoxylate	10	24.4	7	20.0	0.78
loperamide or diphenoxylate	17	41.5	8	22.9	0.32

should incorporate preclinical testing before proceeding to a clinical setting.

In our analysis, the administration of probiotics was associated with a significantly reduced incidence of diarrhea in colostomy patients. We can't assess if this could be related to a decrease in bowel beta-glucuronidase activity due to probiotics and/or if this is achieved by another mechanism. While the incidence of diarrhea in colostomy patients on the placebo arm and/or grade ¾ diarrhea was not different compared to the whole study population, this was dramatically reduced on the probiotic arm. While shorter bowel length may be a contributing factor, it is also possible that differences in microbiome composition could be influencing this observation. To better understand this phenomenon, future studies should investigate the pre- and post-treatment composition of the gut microbiota, as well as measure beta-glucuronidase activity. Animal models showed that the microbiome composition in colostomy is different compared to normal bowel (26). In a rat model with left colostomy, a significant impact on the growth curve of rats was observed. Analysis of the intestinal microbiota indicated that colostomy primarily influenced the cecal microbiota rather than the colonic microbiota. Notably, there was an increase in the number of enterococci in both the ileum and cecum and elevated levels of cecal lactobacilli, contributing to the promotion of lactic acid bacteria in colostomized rats. Interestingly, there were no substantial differences in the translocation of intestinal bacteria to internal organs (spleen, kidneys, lungs, or liver) among colostomized, laparotomized, and control rats, regardless of their diet. The administration of heat-killed *Lactobacillus acidophilus* strain LB (inactive probiotic bacteria) exhibited a tendency to stimulate bifidobacteria, potentially influenced by culture-medium fermentation substances in the pharmaceutical product. However, this stimulatory effect was abolished by laparotomy and colostomy. Additionally, a trend towards a probiotic-like effect, unaffected by colostomy, was observed, as counts of lactobacilli tended to increase in both the cecum and colon of all animals fed with *Lactobacillus acidophilus* LB (26).

In CRC patients with colostomy, differences in microbial composition were observed as well, showing a reduction in anaerobic bacteria, notably affecting *Alistipes, Akkermansia, Intestinimonas*, and methane-producing archaea. Gene function analysis indicated an underrepresentation of methane and short-chain fatty acid production in patients with a stoma. Moreover, the presence of a stoma correlated with overall decreased taxonomic diversity but increased diversity in the KEGG ((Kyoto Encyclopedia of Genes and Genomes) pathway. Based on the results, patients with a stoma exhibit diminished levels of beneficial microbes for cancer immunotherapy. This study underscores that a stoma can significantly alter both taxonomic and functional profiles in fecal microbiota, emphasizing its potential as a confounding factor in fecal microbiota analyses (27). Accordingly, patients with low vs. high-output ileostomy displayed differences in microbiota composition, particularly in the percentage of Bacteroidota between the high-output and low-output groups (14.8% vs 0.5%; p=0.01) (28). Another study investigated the effects of a probiotic formula (Ecologic®825) on the adult human small intestinal ileostoma microbiota. The findings indicated that supplementation with the probiotic formula reduced the growth of pathobionts, such as *Enterococcaceae* and *Enterobacteriaceae*, and decreased ethanol production. These changes were associated with significant alterations in nutrient utilization and resistance to perturbations. The probiotic-mediated alterations, which coincided with an initial increase in lactate production and a decrease in pH, were followed by a sharp increase in the levels of butyrate and propionate (29).

This pooled analysis, beyond several advantages, has some limitations as well. Firstly, clinical trials utilize different probiotic formulas, contributing to the heterogeneity of trials. Moreover, any of the utilized probiotic formulas underwent preclinical testing in animal models of irinotecan-induced diarrhea. Both trials lack compliance measurement as well as assessment of gut colonization by probiotic formula and/or the measurement of stool beta-glucuronidase activity or another potential biomarker of probiotic efficacy. Despite the pooled analysis of the two trials, the statistical power of several subgroups remains low due to the small sample size of the first trial. However, this analysis enables us to confirm the results of probiotic benefit in patients with colostomy as there was only a trend of benefit in the Probio-SK-005 study (18).

Conclusions

In conclusion, this combined analysis suggests that probiotics could be beneficial in irinotecan-induced diarrhea prevention in colorectal cancer patients with colostomy. We propose that the preservation of healthy microbiota composition could be the simple, effective, and nontoxic approach to reduce gastrointestinal toxicity of irinotecan-based chemotherapy. Future research should prioritize mechanistic studies to investigate the link between stool beta-glucuronidase activity and the risk of irinotecan-induced diarrhea. It is also essential to evaluate various probiotic formulas and fecal microbiome transfer strategies to reduce the incidence of chemotherapy-associated diarrhea. However, one major challenge is that most current approaches have been one-size-fits-all, neglecting the unique composition of an individual's original microbiome, its colonization resistance, dietary influences, concomitant medications, and host factors that can all impact the microbiome. To address this complexity, it's crucial to integrate broad translational research into intervention studies, collecting and characterizing biological samples from various time points to understand the intricate interaction between microbiome modification approaches, biomarkers of change, and clinical endpoints. This will help optimize treatment strategies and improve patient outcomes. Until the availability of new pre- and clinical data in this setting, we suggest that the administration of probiotics formulas containing *Lactobacillus* and *Bifidobacterium* strains in colostomy patients treated with irinotecan-based chemotherapy seems prudent. However, there is no evidence to support the role of probiotic administration in unselected populations aimed at reducing irinotecan-induced diarrhea.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethical Committee of National Cancer Institute, Bratislava, Slovakia. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. BK: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. JC: Investigation, Writing – original draft, Writing – review & editing. RD: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. MR: Investigation, Supervision, Writing – original draft, Writing – review & editing. BBy: Investigation, Writing – original draft, Writing – review & editing. PK: Investigation, Writing – original draft, Writing – review & editing. SJ: Investigation, Writing – original draft, Writing – review & editing. SP: Investigation, Writing – original draft, Writing – review & editing. VV: Investigation, Writing – original draft, Writing – review & editing. MW: Investigation, Writing – original draft, Writing – review & editing. MS: Investigation, Writing – original draft, Writing – review & editing. BBr: Investigation, Writing – original draft, Writing – review & editing. DSu: Investigation, Writing – original draft, Writing – review & editing. SC: Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing. DSv: Data curation, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. LD: Conceptualization, Formal analysis, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

RD is employed by S&D Pharma.

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The influence of perilipin 5 deficiency on gut microbiome profiles in murine metabolic dysfunction-associated fatty liver disease (MAFLD) and MAFLD-hepatocellular carcinoma

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Introduction: Metabolic dysfunction-associated fatty liver disease (MAFLD) has emerged as the leading cause of hepatocellular carcinoma (HCC) worldwide. Over the years, Perilipin 5 (PLIN5) has been recognized as a key regulator of both MAFLD and HCC development. In our previous studies we demonstrated that deficiency in *Plin5* reduces the severity of MAFLD and HCC in mice. Interestingly, it has been established that patients with MAFLD and HCC exhibit various changes in their gut microbiome profiles. The gut microbiome itself has been shown to play a role in modulating carcinogenesis and the immune response against cancer.

Methods: Therefore, we conducted a study to investigate the alterations in fecal microbiome composition in wild type (WT) and *Plin5*-deficient (*Plin5*$^{-/-}$) mice models of MAFLD and MAFLD-induced HCC (MAFLD-HCC). We utilized 16S rRNA gene sequencing analysis to profile the composition of gut bacteria in fecal samples.

Results: Notably, we discovered that the absence of *Plin5* alone is already associated with changes in gut microbiota composition. Moreover, feeding the mice a Western diet (WD) resulted in additional microbial alterations. Interestingly, *Plin5*$^{-/-}$ animals exhibited an enrichment of the beneficial taxa *Lactobacillus* in both animal models.

Discussion: Our findings identify *Plin5* as a major regulator of gut microbiota during the development of MAFLD and MAFLD-HCC.

KEYWORDS

microbiome, fatty liver disease, animal models, hepatocellular carcinoma, metabolic syndrome, metabolic dysfunction, microbial diversity

1 Introduction

Within the past decade, there has been increasing evidence of a correlation between the composition of gut microbes and the development of liver diseases (Hrncir et al., 2021). This interaction, in which gut bacteria regulate adaptive immunity, inflammation, metabolism, nutrient absorption, and liver diseases, is known as the gut-liver axis (Albillos et al., 2020). Mechanistically, there are several ways in which the microbiota can regulate and contribute to liver disease. Firstly, pathogenic gut bacteria can overgrow, affect gut permeability, and release lipopolysaccharides, leading to systemic inflammation. Other ways include the release of bacterial metabolites (such as trimethylamine *N*oxide, lactate, choline or ethanol) and the conversion of bile acids into toxic substances (Zhou et al., 2022; Collins et al., 2023). Bacteria have enzymes that convert primary bile acids into secondary bile acids, allowing them to modulate the primary bile acid pool and activate intestinal farnesoid X receptor signaling, a pathway that promotes metabolic dysfunction-associated fatty liver disease (MAFLD) (Collins et al., 2023).

Interestingly, while animal studies have confirmed the causal roles of gut bacteria through coprophagy and fecal transfer studies, human studies have just started to detect microbial signatures that allow discrimination between patients with MAFLD, cirrhosis and healthy individuals (Aron-Wisnewsky et al., 2020). Across the literature, significant differences or even opposing results can be found in the composition of microbial taxa that are relevant in MAFLD, metabolic dysfunction-associated steatohepatitis (MASH) and HCC (Gupta et al., 2022; Albhaisi and Bajaj, 2021). However, some common trends in the composition of the microbiome have been observed at the phylum and genus levels (Aron-Wisnewsky et al., 2020). Generally, an increase in the abundance of *Firmicutes* and *Proteobacteria*, accompanied by a decrease in *Bacteroides* is often observed at the phylum level in MAFLD. Additionally, at the genus level, there is an increase in *Akkermansia*, *Escherichia*, *Ruminococcus* and *Shigella*, while levels of taxa that are often considered beneficial such as *Alistipes*, *Eubacterium* and *Lactobacillus* are reduced. These alterations are often accompanied by a decrease in microbial diversity (Astbury et al., 2020; Jiang et al., 2023).

Perilipin 5 (PLIN5), a member of the perilipin family mostly found in lipid droplets (LD) has recently been studied in the context of MAFLD, MASH and their progression towards HCC. As we and others have previously reported, *Plin5* deficiency can alter the progression of MAFLD by reducing inflammasome formation, hepatic injury, and steatosis (Asimakopoulou et al., 2020; Ma et al., 2021). Furthermore, we have suggested that PLIN5 is a critical factor that drives formation of HCC (Asimakopoulou et al., 2019). Considering that PLIN5 is a crucial regulator of MAFLD-induced HCC (Krizanac et al., 2023) and that its depletion markedly reduces the progress of MAFLD and attenuates MAFLD-HCC formation in two animal models (Mass-Sanchez et al., 2024), the question has arisen about whether these findings are also characterized by specific changes at the gut microbiome level. To investigate this, fecal samples were collected from WT and *Plin5*-deficient mice of MAFLD and MAFLD-HCC models prior to sacrifice, and 16S rRNA gene sequencing analysis was performed to reveal any possible altered microbial diversity.

2 Materials and methods

2.1 Animals

The mice strain targeted disrupted for the *Plin5* gene was generated by inseminating female mice with *Plin5*$^{-/-}$ sperm obtained from the Jackson Laboratory, Bar Harbor, ME, USA using a protocol previously decribed (Kuramoto et al., 2012; Asimakopoulou et al., 2020). Wild type (WT) and *Plin5*$^{-/-}$ mice were bred on a C57BL/6J background and housed at the Institute for Laboratory Animal Science and Experimental Surgery at RWTH-Aachen University, with a constant temperature of 20°C, relative humidity of 50%, and a 12-hr on/off light cycle. Standard cages housed up to four animals of the same genotype and diet. All animals in this study received proper care, and all animal protocols followed the guidelines for animal care approved by German legislation on the protection of animals. The protocols were also approved by the responsible authority of the state of North Rhine-Westphalia (LANUV, Recklinghausen, Germany) under permit no.: 81-02.04.2019.A366.

2.2 Animal model

The two animal models of MAFLD and MAFLD-induced HCC (MAFLD-HCC) and sources of animals used in the study have been previously described (Asimakopoulou et al., 2020; Mass-Sanchez et al., 2024). In brief, the MAFLD-HCC model involved applying 7,12-dimethylbenz(a)anthracene (DMBA) once to the dorsal neck surface of 4-5 day old mice, followed by feeding them a Western diet (WD) (D17010102, Research Diets, Inc., New Brunswick, NJ, USA) for 30 weeks after weaning. In the MAFLD model, the DMBA was replaced with acetone (AC). The WD consisted of a high-fat diet (D17010102, Research Diets, Inc., New Brunswick, NJ, USA) and water *ad libitum* supplemented with glucose/fructose (42 g/L; 55% fructose and 45% glucose), which was renewed every two weeks. Control animals were given a normal diet (ND) (V1534, ssniff Spezialdiäten GmbH, Soest, Germany) and drinking water without the sugar supplement. Animal body weight and food intake were monitored weekly.

2.3 Micro-computed tomography

Micro-computed tomography (μCT) and fat scans in mice were essentially performed as previously described (Kroh et al., 2023).

2.4 Gut microbiome analysis

All animals analyzed were kept in the same room and on the same cage rack to minimize the microbial variability caused by external environmental factors. The fecal samples (n=4 per group) for microbiome analysis were collected directly into Eppendorf tubes from freshly defecated animals to avoid environmental contamination prior to sacrifice. Fecal samples were then snap frozen in liquid nitrogen and stored at -80°C until further processing. Subsequent steps, such as DNA extraction and sequencing, were performed by professionals at the Institute of Medical Microbiology of RWTH University Hospital in Aachen.

2.5 DNA extraction

Microbial DNA was extracted using a slightly modified version of a previously published protocol (Godon et al., 1997). In brief, 600 μL of fecal sample in DNA stabilizer was transferred to autoclaved bead-beater tubes containing 500 mg ± 10 mg of triple-pure 0.1 mm Zirconia/Silica beads (#55D1132-01TP, Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Then, 250 μL 4 M guanidine thiocyanate in 0.1 M Tris pH 7.5 and 500 μL of 5% *N*-laurolylsarcosine in phosphate-buffered saline were added and vortexed. The samples were incubated at 70°C for 60 minutes on a shaker with a low shaking frequency (700 rpm). Bacterial cell walls were disrupted in the Fast Prep-24™ 5G Bead Beating Grinder and Lysis System (MP Biomedicals, Schwerte, Germany) using a bead-beating program consisting of 3 repetitions for 40 seconds at 6.6 m/s. The cooling adapter of the bead-beater was refilled with dry ice between each round. Next, 15 mg of poly(vinylpolypyrrolidone) was added to the samples and the samples were vortexed. The samples were centrifuged at 15,000 x g and 4°C for 3 minutes. The clear supernatants were transferred to new 2 mL Eppendorf tubes and centrifuged again at 15,000 x g and 4°C for 3 minutes. 500 μL of each clear supernatant was transferred into a new 2 mL tube, followed by the addition of 5 μL of RNase solution (10 mg/ml). After incubation at 37°C while shaking (700 rpm) for 20-30 minutes, genomic DNA (gDNA) was extracted using the NucleoSpin® gDNA Clean-up protocol (#740230.50, Machery-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.6 Sequencing

The 16S rRNA gene amplicon profiling was conducted by the Functional Microbiome Research Group at the Institute of Medical Microbiology, RWTH University Hospital Aachen, Aachen, Germany. To begin, library preparation was performed using a pipetting robot (Biomek4000 Beckman Coulter, Krefeld, Germany) for standardized processing. This was done following a previously published protocol (Lagkouvardos et al., 2016). The V3/V4 region of the 16S rRNA genes was amplified in two rounds of PCR in duplicates (15 cycles + 10 cycles) using bacteria-specific primers 341F/785R. Amplicon purification was carried out using the AMPure XP system (Beckman), followed by paired-end modus (PE275) sequencing using a MiSeq system (Illumina, Inc., San Diego, CA, USA) as per the manufacturer's instructions. The final DNA concentration was adjusted to 10 pM with a 15% (v/v) PhiX standard library.

2.7 Microbiome data analysis

Raw read files were processed with UPARSE based analysis (Edgar, 2013) using the IMNGS pipeline (Lagkouvardos et al., 2016). Sequences were de-multiplexed and trimmed to first base with a quality score <3 and then paired. Sequences with <250 and >500 nucleotides and paired reads with an expected rate of 0.002 were excluded. Reads were trimmed by 10 nucleotides on both sides. Pairing, quality filtering and OTU clustering (97% identity) was done by USEARCH 11.0 (Edgar, 2010). Removal of non 16S sequences was done with SortMeRNA v4.2 with SILVA release 138 as a reference (Kopylova et al., 2012). Clustering of Operational taxonomic units (OTUs) was done at 97% sequence similarity. Only OTUs with relative abundance over 0.25% across all samples were kept. Supplementary Table S1 provides an overview of the number of OTUs before and after each IMNGS filtering step. Sequence alignment and taxonomic classification were done by SINA 1.6.1 using the taxonomy of SILVA release 138 (Pruesse et al., 2012). The OTU table from the IMNGS pipeline was used in downstream analyses using the R package Rhea (Lagkouvardos et al., 2016). Rarefaction curves were calculated on OTUs normalized via division by the sum of sequences in a given sample and multiplication by the minimum sum across all samples.

Alpha diversity was calculated based on the Shannon index (Shannon, 2001) and observed OTUs using function richness from R package microbial (Guo and Gao, 2021). Beta diversity analysis between groups of diets was calculated using the Bray-Curtis distance. Principal coordinate analysis (PCoA) ordination based on calculated Bray-Curtis distance was used to visualize the dispersion between the groups. A permutational multivariate analysis of variance (PERMANOVA) using the adonis2 function in the package vegan (Oksanen et al., 2020), was applied to test significant clustering between sample groups. The relative abundances at different taxonomical levels were generated in each sample using the tax_glom command in the R package phyloseq (McMurdie and Holmes, 2013) and shown with stacked bar charts. The taxonomic relative abundances in samples were visualized by heatmap from R package pheatmap (Kolde, 2019). To analyse differential bacterial taxa abundance between diets and genotypes, a linear discriminant analysis (LDA) effect size (LEfSe) method with a threshold of logarithmic LDA score 2.0 and FDR < 0.05 (Segata et al., 2011) was used on the OTU table. Functional analysis of microbiomes was conducted with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (Douglas et al., 2020). PICRUSt2 was used to predict the abundances of functional categories in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2011), orthologs (KO), Enzyme Commission numbers (EC numbers) and metabolic pathways from MetaCyc database (Caspi et al., 2014). ALDEx2 (Fernandes et al., 2014) algorithm was applied to calculate the effect size of KOs and conducted significant testing in the comparison between groups. Due to a low number of significant terms after multiple hypothesis testing, we identified KOs and ECs with an uncorrected *p*-value below 0.1 as distinct using Wilcoxon rank sum. Distinct KOs were used for KEGG over-representation analysis (ORA) with R package clusterPofiler (Yu et al., 2012) and significant terms were reported with Benjamin-Hochberg (BH) adjusted *p*-value < 0.05. Moreover, we calculated the mean proportion of distinct KO terms with error bars showing standard deviation (SD) across all biological samples of the selected pathways involved in the prevention of hepatocarcinogenesis from ORA. R package ggplot2 (Wickham, 2016) was used to construct boxplots and bar charts shown in the microbiome analysis part of the research. The significance was compared using Welch's test, or the Wilcoxon rank sum test with Benjamini-Hochberg correction. Other statistical approaches in bioinformatics analyses have been described above. R version 4.3.3 (R foundation for Statistical Computing).

3 Results

3.1 The loss of *Plin5* reshapes the gut microbiome of animals fed a normal diet

The schematic representation of the time course for the selected models can be seen in Figure 1A. More detailed information regarding the characteristics of these models can be found elsewhere (Mass-Sanchez et al., 2024). When comparing WD-fed animals treated with AC (MAFLD model) to ND-fed animals, it was observed that the former displayed higher weight gain, body fat accumulation and liver fat accumulation (Figures 1B, C). Interestingly, the loss of *Plin5* was found to be beneficial in reducing inflammation caused by MAFLD in this particular context. As anticipated, the MAFLD-HCC model in WT exhibited increased weight gain, tumor formation and steatosis in WD-fed animals when compared to their ND-fed counterparts (Figures 1D, E). Notably, the absence of hepatic tumor formation was observed in the MAFLD-HCC model when *Plin5* deficiency was present.

To analyze whether *Plin5* is implicated in microbiome regulation, fecal samples were collected from animals subjected to the MAFLD and MAFLD-HCC models. 16S rRNA gene sequencing was performed to analyze bacterial composition. Rarefaction curves were generated to detect under-sampled cases. These curves showed that all samples used in the study had a satisfactory level of saturation necessary for the analysis (Figure 2).

Fecal 16S rRNA sequencing results of ND-fed *Plin5*$^{-/-}$ animals treated with either the vehicle AC or DMBA showed that *Plin5* deletion changed the bacterial composition at both the phylum and genus levels in the MAFLD model when compared to WT control littermates. The LDA effect size (LEfSe) was used to calculate the taxa that discriminated between WT and *Plin5*$^{-/-}$ mice (Figures 3A, B). Specifically, the *Proteobacteria*, *Actinobacteriota* and *Deferribacterota* phyla showed increased abundances in *Plin5*$^{-/-}$ mice compared to WT animals. Additionally, there were 17 differences found between the WT and *Plin5*$^{-/-}$ groups at the genus level (Figure 3C). While WT animals displayed higher levels of *Prevotellaceae*, *Roseburia*, and *Oscillibacter* as the top three upregulated genera, *Plin5* knockout animals were characterized by elevated abundances of *Dubosiella*, *Lactobacillus* and *Romboutsia*.

Similar to the MAFLD model, depletion of *Plin5* also changed the bacterial composition at the phylum level in animals that received DMBA. The relative abundance of *Actinobacteriota* greatly increased in *Plin5*$^{-/-}$ animals fed with ND compared to WT controls. Only four bacterial taxa were found to be differentially represented in WT *vs*. *Plin5*$^{-/-}$ animals at the genus level. The loss of *Plin5* resulted in an increase in the abundance of *Ruminococcus* and *Atopobiaceae*, and a decrease in *Sphingomonas* and *Alloprevotella* (Figure 3D). Supplementary Table S2 provides a summary of the shared changes in the microbiome between the MAFLD and MAFLD-HCC models caused by *Plin5* depletion.

3.2 The Western diet leads to specific changes in the gut microbial composition in both models

In the next step, we compared WT animals subjected to the MAFLD and MAFLD-HCC models that were fed either a ND or WD. We observed a significant increase in the levels of *Actinobacteriota*, *Deferribacterota* and *Desulfobacterota*, while there was a decrease in *Cyanobacteria*, *Patescibacteria* and *Campilobacterota* in the animal model subjected to the MAFLD model after being fed WD (Figure 4A). Animals in the MAFLD-HCC model showed a higher abundance of phyla *Firmicutes*,

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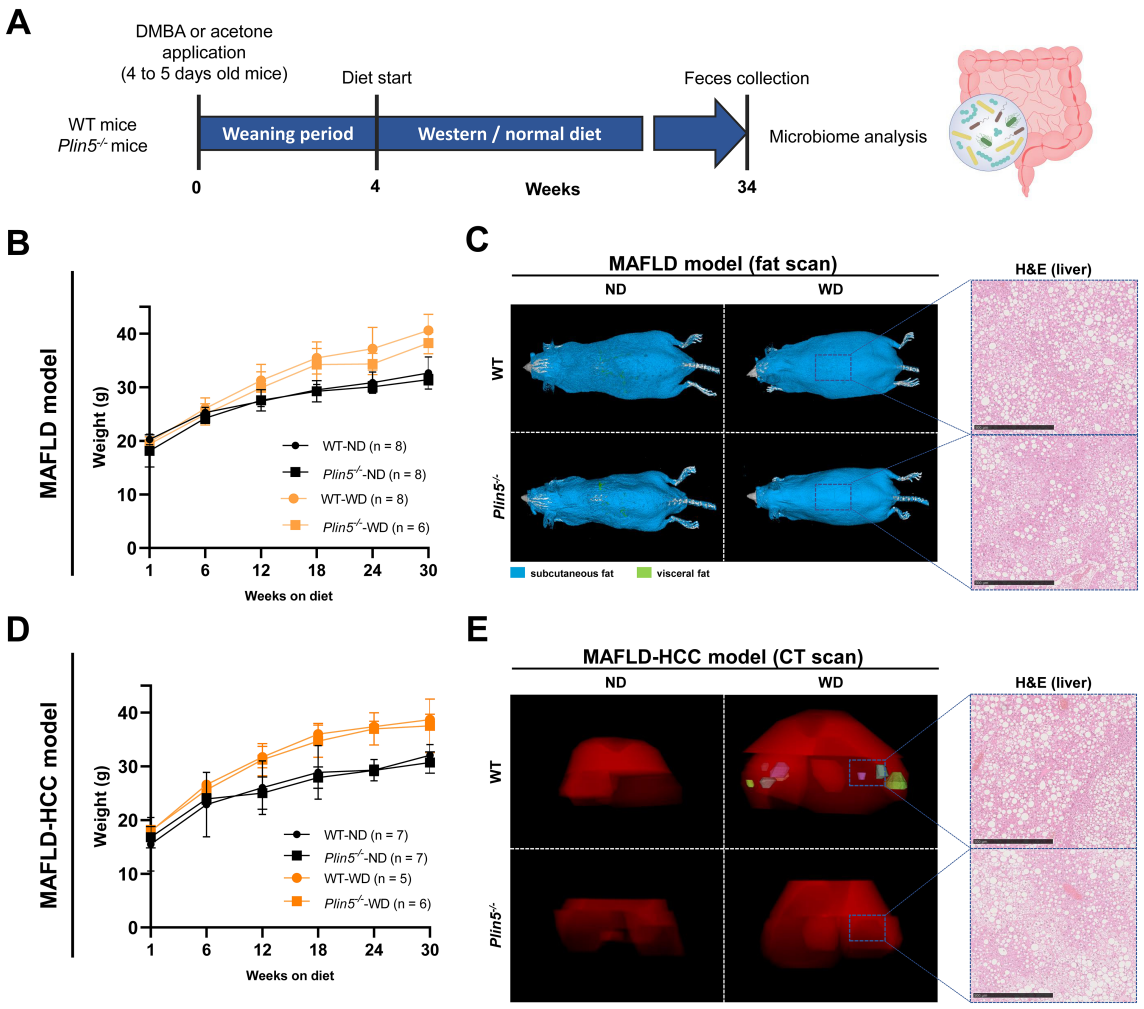

FIGURE 1
MAFLD and MAFLD-HCC models exhibit characteristic changes for fatty liver disease. **(A)** Schematic representation of the MAFLD and MAFLD-HCC models. The treatment with acetone or DMBA was followed by a 4-week weaning period and normal or Western diet feeding for 30 weeks. Fecal samples were collected immediately prior to sacrifice. **(B)** Weight measurements of animals in the MAFLD model. Number of animals per group: WT-ND (n=8); WT-WD (n=8); *Plin5*$^{-/-}$ND (n= 8); *Plin5*$^{-/-}$WD (n= 6) **(C)** Fat scans displaying subcutaneous fat in blue and visceral fat in green. Haematoxylin and eosin (H&E) staining of liver tissues from animals in the MAFLD model. Bars indicate 500 µM. **(D)** Weight measurements of animals in the MAFLD-HCC model. Number of animals per group: WT-ND (n=7); WT-WD (n=5); *Plin5*$^{-/-}$ND (n=7); *Plin5*$^{-/-}$WD (n=6) **(E)** Computed tomography (CT) scans displaying the 3D liver in red and tumors in multiple colors (left panels). H&E staining of liver tissue from animals in the MAFLD-HCC model (right panels). Abbreviations used are: wild type (WT); *Plin5*-deficient (*Plin5*$^{-/-}$); normal diet (ND); Western diet (WD).

Actinobacteriota, and *Desulfobacterota*. Conversely, WD-fed animals had lower abundances of *Cyanobacteria*, *Patescibacteria* and *Bacteroidota* compared to ND-fed animals (Figure 4B).

A comparison at the genus level revealed 44 differentially abundant genera in the MAFLD model. Out of these, 16 were enriched in the WT-WD group, while 28 were more abundant in the WT-ND group (Figure 4C). In the MAFLD-HCC model, 15 bacterial genera were enriched, and 26 genera had decreased abundances in the WT-WD group compared to the WT-ND group (Figure 4D). A total of 27 genera were detected in WT animals that showed alterations in abundance in both the MAFLD and MAFLD-HCC models. The genera enriched in the WT-WD group were *Bacteroides, Faecalibaculum, Lachnospiraceae GCA-900066575, Lactococcus, Romboutsia, Tuzzerella, unknown Atopobiaceae*, and *unknown Desulfovibrionaceae*. The genera that were reduced in abundance were *Odoribacter, Muribaculum, unknown Muribaculaceae, Alloprevotella, Prevotellaceae UCG-001, Alistipes, unknown Gastranaerophilales, Anaeroplasma, unknown RF39, unknown Clostridia UCG-014, unknown Clostridia vadinBB60 group, Eubacterium xylanophilum group, Lachnospiraceae NK4A136 group, Lachnospiraceae UCG-001, Roseburia, Butyricicoccus, Butyricicoccaceae UCG-009, Incertae Sedis*, and *Candidatus saccharimonas* (Supplementary Table S3). The taxa whose increased abundance was observed only in the MAFLD model were *Blautia*, *Rikenellaceae RC9 gut group*, *Parabacteroides*, *Lachnospiraceae UCG-006*, *Clostridium sensu stricto 1*, *Halomonas*, and *Alcaligenes*. Changes observed only in the MAFLD-HCC model were increased abundances of *Dubosiella*, *Lachnoclostridium*, *unknown Peptococcaceae*, *Desulfovibrio*, *Oscillospiraceae NK4A214 group*, and *Escherichia-shigella*.

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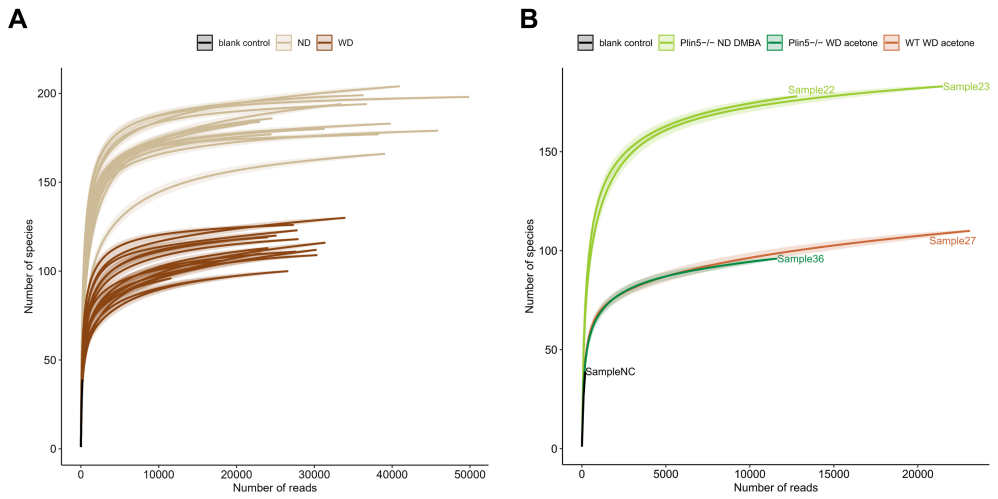

FIGURE 2
Rarefaction curves demonstrate a satisfactory level of saturation in all analyzed samples. **(A)** Rarefaction curves of all samples utilized in the study, including the negative control, are color-coded by the mouse diet **(B)** The figure highlights cases with the least sampling, colored-coded by mouse genotype and administered treatment.

3.3 *Plin5* deficiency reshapes the gut microbiome in the MAFLD model

To determine the impact of *Plin5* deficiency on the microbiome during MAFLD and MAFLD-HCC pathologies, we compared *Plin5*$^{-/-}$ animals treated with either AC or DMBA and fed a WD to corresponding WT controls. Firstly, we analyzed microbial α-diversity in the MAFLD model and found significant differences between ND- and WD-fed mice (Figure 5A). In the MAFLD model, we detected significantly higher observed OTUs and Shannon

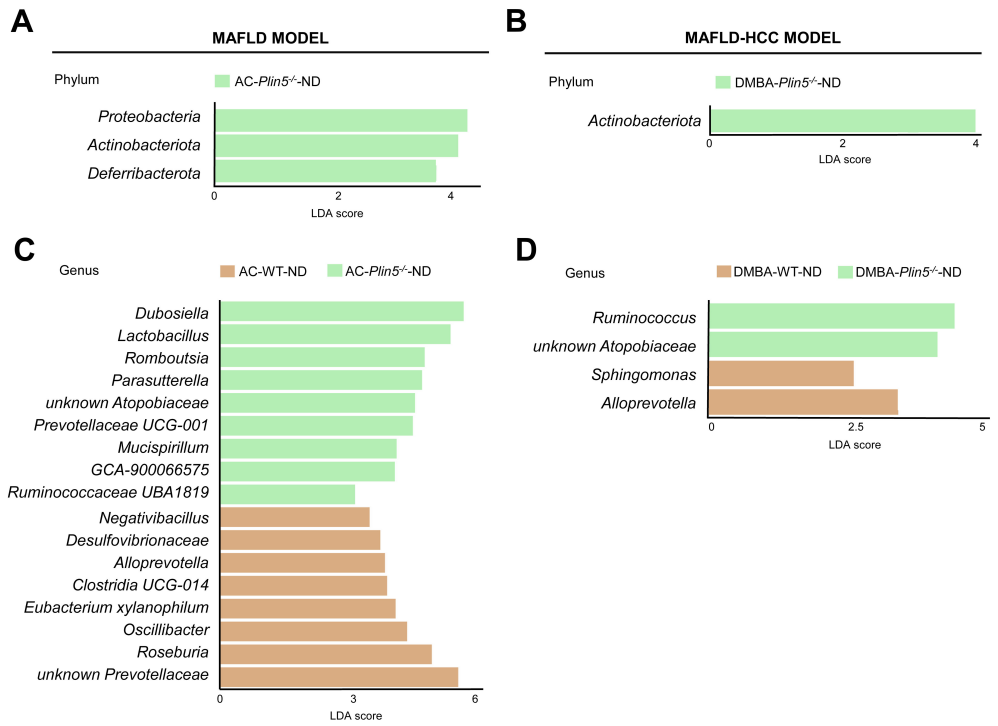

FIGURE 3
Plin5 deletion is sufficient to modify the microbial composition of mice fed a normal diet at the phylum and genus levels. **(A–D)** Linear discriminant analysis (LDA) effect size (LEfSe) was used to compare taxa at the phylum and genus levels in fecal samples between *Plin5*-deficient (*Plin5*$^{-/-}$) and wild type (WT) mice in the MAFLD and the MAFLD-HCC models. *Actinobacteriota* are the common taxa upregulated by the loss of *Plin5* in both animal models. At the genus level, the deficiency of *Plin5* led to changes in the abundances of *unknown Atopobiaceae* and *Alloprevotella*. Abbreviations used are: normal diet (ND); Western diet (WD); 7,12-dimethylbenz(a)anthracene (DMBA); acetone (AC).

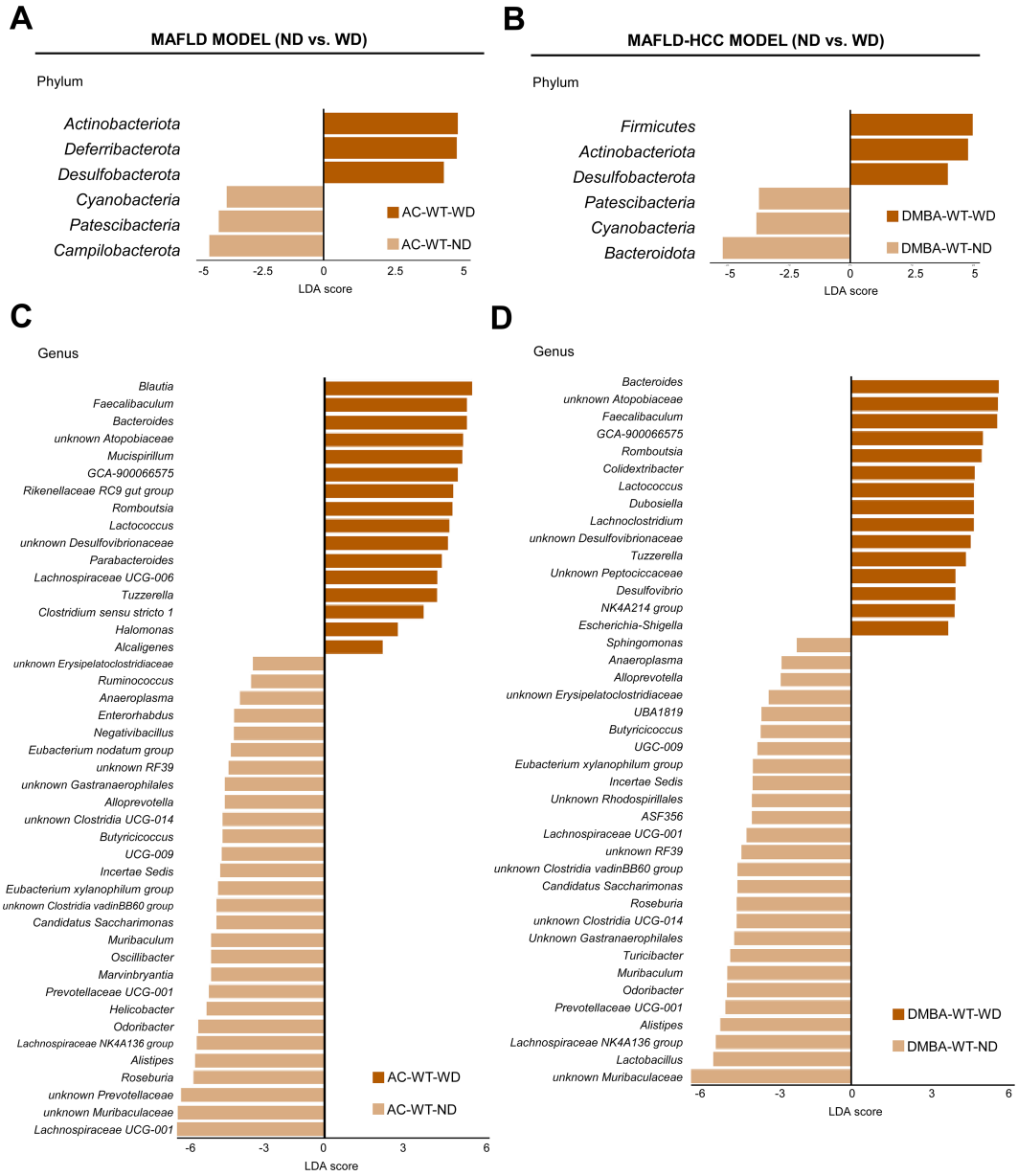

FIGURE 4
The Western diet causes characteristic changes in the gut microbiome in the MAFLD and MAFLD-HCC murine models. **(A)** Linear discriminant analysis (LDA) effect size (LEfSe) was used to compare fecal samples between normal (ND)- and Western diet (WD)-fed wild type (WT) mice at the phylum and genus levels in the **(A, C)** MAFLD model (treated with acetone (AC)) and **(B, D)** MAFLD-HCC model (treated with DMBA). *Actinobacteriota* and *Desulfobacterota* are the common taxa upregulated by the WD in both animal models. At the genus level, more than 15 common changes between the models were detected.

indices for species in both WT and *Plin5*$^{-/-}$ mice on ND compared to mice fed WD, indicating a reduced microbiome diversity caused by WD. Importantly, we did not observe any significant differences in observed OTUs and Shannon indices between genotypes with the same dietary conditions (WT-ND *vs.* *Plin5*$^{-/-}$-ND; WT-WD *vs.* *Plin5*$^{-/-}$WD). Nevertheless, we found a non-statistically significant tendency towards higher diversity after WD in *Plin5*$^{-/-}$ mice compared to WT mice.

Subsequently, β-diversity was assessed and presented as a principal coordinate analysis (PCoA) ordination based on the Bray-Curtis dissimilarity matrix. The PCoA graph clearly showed a separation of all four animal groups in the MAFLD model (WT-ND, WT-WD, *Plin5*$^{-/-}$ND, and *Plin5*$^{-/-}$WD). The most significant clustering was observed on the x-axis, where a clear distinction between clusters representing animals fed ND (on the right) and animals fed WD (on the left) accounted for 50.1% of the differences in microbial composition of the feces (Figure 5B). Two distinct clusters, WT-WD and *Plin5*$^{-/-}$WD, were still present after WD, suggesting a possible role of *Plin5* deficiency in the regulation of the microbiome during MAFLD pathology. Additionally, separate

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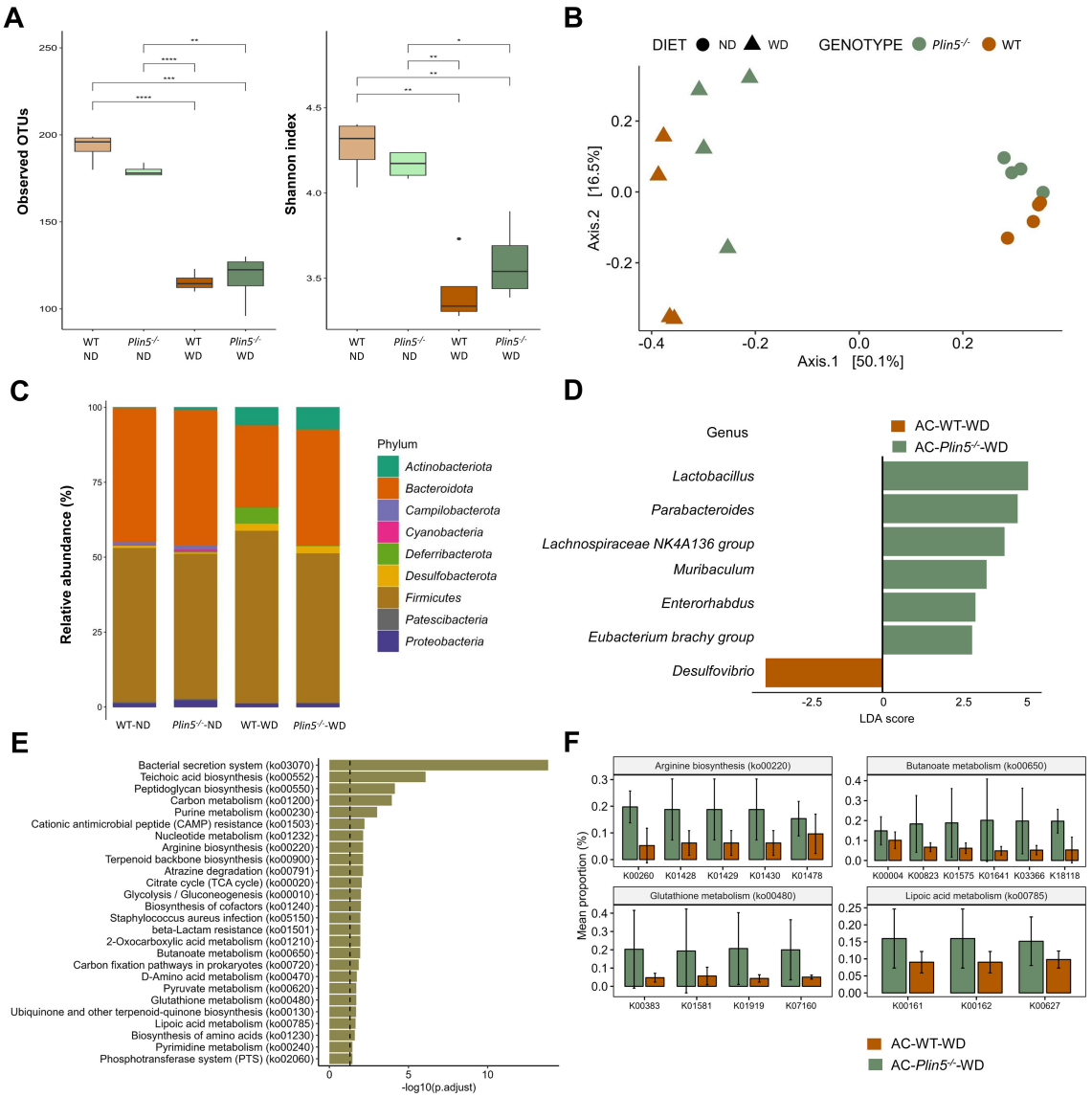

FIGURE 5
Fecal microbiome composition differs between WT and *Plin5*$^{-/-}$ mice in the MAFLD model. **(A)** Assessment of fecal microbiota α-diversity using observed operational taxonomic units (OTUs) (*left*) and Shannon diversity index (*right*) detected a statistically significant reduction in microbial diversity between the normal (ND)- and Western (WD)-fed groups. **(B)** Principal-coordinate analysis (PCoA) of a Bray-Curtis distance generated from fecal bacterial taxa. The four groups are marked as follows: Wild type (WT)-ND: brown points; *Plin5*$^{-/-}$ND: green points; WT-WD: brown triangles; *Plin5*$^{-/-}$WD: green triangles. **(C)** Average relative abundance analysis of fecal samples from the four groups at the phylum level. **(D)** Linear discriminant analysis effect size (LEfSe) and linear discriminant analysis (LDA) based on operational taxonomic units were used to differentiate between WT-WD and *Plin5*$^{-/-}$WD taxa at the genus level. **(E)** Over-representation analysis (ORA) of significantly over-represented PICRUSt2 KEGG pathways (FDR < 0.05). The vertical line represents *p*-value cut-off of 0.05. **(F)** Mean proportion of KEGG Orthology (KO) terms with error bars of selected pathways involved in prevention of hepatocarcinogenesis from ORA. The significant KO were detected across of significant KEGG terms defined at *p*-value of 0.1 (Wilcoxon rank sum). Error bars represent the standard deviation (SD) calculated across all biological replicates. Abbreviation are: acetone (AC), normal diet (ND), wild type (WT), linear discriminant analysis (LDA). *: $p < 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$.

clustering of WT-ND and *Plin5*$^{-/-}$ND was observed on the y-axis, confirming that the loss of *Plin5* is responsible for 16.1% of the microbial diversity.

Moreover, the analysis of fecal microbiota abundance in ND-fed animals revealed that the most common bacterial phyla in WT and *Plin5*$^{-/-}$ were *Actinobacteriota*, *Bacteroidota*, *Firmicutes*, *Proteobacteria* and *Campilobacterota*, respectively (Figure 5C). When switched to a WD, *Plin5*$^{-/-}$ animals, like WT animals, showed a decrease in the relative abundances of *Campilobacterota*, *Cyanobacteria* and *Patescibacteria* as well as an increase in *Actinobacteriota* and *Desulfobacterota* levels (Supplementary Figure S1A). However, the increase in *Deferribacterota* (cf. Figure 4B), which was observed in WT-WD animals, was not detected in the *Plin5*$^{-/-}$WD group. Therefore, similar changes at the phylum level were observed in both WT and *Plin5*$^{-/-}$ animals in the MAFLD model.

We performed LEfSe on multiple taxonomical levels and discovered differences in 7 bacterial taxa at the genus level (Figure 5D). The group of WT-WD animals exhibited a significantly higher abundance of

Desulfovibrio compared to *Plin5*$^{-/-}$WD. Conversely, the loss of *Plin5* resulted in an upregulation of *Lactobacillus, Parabacteroides, Lachnospiraceae NK4A136 group, Muribaculum, Enterorhabdus*, and *Eubacterium brachy group*.

Furthermore, KEGG pathway analysis revealed the deregulation of several biological pathways following *Plin5* ablation. The top five pathways, in terms of statistical significance, were clustered around the bacterial secretion system, teichoic acid biosynthesis, peptidoglycan biosynthesis, carbon and purine metabolism (Figure 5E). From the over-represented KEGG pathways we selected those with known role in stopping or slowing progression from steatosis to hepatocarcinogenesis (Figure 5F) and all of the genes are more increased in *Plin5*$^{-/-}$WD animals than in WT-WD.

3.4 *Plin5* deficiency alters the microbial composition in a MALFD-HCC model

The same analysis was performed on the sequencing data from the MAFLD-HCC model, as was done for the MAFLD model. Firstly, the fecal microbiota diversity of WT and *Plin5*$^{-/-}$ animals in the MAFLD-HCC model was assessed through α-diversity analysis using observed OTUs and Shannon. The data showed that the diversity of the fecal microbiome tended to decrease after WD in both WT and *Plin5*$^{-/-}$ animals, as indicated by the median values of observed OTUs and Shannon diversity index (Figure 6A). The reduction in observed OTUs was statistically significant for both WT and *Plin5*$^{-/-}$ animals, while Shannon showed only a tendency towards reduced diversity. Importantly, the microbial diversity was significantly higher in *Plin5*$^{-/-}$ WD-fed animals compared to WT WD-fed animals.

PCoA ordination, based on a Bray-Curtis dissimilarity matrix, displayed a distinct separation of various animal groups (Figure 6B). On the x-axis, there was a differentiation of clusters representing animals fed a ND on the left, compared to those fed a WD on the right. This difference accounted for 52% variation in microbial diversity, indicating that the most significant variations in microbial composition between the four groups were caused by the WD. Additionally, there was a noticeable separation of animals based on their genotype on the y-axis, which explained an 11.6% difference in diversity between the WT-ND and the *Plin5*$^{-/-}$ND group. However, this clear separation in gut microbial composition between WT and *Plin5*$^{-/-}$ genotypes disappeared after the introduction of the WD. Remarkably the difference in fecal microbial composition was more pronounced between animal groups with different diets than between groups with different genotypes.

When assessing the abundance of bacterial taxa at the phylum level, we found that *Bacteroidota*, *Firmicutes* and *Proteobacteria* were the dominant phyla in WT-ND and *Plin5*$^{-/-}$ND animals (Figure 6C). The relative abundance of *Campilobacterota*, *Deferribacterota*, *Desulfobacterota*, *Actinobacteriota*, and *Firmicutes* increased in *Plin5*$^{-/-}$ animals after WD (Supplementary Figure S1). Compared to WT-WD animals, the *Plin5*$^{-/-}$WD group showed a statistically significant increase in the abundance of *Campilobacterota* (Supplementary Figure S1).

To further explore the difference in microbial composition between groups, LefSe analysis at the genus level was performed, revealing several statistically significant differences among the four groups (Figure 6D). Notably, the *Plin5*$^{-/-}$WD group had significantly higher abundances of *Lactobacillus* and *Romboutsia*, along with a slightly lower increase in *Prevotellaceae*, *Helicobacter*, *Erysipelatoclostridiaceae*, *Rhodospirillales*, *Butyricicoccaceae UCG-009*, and *Ruminococcaceae UBA1819*.

Lastly, KEGG analysis showed deregulation of several terms in *Plin5* null mice, with the top five in significance being cell cycle–Caulobacter, terpenoid backbone synthesis, glyoxylate and dicarboxylate metabolism, butanoate metabolism and atrazine degradation (Figure 6E). *Plin5*$^{-/-}$WD animals have significantly increased butanoate metabolism (Figure 6F) compared to WT-WD in MAFLD-HCC model.

3.5 The microbiome of MAFLD and MAFLD-HCC models in WT mice differs in bacterial taxa at the genus level

Finally, we conducted a search for differences in bacterial composition between fecal samples from WT animals belonging to the MAFLD and MAFLD-HCC model. The PCoA analysis showed clustering of three groups: DMBA-ND, AC-ND, and a cluster consisting of WD-fed animals from both animal models (Figure 7A). We see reduction in both Observed OTUs and Shannon index in groups fed a WD was observed (Figure 7B). Interestingly, while no statistically significant difference in Shannon index was observed between WD-fed groups, DMBA-WD group had significantly lower level of Observed OTUs. Additionally, the LDA analysis on the phylum level revealed that *Firmicutes*, *Campilobacterota* and *Desulfobacterota* were enriched in the AC-ND group compared to the DMBA-ND group, while *Bacteroidota* were enriched in the DMBA-ND group (Supplementary Figure S2). Interestingly, there were no differences in microbiome at the phylum level between the AC- and DMBA-treated WT-WD groups. However, more differences were detected at the genus level, with the ND-fed groups differing in 29 bacterial taxa and the WD-fed animals differing in four taxa (Figures 7C, D). Specifically, WD-fed animals in the MAFLD-HCC model had a high abundance of *Enterorhabdus* and *Oscillospiraceae NK4A214 group*, while the abundance of *unknown Rhodospirillales* and *Clostridium sensu stricto 1* was enriched in animals of the MAFLD model.

4 Discussion

Microbiota plays a fundamental role in gut and liver health (Schwenger et al., 2019). Significant changes in the microbial composition of the gut have been associated with the development and progression of liver diseases (Hrncir et al., 2021), and specifically in the pathogenesis of HCC and the shaping of the tumor microenvironment (Chen et al., 2023; Liu et al., 2023). In this study, we aimed to characterize the microbial changes in the gut during the pathology of MAFLD and MAFLD-HCC in two murine models. Additionally, we assessed the impact of *Plin5* deficiency on the gut microbiome in the same models.

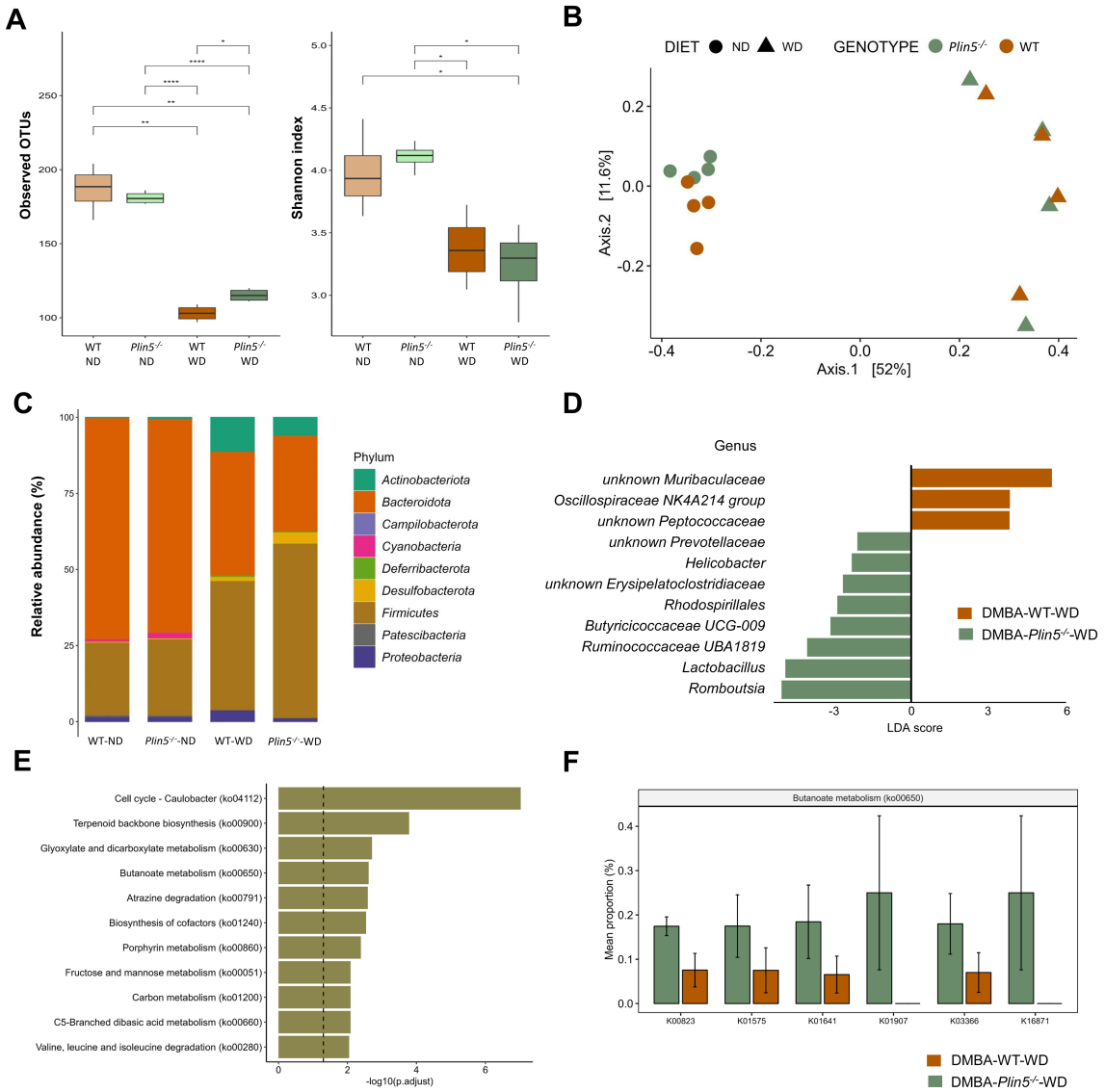

FIGURE 6
Fecal microbiome composition differs between WT and *Plin5*$^{-/-}$ mice in the MAFLD-HCC model. **(A)** α-diversity assessment of fecal microbiota using Observed OTUs (*left*) and Shannon diversity (*right*) index detected a statistically significant reduction in microbial diversity between ND and WD-fed groups of MAFLD-HCC. **(B)** Principal-coordinate analysis (PCoA) of a Bray-Curtis distance generated from fecal bacterial taxa. Four groups are marked as follows: WT-ND: brown points; *Plin5*$^{-/-}$ND: green points; WT-WD: brown triangles; *Plin5*$^{-/-}$WD: green triangles. **(C)** Average relative abundance analysis of fecal samples from the four groups at the phylum level. **(D)** Linear discriminant analysis effect size (LEfSe) and linear discriminant analysis (LDA) based on operational taxonomic units were used to differentiate between WT-WD and *Plin5*$^{-/-}$WD taxa at the genus level. **(E)** Over-representation analysis (ORA) of significantly over-represented PICRUSt2 KEGG pathways (FDR < 0.05). The vertical line represents *p*-value cut-off of 0.05. **(F)** Mean proportion of KEGG Orthology (KO) terms with error bars of selected pathways involved in prevention of hepatocarcinogenesis from ORA. The significant KO were detected across significant KEGG terms defined at a *p*-value of 0.1(Wilcoxon rank sum). Error bars represent the standard deviation (SD) calculated across all biological replicates. Abbreviations used are: operational taxonomic unit (OTU), 7,12-Dimethylbenz(a)anthracene (DMBA), normal diet (ND), wild type (WT), linear discriminant analysis (LDA). *: $p < 0.05$, **: $p \leq 0.01$, ****: $p \leq 0.0001$.

Firstly, it was assumed that the loss of *Plin5* would only alter microbial composition in WD conditions, based on the fact that *Plin5*$^{-/-}$ animals do not differ from WT mice when fed with ND (Wang et al., 2015; Kuramoto et al., 2012). However, this assumption was disproven in both MAFLD and MAFLD-HCC models through LDA scoring, which detected differences in several bacterial taxa between ND-fed WT and knockout animals. Additionally, PCoA analysis showed that ND-fed WT and *Plin5*$^{-/-}$ animals clustered separately. Interestingly, *Plin5*$^{-/-}$ animals in both models had an increased abundance of *Actinobacteriota* that play a pivotal role in maintaining intestinal homeostasis (Binda et al., 2018). At the genus level, *Atopobiaceae*, that promote cardiometabolic health (Vinke et al., 2017), displayed increased abundance in *Plin5*$^{-/-}$ animals in both models. Furthermore, *Alloprevotella*, which has been found to be significantly enriched in fecal samples of patients with irritable bowel syndrome (Tang

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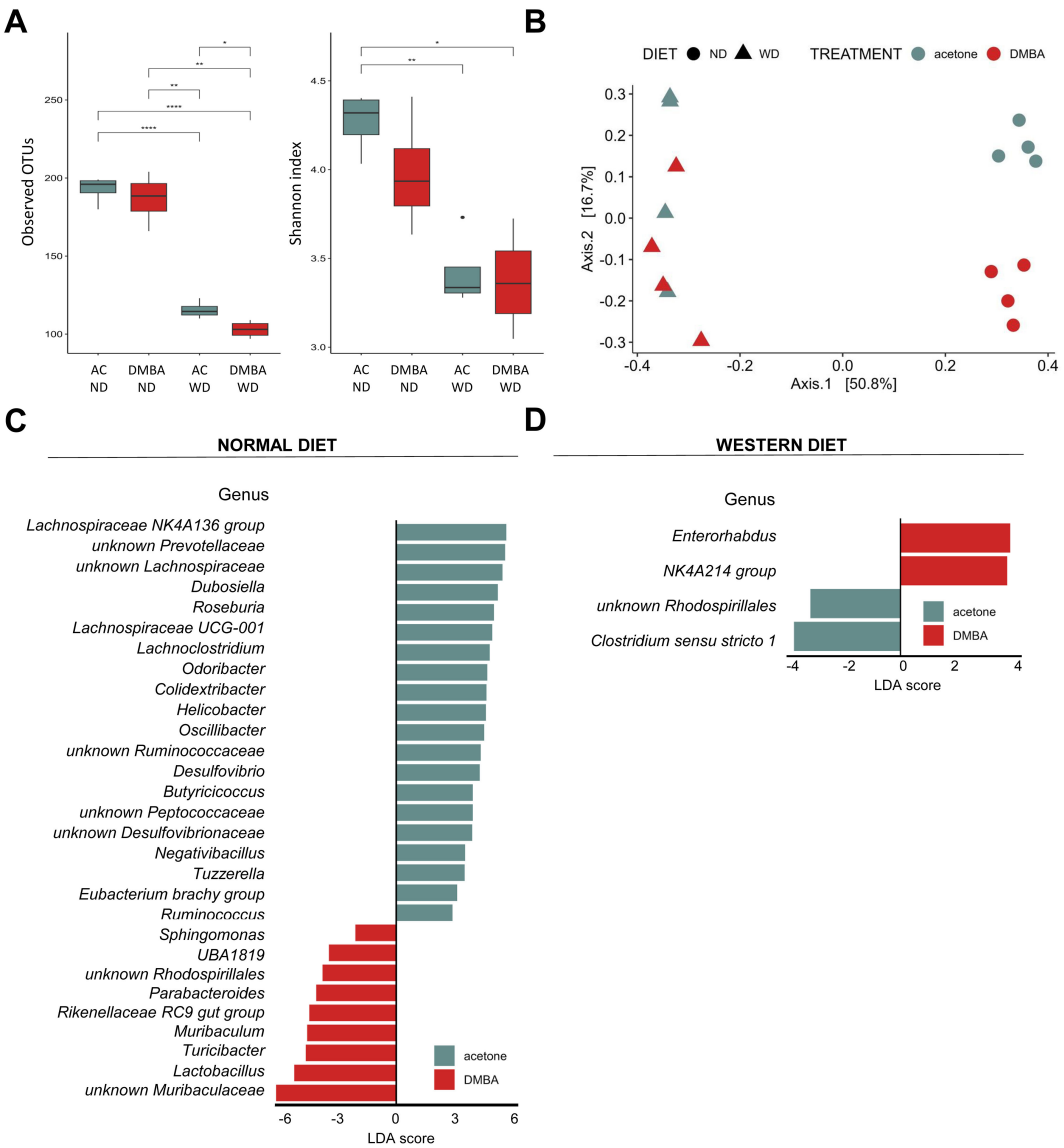

FIGURE 7
Fecal microbiome composition differs between the MAFLD and MAFLD-HCC models in WT mice. **(A)** α-diversity assessment of fecal microbiota using Observed OTUs (*left*) and Shannon diversity (*right*) index between the four animal groups. Groups are: AC-ND, animals that received acetone and a normal diet; DMBA-ND, animals that received DMBA and a normal diet; AC-WD, animals that received acetone and a Western diet; DMBA-WD, animals that received DMBA and a Western diet. **(B)** Principal-coordinate analysis (PCoA) of a Bray-Curtis distance generated from fecal bacterial taxa. The four groups are marked as follows: acetone-ND: cyan points; DMBA-ND: red points; acetone-WD: cyan triangles; DMBA-WD: red triangles **(C, D)** Differences in microbiome at the phylum and genus levels between the different groups subjected either to **(C)** normal or **(D)** Western diet. *: $p < 0.05$, **: $p \leq 0.01$, ****: $p \leq 0.0001$.

et al., 2023) was reduced in *Plin5*$^{-/-}$ mice. Taken together, the data suggests that *Plin5* is a critical factor that impacts the microbial gut content in healthy organisms.

Secondly, we analyzed the microbial changes observed in the MAFLD and MAFLD-HCC models. To do this, we compared WD-fed WT animals from both models to their corresponding ND-fed controls. As others have previously reported, the expected changes included an increase in the abundance of *Firmicutes* and *Proteobacteria*, along with a decrease in *Bacteroidota* (Murphy et al., 2015; Gupta et al., 2022). However, the MAFLD model did not exhibit the same changes. Interestingly, both models did show an increase in *Actinobacteriota* and *Desulfobacterota*, as well as a decrease in *Cyanobacteria* and *Patescibacteria*. Therefore, these common changes in *Actinobacteriota*, *Desulfobacterota*, *Cyanobacteria* and *Patescibacteria*, could be considered a general microbiome signature associated with fatty liver pathologies.

Furthermore, we confirmed that the diversity in microbial composition of feces was reduced after WD in both murine

models, which was consistent with previous studies on MAFLD/MASH (Astbury et al., 2020; Jiang et al., 2023; Behary et al., 2021; Porras et al., 2017). Interestingly, unlike WT animals whose microbial diversity was significantly lower following WD, *Plin5*-deficient animals fed WD showed a tendency towards higher OTU diversity than WD-fed WT littermates. In the MAFLD-HCC model, the loss of *Plin5* in WD-fed mice preserved the level of OTU diversity compared to WD-fed WT animals, indicating that *Plin5* acts as a protective factor in preserving microbial diversity during fatty liver disease. In this regard, differences were observed between WD-fed WT and *Plin5*-deficient animals on several taxonomic levels in both models. While the most significant differences between the groups resulted from WD feeding in both models, WT and *Plin5*-null mice fed a ND showed more than 10% differences indicating that *Plin5* is indeed an important regulator of microbial gut composition.

The most significant difference found as a common denominator in both animal models is the significantly higher abundance of the *Lactobacillus* genus present in *Plin5*-deficient animals (Figure 8). Levels of *Lactobacillus* have been found to be increased in cirrhotic patients and MAFLD in multiple studies (Ponziani et al., 2019; Zhu et al., 2023). However, several reports have suggested that *Lactobacillus* acts as a protector of the intestinal barrier and a factor that attenuates the progression of MAFLD by lowering cholesterol and steatosis (Huang and Zheng, 2010; Yu et al., 2015; Lee et al., 2021; Li et al., 2021). In line with this, supplementation of *Lactobacillus lactis* and *Pediococcus pentosaceus* effectively normalized weight ratio, MAFLD activity score, biochemical markers, cytokine expression and gut-tight junction by modulating and reprogramming the gut metagenomic and metabolic environment, thus highlighting its protective effects (Yu et al., 2021). However, the exact mechanism of *Lactobacillus* regulation via PLIN5 still remains to be elucidated.

Furthermore, one study reported that *Lactobacillus* and *Bifidobacterium* improved hepatic steatosis and fibrosis in high-fat diet-fed mice. This improvement was linked to a decrease in the abundance of *Desulfovibrio* in feces, which aligns with the results we obtained in the MAFLD model (Lin et al., 2022). However, besides *Lactobacillus*, several other bacterial genera are enriched in *Plin5*-deficient animals in both models. Interestingly, increased abundances of *Romboutsia* have been detected *Plin5*-deficient animals in MAFLD-HCC model, despite their previous association with the severity of MAFLD and type 2 diabetes (Gu et al., 2022; Si et al., 2021). Similarly, there are conflicting results regarding the association between increased abundance of *Helicobacter* and MAFLD (Wijarnpreecha et al., 2018; Liu et al., 2021; Porras et al., 2017). In MAFLD model, *Parabacteroides* which show higher prevalence in cirrhotic patients, as well as an increase in MAFLD and MASH (Ponziani et al., 2019; Aron-Wisnewsky et al., 2020), are found in *Plin5*-deficient animals. In the case of *Enterorhabdus*, which is also abundant in *Plin5*$^{-/-}$WD-fed animals, previous research has proven that it is enriched in healthy controls, rather than HCC patients (Bian et al., 2022). Consistent with our hypothesis that *Plin5* deficient animals on WD are healthier than WT controls, genera *Oscillospiraceae NK4A214* that has been identified previously as a prognostic marker for obesity, various metabolic disorders and inflammatory bowel diseases was more enriched in WT animals of the MAFLD model (Burakova et al., 2022).

For functional analysis, we identified changed metabolic pathways involved in lipid and carbohydrate metabolism such as terpenoid backbone synthesis, glyoxylate and dicarboxylate pathway and carbon metabolism in the MAFLD and MAFLD-HCC WD models when *Plin5* was knocked-out. Pathways associated with regulation of the tumor progression and microenvironment by exhibiting anti-inflammatory and anti-tumorigenic properties were more detected in *Plin5*-deficient animals. In both disease models we detected enrichment in butanoate metabolism in *Plin5*-deficient compared to WT animals. Butanoate or butyrate metabolism is one of important metabolism involved in production of short chain fatty acids (SCFAs), butyrate. Compelling evidence through multiple studies have shown that increase in butyrate-producing bacteria, such as *Lachnospiraceae NK4A136 group*, which we found in this study in

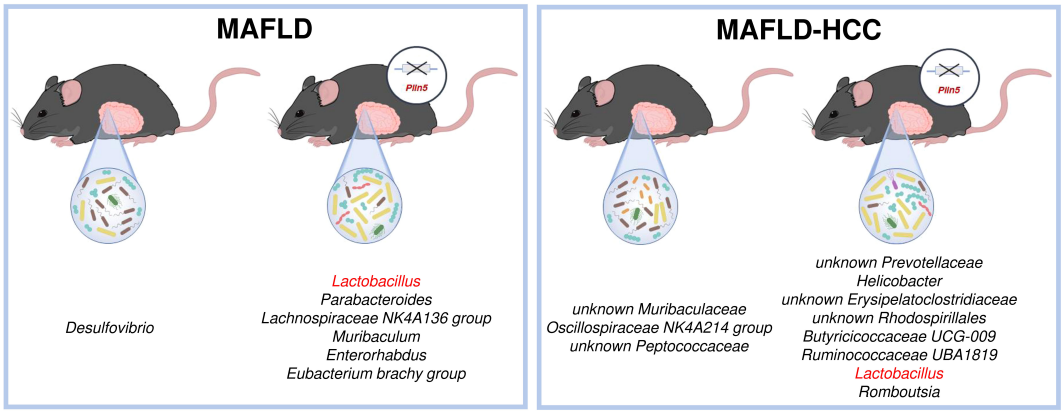

FIGURE 8
Schematic overview of the most significant differences between wild type and *Plin5*$^{-/-}$ mice in the MAFLD (*left*) or MAFLD-HCC (*right*) models. In both models, *Lactobacillus* is found in higher abundance in the *Plin5*$^{-/-}$ group compared to the wild type group.

the MAFLD model, and butyrate production attenuates steatohepatitis by improving intestinal barrier function, up-regulating glucagon-like peptide-1 receptor (GLP-1R) expression, and down-regulating inflammatory signaling as well as oxidative damage in the liver (Zhu et al., 2023; Zhou et al., 2017, 2018; Jin et al., 2015; Endo et al., 2013). Overall, we detect more antioxidant enriched pathways in *Plin5*-deficient animals in MAFLD than MAFLD-HCC animal models. For instance, antioxidant enriched pathway in *Plin5*-deficient animals was lipoic acid metabolism. It has been shown that lipoate acts as antioxidants either by radical quenching or indirectly by recycling other antioxidants such as vitamin C, vitamin E, coenzyme Q10, and glutathione (Spalding and Prigge, 2010; Kagan et al., 1992; Scholich et al., 1989; Xia et al., 2001; Jocelyn, 1967). We also found glutathione metabolism to be increased in *Plin5*-deficient animals. Another interesting pathway increased in *Plin5*-deficient animals within the MAFLD model was arginine biosynthesis. Recent findings highlighted that increased production of l-arginine by *Lactobacillus plantarum* was a good potential treatment for NAFLD (Kim et al., 2023). Taken together these results imply a positive shift in microbial metabolic pathways towards reducing progression and attenuating more predominantly the MAFLD state but also the MALFD-HCC states.

However, as we are lacking detailed serum measures of circulating bacterial products and mechanistic insight regarding the mode of action of each bacterial genera in our models, it is challenging to conclude whether increase in particular bacterial taxa is a body's defense mechanism or opportunistic bacteria spreading with detrimental effects especially for certain taxa with conflicting literature results. Therefore, it would be of utmost importance to focus future research on characterizing the impact of particular bacterial taxa on the progression of MAFLD or HCC.

Lastly, we did not observe any significant variations in bacterial composition in WT animals fed a WD and subjected to the two models. Although the models differ in four bacterial genera, namely *Enterorhabdus*, *Oscillospiraceae NK4A214 group, unknown Rhodospirillales*, and *Clostridium sensu stricto* 1, none of them were exclusively associated with MAFLD or MAFLD-HCC. In summary, the data suggests that the MAFLD and MAFLD-HCC models exhibit similar microbial signatures, making it difficult to distinguish between them.

While this study did not provide a mechanistic insight into how the absence of *Plin5* affects the gut microbiome, another member of the perilipin family has been examined in the context of diet-induced changes in the microbiome. The study indicated that the removal of perilipin 2 (*Plin2*) alters the expression of microbial enzymes, leading to the production of energy and components necessary for cell growth (Xiong et al., 2017). Given the similarities in function and structure between *Plin2* and *Plin5*, we hypothesized that a similar mechanism could explain the observed changes in the microbiome of *Plin5*$^{-/-}$ mice. However, limitations of this study, such as the absence of metatranscriptomics, could be utilized to further understand the genotype-dependent changes observed.

Dietary intervention in murine models has been shown to affect microbiome composition, demonstrating similarities with human studies (Nguyen et al., 2015). Therefore, our report can serve as a reference for a better understanding of microbiome composition in liver pathologies associated with MAFLD. Additionally, we have conducted a thorough analysis that indicates minimal differences between our models, emphasizing the importance of distinguishing between HCC cases of MAFLD and non-MAFLD origin. Despite the challenges, future research should prioritize human studies to investigate host-microbiota interactions and elucidate the role of *Plin5*. This research could be instrumental in the development of future therapeutic approaches in liver pathologies.

Our current data may not directly translate to clinical implications, but it does provide insight into potential therapeutic options for liver disease. One discovery we made is that *Plin5* plays a role in disrupting the microbiome population in both MAFLD and HCC. This suggests that developing pharmacological inhibitors or knockout therapies targeting Plin5 could help prevent the advancement of liver disease. Additionally, our analysis of the microbiome in two liver pathologies revealed that the *Lactobacillus* genus may have a beneficial impact on the progression of MAFLD to HCC. A recent study also found that supplementing *Lactobacillus acidophilus* can suppress NAFLD to HCC progression in mouse models (Lau et al., 2024). However, further research involving human samples and cohorts is needed to confirm these findings.

5 Conclusions

This study identifies PLIN5 as a crucial regulator of gut microbiota composition during the development of MAFLD and its progression to HCC. Our findings demonstrate that *Plin5* deficiency leads to significant shifts in gut microbiota, including an increase in beneficial taxa like *Lactobacillus*, which has been linked to improved liver function and reduced disease severity. Moreover, the Western diet exacerbated microbial alterations, indicating the complex interaction between diet, genetic factors, and the microbiome in liver disease progression. The broader implications of these findings suggest that targeting PLIN5 could serve as a potential therapeutic strategy to modulate gut microbiota and mitigate liver disease progression. By identifying specific microbial signatures linked to *Plin5* deficiency, this study provides a foundation for future research exploring gut-liver interactions and their impact on MAFLD and MAFLD-HCC. These insights are valuable not only for understanding the pathophysiology of liver diseases but also for developing targeted microbiome-based therapies. However, despite these insights, the study has notable limitations that should be considered when interpreting the results. A key limitation is the absence of bacterial metabolites analysis, which restricts our ability to fully understand the functional implications of the observed microbial changes. Without direct measurement of metabolites, such as short-chain fatty acids or bile acids, the exact mechanisms by which *Plin5* influences liver disease through gut microbiota remain speculative.

In conclusion, the study underscores the importance of *Plin5* as a key regulator of gut microbiota in the context of liver disease and highlights the potential of microbiome modulation as a therapeutic avenue. Future research should focus on validating these findings in human studies and exploring the therapeutic potential of manipulating gut microbiota to prevent or treat liver disease.

Data availability statement

The raw microbiome 16S rRNA sequencing data generated in this study have been deposited in the Sequence Read Archive (SRA) under the accession number PRJNA1157013.

Ethics statement

The animal study was approved by LANUV, Recklinghausen, Germany. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MK: Investigation, Visualization, Writing – original draft, Writing – review & editing. PŠ: Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing. PMS: Formal analysis, Investigation, Visualization, Writing – review & editing. RK: Resources, Validation, Writing – review & editing. DM: Investigation, Writing – review & editing. TL: Methodology, Resources, Validation, Writing – review & editing. AA: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. RW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, 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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1443654/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Abundances of bacterial taxa.

SUPPLEMENTARY FIGURE 2
Linear discriminant analysis (LDA) at the phylum level.

SUPPLEMENTARY TABLE 1
Changes in number of detected OTUs before and after each IMNGS pipeline filtering step.

SUPPLEMENTARY TABLE 2
Changes in shared bacterial taxa caused by *Plin5*$^{-/-}$ deletion in normal diet-fed mice of MAFLD and MAFLD-HCC models.

SUPPLEMENTARY TABLE 3
Changes in shared bacterial taxa in wild type animals subjected to the MAFLD and MAFLD-HCC models.

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Intestinal microbiome changes and mechanisms of maintenance hemodialysis patients with constipation

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Background: Constipation is a common symptom in maintenance hemodialysis patients and greatly affects the quality of survival of hemodialysis patients. Fecal microbiota transplantation and probiotics are feasible treatments for functional constipation, but there is still a gap in the research on the characteristics of gut flora in patients with maintenance hemodialysis combined with constipation. The aim of this study is to clarify the characteristics of the intestinal flora and its changes in maintenance hemodialysis patients with constipation.

Methods: Fecal samples were collected from 45 participants, containing 15 in the maintenance hemodialysis constipation group,15 in the maintenance hemodialysis non-constipation group and 15 in the healthy control group. These samples were analyzed using 16S rRNA gene sequencing. The feature of the intestinal microbiome of maintenance hemodialysis constipation group and the microbiome differences among the three groups were elucidated by species annotation analysis, α-diversity analysis, β-diversity analysis, species difference analysis, and predictive functional analysis.

Results: The alpha diversity analysis indicated that maintenance hemodialysis constipation group was less diverse and homogeneous than maintenance hemodialysis non-constipation group and healthy control group. At the genus level, the top ten dominant genera in maintenance hemodialysis constipation group patients were Enterococcus, Escherichia-Shigella, Bacteroides, Streptococcus, Bifidobacterium, Ruminococcus_gnavus_group, Lachnospiraceae_unclassified, Faecalibacterium, Akkermansia and UCG-002. Compared with non-constipation group, the Enterococcus, Rhizobiales_unclassified, Filomicrobium, Eggerthella, Allobaculum, Prevotella_7, Gordonibacter, Mitochondria_unclassified, Lachnoanaerobaculum were significantly higher in constipation group ($p<0.05$). Compared with non-constipation group, the Kineothrix, Rhodopirellula, Weissella were significantly lower in constipation group ($p<0.05$). The predictive functional analysis revealed that compared with non-constipation group, constipation group was significantly enriched in pathways associated with pyruate metabolism, flavonoid biosynthesis.

Conclusions: This study describes for the first time the intestinal microbiome characteristics of maintenance hemodialysis patients with constipation. The results of this study suggest that there is a difference in the intestinal flora between maintenance hemodialysis patients with constipation and maintenance hemodialysis patients without constipation.

KEYWORDS

Constipation, gut microbiome, 16S rRNA, Maintenance haemodialysis (MHD), Intestinal biomarker

1 Introduction

Renal failure is defined as a glomerular filtration rate <15 ml/min/1.73 m2, which can be treated with renal replacement therapy (meaning dialysis or transplantation) or supportive care (Stevens, 2013). Maintenance hemodialysis (MHD) is one of the main treatments used for end-stage renal disease (ESRD), with approximately 89% of dialysis patients receiving hemodialysis (HD) worldwide (Pecoits-Filho et al., 2020). As of 31 December 2017, 62.7% of the 746,557 prevalent cases of ESRD in the US were HD patients. In Europe and worldwide, there are approximately 350,000 and 3 million HD patients, respectively, and numbers are anticipated to over 5.4 million globally by 2030, not including the large number of patients who are unable to access HD treatment for economic reasons (Basile et al., 2021).

A previous report showed patients on MHD are more likely to suffer from varying degrees of gastrointestinal symptoms, such as constipation, nausea and vomiting, loss of appetite, and bloating, etc., of which constipation is one of the very frequent complications, with an incidence of 53% (Murtagh et al., 2007). The occurrence of constipation is mainly attributed to dietary restrictions, lack of exercise, use of calcium-phosphorus binding agents and potassium-lowering resins, accumulation of toxins in the body, the suppression of the urge to defecate during HD, and dysbiosis of intestinal flora (Yasuda et al., 2002). Constipation has a number of adverse effects on MHD patients, including inadequate nutritional intake, negative emotions such as sadness and anxiety, increased risk of hypotension during dialysis, cardiovascular accidents, and even increased accumulation of toxins such as creatinine and urea nitrogen in ESRD patients (Zhang JJ and Shen, 2018). Clinical solutions to the problem of constipation in maintenance hemodialysis patients have gained attention. Osmotic and excitatory laxatives are considered first-line drugs for the relief of constipation in adults (Krogh et al., 2017). Osmotic laxatives are widely used in chronic kidney disease (CKD), but their role may be limited, especially in HD patients. Moreover, osmotic laxatives containing magnesium and sodium may induce adverse renal and metabolic disorders (Heher et al., 2008). Therefore, contact laxatives are commonly used in HD patients suffering from constipation. However, diarrhea, dehydration and electrolyte disorders are common side effects of contact laxative use (Oster et al., 1980). One study found a significant dose- and duration-dependent relationship between contact laxative use and increased risk of arteriovenous fistula maturation failure (Hoang Anh et al., 2022). Additionally, long-term laxative use can increase the risk of adverse cardiovascular events and death (Kubota et al., 2016; Hoppe et al., 2019; Honda et al., 2021).

Research has indicated that intestinal flora dysbiosis is an important cause of the occurrence and development of constipation, and conversely, constipation will make the patient's gut microbiota dysbiosis, which is a mutually reinforcing process (Huang, 2020; Lydia et al., 2022). Individuals with CKD or ESRD have a unique microbial community in the gut that includes an overgrowth of duodenal and jejunal bacteria, an overgrowth of certain aerobic bacteria, and altered genera of commensal bacteria, leading to a dysfunctional gut ecosystem (Vaziri et al., 2013; Ramezani et al., 2016), and the development of chronic gastrointestinal symptoms such as constipation and loss of appetite. There have been a number of studies on the use of synbiotics to reduce uremic toxins and relieve constipation. However, there is a wide variation in the effect obtained using different types of synbiotics, suggesting the value of specifying the type, dose and duration of synbiotics (Nakabayashi et al., 2011; Rossi et al., 2016; Cosola et al., 2021; McFarlane et al., 2021; Nguyen et al., 2021). Therefore, the fecal microbiota analysis is need to be performed to determine the most suitable synbiotics for MHD patients. In addition, numerous clinical Trials have demonstrated that fecal microbiota transplantation (FMT) reduces functional constipation. However, to the best of our knowledge, no study

Abbreviations: MHD, Maintenance hemodialysis; ESRD, end-stage renal disease; HD, hemodialysis; CKD, chronic kidney disease; FMT, fecal microbiota transplantation; TCM, Traditional Chinese Medicine; MHDCG, maintenance hemodialysis constipation group; MHDNCG, maintenance hemodialysis non-constipated group; HCG, healthy control group; CTAB, cetyltrimethylammonium bromide; PCR, polymerase chain reaction; ASV, Amplicon Sequence Variant; LEfSe, Linear discriminant analysis Effect Size; OUT, operational taxonomic unit; 5-HT, 5-hydroxytryptophan; SCFAs, short-chain fatty acids; OXPHOS, oxidative phosphorylation; GABA, gamma-aminobutyric acid.

has reported the characteristics of gut flora in maintenance hemodialysis patients with constipation. Therefore, this research gap is urgent to be addressed.

The aim of this study was to examine the differences in the distribution and abundance of intestinal microbiome among the three groups, including maintenance hemodialysis patients with constipation, maintenance hemodialysis patients without constipation and healthy individuals. And to identify the dominant intestinal flora in MHD patients with constipation, so as to explore possible mechanisms of MHD patients with constipation.

2 Methods

2.1 Study design

In this study, a prospective clinical cohort study was conducted to analyze the bacterial flora in the stools of maintenance hemodialysis patients with constipation, and hemodialysis patients who were not constipated and normal healthy individuals were used as controls. This study starts in January 2023 and lasts for 1 year. All samples in this study were obtained from the feces of participants in Hangzhou, China.

2.2 Study participants

Based on the inclusion and exclusion criteria, 336 patients undergoing maintenance hemodialysis at the Hemodialysis Centre of Hangzhou Hospital of Traditional Chinese Medicine (TCM) were screened. A questionnaire survey of constipation-related symptoms was conducted on these 336 patients. Then, 100 MHD patients who met the Rome-IV diagnostic criteria for constipation were screened out (Supplementary Material 1). From these 100 patients, 15 was randomly selected as the maintenance hemodialysis constipation group (MHDCG). And the same number of people were set up in the maintenance hemodialysis non-constipated group (MHDNCG) and the healthy control group (HCG) respectively. The HCG came from Hangzhou Hospital of Traditional Chinese Medicine and were older than 18 years old, and the results showed that they were in good health, had no gastrointestinal symptoms, and had not taken any medications in the past month. The study was ethically reviewed by the Research Ethics Committee of Hangzhou Hospital of TCM.

The inclusion criteria were as follows:

1. Maintenance hemodialysis treatment for more than 3 months with stable condition;
2. Patients older than 18 years of age;
3. Maintenance hemodialysis patients who were conscious, had no language communication barriers; gave informed consent and signed the Patient Informed Consent Form.

The exclusion criteria were as follows:

1. Those who have had infections or stress conditions such as abdominal pain and diarrhea, coughing and sputum in the last month, and who have used antibiotics;
2. History of taking probiotics, prebiotics, antibiotics or proton pump inhibitors within the last 1 month;
3. Presence of organic gastrointestinal disorders (e.g. irritable bowel syndrome, intestinal obstruction, intestinal adhesions, etc.), or history of gastrointestinal disorders (e.g. tumors, ulcerative colitis, etc.), or history of gastrointestinal surgery.
4. Patients with incomplete clinical information or who did not provide a fecal specimen on request.

2.3 Fecal sample collection and DNA extraction

Fresh fecal specimens were collected from the selected individuals. The fecal specimens were excreted into the aseptic stool tubes and moved to a −80°C refrigerator for storage within 2 h after sampling. The total DNA of microbiome was extracted by cetyltrimethylammonium bromide (CTAB) method, and the purity and concentration of DNA were detected by agarose gel electrophoresis.

2.4 16S rRNA gene targeted amplification and sequencing

The use of different primers for polymerase chain reaction (PCR) amplification was selected based on the different project, the PCR products were purified from AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). PCR amplification products were detected by 2% agarose gel electrophoresis. The purified PCR products were evaluated using Agilent 2100 Bioanalyzer (Agilent, USA) and Illumina (Kapa Biosciences, Woburn, MA, USA) library quantification kits, and the acceptable library concentration should be above 2nM.The qualified sequencing libraries (Index sequences are not reproducible) were diluted in a gradient, mixed according to the required sequencing volume in the appropriate ratio, and denatured by NaOH to single-stranded for sequencing;2×250bp double-end sequencing was carried out using the NovaSeq 6000 Sequencer, and the corresponding reagent was NovaSeq 6000 SP Reagent Kit (500 cycles).

2.5 Statistical analysis

Based on the Amplicon Sequence Variant (ASV) sequence files, the SILVA database was used to annotate the species with the NT-16S database, and the abundance of each species in each sample was counted according to the ASV abundance table. Based on the obtained ASV feature sequences and ASV abundance tables, alpha and beta diversity analyses were performed. The alpha diversity analysis was based on the observed_species, shannon, simpson, chao1, pielou_e indices to assess the intra- and inter-group diversity. Beta diversity was assessed by calculating the weighted_unifrac distance, and PCA and PCoA analyses were used to assess the diversity between groups. Linear discriminant

analysis Effect Size (LEfSe) analysis was used to find biomarkers. Different statistical methods were selected for species difference analyses according to the samples: Fisher's exact test was used for samples without biological replicates comparison; Mann-Whitney Utest, for comparison of differences between two groups of samples with biological replicates; Kruskal-Wallis test, comparison between multiple groups of samples with biological replicates. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) v2.2.0b was used to predict potential metagenome functionality based on 16S ASV content for functional analysis. The ASV table and corresponding representative sequences were aligned (NSTI cut-off value of 2) to a reference phylogenetic tree, and the software predicted functional gene families and copy numbers for each specific ASV. The resulting output generated an abundance profile of pathways based on the KEGG database. Differential pathways between groups were identified and presented using STAMP software with t-test. Benjamini-Hochberg FDR-adjusted p values <0.05 were considered significant.

3 Results

3.1 General characteristics of all participants

In the comparison of general clinical data, MHDCG and MHDNCG were not statistically different in terms of age, gender, ultrafiltration quality and medications taken (p>0.05). MHDCG and HCG were statistically different in age (p=0.003) and not statistically different in gender (p>0.05). There was no statistically significant difference in age and gender for MHDNCG and HCG (p>0.05). In the comparison of test result, MHDCG and MHDNCG did not show statistical difference in blood urea nitrogen, blood creatinine, blood calcium, blood phosphorus and blood uric acid (p>0.05). No statistically significant difference in blood calcium was observed for MHDCG as well as MHDNCG compared to HCG (p>0.05). MHDCG and MHDNCG were statistically different in blood phosphorus compared to HCG (p=0.007, p=0.000) (Table 1).

3.2 Species annotation analysis

Using the operational taxonomic unit (OTU) Venn diagram, this study found that there were 1246 OTUs between the MHDCG and MHDNCG groups, 1752 OTUs specific to the MHDCG group, and 1511 OTUs specific to the MHDNCG group. There were 1164 OTUs between the MHDCG and HCG groups, 1834 OTUs specific to the MHDCG group, and 1578 OTUs specific to the HCG group. There were 1234 OTUs between the MHDNCG and HCG groups, 1523 OTUs specific to the MHDNCG group, and 1508 OTUs specific to the HCG group. This tentatively suggests that there are differences in the distribution of intestinal microbiome between MHDCG and MHDNCG, and that the difference in intestinal microbiome species between MHDCG and HCG is greater than that between MHDNCG and HCG (Figure 1).

3.3 Species diversity analysis

3.3.1 α-diversity analysis

According to the dilution curve of alpha diversity analysis, combined with Chao1 index and observed_species, this study found that the gut microbial community of MHDCG and MHDNCG contained fewer species than that of the HCG group. Combined with the Shannon index and Simpson index, we found the diversity of gut microbial organisms in MHDCG was lower than that of MHDNCG and HCG. Combined with the Shannon index and

TABLE 1 General characteristics of participants.

	MHDCG (N=15)	MHDNCG (N=15)	HCG (N=15)	t	p
Age(year)	69.20 ± 8.64	62.13 ± 11.39	59.33 ± 8.08	t^a=1.915; t^b =3.231; t^c =0.777	p^a=0.066; p^b=0.447; p^c=0.444
Sex (male/female)	10/5	9/6	8/7	/	p^a=0.500; p^b=0.710; p^c=1.000
Blood phosphorus(mmol/L)	1.57 ± 0.35	1.72 ± 0.37	1.26 ± 0.21	t^a=-1.131; t^b= 2.972; t^c= 4.223	p^a=0.267; p^b=0.007; p^c=0.000
Blood calcium (mmol/L)	2.26 ± 0.12	2.26 ± 0.17	2.35 ± 0.18	t^a=-0.025; t^b=-1.668; t^c=-1.422	p^a=0.981; p^b=0.106; p^c=0.166
Blood creatinine(μmol/L)	787.80 ± 204.05	845.53 ± 211.74	/	t^a=-0.760	p^a=0.453
Blood uric acid (μmol/L)	441.07 ± 123.71	436.13 ± 86.33	/	t^a=0.127	p^a=0.90
Blood urea nitrogen (μmol/L)	22.61 ± 6.67	20.53 ± 8.03	/	t^a=0.771	p^a=0.447
Ultrafiltration quality(Kg)	1.89 ± 0.72	1.99 ± 0.93	/	t^a=-0.351	p^a=0.729
Taking medication that affects bowel movements (Yes/No)	2/13	5/10	/	/	p^a=0.195

a: MHDCG vs MHDNCG; b: MHDCG vs HCG; c: MHDNCG vs HCG.

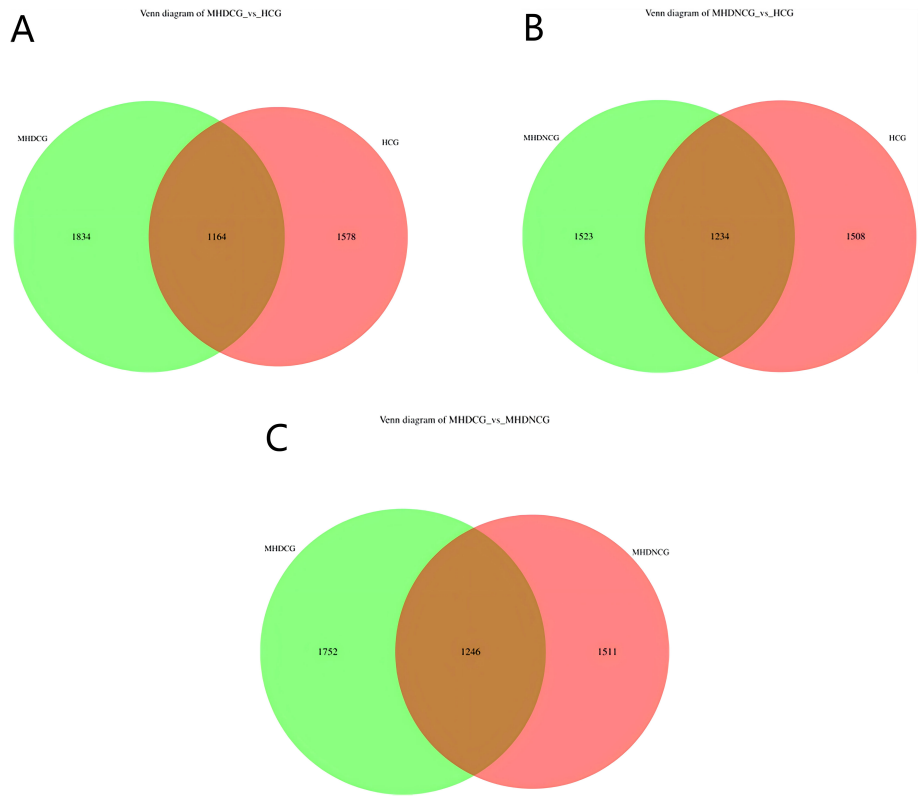

FIGURE 1
OTU Venn diagram. **(A)** There were 1164 OTUs between the MHDCG and HCG groups, 1834 OTUs specific to the MHDCG group, and 1578 OTUs specific to the HCG group. **(B)** There were 1234 OTUs between the MHDNCG and HCG groups, 1523 OTUs specific to the MHDNCG group, and 1508 OTUs specific to the HCG group. **(C)** There were 1246 OTUs between the MHDCG and MHDNCG groups, 1752 OTUs specific to the MHDCG group, and 1511 OTUs specific to the MHDNCG group.

pielou-e index curve, we found the uniformity of gut microbial organisms in MHDCG was lower than that of MHDNCG and HCG. In summary, our study suggests that MHDCG have lower diversity and homogeneity than MHDNCG and HCG (Figure 2).

3.3.2 β-diversity analysis

Both PCoA and PCA analysis revealed no significant difference in the species' variety and abundance between MHDCG, MHDNCG and HCG in Beta diversity analysis ($p=0.864$, $p=0.825$). This suggests that there may be no difference in the variety and abundance of species of the intestinal flora of MHDCG and MHDNCG and HCG (Figure 3).

3.4 Species difference analysis

3.4.1 Species composition heat map

Cluster analyses were used to distinguish between high and low abundance taxonomic units in the community composition of the top30 within each group at the phylum level. Cross-sectional comparisons via species composition heatmaps revealed less overlap in top30 species abundance between the MHDCG, MHDNCG and HCG groups, tentatively suggesting that that the distribution of gut microbes differed among the three groups at the phylum level (Figure 4).

3.4.2 Comparison of species abundance

At the phylum level, the dominant phyla for MHDCG, MHDNCG and HCG were all Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota and Verrucomicrobiota. The abundance of Verrucomicrobiota in MHD was significantly higher than that in HCG, while the abundance of Desulfobacterota was significantly lower than that in HCG ($p<0.05$). There was no significant difference between MHDCG and MHDNCG (Figure 5).

At the genus level, the top 10 dominant genera in the MHDCG group were Enterococcus, Escherichia-Shigella, Bacteroides, Streptococcus, Bifidobacterium, Ruminococcus_gnavus_group, Lachnospiraceae_unclassified, Faecalibacterium, Akkermansia and UCG-002. MHDCG showed a significant increase in abundance of Enterococcus, Eggerthella, Ruegeria, Dubosiella, Akkermansia, Filomicrobium, Prevotella_7, Altererythrobacter, CAG-352, Herminiimonas, and significant decrease in abundance of Agathobacter, Haemophilus, Bilophila, Erysipelotrichaceae_UCG-003, Parasutterella, Lachnospiraceae_UCG-006, Leuconostoc, Coprobacter, Dialister, Kineothrix, Butyricicoccus, Romboutsia, Enterobacter and Klebsiella, which compared to HCG($p<0.05$). MHDNCG showed a significant increase in abundance of CAG-352, Neisseria, Akkermansia, Lachnospiraceae_unclassified, and a significant decrease in abundance of Bilophila, Haemophilus, Coprococcus, Citrobacter, Lactiplantibacillus, Alysiella, Leuconostoc, Selenomonas, Erysipelotrichaceae_UCG-003, Lacticaseibacillus,

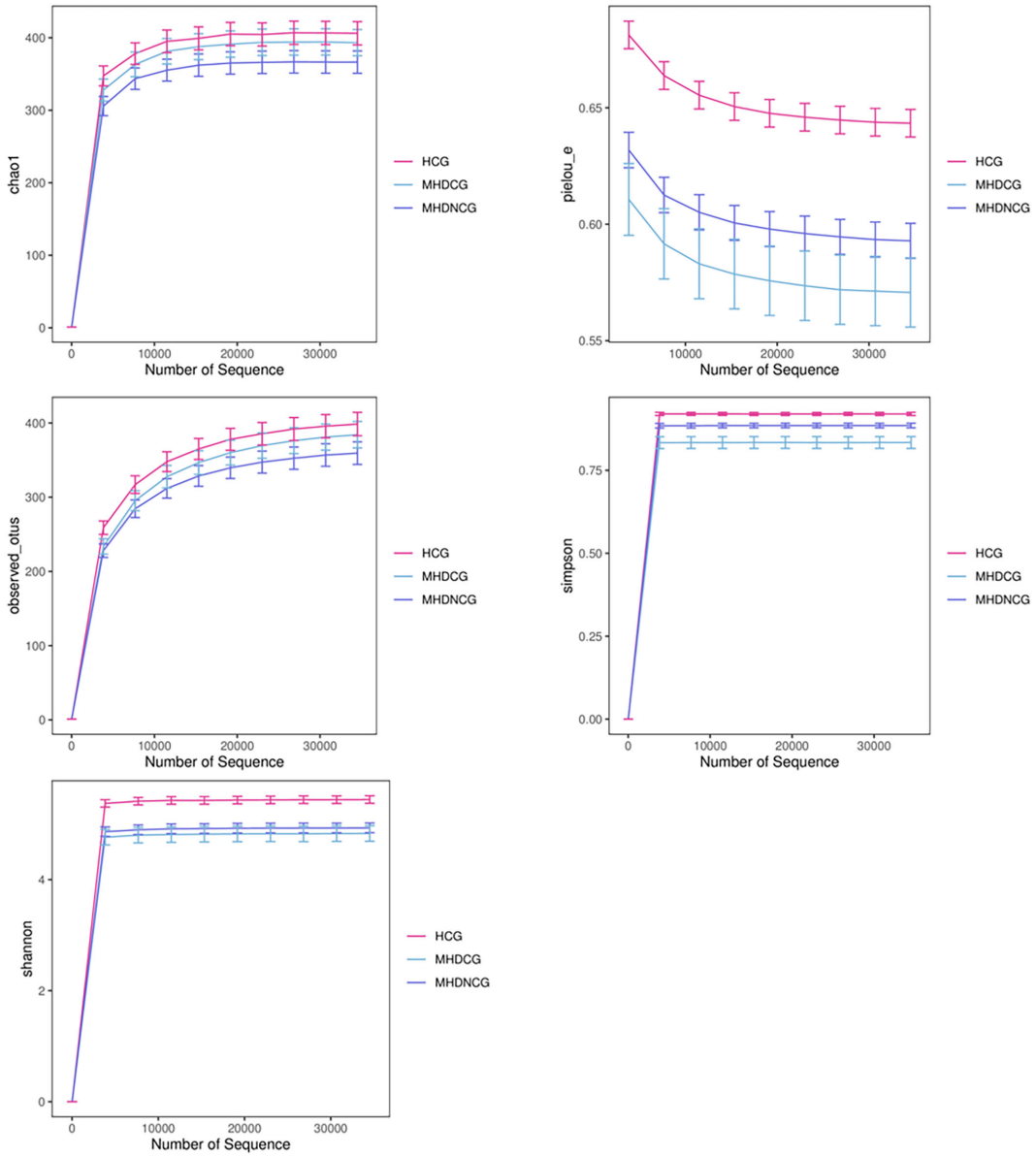

FIGURE 2
α-diversity analysis. The figures of Chao1 index and observed_species showed that the gut microbial community of MHDCG and MHDNCG contained fewer species than that of the HCG group. The figures of Shannon index and Simpson index showed that the diversity of gut microbial organisms in MHDCG was lower than that of MHDNCG and HCG. The figures of Shannon index and pielou-e index curve showed that the uniformity of gut microbial organisms in MHDCG was lower than that of MHDNCG and HCG.

Enterobacter, Coprobacter and Neobitarella, which compared to HCG ($p<0.05$). Compared with MHDNCG, the Enterococcus, Rhizobiales_unclassified, Filomicrobium, Eggerthella, Allobaculum, Prevotella_7, Gordonibacter, Mitochondria_unclassified, Lachnoanaerobaculum were significantly higher and the Kineothrix, Rhodopirellula, Weissella were significantly lower in MHDCG ($p<0.05$) (Figure 6).

3.4.3 LEfSe analysis

The abundance difference analysis plots for the MHDCG and MHDNCG groups illustrate that 7 gut microbial taxa were significantly more abundant in the MHDNCG group samples, and 20 gut microbial taxa were significantly more abundant in the MHDCG group samples. At the genus level, MHDNCG has more abundance in Rhodopirellula and Weissella. MHDCG has more abundance in Rhizobiales, Prevotella_7, Allobaculum, Filomicrobium, Lachnoanaerobaculum, Mitochondria, Enterococcus (Figure 7).

3.5 Predictive functional analysis

The species' functions in the gut microbiota of both the groups were predicted and analyzed based on the amplified sequencing data, using the PICRUSt2 analysis tool. The PICRUSt2 analysis revealed

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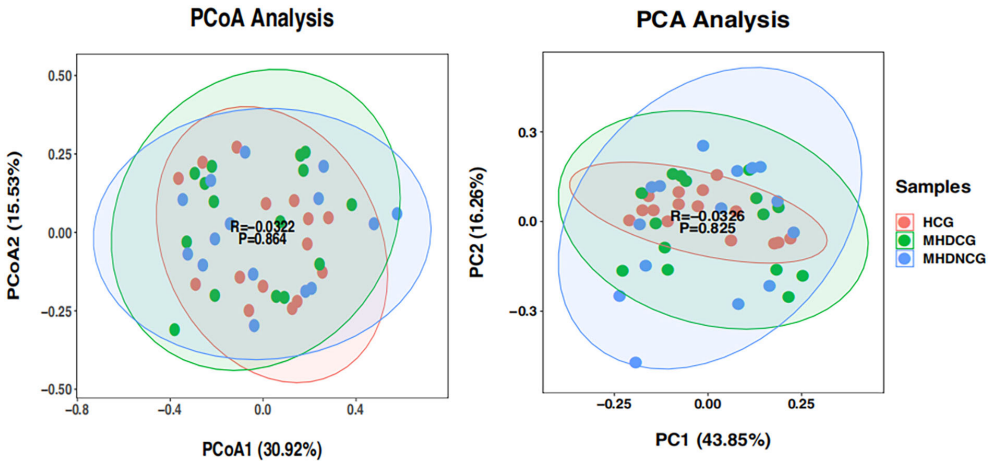

FIGURE 3
β-diversity analysis. PCoA and PCA analysis revealed no significant difference in the species' variety and abundance between MHDCG, MHDNCG and HCG in Beta diversity analysis (p=0.864, p=0.825).

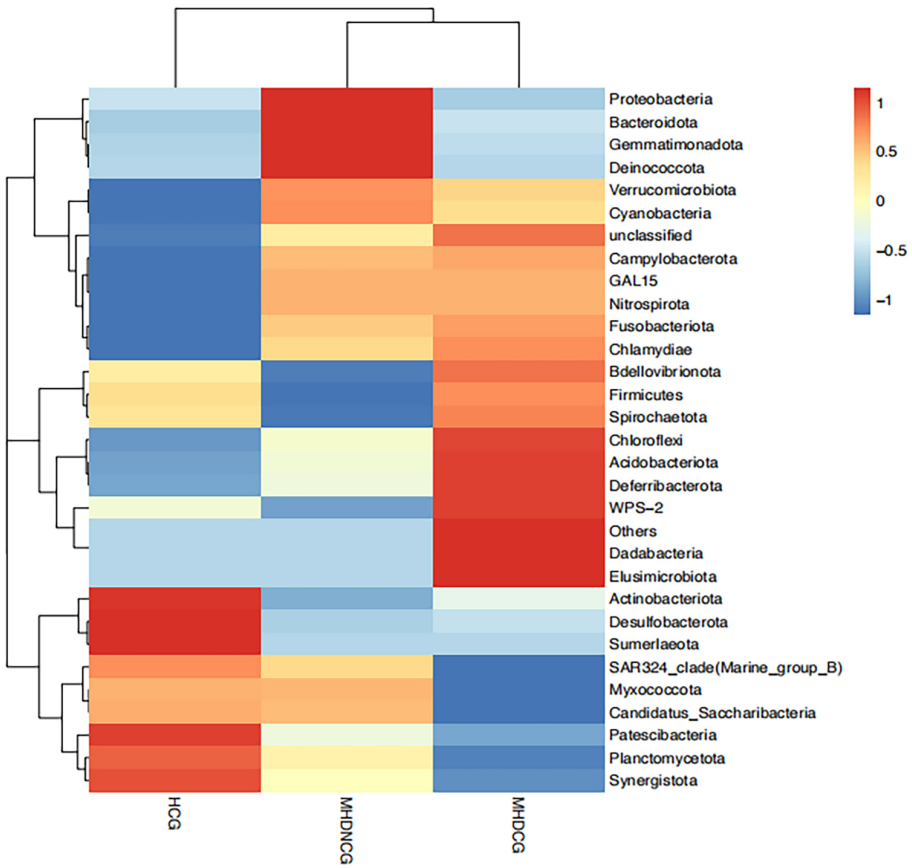

FIGURE 4
Species composition heat map. Species composition heatmaps revealed less overlap in top30 species abundance between the MHDCG, MHDNCG and HCG groups.

several potential pathway alterations in the microbial communities. Compared with MHDNCG, MHDCG was significantly enriched in pathways related to pyruate metabolism and flavonoid biosynthesis (Figure 8).

4 Discussion

Maintenance hemodialysis is the most commonly used treatment for uremia, which significantly prolongs the survival of

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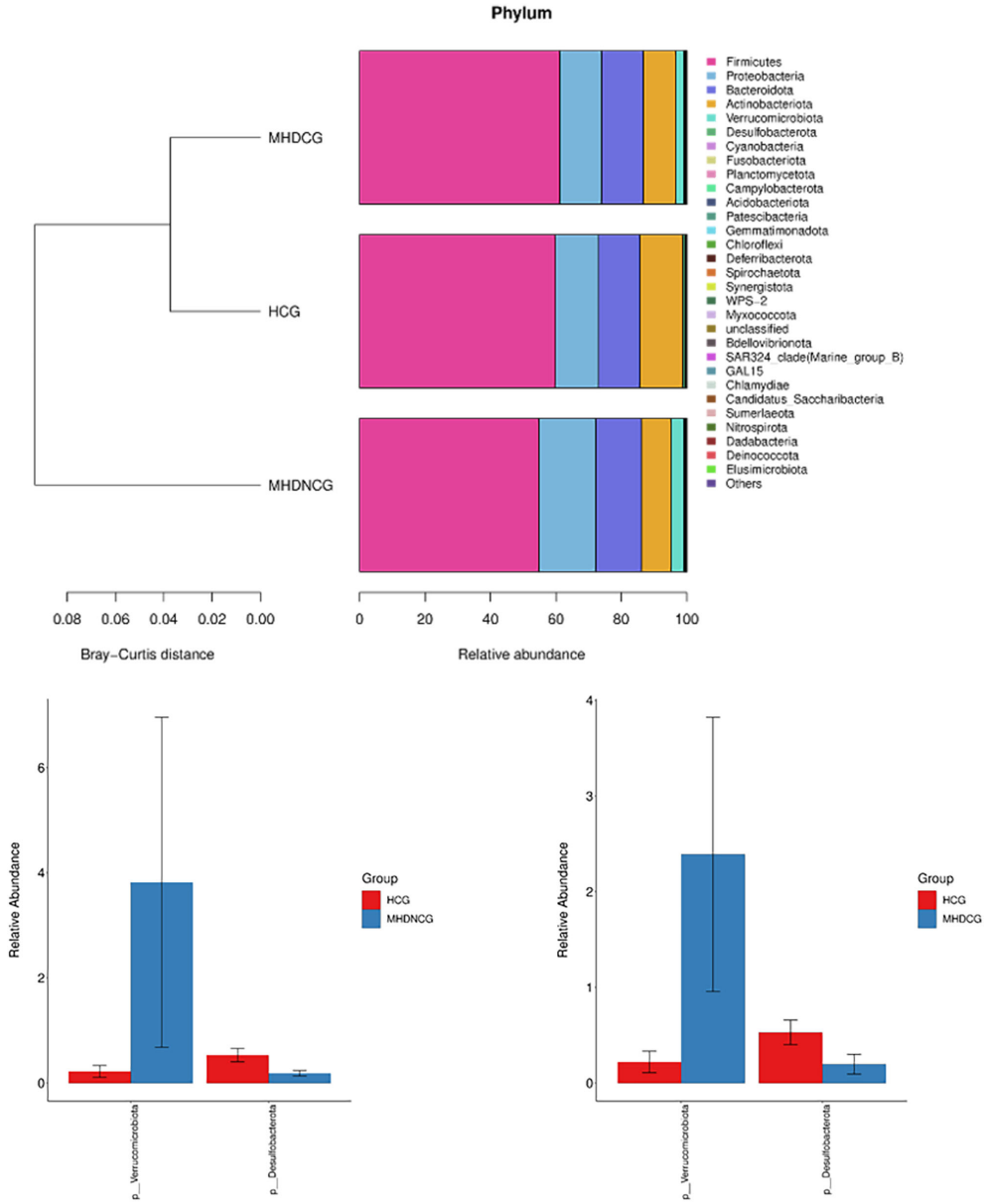

FIGURE 5
Comparison of species abundance at the phylum level. At the phylum level, the dominant phyla for MHDCG, MHDNCG and HCG were all Firmicutes, Proteobacteria, Bacteroidota, Actinobacteriota and Verrucomicrobiota. The abundance of Verrucomicrobiota in MHD was significantly higher than that in HCG, while the abundance of Desulfobacterota was significantly lower than that in HCG ($p<0.05$).

ESRD patients. However, this treatment is inevitably accompanied by a variety of complications that plague patients. Specifically, hemodialysis patients are prone to chronic gastrointestinal diseases such as constipation due to the inability of the kidneys to completely remove toxins, uric acid, oxalic acid and other substances, resulting in the accumulation of toxins in the intestinal tract, accompanied by changes in intestinal function and microbiota. A recent systematic evaluation showed that the most prevalent gastrointestinal symptom in patients receiving dialysis for ESRD was constipation, with prevalence ranging from 1.6% to 71.7% in HD patients (Zuvela et al., 2018).

Gut microorganisms are crucial to the health of the host and the occurrence and development of diseases. The total gut microbial genome is known as the "second human genome", and tens of thousands of diverse gut microbial communities inhabit the human gut, which are involved in a variety of physiological activities of the host and play an important role in the health of the human body. In recent years,16SrRNA and macro-genomics sequencing technologies have been widely used to the research of human intestinal microbiome, especially to explore the association between intestinal microbiome and cardiovascular, neurological, renal and other diseases. Gastrointestinal disorders are even more closely linked to intestinal flora. The process of digestion and absorption of food in the body and its eventual transformation into feces cannot be achieved without the help of bacteria in the gastrointestinal tract. Therefore, we speculate that the occurrence of constipation in hemodialysis patients

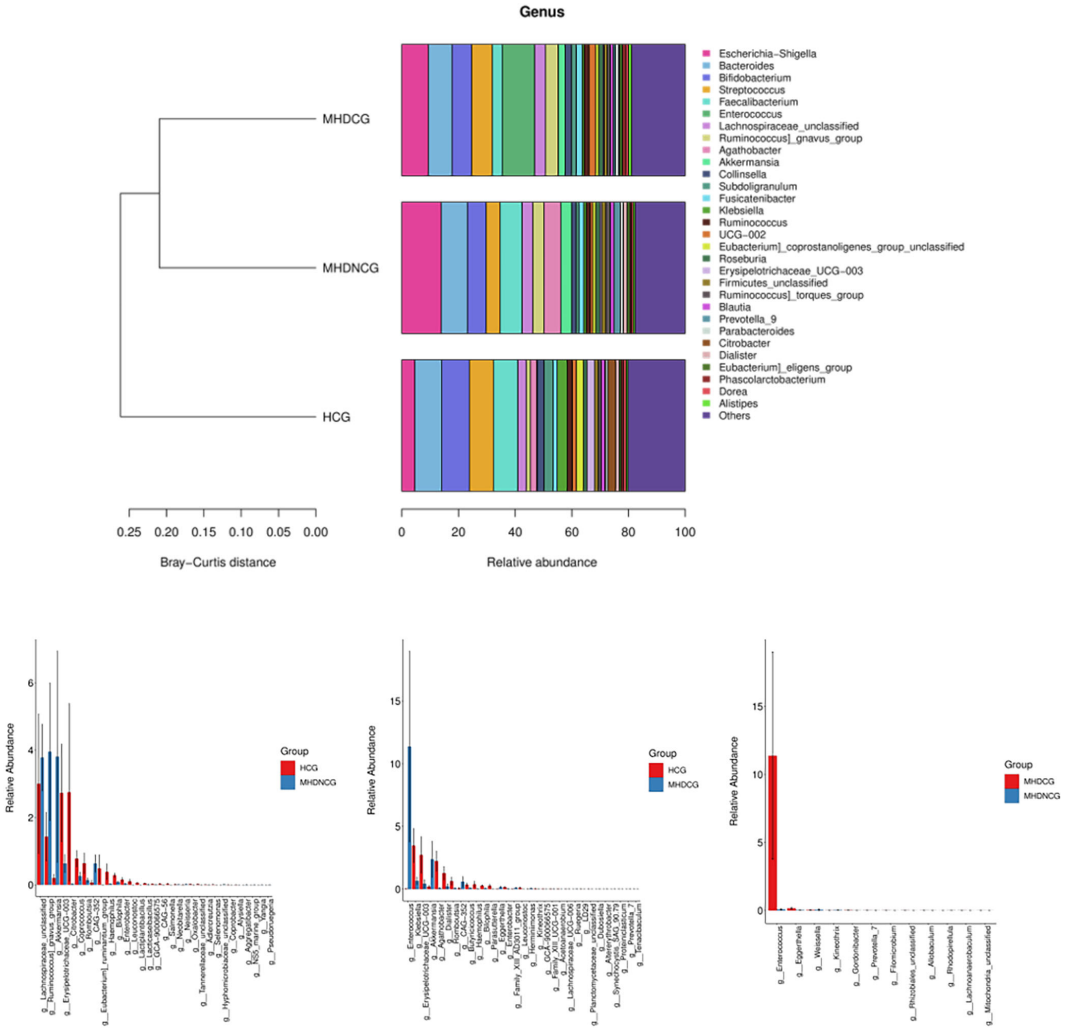

FIGURE 6
Comparison of species abundance at the genus level. At the genus level, the top 10 dominant genera in the MHDCG group were Enterococcus, Escherichia-Shigella, Bacteroides, Streptococcus, Bifidobacterium, Ruminococcus_gnavus_group, Lachnospiraceae_unclassified, Faecalibacterium, Akkermansia and UCG-002. At the genus level, there were significant differences in the abundance of many species.

may also be related to abnormal intestinal flora. Our trial used 16SrRNA sequencing to analyze the diversity of gut microorganisms in Chinese patients on maintenance hemodialysis with constipation. Macrogenomic studies have shown that Bacteroidota and Firmicutes are the two most dominant bacterial types among human microorganisms, followed by other bacteria such as Proteobacteria, Actinobacteriota, Fusobacteria and Verrucomicrobiota (Adak and Khan, 2019). Our study also demonstrated that Bacteroidota, Firmicutes, Proteobacteria, Actinobacteriota and Verrucomicrobiota are the major phylums in hemodialysis patients and normal healthy individuals. This suggests that to some extent the human gut flora is stable and similar. However, we found that MHDCG had a reduced diversity of gut flora and a heterogeneous distribution of flora compared to the MHDNCG and the HCG, suggesting that the distribution of the gut microbial community is shifted to some extent in patients of MHDCG.

In our study, some potentially pathogenic bacteria (Enterococcus, Eggerthella, Gordonibacter) were more abundant in the constipated group. These bacteria are often responsible for causing inflammation and infection. Studies have shown that Enterococcus exhibit intrinsic resistance to many antimicrobial drugs, such as compound-boosted sulfonamides, cephalosporins, clindamycin, and low-concentration aminoglycosides. Enterococci are regarded as one of the most important hospital infection pathogens among gram-positive bacteria, and their infections are most commonly urinary tract infections (Pan SX, 2008). Gordonibacter is an opportunistic pathogen whose increased abundance is associated with the emergence of enteritis (Pei et al., 2019). The relative abundance of Gordonibacter was significantly and positively correlated with the levels of inflammatory factors such as IL-1, IL-6 and IL-8 (Zhang et al., 2023). Eggerthella can cause bloodstream infections and is considered an opportunistic human pathogen. Eggerthella colonization promotes intestinal Th17 activity and thus induces intestinal inflammation (Alexander et al., 2022). These bacteria raise the chances of inflammation in MHDCG patients. There have been many

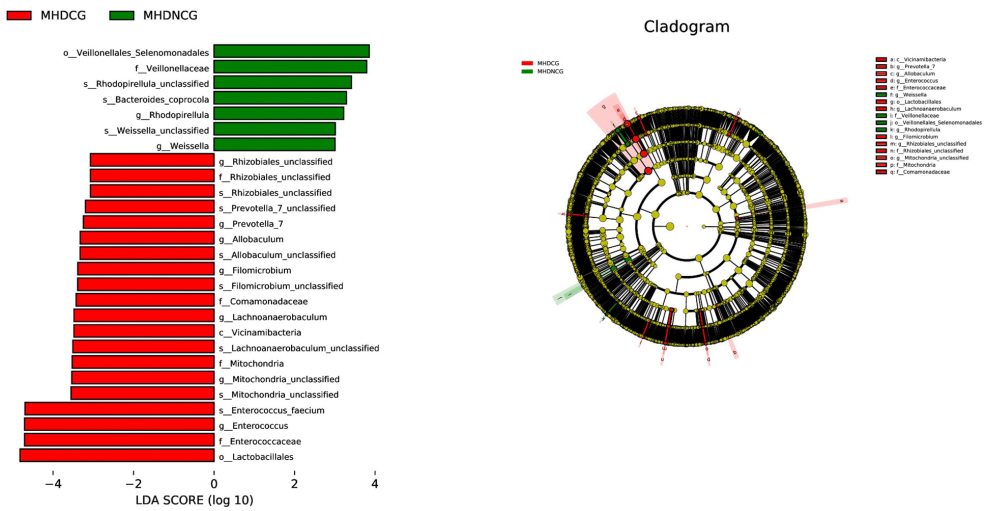

FIGURE 7
LEfSe analysis. The abundance difference analysis plots for the MHDCG and MHDNCG groups illustrate that 7 gut microbial taxa were significantly more abundant in the MHDNCG group samples, and 20 gut microbial taxa were significantly more abundant in the MHDCG group samples.

studies showing a correlation between constipation and inflammation. Mean IL-6, IL-12 and neopterin levels were significantly higher in constipated children than in healthy controls (Cıralı et al., 2018). Constipation correlated with serum IgA antibody titers, while serum IgA levels were positively correlated with ESR values (Strati et al., 2016). In recent years, it has been found that there are structural and functional changes in the intestinal mucosal barrier in patients with chronic constipation, so its role in the pathogenesis of constipation has received widespread attention (Ohkusa et al., 2019). A variety of factors, including bacterial infection (Bischoff et al., 2014), can cause damage to the intestinal mucosal function, increasing intestinal permeability, the intestinal tract loses its normal barrier function, and pathogenic bacteria can take advantage of the situation, resulting in disorders of intestinal flora and abnormal bowel function. Thus, hemodialysis patients with low immunity are more likely to stimulate an inflammatory response once they are attacked by these disease-causing bacteria, leading to intestinal dysfunction and triggering a series of diseases such as constipation.

An animal study found Enterococcus abundance negatively correlates with 5-hydroxytryptophan (5-HT) levels (Hao et al., 2023). Another study found Prevotella to be negatively associated with 5-HT levels (Yang et al., 2023). 5-HT is a prominent neurotransmitter that is highly abundant in the gut and is the driver of the peristaltic reflex (Heredia et al., 2013). And our findings found elevated Enterococcus, Prevotella in MHDCG, which is consistent with the above findings. This suggests that Enterococcus, Prevotella may reduce 5-HT levels, which exacerbates the process of constipation in hemodialysis patients. An RCT of critically ill patients found that exogenous probiotics reduced Enterococcus abundance and effectively relieved constipation (Wang et al., 2021). Similarly, another study found that treatment with herbs reduced Prevotella abundance, activated the 5-HT-cAMP-PKA signaling pathway and improved gastrointestinal motility (Wang et al., 2023). This reminds to us that reducing the abundance of Enterococcus and Prevotella by probiotics, FMT, etc., or activating the 5-HT pathway by 5-HT 4-receptor agonists, may be one of the ways to treat constipation in patients undergoing hemodialysis.

Eggerthella has been associated with autoimmune diseases such as asthma (Wang et al., 2018), multiple sclerosis (Cekanaviciute et al., 2017), inflammatory bowel disease (Alexander et al., 2022)and rheumatoid arthritis (Chen et al., 2016). An animal experiment verifies the role of Eggerthella in the production of serum uremic toxins and in aggravating the development of renal disease (Wang et al., 2020). Another study (The effect of constipation on urinary toxin levels in patients with chronic kidney disease and the evidence of Chinese medicine, 2023) showed that constipation can exacerbate urinary toxin accumulation in CKD patients, and the more severe the constipation, the greater the urinary toxin accumulation. The gut and kidneys are closely related pathophysiologically. Increased intestinal permeability allows a variety of pathogens to enter the bloodstream, leading to aberrant immune activation and facilitating the progression of renal disease, while deterioration of renal function leads to the accumulation of toxins in the body, causing disturbances in the internal environment and exacerbating the disruption of the intestinal barrier. Combining the above studies with ours, we hypothesize that Eggerthella may cause constipation and exacerbate the accumulation of urinary toxins.

The gut microbiota regulates peristalsis by the release of microbial metabolites or fermentation end products. The three main groups of bacterial metabolites include Bas, short-chain fatty acids (SCFAs) and tryptophan metabolites. SCFAs are major metabolites produced by specific colonic anaerobes after fermentation of dietary fiber and resistant starch. Short-chain fatty acids stimulate the production of glucagon-like peptide-1 and peptide YY, modulate the release of 5-HT, and increase gastrointestinal motility. SCFAs mainly consist of acetic acid,

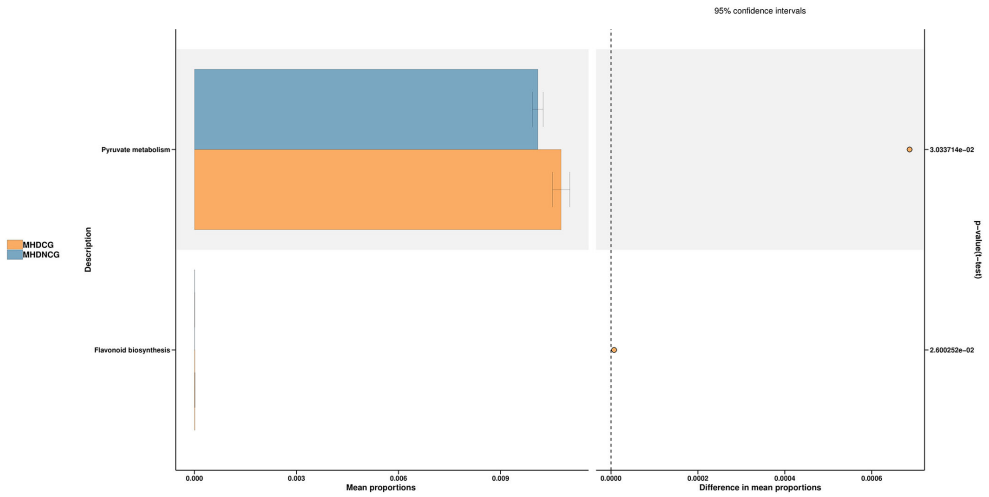

FIGURE 8
Predictive functional analysis. The PICRUSt2 analysis revealed that compared with MHDNCG, MHDCG was significantly enriched in pathways related to pyruate metabolism, flavonoid biosynthesis.

propionic acid, and butyrate, which also directly modulates the enteric nervous system and controls gastrointestinal motility (Soret et al., 2010). It has been reported that butyrate is mainly produced by Lachnospiraceae, Ruminococcaceae, Faecalibacterium, prausnitzii, Prevotella, Roseburia, and Clostridium (Pryde et al., 2002). However, our study found no significant difference in the abundance of these bacteria between the two groups, except that Prevotella was elevated in MHDCG. In addition, there was an increase in Allobaculum and Lachnoanaerobaculum in MHDCG, which are also capable of producing butyrate. It seems contrary that butyrate promotes intestinal peristalsis, whereas butyrate-producing bacteria are instead increased in MHDCG. This may be due to the differences in race, geography, and underlying disease between our experimental population and the participants in the above studies. However, there are also studies that showed non-physiological high levels of short-chain fatty acids in the gut may lead to gastrointestinal symptoms, including a constipated state, by altering the secretion of mucin secretions from cuprocytes (Barcelo et al., 2000) and inhibiting intestinal smooth muscle contraction (Squires et al., 1992) mediated by the release of peptide YY from enteroendocrine cells (Cherbut et al., 1998). Based on the above studies, we hypothesized two possible mechanisms for the elevation of short-chain fatty acid-producing bacteria (Prevotella, Allobaculum, Lachnoanaerobaculum) in MHDCG. First, constipation leads to an increase in retained dietary fiber and resistant starch in the colon, causing compensatory elevation of short-chain fatty acid-producing bacteria. Second, the abnormal elevation of short-chain fatty acids leads to constipation. Colonic epithelial cell mitochondria can catalyze butyrate to nicotinamide adenine dinucleotide (NADH) to participate in the oxidative phosphorylation (OXPHOS) process. OXPHOS produces high oxygen consumption and maintains a hypoxic environment in the intestinal lumen, which helps to maintain a predominantly anaerobic gut microbial community (Litvak et al., 2018). In our study, both mitochondria and butyrate-producing bacteria were elevated in MHDCG, which seems to be a process of interaction. In addition to this, an important function of mitochondria is to supply energy to the cell, and the increase in mitochondria may be an adaptive response caused by the increased functional load of the gut (Qiao et al., 2020; Choi et al., 2023).

Our study found that pyruvate metabolism was stronger in MHDCG than in MHDNCG. An animal experiment shows significantly higher levels of pyruvate metabolism in the intestinal flora of a rat model of constipation (Study on the effects of fluid-enhancing soup on intestinal flora and host metabolism in rats with fluid deficiency and senile constipation, 2018), and a serum metabolic profiling study also showed significant enrichment of the pyruvate metabolic pathway in constipated patients (Xu et al., 2022). These findings are consistent with our results. The pyruvate metabolism pathway belongs to energy metabolism, and the high expression of this pathway suggests that constipation may lead to accelerated carbohydrate metabolism and increased energy expenditure. We also found a significant enrichment of flavonoid biosynthesis pathways in MHDCG compared to MHDNCG. Recent studies have shown that flavonoids and their derivatives can be antioxidants, reduce low-density lipoprotein, inhibit thrombosis, inhibit tumors, protect liver cells, anti-fatigue and other effects (Physiological functions of soybean isoflavones and their application prospects, 2002; Yaru et al., 2022). Flavonifractor uses gamma-aminobutyric acid (GABA) as a growth substrate, and hence reduces the amount of GABA in the gut (Strandwitz et al., 2019). GABA has a variety of regulatory roles in the intestinal tract, including promoting peristalsis and gastric emptying (Hyland and Cryan, 2010). Thus, enrichment of flavonoid biosynthesis pathways may cause constipation. However, there are study showing that flavonoids in *Amomi Fructus* improved constipation symptoms in mice by modulating the gut microbiota and related metabolites (Hu et al., 2023), contrary to our findings. It is possible that this is an effect of the differences between the human and mice species. There is a lack of reports on the association between flavonoid

biosynthesis pathways and constipation, and the association needs to be further investigated.

There are potential limitations to this study. First, although this study characterized the microbial composition of MHDCG patients, the population included in this study was limited, all from Hangzhou, China. Ethnic and geographic limitations may have biased the results somewhat. Second, due to the strict conditions of sample collection and preservation, we only collected 15 samples from each group. Third, although our statistical results showed no statistical difference between MHDCG and MHDNCG in the use of medications affecting intestinal motility, patients' use of lactulose and polyethylene glycol-4000 all have the potential to affect intestinal microbes (Bouhnik et al., 2004). In our study, the interference of these drugs was not completely avoided. In addition, we lacked metabolomics studies, and the mechanism between differential flora and maintenance hemodialysis constipation was not adequately investigated. We look forward to more multi-center, large-sample studies on the intestinal flora of patients with maintenance hemodialysis constipation in the future.

5 Conclusions

This study describes for the first time the gut microbiome characteristics of patients with maintenance hemodialysis constipation. The results showed that MHDCG differed significantly from MHDNCG and HCG in the abundance of some intestinal bacteria, which may be the direction for future FMT treatment. By studying these differential bacteria, we believe that constipation in hemodialysis patients may be associated with a variety of mechanisms, including inflammatory responses induced by potentially pathogenic bacteria, decreases in 5-HT, interactions between urinary toxins and intestinal function, and abnormal increases in short-chain fatty acids. However, there are limitations in this study and the understanding of the mechanisms of constipation in hemodialysis patients lacks direct evidence, which needs to be further explored by more multi-center and large-sample studies in the future.

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.ncbi.nlm.nih.gov/, PRJNA1159710.

Ethics statement

The study was ethically reviewed by the Research Ethics Committee of Hangzhou Hospital of TCM. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

AZ: Writing – original draft. SC: Writing – original draft. YQZ: Writing – original draft. XZ: Writing – original draft. MW: Writing – original draft. BL: Writing – original draft. YZ: Writing – original draft. XX: Writing – original draft. HL: Funding acquisition, Project administration, Writing – review & editing. FZ: Funding acquisition, Project administration, Writing – review & editing. RL: 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.2024.1495364/full#supplementary-material

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Association of *Fusobacterium nucleatum* infection with colorectal cancer in Kazakhstani patients

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Objectives: *Fusobacterium nucleatum* is a gram-negative anaerobic bacillus associated with colorectal cancer (CRC). We aimed to determine the abundance of *F. nucleatum* and other CRC-associated bacteria using quantitative real-time polymerase chain reaction (qPCR) analysis to detect the possible correlations between tumor and normal tissues and the relationships between patients' clinical characteristics, diet, and CRC-associated bacteria.

Methods: A total of 249 biopsy samples of tumor and paired normal tissues were collected from patients with CRC. Biopsy samples were screened for detection of *F. nucleatum* using qPCR targeting *nusG* gene. *Bacteroides fragilis*, *Escherichia coli*, and *Streptococcus gallolyticus* were also detected in the samples using species-specific genes.

Results: The frequencies of detection of *F. nucleatum* in the tumor and normal tissues of patients with CRC were 43.37 and 24.1%, respectively ($P < 0.05$). Statistical analysis using cycle threshold (Ct) values from qPCR data and clinical characteristics showed that tumor size, tumor location, and processed meat consumption were significantly correlated with the abundance of *F. nucleatum* ($P < 0.05$). The significance of the prevalence of *B. fragilis* and *E. coli* in tumor tissues was marginally higher than that in normal tissues ($P < 0.1$), and the consumption of processed/red meat affected the prevalence of these bacteria ($P < 0.05$).

Conclusions: Our results showed an association between the presence of *F. nucleatum* in tumor tissues and CRC, indicating that *F. nucleatum* may be a potential marker for CRC diagnosis. *F. nucleatum* is enriched in CRC tissues and is associated with CRC development.

KEYWORDS

abundance, colorectal cancer, Fusobacterium nucleatum, Kazakhstan, qPCR

1 Introduction

Fusobacterium nucleatum is a gram-negative anaerobic bacillus present in the oral microbiota and is associated with colorectal cancer (1, 2). CRC is the third most common cancer and the second leading cause of cancer-related mortalities worldwide. *F. nucleatum* has gained attention in recent years because of its potential role in CRC development (3, 4). Various risk factors influence the development of cancer, including age, family history of the disease, inherited genetic conditions (such as Lynch syndrome and familial adenomatous polyposis), personal history of inflammatory bowel disease (such as Crohn's disease or ulcerative colitis), obesity, physical inactivity, smoking, heavy alcohol consumption, and a diet high in red and processed meats and low in fiber. Studies have shown that dietary patterns play a significant role in the development of colorectal cancer (5). Certain diets, identified through the empirical dietary inflammatory pattern (EDIP) assessment, have been linked to increased intestinal inflammation and a higher risk of *F. nucleatum*-positive colorectal carcinomas (6). Diet-induced intestinal inflammation alters the gut microbiome, promoting colorectal carcinogenesis. A high consumption of red and processed meats has been associated with an increased risk of colorectal cancer, potentially due to carcinogens such as nitrates, nitrites, and heterocyclic amines (7). Environmental factors, including dietary habits and antibiotic use, may also affect the behavior of *F. nucleatum* in the colon. On the other side, the roles of intestinal microorganisms in initiating and promoting the development of colorectal cancer are becoming increasingly well understood. There is a complex relationship between gut microbiota and colorectal cancer. Recent research has identified *Streptococcus gallolyticus*, enterotoxigenic *B. fragilis*, *F. nucleatum*, and *E. coli*, as potential pathogens associated with colorectal cancer (8). Although intestinal microbiota varies among individuals, certain bacterial species have been consistently linked to colorectal cancer. *S. gallolyticus*, a gram-positive cocci, is a reported risk factor for CRC (9). Enterotoxigenic *B. fragilis* (ETBF), which produces *B. fragilis* toxin (BFT), is known to cause diarrhea and contribute to inflammatory bowel disease (IBD) (10). Similarly, *E. coli*, a gut commensal bacterium, has been found to colonize the colonic by mucosa-associated *E. coli* at higher levels in colorectal cancer patients compared to healthy individuals (11, 12). However, the response to these risk factors may vary depending on the ethnicity and geographical location, thereby affecting the distribution and prognosis of CRC.

Although *F. nucleatum* is a common inhabitant of the human oral cavity, its abundance is elevated in colorectal tumors and adjacent tissues of patients with CRC (13, 14). Several studies have suggested a potential link between *F. nucleatum* and CRC (1, 15). This bacterium has been reported to promote inflammation, impair immune responses, alter tumor microenvironment, promote resistance to chemotherapy, and facilitate tumor growth and metastasis in preclinical models (16, 17). Additionally, *F. nucleatum* has been associated with a poor prognosis in patients with CRC (18). The presence of *F. nucleatum* in colorectal tissues has led to an interest in its potential as a diagnostic marker or therapeutic target for CRC. However, the role of *F. nucleatum* in the development of CRC remains unclear, for several reasons.

The gut microbiome is highly complex, comprising a diverse range of microorganisms. While *F. nucleatum* is more abundant in the tumors of some colorectal cancer (CRC) patients, its presence alone may not be sufficient to initiate cancer. The interactions between *F. nucleatum*, other microbes, and the host immune system may affect its potential role in cancer development, making it difficult to determine its precise contribution. While studies have linked *F. nucleatum* to colorectal cancer, it remains unclear whether the bacterium directly causes cancer development or if its presence results in changes in the tumor microenvironment. Whether *F. nucleatum* is a driver or merely a bystander in colorectal cancer continues to be an area of active investigation. Further research is needed to fully understand its role in CRC development, explore the clinical implications, and clarify the role of *F. nucleatum* — as a target, a biomarker, or a secondary by-product of tumor development. However, to our knowledge, the effect of local diet, demographics, and clinical characteristics of patients on the specific gut bacteria, comprising *F. nucleatum*, has not been investigated thoroughly in Kazakhstan.

In this study, we aimed to determine the abundance of *F. nucleatum* and other CRC-associated bacteria using quantitative real-time polymerase chain reaction (qPCR) analysis to detect the possible correlations between tumor and normal tissues, as well as relationships between patients' clinical characteristics, diet, with CRC-associated bacteria.

2 Materials and methods

2.1 Patients

A total of 83 patients with histologically confirmed colorectal adenocarcinoma (39 men and 44 women; age range, 26–86 years) who underwent surgical resection at the National Research Oncology Center, Astana, Kazakhstan, between October 2022 and April 2024 were included in this study. Patients who had other oncological diseases, received preoperative radiation or chemotherapy, and/or had distant metastases were excluded. Biopsies were obtained from carcinoma tissues (CTs), adjacent normal tissues (ATs), and distant normal tissues (NTs, 10 cm beyond the cancer margins) of patients with CRC. In total, 249 tissue biopsy samples were collected in tubes containing 20% sucrose. Culture and DNA extraction for qPCR were performed within 2 h of tissue collection, and the remaining tissues were stored in a deep freezer (-80 °C) until use. All 83 patients were included in the qPCR study.

2.2 Ethics approval

The study protocol complied with the Declaration of Helsinki and was approved by the local ethics committee of the National Center for Biotechnology of the Ministry of Health of the Republic

of Kazakhstan (Extract from Protocol No. 1, dated 04/01/2022). All methods were performed according to the relevant guidelines and regulations. Written informed consent was obtained from all participants.

2.3 Detection of CRC-associated bacteria using qPCR

DNA was extracted from the colon tissue samples using the QIAamp DNA Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration and purity were recorded using a NanoDrop spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific, USA). Specific genes were amplified by qPCR using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA) to detect four CRC-associated bacteria, namely, *F. nucleatum*, *Bacteroides fragilis*, *Escherichia coli*, and *Streptococcus gallolyticus*. The reaction mixture consisted of 5 µL SYBR Green (Biolabmix, Russia), 0.5 µL each of the specific primer pair (10 µM), and 50 ng/µL DNA template in a total reaction volume of 10 µL. *F. nucleatum* subsp. *nucleatum* (accession no. SRR24390575) and three clinical isolates (enterotoxigenic *B. fragilis* [ETBF], *E. coli*, and *S. gallolyticus*) were used for qPCR quality control. The clinical isolates were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and *16S rRNA* sequencing. The cycle threshold (Ct) values for *F. nucleatum* and other bacteria were normalized to the amount of human DNA in each reaction mix using a primer set for the reference gene, the solute carrier organic anion (*SLCO*) transporter, as described previously (19). All assays were performed in duplicates, and the results were averaged. The fold changes of *F. nucleatum* abundance in diseased tissues over that in the matched normal colorectal tissues was calculated as $2^{-\Delta\Delta Ct}$.

Previously published primers with the following sequences were used: *F. nucleatum* forward primer, 5′-ACCCTCGTGTATGG TATGAAGT-3′; *F. nucleatum* reverse primer, 5′-TCAGCAAC TTGTCCTTCTTGA-3′ (19); *SLCO* forward primer, 5′-ATCCCC AAAGCACCTGGTTT-3′; *SLCO* reverse primer, 5′-AGAGGC CAAGATAGTCCTGGTAA-3′ (19). The following primers were used to detect specific bacteria: *Bacteroides* forward primer, 5′-GGACATTTGGGAGTTCAGGAC-3′; *Bacteroides* reverse primer, 5′-TGCTTTTCTGATCTCTTCGGC-3′; *Streptococcus* forward primer, 5′-GGGAATTGTTATCGCCTGAA-3′; *Streptococcus* reverse primer, 5′-GTGCCAAAATTGGTGCTTTT-3′; *E. coli* forward primer, 5′-CTGATAGCGCGTGACAAAAA-3′; *E. coli* reverse primer, 5′-GGCACAGCACATCAAAGAGA-3′.

2.4 Statistical analysis

F. nucleatum levels determined by qPCR are given as $2^{-\Delta Ct}$, where ΔCt is the median of the difference in Ct between the test and reference genes. This relative quantification (RQ) was log-transformed to be analyzed as $\log2(1/2^{-\Delta Ct})$. The ratio of *F. nucleatum* levels between tumor and matched normal colorectal tissues is given as the fold increase, $2^{-\Delta\Delta Ct}$, where ΔΔCt is the median of the difference between ΔCt of diseased and ΔCt of normal tissues.

All statistical analyses were performed using the R software (https://www.r-project.org, version 4.2.0; the RStudio 2022.02.2). Continuous data are expressed as medians (25^{th} percentile, 75^{th} percentile) that were calculated using Student's t-test of independent or paired samples. The Mann–Whitney (Wilcoxon) test was used to compare the results for two non-paired groups. The Kruskal–Wallis test was used to compare the median levels of *F. nucleatum* between more than two groups, such as different cancer stage subgroups. Categorical variables were analyzed using Fisher's exact test. A value of $P < 0.05$ was defined as statistically significant, and a P-value between 0.05 and 0.1 was considered marginally significant.

3 Results

3.1 Clinical characteristics of patients

The clinical features of 83 patients with CRC and 249 biopsy samples were examined (Supplementary Table S1).

3.2 Frequency of occurrence of CRC –associated bacteria as determined using qPCR

The prevalence of the four CRC-associated bacteria was examined in the CTs, ATs, and NTs of patients with CRC using qPCR (Table 1). *F. nucleatum* was most frequently detected in CTs and slightly less frequently in ATs compared to that in the NTs of patients with CRC (43.4, 27.7, and 24.1%, respectively; P = 0.02).

TABLE 1 Frequency of colorectal cancer (CRC)-associated bacteria, as determined by quantitative real-time polymerase chain reaction (qPCR).

Bacteria	No. (%) of patients positive for qPCR			P value[a]
	CT (n=83)	AT (n=83)	NT (n=83)	
Fusobacterium nucleatum	36 (43.37)	23 (27.71)	20 (24.10)	0.02*
Bacteroides fragilis	40 (48.19)	31 (37.35)	25 (30.12)	0.06
Escherichia coli	57 (68.67)	53 (63.86)	43 (51.81)	0.07
Streptococcus gallolyticus	3 (3,61)	0 (0)	1 (1.20)	0.33

[a]P-values were calculated using Fisher's exact test for count data. *Statistically significant at $P < 0.05$.

However, marginally significant differences in the prevalences of *E. coli*, and *B. fragilis* in CTs were higher than in ATs, and NTs of patients with CRC (P = 0.07, and P = 0.06, respectively). No significant difference in the prevalence of *S. gallolyticus* was observed among the tissues of patients with CRC.

3.3 Correlation between *F. nucleatum* infection and clinical characteristics of patients with CRC

Compared with that in the matched normal tissues, the *F. nucleatum* load was significantly overrepresented in 75 of 83 (90.36%) CRC samples (Figure 1). The median abundance of *F. nucleatum*, as determined by $2^{-\Delta\Delta Ct}$, was significantly greater in the tumor samples (19.4 [2.4–326.7]) than that in the matched normal controls (4.39 [0.99–28.26]; P = 0.001).

The associations between the clinical variables of the patients and *F. nucleatum* infection are summarized in Table 2. In total, 58 of the 83 (69.9%) CRC cases were localized in the distal part of the large intestine. The *F. nucleatum* level, expressed as fold changes ($2^{-\Delta\Delta Ct}$; cancer versus normal tissues), in the distally located CRC (390.76 [28.78–3062.55]) was significantly higher than that in the proximally located CRC (27.19 [4.42–427.08]; P < 0.05). Distally located CRC was observed in 53 out of 75 (70.7%) patients with *F. nucleatum* over-abundance (fold change > 1) and five out of eight (62.5%) patients with *F. nucleatum* under-abundance (fold change < 1; P > 0.05). No significant association was observed between *F. nucleatum* infection and other clinical variables, such as patients' sex, age, pathological differentiation, infiltration depth, lymph node metastasis, and cancer stage (P > 0.05; Table 2).

3.4 Prevalence of *F. nucleatum* across different tumor stages and tissue types

We examined the relationship between *F. nucleatum* positivity and clinicopathological features of patients with CRC. Patients with CRC were categorized according to tumor stage as early stage (I/II) or late stage (III/IV). *F. nucleatum* was detected at similar frequencies in both the early (51%) and late (47%) stages; however, this difference was not statistically significant (Figure 2A). Regarding tissue type, the prevalence of *F. nucleatum* was significantly higher in CTs (43.4%) compared to that in ATs (27.7%) and NTs (24.1%) in patients with CRC. No significant differences were observed between the AT and NT groups (Figure 2B). In *F. nucleatum*-positive cases, the Ct values obtained by qPCR were significantly lower in CTs than those in other tissue types (Mann–Whitney U test; P < 0.0001).

3.5 qPCR analysis

Duplicate qPCR assays were conducted for detection of the four bacterial genera in the three types of CRC tissue samples. In total, 996 Ct values were generated and used for further statistical analyses. Comparison of CTs with normal tissues of patients with CRC revealed that the Ct values for *Fusobacterium* and *Escherichia* were significantly different after false discovery rate correction for multiple testing (Figure 3). Regarding the clinical characteristics with continuous values, tumor sizes in patients with *Fusobacterium*-positive CRC were significantly larger than those in patients with *Fusobacterium*-negative CRC (4.75 ± 2.33 vs. 3.27 ± 1.92, respectively; P = 0.04); similarly, tumor sizes in patients with *Bacteroides*-positive CRC were significantly larger than those in patients with *Bacteroides*-negative CRC (4.66 ± 2.28 vs. 1 ± 0, respectively; P = 2.2e - 16; Table 3). Importantly, patients with *Fusobacterium*-positive CRC consumed significantly higher amounts of processed meat than patients with *Fusobacterium*-negative CRC (31.64 ± 47.76 vs. 8.75 ± 18.08, respectively; P = 0.02; Table 3). Moreover, patients with *Bacteroides*-positive CRC consumed significantly higher amounts of processed, red, and total meat than patients with *Bacteroides*-negative CRC (29.32 ± 46.02 vs. 5 ± 7.07, P = 0.02; 175.78 ± 114.12 vs. 62.5 ± 17.68, P = 0.001; 258.78 ± 203.7 vs. 125 ± 35.36, P = 0.02, respectively; Table 3). Patients with *Escherichia*-positive CRC consumed significantly higher

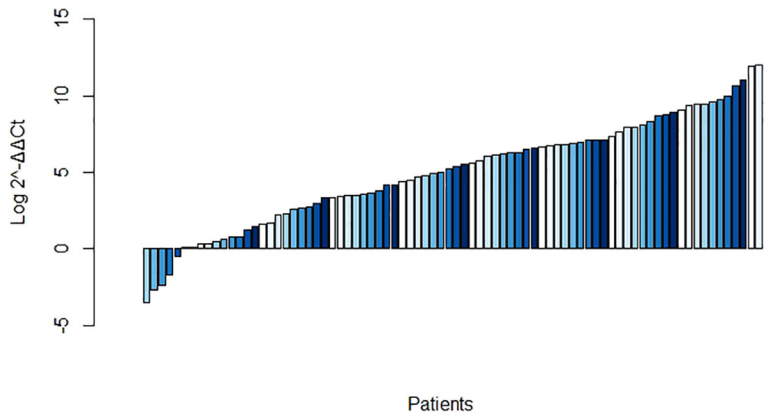

FIGURE 1
Log abundance of *Fusobacterium nucleatum* in colorectal cancer (CRC) tissues compared to that in matched normal tissues (n = 83).

TABLE 2 Association of *Fusobacterium nucleatum* infection with clinicopathological variables in a cohort of 83 patients.

	n	Fold changes between cancer and normal tissues [2-ΔΔCt (median)]	P value
Gender			
Male Female	39 44	149.55 (4.64 - 1360.26) 175.76 (27.72 - 1056.83)	0.08
Age (yr)			
<65 ≥65	45 38	178.59 (5.21 - 2834.86) 98.03 (10.53 - 959.24)	0.75
Location of CRC			
Proximal Distal	25 58	27.19 (4.42 - 427.08) 390.76 (28.78 - 3062.55)	0.03*
Differentiation			
Moderately and high 2 Low 3	32 51	37.28 (4.76 - 1027.88) 250.97 (14.89 - 1201.12)	0.72
Tissue infiltration			
T1 + T2 T3 + T4	15 67	23.20 (3.16 - 1390.43) 231.4 (10.7 - 1145.8)	0.49
Lymph node metastasis			
N0 N1 + N2	46 37	184.17 (10.19 - 2610.87) 149.55 (9.65 - 976.88)	0.22
Stage			
I II III IV	11 34 36 1	27.19 (9.19 - 1599.99) 412.34 (10.36 - 2828.17) 164.07 (8.12 - 986.53) 84.76 (84.76 - 84.76)	0.99

*Statistically significant at $P < 0.05$.

amounts of processed and total meat than patients with *Escherichia*-negative CRC (29.5 ± 45.93 vs. 0 ± 0, P = 1.228e - 5; 260.5 ± 202.28 vs. 65 ± 49.49, P = 0.04, respectively; Table 3). Furthermore, the body mass indices (BMIs) of patients with *Streptococcus*-positive and *Streptococcus*-negative CRC were significantly different (27.4 ± 4.74 vs. 25.52 ± 3.16, respectively; P = 0.04; Table 3). Regarding the clinical characteristics with binomial values, *Streptococcus* was marginally associated with sex (P = 0.05; Table 4), whereas *Fusobacterium* was associated with tissue infiltration, although the difference in CTs was marginally significant (P = 0.11; Supplementary Table S2).

The tumor sizes were significantly larger in patients with *F. nucleatum*-positive CRC than those in patients with *F. nucleatum*-negative CRC (P = 0.04; Supplementary Figure S1A). With respect to tumor location, the *F. nucleatum* Ct values (fold change) were significantly higher in the descending colon (P < 0.03; Supplementary Figure S1B) than those in other parts of the colon. The tumor stage was not significantly correlated with the presence of specific bacteria. Regarding CTs, comparison of *F. nucleatum* fold-change values (Ct values) revealed that qPCR-positive cases had higher fold-change abundance values than those of qPCR-negative cases, and these values were significantly differ (P < 0.05; Figure 4).

4 Discussion

Till date, the role of *F. nucleatum* in the development of CRC remains unclear. Determining the etiology of CRC can lead to the development of preventive and therapeutic strategies. In this study, we investigated the association between CRC, specific gut bacteria, clinical characteristics, and diet to determine the role of specific microbes in CRC development and the relationship between microbiota and red/processed meat in CRC. We determined the abundance of CRC-associated bacteria using qPCR and the statistical correlations between the clinical characteristics and outcomes of colon infections caused by the most common species that infect the colon. One of the important findings of our study is that among the infections caused by the four species analyzed, *F. nucleatum* infection is a serious and common infection. *F. nucleatum* has drawn attention for its possible link to CRC; however, infections caused by this species have been extensively studied and documented in case reports and large series of reports (20).

The gut bacteria play a significant role, and dysbiosis can lead to colonic carcinogenesis via chronic inflammatory mechanisms (21). Microbial dysbiosis can alter the host gene expression and inflammatory responses, creating a microenvironment that promotes cancer development. Several studies have shown a significant increase in the numbers of *F. nucleatum*, *S. gallolyticus*, *E. coli*, and *B. fragilis* in patients with adenomas or adenocarcinomas compared with those in healthy individuals (22).

Enterotoxigenic *B. fragilis* contributes considerably to the development and progression of CRC via its toxin-mediated effects on colonic cells and the immune system (23). Further, our study reported a 48.2% carriage rate of *B. fragilis* in patients with CRC using CT samples and a rate of 37.4% using ATs; and a 30.1% colonization rate was observed in normal tissue samples from patients with CRC, with a marginally significant difference between tissue types (P = 0.06; Table 1). However, we observed a marginally significant difference in the prevalence of *B. fragilis* between the CT and NT samples (P = 0.06; Figure 3), with the prevalence in CT (48.2%) being significantly lower than that reported in a previous study (86%) (24). A recent qPCR study revealed that only 6.1% of CRC cases tested positive for ETBF. Additionally, *B. fragilis* has been associated with good outcomes in patients with stage II and III CRC after curative resection (25). Another study found an association between fecal ETBF and CRC, with *B. fragilis* present in 58.3% of CRC cases compared to 26.6% occurrence of *B. fragilis* in controls (P < 0.05). Furthermore, the presence of *B. fragilis* in patients with stage III CRC was significantly higher than that in patients with stage I and II CRC (P < 0.05) (23). Therefore, further studies are required to determine the prevalence and distribution of ETBF.

Certain strains of intestinal *E. coli* can potentially affect the onset and progression of CRC by utilizing virulence factors and

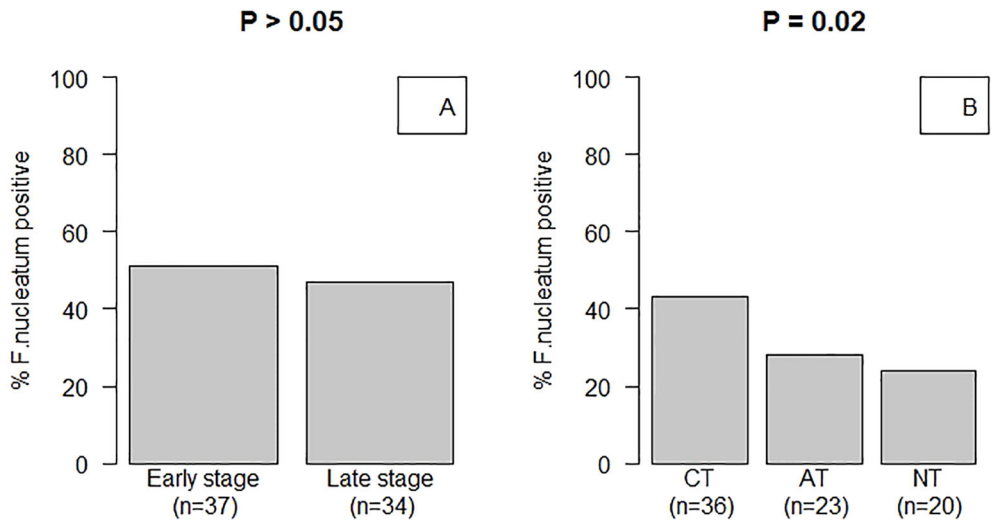

FIGURE 2
Fusobacterium nucleatum infection status of patients with CRC. **(A)** Patients with CRC were classified based on tumor stage: stages I/II were classified as early stage, and stages III/IV were classified as late stage. The prevalence of *F. nucleatum* was not significantly different between patients with early and late stage CRC (51 vs 47%, respectively; Fisher's exact test, P > 0.05). **(B)** Carcinoma tissue (CT), adjacent normal tissue (AT), and normal tissue (NT) samples were collected from non-CRC sites from patients with CRC. *F. nucleatum* was significantly more prevalent in CTs (43.4%) compared to that in ATs (27.7%) and NTs (24.1%) of patients with CRC (pairwise Fisher's exact test: CT vs. AT, P = 0.08; CT vs. NT, P = 0.04; AT vs. NT, P = 0.72). Additionally, *F. nucleatum* was observed in significantly higher numbers in CTs compared to that in NTs of patients (P < 0.02).

inflammatory pathways. Mucosa-associated *E. coli* strains are found more frequently in CRC biopsies than in healthy individuals (26). The *uidA* gene, which encodes beta-glucuronidase in *E. coli*, was used to determine total *E. coli* DNA concentrations (27). The presence of *clbB* gene a part of the *pks* island of *E. coli*, in patients with CRC might indicate an association between *E. coli* and CRC. Recent studies show that some *E. coli* strains possessing a gene cluster named the *pks* island might have a causative role in the development of human colorectal cancer (CRC). However, the results from the Japanese population showed that the prevalence of *pks*-positive *E. coli* was not significantly higher in CRC patients compared to controls (27). Deletion of the polyketide synthase (pks) genotoxic island from *E. coli* NC101 decreased tumor multiplicity and invasion in AOM/Il10−/− mice, without altering intestinal inflammation (28). Mucosa-associated pks + *E. coli* was found in a significantly high percentage of IBD and CRC patients. This suggests that colitis can promote tumorigenesis in mice, by altering microbial composition and inducing the expansion of microorganisms with genotoxic capabilities (28). In our study, the

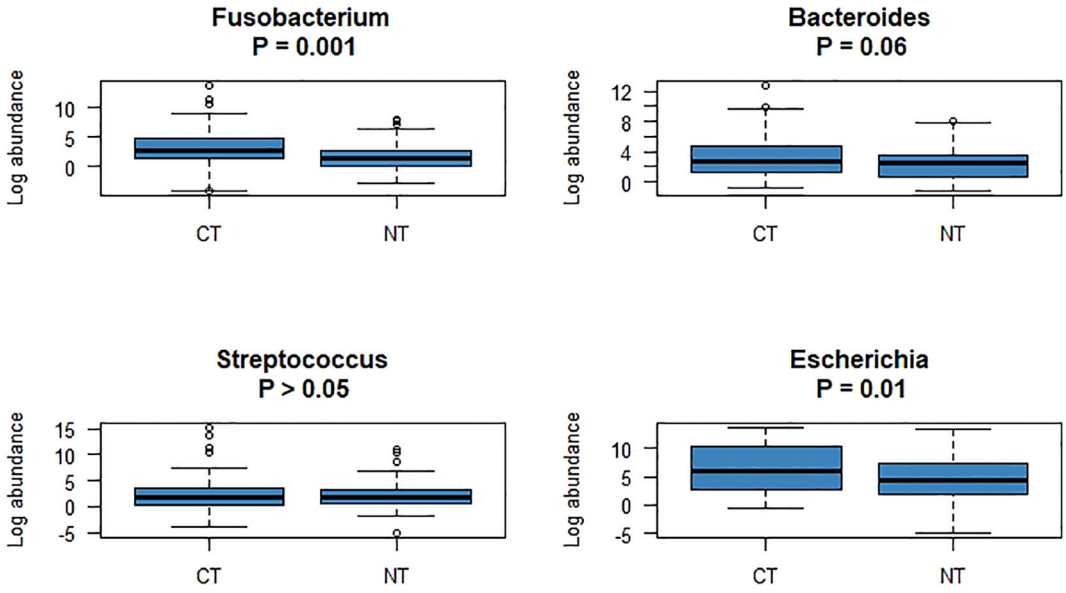

FIGURE 3
The abundances of four bacteria, which were significantly different between CTs and NTs, were compared using R software.

TABLE 3 Variations in epidemiological characteristics with continuous values based on the association of CRC with the four Ct values.

Bacteria	Relative abundance ≥1%	Age	BMI	Tumor size	CEA	Processed meat consumption, g/day	Red meat consumption, g/day	Total meat consumption, g/day
Fusobacterium nucleatum	Positive	61.36 ± 10.81	27.06 ± 4.60	4.75 ± 2.33	87.29 ± 371.32	31.64 ± 47.76	167.8 ± 109.68	252.74 ± 208.92
	Negative	64.81 ± 14.39	25.67 ± 2.78	3.27 ± 1.92	15.69 ± 25.48	8.75 ± 18.08	195.0 ± 141.15	269.5 ± 161.63
	P-value	0.46	0.18	0.04*	0.33	0.02*	0.59	0.78
Bacteroides fragilis	Positive	61.71 ± 11.47	26.97 ± 4.46	4.66 ± 2.28	NA	29.32 ± 46.02	175.78± 114.12	258.78 ± 203.73
	Negative	64.66 ± 5.77	24.31 ± 2.06	1.00 ± 0	NA	5.00 ± 7.07	62.50 ± 17.68	125.00 ± 35.36
	P-value	0.48	0.14	2.2e-16*	NA	0.02*	0.001*	0.02*
Streptococcus gallolyticus	Positive	61.60 ± 11.52	27.40 ± 4.74	4.55 ± 2.55	100.72 ± 402.03	25.44 ± 43.06	173.97 ± 118.37	243.30 ± 213.22
	Negative	62.39 ± 10.95	25.52 ± 3.16	4.60 ± 1.70	13.81 ± 22.66	34.25 ± 50.29	168.00 ± 107.96	281.81 ± 175.87
	P-value	0.77	0.04*	0.92	0.31	0.51	0.85	0.43
Escherichia coli	Positive	62.0 ± 11.37	26.89 ± 4.46	4.59 ± 2.35	NA	29.5 ± 45.93	176.05 ± 113.55	260.5 ± 202.28
	Negative	54.5 ± 4.95	26.24 ± 3.04	3.75 ± 0.35	NA	0.0 ± 0	55.00 ± 63.64	65.0 ± 49.49
	P-value	0.25	0.81	0.08	NA	1.228e-05*	0.19	0.04*

*Statistically significant at $P < 0.05$.

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TABLE 4 Variations in epidemiological characteristics with binary values based on the association of CRC with the four Ct values.

Bacteria	Relative abundance ≥1%	Sex		Diabetes		Smoking		Alcohol		Hypertension		Tumor location		MSI		Nationality		Degree of different	
		M	F	Yes	No	Yes	No	Yes	No	Yes	No	Pro	Dis	Low	High	Asians	Europeans	GII	GIII
Fusobacterium nucleatum	Positive	35	37	12	60	14	58	2	69	33	39	23	49	44	6	47	24	27	45
	Negative	4	7	3	8	1	10	0	11	6	5	2	9	8	0	10	1	5	6
	P-value	0.53		0.41		0.68		1		0.75		0.49		0.58		0.159		0.74	
Bacteroides fragilis	Positive	38	42	15	65	15	65	2	77	33	37	25	55	51	6	55	24	30	50
	Negative	2	1	0	3	0	3	0	3	1	2	0	3	1	0	2	1	2	1
	P-value	0.61		1		1		1		1		0.55		1		1		0.55	
Streptococcus gallolyticus	Positive	24	36	13	47	12	48	1	58	30	30	18	42	35	5	40	19	24	36
	Negative	15	8	2	21	3	20	1	22	9	14	7	16	17	1	17	6	8	15
	P-value	0.05*		0.22		0.54		0.48		0.46		1		0.65		0.79		0.80	
Escherichia coli	Positive	39	42	15	66	15	66	2	78	39	42	25	56	50	6	56	24	32	49
	Negative	0	2	0	2	0	2	0	2	0	2	0	2	2	0	1	1	0	2
	P-value	0.49		1		1		1		0.49		1		1		0.519		0.52	

*Statistically significant at $P < 0.05$.

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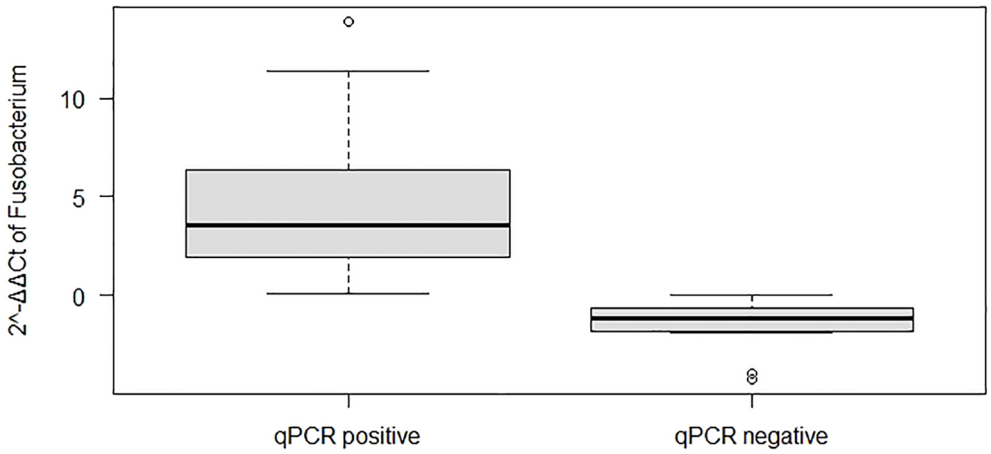

FIGURE 4
Boxplots showing the abundance of *Fusobacterium nucleatum* based on quantitative real-time polymerase chain reaction (qPCR) analysis and its positivity status.

prevalence of total *E. coli* in patients was significantly different between the CT and NT groups (P = 0.01; Figure 3). The frequency of *E. coli* among the tissue types was also significantly different (P = 0.04; Table 1). Our results indicate a potential relationship between total *E. coli* and CRC.

The prevalence of *S. gallolyticus* was investigated using qPCR with primers specific for superoxide dismutase (*sodA*) (29). Patients demonstrate higher levels of this bacterium than observed in healthy individuals (30). Colonic cells incubated with *S. gallolyticus* showed elevated levels of β-catenin, c-MYC, and proliferating cell nuclear antigen (PCNA), which are transcription factors linked to cancer development. Additionally, in mice, administration of *S. gallolyticus* results in a greater number of tumors, increased tumor burden, higher dysplasia grade, and enhanced cell proliferation and β-catenin staining in colonic crypts compared to that in mice treated with control bacteria (30). However, CRC-specific conditions such as elevated bile acid concentrations may also encourage *S. gallolyticus* colonization and perpetuate high levels of this bacterium in the gut. In our study, *S. gallolyticus* was not commonly found in patients with cancer (Table 1); however, *S. gallolyticus* was associated with BMI (Table 3).

Recently, *F. nucleatum* has gained attention as a potential cause of CRC (1, 14, 16). Although the role of *F. nucleatum* in CRC pathogenesis remains incompletely understood, four mechanisms have been proposed to explain its involvement. 1) Promotion of cell proliferation via WNT signaling through the interaction between *FadA* (adhesin A) and E-cadherin; *F. nucleatum* expresses proteins like *FadA* and *Fap2*, which allow it to adhere to host epithelial cells (31). *FadA* binds to E-cadherin on host cells, triggering β-catenin signaling pathways (32). Activation of the β-catenin signaling pathway through *FadA* binding leads to increased expression of oncogenes and enhanced WNT signaling, which promotes uncontrolled cell division and tumor growth (33). 2) Evasion of antitumor immune responses through the interaction of galactose-inhibitable autotransporter adhesion (Fap2) with T cell immunoreceptors containing immunoglobulin (Ig) and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT); *F. nucleatum* uses the Fap2 protein to bind to TIGIT, an inhibitory receptor on T cells and natural killer cells (34). This interaction reduces immune surveillance, allowing cancer cells to evade detection. TIGIT has been linked to the exhaustion of natural killer cells and T cells in CRC. 3) Binding to tumors and increase in colonization through Fap2 and galactose-N-acetylgalactosamine (Gal-GalNAc) interactions; Fap2 interacts with Gal-GalNAc sugars on cancer cells, facilitating bacterial adhesion specifically in CRC tissues (35). After adhesion, *F. nucleatum* can penetrate epithelial cells, disrupting their integrity and contributing to the development of chronic infection. 4) Contribution to chemoresistance via lipopolysaccharide and toll-like receptor mechanisms (17, 18, 20). *F. nucleatum* induces chronic inflammation, which is a well-known driver of cancer. Lipopolysaccharide, a component of the bacterial cell wall, binds to TLR4 (Toll-like receptor 4) on immune and epithelial cells, triggering NF-κB signaling, leading to the production of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and IL-17 (36). These cytokines create a pro-inflammatory tumor microenvironment, promoting cancer cell proliferation, angiogenesis, and resistance to apoptosis (37).

Fusobacterium species are obligate anaerobes that pose challenges for isolation using culture methods. In this study, the prevalence of *F. nucleatum* in the CTs of patients analyzed using qPCR (43.37%) was significantly higher than that obtained using anaerobic cultures (9.6%; data not shown). Hence, non-culture-dependent detection techniques such as qPCR analysis could be crucial for screening *Fusobacterium* species or investigating its epidemiology in a population during CRC progression (38). The presence of *F. nucleatum* was significantly correlated with the location of CRC (Table 2) (39, 40). These findings suggest that *F. nucleatum* plays a role in the early stages of CRC development. One review suggested an association between *F. nucleatum* and carcinomas at various stages of CRC progression. Analysis of *F. nucleatum* abundance by tissue type indicated a higher prevalence of bacteria in CTs than that in ATs and NTs (Figure 2B). This

observation aligns with those of previous studies indicating that elevated *Fusobacterium* colonization levels are associated with CRC (14). Certain microorganisms, such as *F. nucleatum* and *E. coli*, are prevalent in the colonic mucosa and have the potential to accelerate cancer progression and malignancy.

Conversely, diets high in red and processed meat have been associated with CRC development (41). However, the intricate metabolic and inflammatory mechanisms underlying the association between diet and cancer remain unclear. The primary carcinogenic factors associated with the consumption of red and processed meat include heme compounds, heterocyclic amines, nitrosamines, and undigested proteins (42). In addition to the direct carcinogenic effects, these molecules can alter gut microbiota, thereby influencing gene expression and disrupting colorectal epithelial cell homeostasis, which may promote the development of CRC (43).

This study had a few limitations. First, we did not incorporate innovative concepts of molecular science into the study design. Second, we did not include the gene clbB of *E. coli* in our study, which has a strong association with CRC. Longitudinal studies are needed to establish the association of pks-positive *E. coli* infection with colorectal cancer in our population. Overall, our results captured the primary characteristics of the Kazakhstani population. However, further large-scale studies are required to validate these findings.

In conclusion, our findings indicate that *F. nucleatum* is prevalent in CRC tissues and is present in different tissue types in both early and late stages of CRC. Moreover, we found a positive association between *F. nucleatum* abundance, tumor size, tumor location, and processed meat consumption in patients with CRC. The findings presented here emphasize the role of *F. nucleatum* in the tumorigenesis and progression of CRC. Further investigation is required to identify the genetic and phenotypic diversity of *F. nucleatum* colonizing tumors, which contribute to the initiation of CRC. No ethnic differences were observed with regard to this association. To the best of our knowledge, this is the first report on the association between *F. nucleatum* and CRC in Kazakhstani patients. Our findings suggest that *F. nucleatum* may be a potential marker for CRC diagnosis.

Data availability statement

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

Ethics statement

The studies involving humans were approved by The local ethics committee of the National Center for Biotechnology of the Ministry of Health of the Republic of Kazakhstan (Extract from Protocol No. 1, dated 04/01/2022). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

GK: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. BK: Formal analysis, Methodology, Writing – original draft. AG: Methodology, Writing – review & editing. TU: Methodology, Writing – original draft. DA: Methodology, Writing – review & editing. PT: Writing – review & editing, Methodology. MM: Data curation, Writing – original draft. SK: Data curation, Writing – original draft. SS: Data curation, Writing – original draft. AK: Data curation, Writing – original draft.

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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/fonc.2024.1473575/full#supplementary-material

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Deciphering the gut microbiota's role in diverticular disease: insights from a Mendelian randomization study

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Background: Previous studies have indicated a potential association between gut microbiota and diverticular disease. However, the precise nature of this relationship remains unclear. In light of this, we decided to use a bidirectional two-sample Mendelian randomization (MR) study to investigate the causal relationship between gut microbiota and intestinal diverticular disease in greater depth.

Methods: To investigate the potential causal relationship between gut microbiota and intestinal diverticular disease, we conducted a two-sample MR study in a European ancestry. Genetic instrumental variables for gut microbiota were obtained from a genome-wide association study (GWAS) involving 5,959 participants. Summary statistics for intestinal diverticular disease were sourced from the IEU Open GWAS project, which included data from 5,193 cases and 457,740 controls. The analysis was primarily conducted using the inverse variance weighted method, with additional sensitivity analyses to assess the robustness of the findings.

Results: With regard to the findings, 11 microbial taxa were identified as having a potential causal relationship with intestinal diverticular disease. Specifically, the microbial taxa Caryophanales, *Paenibacillaceae*, *Herbinix*, *Turicibacter*, *Turicibacteraceae*, and *Staphylococcus fleurettii* were found to be positively associated with the risk of developing intestinal diverticular disease, while Chromatiales, *Arcobacter*, *Herbidospora*, *Ligilactobacillus ruminis*, and *Megamonas funiformis* were found to be negatively associated with the risk. Further reverse MR analysis did not reveal a reverse causal effect between these microbial taxa and intestinal diverticular disease.

Conclusion: Our MR analyses revealed a potential causal relationship between certain gut microbiota and intestinal diverticular disease, which may provide new directions for future intestinal diverticular disease prevention and treatment strategies.

KEYWORDS

gut microbiota, instrumental variables, diverticular disease, Mendelian randomization, GWAS

1 Introduction

Diverticular disease, a prevalent gastrointestinal disorder, is particularly significant in Western countries, with prevalence rates as high as 30% in people in their 50s and more than 70% in people older than 80 (Everhart and Ruhl, 2009). In the United States, diverticular disease results in significant healthcare utilization, with more than 1.7 million outpatient visits and over 300,000 hospitalizations annually. Additionally, it leads to approximately 38,740 30-day readmissions and 4,780 deaths, contributing to healthcare expenditures of around $9 billion annually (Peery et al., 2022).Typical symptoms of the disease include abdominal pain, diarrhea, and bleeding. Depending on the severity, diverticular disease can be subdivided into simple diverticulosis (presence of symptoms but no evidence of inflammation), diverticulitis, and diverticular hemorrhage (Tursi et al., 2020). It is important to note that diverticulitis may occur in approximately 10% to 25% of patients with diverticular disease (Strate and Morris, 2019). Therefore, studying the pathogenesis of diverticular disease (especially intestinal diverticular disease) can provide valuable references for better prevention and treatment, holding significant clinical and social value.

The gut microbiota is a complex and diverse ecosystem composed of bacteria, fungi, viruses, and other microorganisms (Lozupone et al., 2012). These microbes co-evolved with the host and play crucial roles in regulating metabolism (Hacquard et al., 2015), immunity (Hooper et al., 2012), nervous system function (Cryan and Dinan, 2012), and maintaining the intestinal barrier (Adak and Khan, 2019), earning the title of the body's "second genome". However, when the gut microbiota is affected by internal and external factors, an imbalance may occur, leading to reduced microbial diversity or a disrupted balance between commensal and pathogenic bacteria (Ha et al., 2014). Studies have shown that such imbalance is closely associated with various diseases, particularly gastrointestinal disorders like intestinal diverticular disease (Canakis et al., 2020; Quaglio et al., 2022; Marasco et al., 2023). For instance, research indicates that a decrease in *Clostridium* cluster IV bacteria, known for their anti-inflammatory properties, is associated with the development of intestinal diverticular disease (Barbara et al., 2017). Another prospective study found that a decline in gut microbiota diversity, coupled with a reduction in commensal bacterial families and genera (such as *Faecalibacterium* and *Ruminococcus*) and an increase in potentially pathogenic bacteria (such as *Fusobacteria*), may be linked to a higher risk of diverticulitis (Mj et al., 2022). These commensal bacteria ferment undigested dietary fiber, producing metabolites such as short-chain fatty acids (SCFAs) that not only provide energy but also protect the gut by supporting the intestinal barrier and regulating the immune system (Arumugam et al., 2011; Vinolo et al., 2011). In contrast, pathogenic bacteria release enterotoxins that disrupt tight junctions in intestinal epithelial cells, weakening the barrier function (Hecht et al., 1992). These changes collectively may damage the structure and function of the intestinal wall, thereby increasing the risk of intestinal diverticular disease (Tursi, 2016). Although a few studies (Jones et al., 2018) have suggested that there may be no direct relationship between the composition of the gut microbiota and intestinal diverticular disease, the potential impact of microbiota alterations on this condition is increasingly recognized as more research emerges. Given the lack of conclusive causal evidence, further studies remain essential.

Mendelian randomization (MR) is widely recognized for its reliability as a statistical method. The core of the method is to utilize genetic variations as instrumental variables (IVs), which in turn provide an effective assessment of the causal relationship between exposure and outcome. In comparison to traditional observational studies, MR offers significant advantages in reducing bias, avoiding confounders, and guarding against reverse causality. Currently, MR has been applied in numerous fields, and has demonstrated its unique value in exploring the causal relationship between gut microbiota and various diseases, including appendicitis, anxiety disorders, and lymphoma (Wang et al., 2023; Liang et al., 2024; Li et al., 2024).

To date, however, few studies have employed MR to explore the causal relationship between gut microbiota and diverticular disease, particularly intestinal diverticular disease. To address this gap, our study leverages summary statistics from genome-wide association studies (GWAS) and applies a bidirectional two-sample MR approach. This methodology enables us to rigorously test the potential association between gut microbiota and intestinal diverticular disease, enhancing both the stability and reliability of our findings.

2 Materials and methods

2.1 Study design

In the forward MR analysis, we investigated the potential influence of gut microbiota on the development of intestinal diverticular disease. In reverse MR, we examined the effect of intestinal diverticular disease on gut microbiota. In this process, each gut microbiota and intestinal diverticular disease-associated single-nucleotide polymorphisms (SNP) serve as IVs for inferring causal effects between them. To ensure the validity of the IVs, the study had to fulfill the three core assumptions of MR (Figure 1). These assumptions are as follows: 1. The "correlation" assumption, where IVs are strongly associated with exposure factors; 2. The "independence" assumption, IVs are not associated with confounders; 3. The "exclusivity" assumption, where IVs are not associated with the outcome.

2.2 Data sources

The GWAS data on gut microbiota were obtained from the FINRISK 2002 study in Finland, a large population-based prospective cohort study (Qin et al., 2022). The study conducted a comprehensive genetic analysis of fecal samples from 5,959 participants. This entailed an exhaustive sample collection and analysis process, which covered 2,801 microbial taxa and 7,967,866 human genetic variants. Following rigorous statistical analysis and screening, the study identified 471 gut microbiota taxonomic groups, including 11

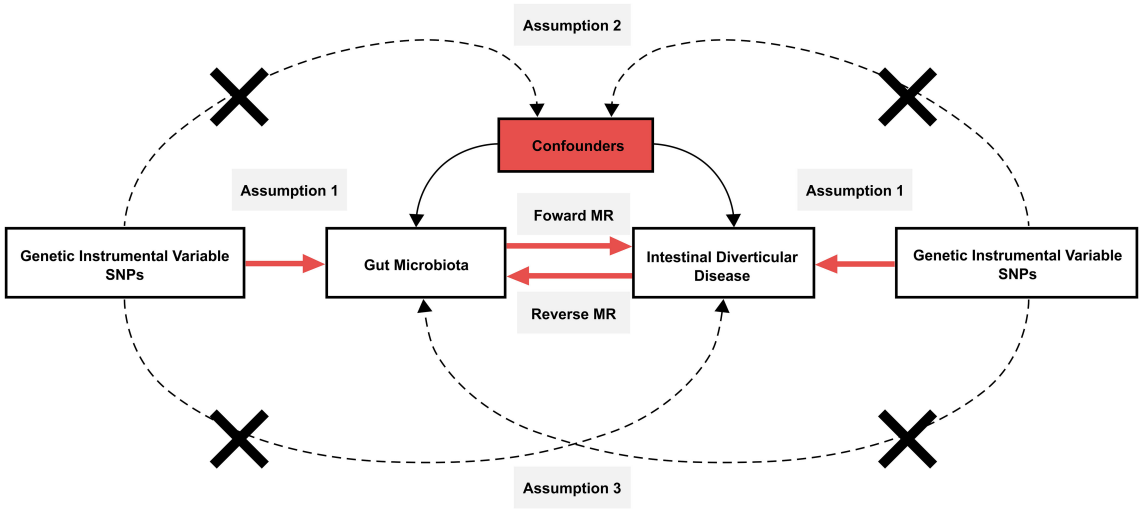

FIGURE 1
Schematic design of Mendelian randomization. Mendelian randomization requires valid genetic instrumental variables satisfying three assumptions.

phyla, 19 classes, 24 orders, 62 families, 146 genera, and 209 species. Following further data cleansing, taxonomic units that could not be accurately identified were eliminated, and 410 units were included as the main subjects of the study.

The GWAS data for intestinal diverticular disease were obtained from the IEU Open GWAS program "ukb-b-14796" (https://gwas.mrcieu.ac.uk/). The study covered 462,933 people, of which 5,193 were intestinal diverticular disease patients and 457,740 were controls. It is worth mentioning that the population participating in the above study was of European ancestry (Table 1).

2.3 Selection of IVs

To ensure the accuracy of the causal conclusion between gut microbiota and intestinal diverticular disease, we extracted SNPs that were significantly associated with exposure as IVs. The final screened SNPs were required to satisfy the following conditions: 1) Threshold $p < 5x10^{-6}$; 2) Consistent with linkage disequilibrium (LD) with $R^2 < 0.001$ and LD > 10,000; 3) *F*-statistic > 10. In addition, we utilized the LDlink to exclude SNPs that may be significantly associated with potential confounders (https://ldlink.nih.gov/?tab=home).

TABLE 1 Data sources and information used in this study.

Variable	ID	Sample size	Web resource
Gut Microbiota	PMID: 35115689	5,959	https://www.nature.com/articles/s41588-021-00991-z
Intestinal Diverticular Disease	ukb-b-14796	462,933	https://gwas.mrcieu.ac.uk/datasets/ukb-b-14796/

2.4 Data analysis

In assessing the causal relationship between exposure and outcome, we used a variety of MR methods, including inverse variance weighted (IVW), MR-Egger, weighted median, simple mode, weighted mode, leave-one-out sensitivity analysis, and MR-PRESSO. where IVW was used as the main analytical method to derive a combined causal estimate by combining the Wald ratios of all IVs based on the assumption that all IVs are valid variables (Burgess et al., 2023). At the same time, we corrected the p-values using the Bonferroni method by setting different significance p-values at different classification levels (phylum $p< 4.545\times10^{-03}$, class $p< 2.632\times10^{-03}$, order $p< 2.083\times10^{-03}$, family $p< 8.065\times10^{-04}$, genus $p< 3.425\times10^{-04}$, species $p< 2.392\times10^{-04}$) (Sedgwick, 2014). If the p-value is between the above significance p-value and 0.05, we consider that they have a potential causal relationship. MR-Egger and MR-PRESSO can be used to detect horizontal pleiotropy ($p< 0.05$). When the intercept of MR-Egger is not zero, it may imply the existence of horizontal pleiotropy, which may violate the basic assumptions of MR analysis. Leave-one-out sensitivity analysis was used to assess the degree of dependence of the results on a single IV by removing each SNP one by one and rerunning the MR analysis to observe the stability of the results. The Q-statistics of IVW and MR-Egger were used to assess the degree of heterogeneity among IVs. The presence of heterogeneity was indicated when the p-value of the heterogeneity test was less than 0.05. All analyses were based on "TwoSampleMR", "MRPRESSO", "ggplot2", "foreach" and "foreach" software packages in R version 4.3.2.

3 Results

3.1 Selection of IVs

Based on the initially set criteria, we screened 410 gut microbiota taxonomic groups and intestinal diverticular disease

for suitable SNPs as IVs, and the detailed results are shown in Supplementary Data Sheets 1–3.

3.2 MR results of the effect of gut microbiota on intestinal diverticular disease

According to the results of the IVW analysis, there was a potential causal association between 11 microbial taxa and intestinal diverticular disease ($p < 0.05$) (Figure 2). Specifically, there was a positive correlation between increasing abundance of Caryophanales and diverticular disease risk at the order level (OR 1.031, 95%CI 1.029-1.049, p=0.001), whereas Chromatiales was negatively associated with diverticular disease risk (OR 0.991, 95%CI 0.983-1.000, and p=0.038). At the family level, *Paenibacillaceae* (OR 1.011, 95%CI 1.002-1.019, p=0.013) and *Turicibacteraceae* (OR 1.003, 95%CI 1.000-1.005, p=0.036) were positively associated with diverticular disease risk. At the genus level, *Herbinix* (OR 1.007, 95%CI 1.000-1.017, p=0.049) and *Turicibacter* (OR 1.002, 95%CI 1.000-1.004, p=0.046) were positively associated with the risk of diverticular disease, while *Arcobacter* (OR 0.994, 95%CI 0.990-0.999, p=0.222) and *Herbidospora* (OR 0.995, 95% CI 0.990-1.000, p=0.039) were negatively associated with diverticular disease risk. At the species level, *Staphylococcus fleurettii* (OR 1.002, 95%CI 1.000-1.004, p=0.035) was positively associated with the risk of diverticular disease, while *Ligilactobacillus ruminis* (OR 0.997, 95%CI 0.995-0.999, p= 0.006) and *Megamonas funiformis* (OR 0.997, 95%CI 0.995-0.999, p=0.012) were negatively associated with diverticular disease risk. Furthermore, the results of other MR methods are provided in the Supplementary Table 1, and the OR in these results are consistent with those of IVW. In order to more visually demonstrate the causal relationship between these microbial taxa and intestinal diverticular disease, we plotted a scatter plot (Figure 3).

3.3 Heterogeneity and pleiotropy of IVs

We used MR-Egger intercept and MR-PRESSO to detect pleiotropy of all IVs, and the results showed nonexistent pleiotropy (p>0.05). Cochran's Q test did not find significant heterogeneity of IVs (p>0.05) (Table 2). Finally, in order to present the results of the study visually, we used leave-one-out sensitivity analysis, forest plot and funnel plot to visualize the results (Supplementary Figures 1–3).

3.4 Reverse MR results

To further explore whether there was a causal effect of intestinal diverticular disease on the above 11 microbial taxa, we further performed reverse MR analysis. Finally, they were not found to be statistically associated in the IVW method (Supplementary Table 2).

4 Discussion

This study is the first of its kind to use MR to explore potential causal links between gut microbiota and intestinal diverticular disease. Based on genomic data from 5,959 individuals, we systematically analyzed the possible roles of 410 microbiota taxonomic groups in the pathogenesis of intestinal diverticular disease. Ultimately, our study revealed potential associations between changes in the abundance of 11 microbial taxa and intestinal diverticular disease. Specifically, an increased abundance of the microbial taxa Caryophanales, *Paenibacillaceae*, *Herbinix*, *Turicibacter*, *Turicibacteraceae*, and *Staphylococcus fleurettii* may promote the development of intestinal diverticular disease. In contrast, microbial taxa such as Chromatiales, *Arcobacter*, *Herbidospora*, *Ligilactobacillus ruminis*, and *Megamonas funiformis* demonstrated a potential protective effect against intestinal diverticular disease.

The gut microbiota is a vast assemblage of microorganisms that inhabit the human gut. It is comprised of hundreds of millions of microorganisms that work in concert to form a complex ecosystem (Sandler et al., 2002). They are mainly composed of the phylum Firmicutes and Bacteroidota, with a few belonging to the phylum Actinomycetota, Fusobacteria, and

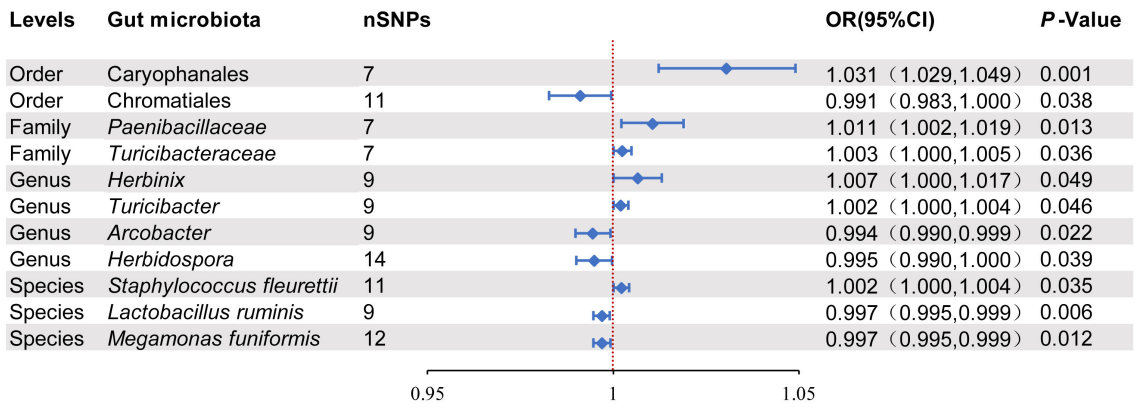

FIGURE 2
Positive MR results of causal links between gut microbiota on intestinal diverticular disease. SNP, Single-nucleotide polymorphism; OR, odds ratios; CI, Confidence interval.

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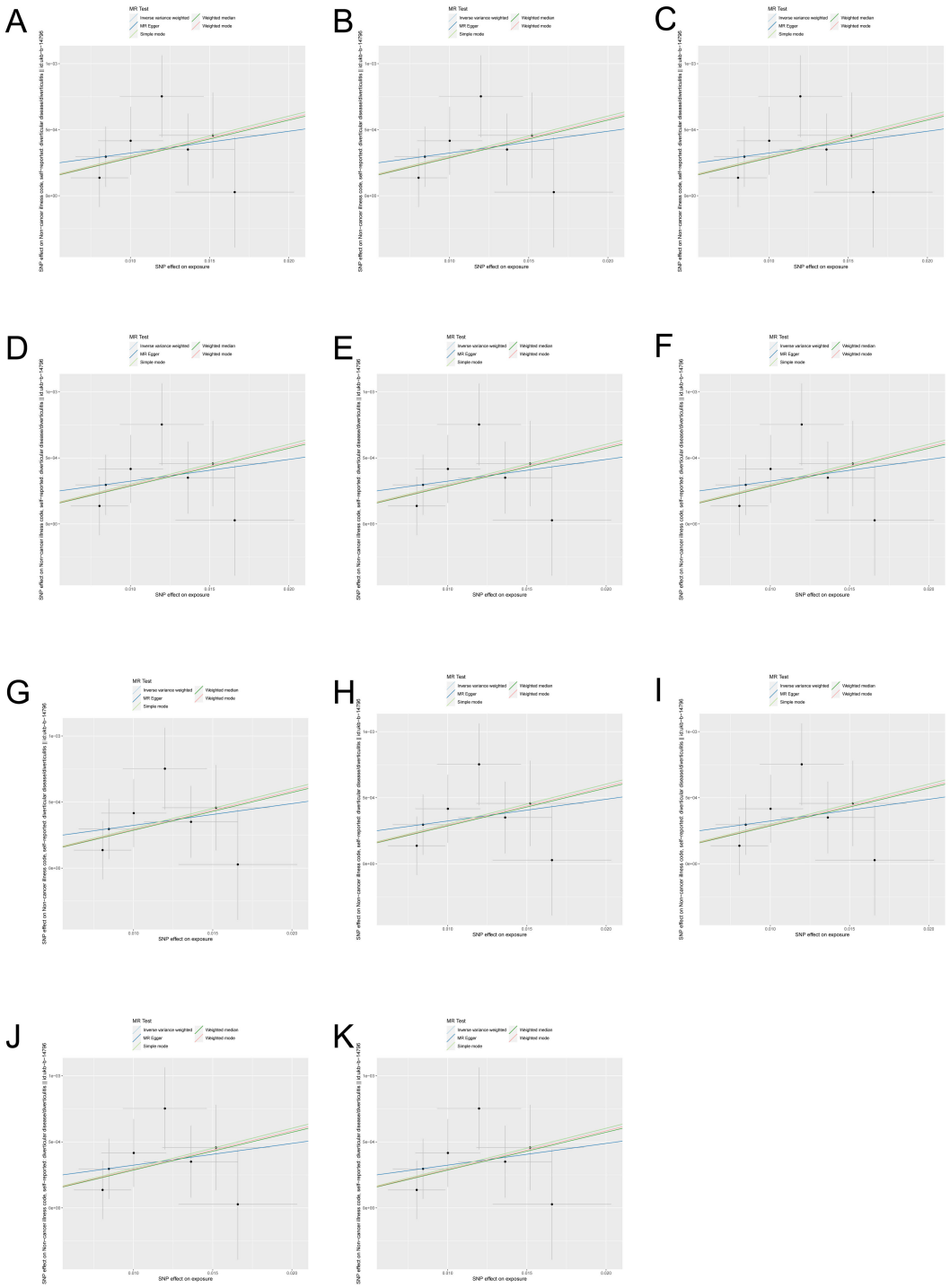

FIGURE 3
Scatter plots of gut microbiota with causal effects on intestinal diverticular disease. **(A)** Caryophanales; **(B)** Chromatiales; **(C)** *Paenibacillaceae*; **(D)** *Turicibacteraceae*; **(E)** *Herbinix*; **(F)** *Turicibacter*; **(G)** *Arcobacter*; **(H)** *Herbidospora*; **(I)** *Staphylococcus fleurettii*; **(J)** *Ligilactobacillus ruminis*; **(K)** *Megamonas funiformis*.

Pseudomonadota, among others (Eckburg et al., 2005). This microbiota composition is highly dynamic and influenced by factors such as age, diet, medication, lifestyle, and environmental exposures, which can all lead to shifts in microbial balance, often referred to as dysbiosis (Rinninella et al., 2019). Dysbiosis has been implicated in a range of systemic diseases, spanning neurological conditions like Parkinson's disease (Wang et al., 2024), gastrointestinal disorders like inflammatory bowel disease (Lloyd-Price et al., 2019) and diverticular disease, and metabolic and immune-related diseases such as obesity (Le Chatelier et al., 2013) and autoimmune disorders (Alkader et al., 2023).

TABLE 2 Heterogeneity and horizontal pleiotropy of IVs.

Exposure	Heterogeneity				Pleiotropy		
	MR-Egger		IVW		MR-Egger		MR-PRESSO
	Q	P-value	Q	P-value	Intercept	P-value	P-value
Caryophanales	3.227	0.665	3.379	0.760	$1.590x10^{-04}$	0.713	0.802
Chromatiales	5.933	0.747	6.101	0.807	$1.239x10^{-04}$	0.691	0.812
Paenibacillaceae	4.141	0.529	4.435	0.618	$-2.539x10^{-04}$	0.611	0.649
Turicibacteraceae	1.976	0.853	2.185	0.902	$-2.708x10^{-04}$	0.666	0.902
Herbinix	11.312	0.126	11.353	0.182	$1.030x10^{-04}$	0.878	0.233
Turicibacter	3.259	0.860	3.368	0.909	$-1.895x10^{-04}$	0.751	0.925
Arcobacter	6.597	0.472	6.598	0.581	$-1.331x10^{-05}$	0.982	0.637
Herbidospora	10.246	0.594	10.473	0.655	$-1.550x10^{-04}$	0.642	0.648
Staphylococcus fleurettii	10.721	0.295	11.510	0.319	$-2.717x10^{-04}$	0.437	0.340
Lactobacillus ruminis	7.089	0.420	7.343	0.500	$1.791x10^{-04}$	0.632	0.544
Megamonas funiformis	4.504	0.922	4.504	0.953	$3.703x10^{-06}$	0.990	0.957

A study conducted in Italy observed a notable increase in the abundance of the phylum Firmicutes in patients diagnosed with intestinal diverticular disease, particularly within the family *Ruminococcaceae*, with levels exceeding twice those observed in the general population (Lopetuso et al., 2018). In our study, an increased abundance of five Firmicutes (Caryophanales, *Paenibacillaceae*, *Herbinix*, *Turicibacter*, and *Staphylococcus fleurettii*) was associated with an increased risk of intestinal diverticular disease, whereas two Pseudomonadota (Chromatiales and *Arcobacter*) demonstrated a protective effect against intestinal diverticular disease. Further, an analysis of fecal samples from 28 patients with diverticulosis revealed a link between diverticulitis and increased abundance of *Pseudobutyrivibrio*, *Bifidobacterium*, and *Christensenellaceae* (Kvasnovsky et al., 2018). This supports the observed pathogenic role of *Herbinix* in intestinal diverticular disease, given its familial association with *Pseudobutyrivibrio* (both belonging to *Lachnospiraceae*). Although the odds ratios of these microbiota are not particularly large, it is important to note that their cumulative effect, when acting synergistically, could still have a meaningful impact on the development of intestinal diverticular disease. Furthermore, the majority of microbiota positively associated with intestinal diverticular disease in this study belonged to the phylum Firmicutes, which aligns with previous studies linking Firmicutes bacteria to this disease. This consistency further reinforces the reliability of our findings.

Another descriptive, cross-sectional study showed a trend toward a decrease in the number of *Clostridium* cluster IX and *Lactobacillaceae* in symptomatic intestinal diverticular disease patients, which is consistent with the protective effect of *Ligilactobacillus ruminis* against intestinal diverticular disease found in our study (Barbara et al., 2017). This mechanism may be related to the ability of these bacteria to produce SCFAs (including acetic, propionic, and butyric acids). SCFAs play a multifaceted role in supporting intestinal health. They not only activate anti-inflammatory factors, such as IL-10, which help modulate immune responses, but also stimulate B cells to produce immunoglobulin A (IgA) (Hecht et al., 1992). This production of IgA is essential for reinforcing the gut's immune defense, as IgA binds to pathogens and toxins, preventing them from penetrating the intestinal lining. In line with this, *Megamonas funiformis*, identified in our study as a protective bacterial group, demonstrates similar beneficial functions. Originally isolated from healthy human feces, *Megamonas funiformis* has recently been shown to alleviate fatty liver disease associated with metabolic dysfunction through its production of propionic acid (Sakon et al., 2008; Yang et al., 2023). This finding suggests it may exert comparable protective effects in intestinal diverticular disease by enhancing gut health and resilience. These insights reinforce the potential application of specific "probiotics" in treating or managing intestinal diverticular disease.

Staphylococcus fleurettii belongs to the genus *Staphylococcus*. Although there is a relative paucity of studies on its specific association with intestinal diverticular disease, *Staphylococcus* are widely recognized to be strongly associated with a variety of infections involving multiple sites such as the intestinal tract, urinary tract, and skin (Trautner and Darouiche, 2004; Raineri et al., 2022; Severn and Horswill, 2023). In addition, it is also worth noting that *Staphylococcus fleurettii* was initially isolated from goat cheese, a finding that further corroborates speculation in previous studies about a potential link between dietary patterns and intestinal diverticular disease (Lemes et al., 2021). *Herbidospora* has been isolated primarily from soil and plant samples. Although studies on its functionality in the gut are still insufficient, scientific studies have found that its subspecies, such as *Herbidospora daliensis*, have significant anti-inflammatory properties (Kudo et al., 1993; Chen et al., 2022). Based on this finding, it is reasonable to hypothesize that *Herbidospora* may have a protective effect against intestinal diverticular disease.

This study has significant advantages. First, this study is based on MR analysis of large-scale GWAS data, which effectively overcomes the limitations of insufficient sample size, confounding factor interference and reverse causation in observational studies. Second, by selecting a study population of European origin, this study effectively reduces the influence of ethnic differences on the study results.

However, there are several limitations to this study. First, the research focuses exclusively on a European cohort, which may limit the generalizability of the findings to other populations. Additionally, we acknowledge that the pathogenic mechanisms of intestinal diverticular disease may vary depending on its location and type. Due to data limitations, our study was unable to further differentiate between specific locations or types of intestinal diverticular disease. However, the majority of the data are based on patients with colonic intestinal diverticular disease, meaning that the results primarily reflect the relationship between gut microbiota and colonic intestinal diverticular disease. Finally, the lack of detailed information on age and lifestyle factors restricted further stratified analyses. Future research should aim to validate these findings, address these limitations, and explore in more detail the biological mechanisms underlying the relationship between gut microbiota and intestinal diverticular disease.

5 Conclusion

This study, employing a bidirectional two-sample MR approach, identifies potential causal relationships between 11 gut microbiota taxa and intestinal diverticular disease. The findings suggest that variations in microbiota abundance may influence the onset and progression of intestinal diverticular disease, with some taxa providing a protective effect and others increasing risk. Future research should validate these results across diverse ethnic groups and regions (such as Asia and Africa) to assess the impact of racial and geographic differences on the gut microbiota–disease relationship. Integrating functional genomics and experimental studies will be essential to further investigate the role of symbiotic and pathogenic bacteria in the pathogenesis of intestinal diverticular disease and gut ecological balance. Additionally, considering lifestyle factors, dietary habits, and their interactions with microbiota will be crucial in understanding their influence on disease risk. The effect of gut microbiota on intestinal diverticular disease in various sites and forms should also be explored.

In summary, this study expands our understanding of the gut microbiota–intestinal diverticular disease relationship, providing valuable insights for the development of personalized treatment and prevention strategies.

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

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

BZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DC: Data curation, Writing – review & editing. HZ: Data curation, Writing – review & editing. SL: Data curation, 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.2024.1460504/full#supplementary-material

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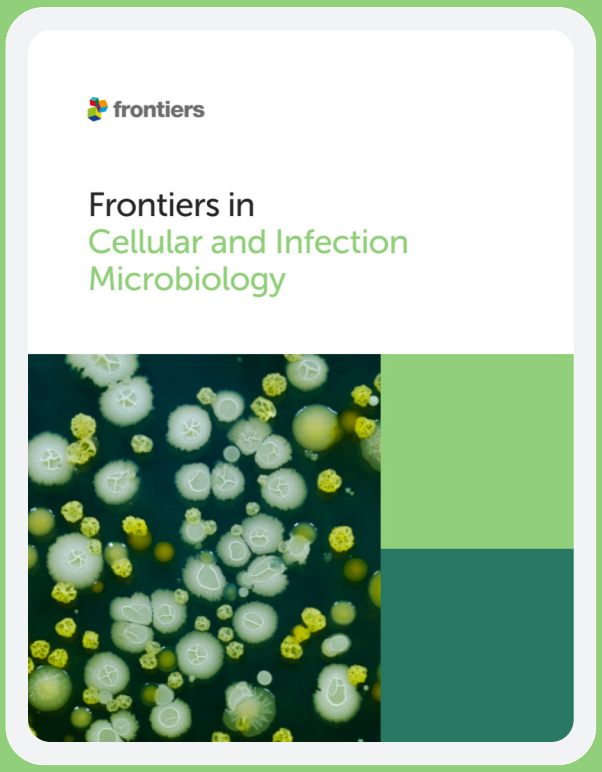

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