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Gut microbiota in health and disease

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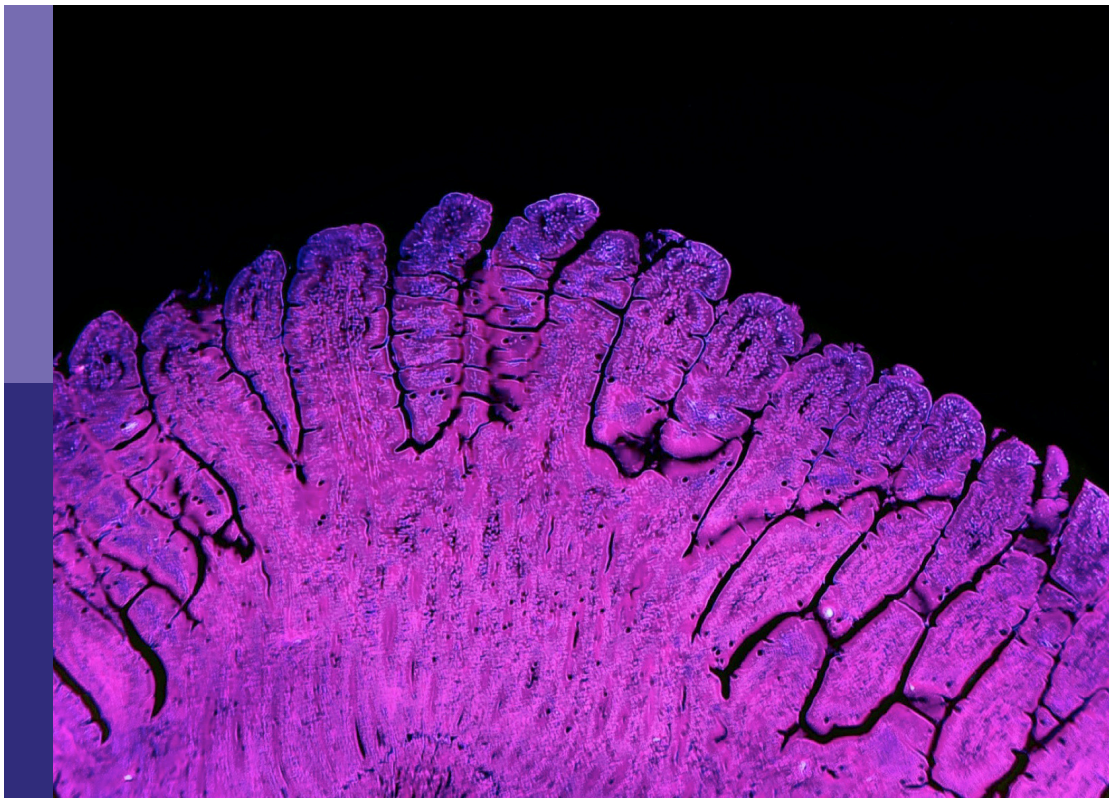

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Gut microbiota in health and disease

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Editorial: Gut microbiota in health and disease

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Editorial on the Research Topic
Gut microbiota in health and disease

In recent years, terms such as microbiota and microbiome have gained prominence in pre- and clinical research, reflecting the growing understanding of its importance for human health and disease. But what is its meaning, and why does it attract so much attention?

The microbiota refers to the set of microorganisms, such as bacteria, fungi, viruses, and protozoa, living in a given environment, including the human body and, most importantly, the gastrointestinal tract (GIT). On the other hand, the microbiome represents not only the microorganisms *per se* but also their genetic material and interactions with the host organism (1, Almeida et al.).

The gut contains the largest population of microorganisms in the human body, with more than 100 trillion microorganisms and between 2 and 20 million microbial genes. These numbers correspond to approximately 200 g of body weight, the equivalent of a medium-sized mango. Thus, can we disregard more than half of our non-human cells (microbiota) and 99% of genes (microbiome) that coexist in our body? The microbiota composition is dynamic throughout life. It begins in intrauterine life with the transfer of bacteria from mother to fetus through the placenta, which appears to be definitively established by 3-4 years of age. With aging, microbiota enters a less diversified and stable state (1, Almeida et al.).

The healthy GI microbiota comprises more than 160 species of bacteria, of which Firmicutes and Bacteroidetes phyla represent more than 90%. Firmicutes are mainly composed of *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, and *Ruminococcus* genera; Bacteroidetes are composed of the *Bacteroides* and *Prevotella* genera. Other phyla include Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (Serpa).

As aforementioned, a set of bacteria is common to all healthy humans. However, like a fingerprint, the microbiota is unique to each individual, being influenced by several modifiable factors (e.g., breastfeeding, eating habits, lifestyle, and antibiotics) and non-modifiable factors (e.g., genetics, GIT anatomy, gestational age, type of delivery, and aging). Regarding modifiable factors, gut microbes can influence human health and disease by metabolizing substrates from the diet and host to produce bioactive compounds, including signaling compounds, biological precursors, and toxins (Zhang et al.). For instance, as pointed out by Silva et al., (Almeida et al., and Zhang et al., everyday dietary components are metabolized by the gut microbiota to produce metabolites (e.g., the transformation of choline, lecithin, and carnitine, found in red meat, eggs, fish, and dairy products, into trimethylamine – TMA – and then into trimethylamine N-oxide – TMAO- through gut microbiota metabolism and liver oxidation, respectively) that have been associated with atherosclerosis, arterial hypertension, heart failure, and cerebral infarction (CI).

Furthermore, Silva et al. discussed several gut microbiota-diet interactions. A diet rich in saturated fatty acids and sweet and salty foods modifies the gut microbiota, causing elevated levels of lipopolysaccharides (LPSs) in the circulation, leading to a pro-inflammatory state (metabolic endotoxemia). Conversely, some foods have a positive effect on the gut microbiota, for example, those that elevate short-chain fatty acids (SCFAs) production and the abundance of *Lactobacillus* and *Bifidobacterium* and those that are included in the Mediterranean diet, such as olive oil. Fermented foods, wine and beer, and coffee consumption also positively affect the gut microbiota composition.

From another perspective and still considering the modifiable factors of the gut microbiota, Nobre and Costa evaluated the importance of socioeconomic factors that may affect gut microbiota composition and, thus, influence health and disease status – a new term called "sociobiome". Moreover, in a Dutch study, children residing in urban environments showed a lower abundance of *Bacteroides* and *Alistipes* than those in rural backgrounds. Conversely, in a Mexican study, the microbiota (*Prevotella copri – P. copri*, *Faecalibacterium prausnitzii*, *Rothia muciliginosa*, *Bifidobacterium* spp., and *Mitsuokella*) of children in rural areas had more anti-inflammatory characteristics that may enhance the microbiota resilience and decrease disease susceptibility.

Yet, another factor that remains largely overlooked is the significant diversity of the microbiota in the several subsections of the GIT. Serpa reinforced that different microenvironmental conditions control microbiota representativeness and density, namely, acidity, oxygen availability, the presence of antimicrobial compounds, and the time of transit through the GIT. In addition, the microbiota load increases from the stomach to the colon, creating a complex microbial ecosystem. Several studies describe sample collection from only the "small intestine" or "large intestine." Lawal et al. highlighted evidence of the different microbiota communities of intestinal sub-organs in healthy individuals. These authors emphasized that the microenvironment of the small intestine is less favorable for microbial growth than the colon due to the lower pH, increased concentration of oxygen, and antimicrobial peptides produced by host cells of the epithelial lining of the small intestine. As such, most microbes in the small intestine are fast-growing, facultative anaerobes. Regional differences are particularly noticeable when comparing the segments of the colon because microbial diversity progressively increases from the proximal to the distal colon. The colon is a more conducive habitat for microbiota growth than the small intestine because it has a longer transit time and higher pH, a lower cell turnover, a lower redox potential, and fewer antimicrobials. In this microenvironment, many bacteria in the colon are fermentative, polysaccharide-degrading anaerobes.

Thus, the gut microbiota plays a crucial role in human health and disease. These microorganisms influence not only the digestion and absorption of macro- and micronutrients but also the synthesis of metabolites essential to homeostasis, the modulation of the immune system, and even the ability to influence behavior and mood.

At a global level in the adult population, the group of significant diseases responsible for the most morbidity and mortality each year includes cardio and cerebrovascular diseases, all types of cancer, respiratory diseases (mainly infections), and mental and substance use disorders. Nevertheless, the epidemiology varies significantly across the world. For instance, in low-income countries, communicable diseases tend to rank much higher. This starkly contrasts with high-income countries, where communicable diseases may not be in the top ten, and instead, cardiovascular disease and cancers tend to contribute the largest burden (2).

Mitigating and overcoming this dismal reality is crucial worldwide. In the last few years, we have been trying to investigate better the role of environmental and host microbiota in health and disease. Understanding the cause or consequence of this situation and how to maintain or restore the composition of the gut microbiota will be very helpful in developing new preventive and therapeutic avenues.

Recent studies prove that the balance between the microbial species in the gut microbiota is fundamental for maintaining the body's homeostasis. Dysbiosis, an imbalance (altered abundance and diversity of microbiota) in the so-called healthy microbial community, can lead to increased intestinal permeability, the emergence of opportunistic microorganisms, chronic inflammation, metabolic alterations, and an unfavorable shift in the response of the innate and acquired immune systems. A growing body of proof suggests that dysbiosis is a hallmark of intestinal and several extra-intestinal diseases, such as cardiovascular and neurological disorders, cancer, and many others (1, Silva et al.; Almeida et al.; Serpa; Zhang et al.; Nobre and Costa; Lawal et al.).

In this context, we highlight some of the main points that were explored in this Research Topic that focused on the association between microbiota and different health and disease processes:

1 Immune system modulation

The commensal microbiota has been implicated in regulating a wide range of physiological processes within the GIT and at distant

tissue sites. This "external metabolic organ" interacts with the human innate and adaptative immune systems.

Microbial factors, such as virulence factors and microbe-associated molecular patterns (MAMPs), are primarily responsible for modulating the immune response. Duarte Mendes et al. and Yu et al. reviewed the immune-microbiota cell-cycle crosstalk, using colorectal cancer (CRC) pathogenesis as an explanation model for immune evasion, cancer cell survival, tumor microenvironment modulation, and metastases. The lamina propria beneath the epithelial cells (IECs) harbors immune cells, encompassing the gut-associated lymphoid tissue (GALT), including antigen-presenting cells such as dendritic cells, T cells, and B cells. The several pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs), expressed in IECs and immune cells are thought to recognize MAMPs of commensal bacteria. Thereafter, the dendritic cells are activated by the microbes or by microbe-derived elements (e.g., metabolites, products) via interactions with PRRs. When activated, they travel to the mesenteric lymph nodes and orchestrate the differentiation of naïve T cells into effector T cells, mainly Tregs and helper 17 (Th17). A subset of these cells may migrate back to the intestine or enter the systemic circulation, thus locally and systemically modulating the host's immune system. Additionally, MAMPs or microbe metabolites can also stimulate the immune system through other mechanisms, including stimulation of enteric neurons with the release of neurotransmitters that regulate the immune cell function, secretion of immunoglobulin (namely IgA), and activation of the innate immune response.

Gut microbiota can exert beneficial or detrimental effects on immune response by producing metabolic products and signaling molecules, which influence diverse functions in different organs.

Among these bacteria-derived metabolites, SCFAs have been shown to have several beneficial effects on the organism. As addressed by Serpa, the SCFAs derived from carbohydrates and amino acid fermentation are the most relevant end product to be absorbed in the human gut and used by other bacteria. The most abundant SCFAs are acetate, propionate, and butyrate. SCFAs regulate gut pH, impact the metabolic functions of invasive pathogens, inhibit their growth and reproduction, and suppress the expression of virulence genes in pathogens. The regulatory role of SCFAs in the innate immune system includes pyrin dome-containing protein 3 (NLRP3) inflammasome, receptors of TLR family members, neutrophils, macrophages, natural killer cells, eosinophils, basophils, and innate lymphocyte subsets. The regulatory role of SCFAs in the adaptive immune system includes T-cell subsets, B cells, and plasma cells. Yu et al. described one of the putative anti-inflammatory mechanisms of SCFAs, mainly through butyrate, that involves an enhancement of CD8+ T cell metabolism and their differentiation into memory T cells.

Many members of the gut microbiota are able to produce SCFAs in the colon. *Akkermansia muciniphila* (*A. muciniphila*) is recognized as a key element for producing these metabolites. In a seminal paper, Iwaza et al. reviewed the state of the art of this particular species belonging to the Verrucomicrobia phylum. In 2004, Derrien et al. (3) discovered and isolated *A. muciniphila* from the stool of a healthy individual. *A. muciniphila* relies on mucin for carbon, nitrogen, and energy. The capacity of this bacteria to degrade and use mucin as a unique source of carbon and nitrogen gives it significant importance in the human GIT, allowing other bacteria to survive and grow by using the metabolites resulting from mucin degradation. SCFAs also play a role in the inflammatory status of the host, regulating the immune system and improving the gut barrier function (3).

SCFAs have been demonstrated to be relevant in several pathologies. In CRC, an increased abundance of pathogenic microbes, such as *Fusobacterium nucleatum* (*F. nucleatum*), and a decreased abundance of butyrate-producing bacteria have been observed, resulting in lowered SCFAs levels and enhanced inflammation (Yu et al.). In hypertension, lowered butyrate-producing gut microbial counts and deficient intestinal absorption of SCFAs have been observed (Almeida et al.). Koester et al. also pointed out that *F. nucleatum*, linked to CRC progression and metastasis, has been associated with CpG island methylator phenotype in the female sex. Furthermore, this group investigated the ambivalent role of RET as an oncogene or tumor suppressor in CRC. Their study offered a proof-of-principle that CRC risk-modulating gut microbial effects depend on sex and genetics, and they underscored the importance of evaluating sex as a biological variable in research and of reporting the sexes of both human and non-human study participants.

Zhu et al. reported another example of the anti-inflammatory potential of SCFAs. Their pioneering study found that serum levels of stress-inducible 72-kDa heat-shock protein (HSP72) and zonulin, immunomodulatory and anti-inflammatory proteins, were increased in patients with CI. Accordingly, the upregulation of these proteins was related to specific gut microbiota alterations and the clinical severity of CI. Moreover, the abundance of bacteria *Eubacterium fissicatena* (*E. fissicatena*) and *E. eligens* groups and *Romboutsia* manifested a remarkably positive correlation with serum HSP72. The abundance of bacteria *E. fissicatena* group and *Acetivibrio* had a significantly positive correlation with zonulin levels. The genus *Eubacterium* has been identified to contribute to massive aspects of human health, for most of the family produce SCFAs, especially butyrate.

Nevertheless, the other side of the coin can also happen, and some microbiota-derived metabolites have been linked to an increased risk of certain diseases. TMAO appears to be correlated with cardio and cerebrovascular diseases. (Almeida et al., Zhang et al.) Indeed, bacterial translocation from the gut to the heart and the discovery of bacterial DNA in atherosclerotic plaques led to the gut being considered a potential reservoir of opportunistic microorganisms. According to the narrative review of Almeida et al., relevant data from 19 prospective studies reported that higher levels of TMAO and its precursors were associated with a higher risk of major adverse cardiovascular events and all-cause mortality. Also, there appears to be a graded association between TMAO levels and the risk of subsequent cardiovascular events in patients with recent ischemic stroke. These TMAO inflammatory signals involve NF-κB, NLRP3 inflammasome, MAPK/JNK pathway, and gut microbiota modulation. Although, according to Zhang et al.'s systematic review, which included six studies of acute ischemic stroke and one study of intracerebral hemorrhage, there is limited evidence indicating that high baseline plasma levels of TMAO may be associated with poor IC outcomes.

Moreover, based on the potentially predictive risk of gut microbiota for cardiovascular disease, Almeida et al. and Silva et al. reviewed data describing that there could be an association between leaky gut and higher levels of LPSs in the bloodstream. These endotoxins are released when gram-negative bacteria die and lyse, releasing their content into the surrounding environment. Therefore, LPSs and their derivates act as MAMPs and induce acute and chronic inflammatory responses when entering the bloodstream, as the immune system recognizes these active substances as foreign invaders. Furthermore, Almeida et al. emphasized that gut microbiota can also affect the host's insulin resistance, glucose metabolism, and certain hormone levels, such as leptin and ghrelin, which can lead to increased inflammation or regulate appetite, leading to atherosclerosis.

Still, within the scope of metabolites with potentially harmful effects on health, Serpa described the role of cysteine in microbiota and human cells crosstalk, favoring cancer. Cysteine is a very relevant compound in cancer metabolism that constitutes the main thiol in the biological fluids of cancer patients, which comes from endogenous synthesis, transsulfuration pathway, and protein degradation or by increased intestinal absorption of cysteine intestinal content that originated from diet and microbiota metabolism. In some types of cancer, this amino acid was shown to be a relevant carbon source, sustaining bioenergetics and biosynthesis, and a pivotal source needed for ATP production, cell cancer survival, and disease progression. Moreover, cancer cells that exhibit metabolic dependence on cysteine account for increased glutathione levels and scavenging capacity of reactive oxygen species to cope with oxidative stress, contributing to better antioxidant potential.

These findings reinforce data indicating that microbiota could modulate directly or indirectly immune processes in specific individuals, potentially influencing the predisposition to the risk of some diseases and their clinical course.

2 Microbiota disease signatures

There is an accumulation of evidence that the human gut microbiota plays a role in maintaining health and that dysbiosis is associated with risk for many communicable and non-communicable diseases. Furthermore, microbial signature taxa are being identified for the diagnosis of some diseases, like ulcerative colitis, Crohn's disease, irritable bowel syndrome, depression and anxiety disorders, auto-immune disorders, cancer, and COVID-19 infection, among others.

Lawal et al. highlighted evidence about the variations in the composition of the microbiota communities identified at specific sites along the GIT in healthy individuals and patients with inflammatory bowel diseases (IBD), ulcerative colitis, and Crohn's disease, which are characterized by persistent inflammation and gut damage. IBD patients have different microbiota than healthy individuals (e.g., in the duodenum, the beneficial genera of bacteria *Bifidobacterium* and *Lactobacillus* are notably decreased in IBD, whereas the populations of *Bacteroides* and *Escherichia* genera are increased).

In their review, Bibbó et al. explored the role of the gut-brain axis in depression and anxiety disorders. Indeed, gut microbes can interact with the brain, interfering with behavior through mechanisms such as amino acid metabolism, SCFAs, vagus nerve, endocrine signaling, and immune responses. For instance, a systematic review showed that about 50 bacterial taxa exhibit differences between patients with major depressive disorders and controls.

Finally, several studies have shown that cancer patients often experience changes in the composition of their gut microbiota compared to healthy individuals (1, 4). These changes may be associated with an increased risk of developing cancer.

As earlier mentioned, specific intestinal pathogens, such as *F. nucleatum* or colibactin-producing *Escherichia coli*, are associated with CRC (1–Yu et al.). Chen et al., through Mendelian randomization (MR) analysis, investigate causal associations between gut microbiota and intrahepatic cholangiocarcinoma (ICC), an aggressive liver cancer with a poor prognosis. Genetically predicted increases in *Veillonellaceae*, *Alistipes*, *Enterobacteriales*, and Firmicutes were suggestively associated with higher ICC risk, while increases in *Anaerostipes*, *Paraprevotella*, *Parasutterella*, and *Verrucomicrobia* appeared protective. Bioinformatics analysis revealed that differentially expressed genes near gut microbiota-associated loci may influence ICC through regulating pathways and tumor immune microenvironment.

Parallelly, the gut microbiota has been significantly associated with differentiated thyroid cancer (DTC). However, the causal relationship between the gut microbiota and DTC remains unexplored. Thus, Hu et al. investigated the causal relationship between the gut microbiota and DTC. In this context, four bacterial traits were associated with the risk of DTC (class Mollicutes, phylum Tenericutes, genus *Eggerthella*, and order Rhodospirillales). Additionally, four other bacterial traits were negatively associated with DTC (genus *E. fissicatena* group, genus *Lachnospiraceae* UCG008, genus *Christensenellaceae R-7* group, and genus *Escherichia Shigella*).

Observational epidemiological studies suggested an association between the gut microbiota and breast cancer (BC). Still, it remains unclear whether the gut microbiota causally influences the risk of BC (1, 4). Zhang et al. employed a two-sample MR analysis to investigate this association. The inverse variance-weighted (IVW) MR method examined the causal relationship between the gut microbiota and BC and its subtypes. The IVW estimates indicated that an increased abundance of genus *Sellimonas* was causally associated with an increased risk of estrogen receptor-positive (ER+) BC, whereas an increased abundance of genus *Adlercreutzia* was protective against ER+ BC. For human epidermal growth factor 2 positive (HER2+) BC, an increased abundance of genus *Ruminococcus2* was associated with a decreased risk, whereas an increased abundance of genus *Erysipelatoclostridium* was associated with an increased risk. In a case report, Vilhais et al. described the longitudinal analysis of the gut microbiota of an ER+/HER2- BC patient throughout the therapeutic approach with a 6-month regimen of endocrine therapy (ET) plus a CDK 4/6 inhibitor (CDK4/6i). This clinical case evidenced a shift in gut microbial dominance from Firmicutes to Bacteroidetes primarily due to a noteworthy increase in

the relative abundance of *P. copri* following the treatment course. *P. copri* is an abundant member of the human gut microbiota, whose relative abundance has curiously been associated with positive and negative impacts on several diseases, alongside some pharmacomicrobiomic implications. The link between *P. copri* and different types of cancer remains inexplicable. However, some hypothesize that *Prevotella* genera may be involved in breast disease due to its estrogen-deconjugating enzymatic activity. The role of *P. copri* and other bacterial species capable of metabolizing estrogens in BC, called "estrobolome", is particularly interesting for future research (1, 4).

Parallel to what was observed at the gut level, it also appears that there are specific local microbiota signatures for each type and subtype of cancer. These findings result from a close relationship between the intestine and the primary tumor and/or an environment conducive to the growth of microorganisms at the tumor microenvironment level. Is there a tumor-gut axis? (1, Yu et al., Vilhais et al.5) In this sequence, Vilhais et al. showed in the analysis of the local microbiota of the breast surgical specimen an interestingly high dissimilarity between the residual tumor and respective margins, suggesting markedly different microbial compositions. While the margins revealed a more diverse distribution of microbial species, the tumor's microbial composition was dominated by fewer species, particularly *Streptococcus pneumoniae* and *Atopobium vaginae*. Additionally, the authors described the data of a preclinical study reporting that *Streptococcus* in BC cells can inhibit the RhoA-ROCK signaling pathway to reshape the cytoskeleton and help tumor cells resist mechanical stress in blood vessels, thus promoting hematogenous metastasis.

3 Pharmacomicrobiomics

Finally, inter-individual heterogeneity in drug response is a serious problem that affects the patient's well-being and poses enormous clinical and financial burdens on a societal level. Understanding the role of the gut microbiota in drug response may enable the development of microbiota-targeting approaches that enhance drug efficacy and decrease toxicity. Pharmacomicrobiomics is an emerging field investigating the interplay of microbiota variation and drug response and disposition (absorption, distribution, metabolism, and excretion). Modulating the gut microbiota has the potential to become a very attractive approach to managing drug efficiency toward more personalized medicine (1, 6).

Manipulating the gut microbiota through diet, biotics, or fecal transplantation (FMT) is being investigated as a potential strategy for several diseases. For instance, as Silva et al. mentioned, microbiota-dependent SCFAs production can be enhanced by consuming high-fiber diets such as the Mediterranean one. In a pre-clinical study, Nguyen et al. investigated the effect of an exopolysaccharide (EPS) probiotic molecule produced by the commensal bacterium *Bacillus subtilis* (*B. subtilis*) on BC phenotypes. Although *B. subtilis* is commonly included in probiotic preparations and its EPS protects against inflammatory diseases, it was virtually unknown whether *B. subtilis*-derived EPS affected cancer. Short-term treatment with EPS inhibited the proliferation of specific BC cells, while more extended treatment in mice led to tumor growth. Additional experiments are needed to determine the physiological relevance of EPS on BC, and a favorable risk-benefit ratio is warranted to be implemented in clinical practice.

Silva et al. also addressed a hot topic among researchers and clinicians, the FMT. This procedure is an established treatment for recurrent *Clostridioides difficile* infections (CDIs). Furthermore, FMT is indicated for patients with multiple recurrences of CDI for whom appropriate antibiotic treatments have failed, and it has cure rates of 80%–90%. In addition, it seems promising as a treatment for many other conditions, like IBD, obesity, metabolic syndrome, psychiatric neurological diseases, COVID-19, and cancer (1, 2, 7, Almeida et al., Duarte Mendes et al., Yu et al., Bibbó et al.). This procedure consists of collecting feces from a healthy donor and introducing them into a patient's GIT to treat a certain disease linked with the alteration of the gut microbiota. FMT can be performed through the upper GIT, via a duodenal tube or capsules taken orally, or through the lower GIT via colonoscopy or an enema. The authors discussed the importance of including the dietary patterns of stool donors and receptors in the stool donor screening process and the importance of monitoring receptors' diet to ensure the engraftment and success of the FMT (Silva et al.).

Regarding toxicity, drug–microbial interactions can be categorized into two classes: microbiota modulation of toxicity (MMT) and toxicant modulation of the microbiota (TMM). MMT refers to transforming a drug (chemical) by microbial enzymes or metabolites to modify the chemical in a way that makes it more or less toxic. TMM is a change in the microbiota that results from chemical exposure. An example of MMT could occur by the induction of host-detoxifying enzymes by microbial metabolites that shift the metabolic pathway for a chemical and result in differential toxicity levels (6). Gonçalves-Nobre et al. reviewed some mechanisms, including the irreversible dose-dependent anthracyclines cardiotoxicity related to oxidative stress and the reversible cardiotoxicity with trastuzumab in BC treatment. The authors highlighted that altered gut microbiota composition has been linked to long-term cardiotoxicity. *Bacteroides* spp., *Coriobacteriaceae UGC-002*, and *Dubosiella* have deleterious effects on the myocardium, mainly due to the promotion of inflammation. On the other hand, *Alloprevotella*, *Rickenellaceae RC9*, *Raoultella planticola*, *Klebsiella pneumoniae*, and *E. coli BW25113* can induce cardioprotection predominantly by increasing anti-inflammatory cytokines, promoting intestinal barrier integrity and early metabolization of doxorubicin.

The relationship between microbiota, health, and disease is complex and multifaceted. It involves interactions between microorganisms, inflammatory processes, metabolism, and immune responses. More research is needed to elucidate better these mechanisms, identify optimal interventions, and determine their efficacy and safety in different clinical settings.

Author contributions

DAC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project

administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. PC: Supervision, Validation, Visualization, Writing – review & editing. CC: Supervision, Validation, Visualization, Writing – review & editing. AF: Supervision, Validation, Visualization, Writing – review & editing.

Conflict of interest

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

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The authors declare that no Generative AI was used in the creation of this manuscript.

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The putative role of gut microbiota in cancer: Cysteine is a pivotal coin

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Tumor metabolism is mandatory for the proper adaptation of malignant cells to the microenvironment and the acquisition of crucial cellular skills supporting the systemic spread of cancer. Throughout this journey, the contribution of the gut microbiota to the bioavailability of nutrients supporting the bioenergetic and biosynthetic requirements of malignant cells is an issue. This review will focus on the role of cysteine as a coin that mediates the metabolic crosstalk between microbiota and cancer. The key points enclose the way cysteine can be made available by the microbiota, by degradation of more complex compounds or by *de novo* synthesis, in order to contribute to the enrichment of the colonic microenvironment as well to the increase of cysteine systemic bioavailability. In addition, the main metabolic pathways in cancer that rely on cysteine as a source of energy and biomass will be pointed out and how the interspecific relationship with the microbiota and its dynamics related to aging may be relevant points to explore, contributing to a better understanding of cancer biology.

KEYWORDS

gut microbiota, cancer metabolism, cysteine reliance, cysteine bioavailability, aging-related dynamics

Introduction

In the human organism, several interspecific relationships are constantly in operation, which are established between the different species that make up the microbiota and the human cells of the various organs where it resides. These interspecific relationships are mainly symbiotic in which both partners benefit. This is the case in health, but in disease, there are still some doubts about the role of the microbiota in the pathophysiology, namely, in the context of cancer, at both the organ and systemic levels. Currently, new clues have been proposed, and several studies have been developed to determine the influence of microbiota in cancer initiation, progression, and therapy, as it is extensively reviewed (1–7).

Metabolic adaptation in cancer is undoubtedly an essential requirement for the establishment, growth, and spread of a malignant neoplasm. Cellular plasticity is crucial for the adaptation of the tumor cell to the microenvironment of the organ where carcinogenesis occurs and to the emergence of stress conditions, such as drug exposure. Recent studies prove that cysteine metabolic circuits are a relevant component of the metabolic network, sustaining biosynthesis and bioenergetics and allowing chemoresistance (as reviewed in 8–10). This review intends to confront some of the most recent findings in the field of cysteine metabolism in cancer and the role of the intestinal microbiota in the dynamic balance of the control of cysteine bioavailability and its putative impact on the progression of oncological disease.

Gut microbiota composition, interplay, and aging-related evolution

Microbiota is defined as a group of microorganisms that live in a given environment, and it includes bacteria, fungi, protozoa, and viruses, even though viruses are not living organisms. Considering the fungal community, a minor component of gut microbiota compared to bacteria (11), the prevalent genera are *Saccharomyces*, *Candida*, *and Cladosporium* (11–14), with *Candida albicans* the most frequently found in feces of healthy individuals (15). Nevertheless, *C. albicans*, like other intestinal yeasts (16), also presents an opportunistic behavior pattern, being implicated in the development of some infectious diseases (11). Albeit the main studies dedicated to gut microbiota are focused on bacteria, it is known that fungi are important in microbiota reestablishment and equilibrium, immune control, and gut protection (17, 18). The role of fungi and bacteria in the immune response is similar, and these two populations interact and control their own density (16). Importantly, gut fungi seem to be pivotal not only in gut physiology but also in other organs physiology such as the liver, brain, lungs, and kidney (16). Since fungi present specificities that are not deeply explored in cancer, this review will be mainly focused on the bacterial component of gut microbiota.

The gastrointestinal (GI) microbiota is composed of more than 160 species of bacteria organized in a few phyla, as reviewed by Rinninella etal. (19). Firmicutes and Bacteroidetes phyla represent more than 90% of microbiota, and Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia phyla account for the major part of the remaining 10%. Firmicutes are mainly composed of *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, and *Ruminococcus* genera, and Bacteroidetes are composed of the *Bacteroides* and *Prevotella* genera. GI microbiota is organized from the small intestine to the colon (Figure 1), and, based on mouse studies, the small intestine is dominated by Lactobacillaceae, while in the colon, the following prevail: Prevotellaceae, Lachnospiraceae, and Rikenellaceae (20–22).

Different microenvironmental conditions control microbiota representativeness and density, namely, acidity, oxygen availability, the presence of antimicrobial compounds, and the time of transit through the GI tract (20, 22). These variations allow the establishment of facultative anaerobes in the small intestine and of anaerobes, able to digest carbohydrate fibers, in the colon (20, 22).

Along the gut, there are two different microenvironmental niches, the lumen and the mucinous barrier close to the mucosa (mucosal layer), in which the representativity of bacteria is different (23, 24). The formation of a mucous biofilm near mucosa assembles features favoring certain bacteria proliferation and controlling the preferential consumption of particular organic compounds. The impact of these two different niches (mucosal and luminal) on the dynamics of the same bacterial species is clearly described (25). However, microbiota composition works in an individual-specific manner, although a group of designated core bacteria seems to be represented in most individuals. More studies are needed to explore microbiota dynamics in health and disease, and currently, efforts are being made to define the major features required for microbiota to optimize the host systemic metabolism. Most studies trying to map the microbiota distribution and prevalence in the gut are guided by genomics and transcriptomics (26, 27); they revealed that a group of specific bacterial genes seem to be constantly present in the microbiota pool, suggesting that they are crucial for microbiota physiology and they may consequently benefit human physiology. Nevertheless, genomics and transcriptomics are not fully informative to disclose the bacterial physiology and indicate which main pathways support metabolic functioning; thus, biochemical studies are also needed. In addition, research directed to human microbiota is mandatory, since the majority of studies were developed in animal models, and they may not fully represent the human microbiota or the physiology.

The main contributors to microbiota selection and dynamics are energy and biomass sources from diet and host components. Different studies proved the impact of diet on microbiota, mainly relating to dietary patterns in childhood and the typical diet in different spots of the globe with the relevance for a variety of bacterial genera (21, 27, 28). Nonetheless, these studies predominantly consider the enrichment of the intestinal lumen with simple free sugars and carbohydrate fibers, including also the short-chain fatty acids (SCFAs) resulting from the fermentation of the latter ones (27, 29–32). The prevalence of specific energy sources selects bacterial species and contributes to their distribution since they tend to localize in niches enriched with substrates they can degrade. Therefore, species capable of

degrading mucins are placed in the mucus layer where they can digest carbohydrates and release the simplest sugars to be used by bacteria without the mucolytic ability (33).

Even the gene expression profile of the host, which determines the composition of the mucus layer, contributes to the selection of bacteria. Intestinal mucus is mainly composed of mucin 2 (MUC2), produced by goblet cells, which is an *O*-glycosylated protein (34). The diversity of *O*-glycans ornamenting MUC2 is conditioned by the genotype and the expression profile of genes encoding glycosyltransferases. Interestingly, the host glycosyltransferase expression profile can be modulated by the action of some species, such as *Ruminococcus gnavus*, *Lactobacillus casei*, and *Bacteroides thetaiotaomicron*, which are somehow able to control the colonization of other bacterial species (35–37). Thus, MUC2 represents an important substrate to which bacteria can adhere and proliferate, but it also harbors important energy and biomass sources for microbiota. The gut biochemical fraction of the microenvironment for sure exerts a crucial selective pressure on microbiota, regulating the balance between bacterial species. Furthermore, the symbiosis established between the microbiota members is also pivotal to control bacteria representativeness and density. Some species produce organic compounds to be shared and used by other species; for instance, *Eubacterium hallii* and *Anaerostipes caccae* can produce butyrate from acetate and lactate, respectively, produced and released by *Ruminococcus bromii* and *Lactobacillus* sp. or *Bifidobacterium* sp. (38–40). Afterward, butyrate is used by human cells as a valuable carbon source and also as a modulator of gut homeostasis and mucosa turnover, due to its role as an epigenetics regulator (41–44).

The impact of aging on evolution and changing of gut microbiota representativeness and diversity is controversial. However, it seems that aging-related alterations in molecular composition and architecture of the intestinal mucosa correlate with a decrease in microbiota diversity (reviewed by (45). Multivariate analysis shows a continuous aging advancement of human GI microbiota along with host aging course (46). Together with this, the metabolic capacity and putative contribution to human physiology will also be remodeled. Hence, the capacity of the microbiota to produce SCFA and degrade starch is reduced with aging, while proteolytic capacity is increased (47, 48). This fact can explain the increased inflammatory process in the intestine of elderly people, due to the lack of the protective effect of SCFA (49), mainly butyrate, whereas the increased capacity to degrade proteins can account for the emergence of cancer beneficial conditions, as it will be discussed later. Furthermore, age-related disequilibrium of the microbiota can favor the installation of novel potentially pathogenic microorganisms.

In gut microbiota equilibrium, cysteine is a major nutrient, not only as a metabolic player but also as a controller of certain pathogenic species, which can overtake microbiota, which is the case of *Clostridium difficile*. *C. difficile* is a nosocomial bacterial responsible for antibiotic-related diarrhea, upon the destruction of the normal gut microbiota (50, 51). In normal conditions, other bacteria control *C. difficile* density by the production of antibiotic compounds and by the regulation of microenvironmental levels of controller nutrients (51–53). Cysteine is one of these nutrients since it functions as a growth and metabolism controller (51–53) and as an inhibitor of the synthesis of *C. difficile* toxin (54). *Escherichia coli* also presents a growth pattern sensitive to cyst(e)ine availability; upon the expression of highly efficient cystine importers, *E. coli* becomes sensitive to oxidative stress because bacteria import excessive cystine, but it is not able to properly metabolize cysteine; thus, this metabolic profile endangers *E. coli* survival (55). This behavior can be triggered by other bacteria as an antibiotic mechanism, and when out of control, it can be a threat to the balance of the microbiota. Nevertheless, *E. coli* strains that are able to metabolize cysteine and produce H_2S present oxidative stress and antibiotic resistance (56). Hence, cysteine metabolic ability is an important feature to control bacteria density in gut microbiota.

The main substrates are metabolized by the gut microbiota and contribute to gut enrichment with cysteine and cysteine-related compounds

The focus of this paper is the role of cysteine in microbiota and human cells crosstalk, favoring cancer; therefore, the way cysteine is generated and enriches the gut lumen is an important point to address. Dietary proteins are a source of cysteine, and their digestion occurs along the GI tract, being a considerable proportion (about 10 g) digested in the colon (57). In there, bacteria degrade proteins and use the amino acids for the synthesis of new proteins, peptides, or other organic and inorganic compounds (58). The degradation of the host proteins is also a relevant contribution to cysteine and other amino acid release; for instance, MUC2 presents cysteine-rich domains that are very important for the MUC2-3D structure and the formation of the mucous biofilm (59); thus, MUC2 degradation contributes to cysteine enrichment of gut microenvironment. Moreover, Daniels etal. (60) described that Firmicutes bacteria are able to exclude cysteine from the sequence of cytoplasmic and exported proteins, indicating that this way bacteria are capable of maintaining their resistance to reductant environments since they seem to have acquired an evolutionary skill and they do not rely on disulfide bounds to survive. However, cysteine is used in detoxifying systems, releasing bacteria from damaging compounds, as already described in some Firmicutes genera such as *Staphylococcus*

that used bacillithiol (BSH)-related detoxifying systems (61). Therefore, this detoxification process recycles cysteine and may be one more mechanism accounting for cysteine enrichment of luminal gut fraction.

From carbohydrates, the SCFA is the most relevant end product to be absorbed in the human gut and also to be used by other bacteria. The most abundant SCFA are acetate, propionate, and butyrate, and among them, butyrate plays an important role in human physiology, as it is a valuable energy and biomass source, but it is also an epigenetic regulator, controlling gene expression (62). Importantly, butyrate can also be synthesized from amino acid fermentation (Figure 1); there is a pivotal metabolic link between butyrate and cysteine since cysteine fermentation is a way of butyrate production (63). In health, butyrate is important in cell renewal, but in cancer, butyrate impacts cell proliferation control and activation of cell death. Thereby, butyrate protects the organism from cancer due to its action as a histone deacetylase inhibitor (HDACi) (64).

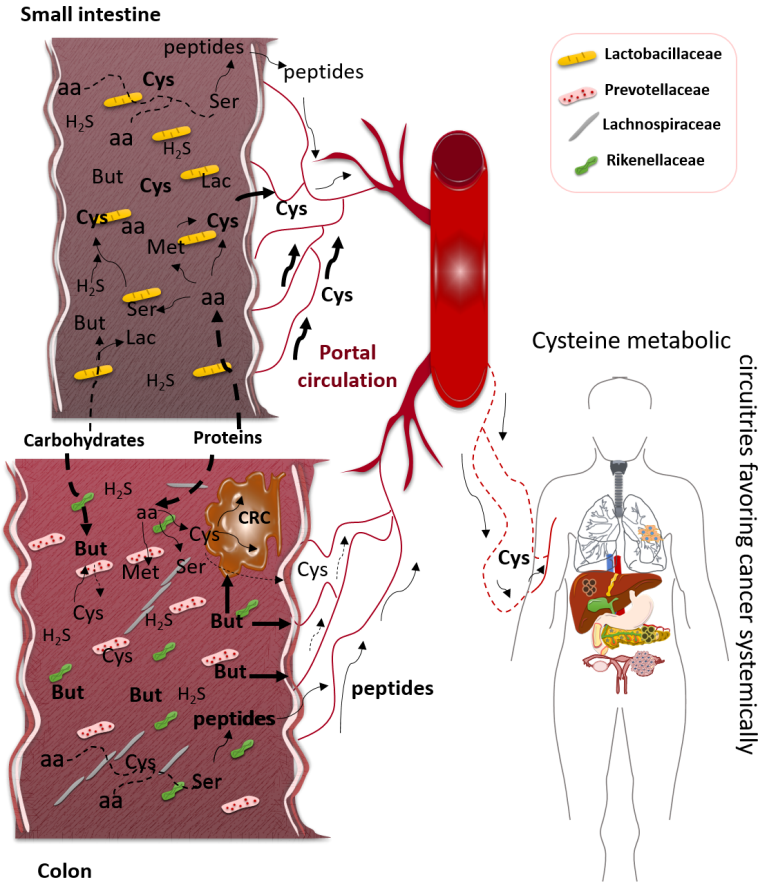

FIGURE 1
Impact of gastrointestinal (GI) microbiota physiology in cysteine bioavailability, favoring cancer. In small intestine, the prevalent bacterial family is Lactobacillaceae, which can degrade some carbohydrates to generate short-chain fatty acids (SCFAs), namely, butyrate (But), and degrade dietary and host proteins to release amino acids (aa), such as serine (Ser), methionine (Met), and cysteine (Cys). In colon, the dominant bacteria families are Prevotellaceae, Lachnospiraceae, and Rikenellaceae, which represent the most SCFA-producing bacteria, here represented by But. Most parts of dietary proteins are degraded in the colon with the release of peptides and free amino acids (aa). These aa, including Cys, will be mainly absorbed in small intestine since the colon mucosa does not present an efficient absorption of free aa. The peptides can be absorbed by the small intestine and colonic mucosae, and after absorption and distribution through the bloodstream, they can constitute a source of aa, including Cys. Cysteine can result from the degradation of proteins or be synthesized from But or Ser reacting with hydrogen sulfide (H_2S) or directly from Met. The bulk of H_2S is Cys-derived, and Cys can also be used to synthesize But. Colorectal cancer (CRC) cells can benefit directly from But, the ones that retain the capacity of metabolizing it and Cys to sustain the metabolic remodeling. Systemically, cancer placed in any organ can benefit from Cys bioavailability, while it enters the blood, to be used as an energy and biomass source, as well as an oxidative stress controller.

However, it is described that cancer cells are sensitive to butyrate, as it will function as a HDACi and induce cancer cell death. Nonetheless, some alterations in the microbiota or the metabolic fitness of cancer cells may affect the butyrate-protective outcome. Of note, colorectal cancer patients present a microbiota with diminished representativeness of butyrate-producing bacteria (65); in addition to this, cancer cells that retain the ability to fully metabolize butyrate or are able to adjust their metabolism due to butyrate exposure can escape from cell death control and benefit from butyrate as a carbon and energy (66, 67). Autophagy has also been demonstrated to underlie butyrate resistance in cancer cells (68). Importantly, the synthesis of cysteine from butyrate and hydrogen sulfide (H_2S) by gut microbiota is not proven, but a study exploring the *in silico* relation of gout arthritis and microbiota physiology indicates that butyrate-producing bacteria are mainly responsible for cysteine production (69). Since other SCFAs, such as acetate, are implicated in cysteine production (69, 70), maybe butyrate can be an undisclosed cysteine source (Figure 1).

Additionally, H_2S, which mainly results from cysteine degradation (71), is a very important player in gut microbiota physiology, and its metabolism can be helpful to understand cysteine microbiota:host interdependency (62) since H_2S is also a substrate for the synthesis of cysteine by microbiota (72). As reviewed by me (9), in humans, cysteine metabolism is deeply connected with one-carbon metabolism having cobalamin (vitamin B12) as a central compound in the intercross spot between folate and methionine cycles. Importantly, 31% and 37% of daily reference intake of, respectively, cobalamin and folate are estimated to come from gut microbiota (73). In this context, serine and methionine, two important players in one-carbon metabolism, are also connected with cysteine synthesis in gut microbiota. It was described that some strains of *L. casei* are able to synthesize cysteine from serine and H_2S (72) and methionine (74). As mentioned before, Lactobacillaceae is a prevalent group of bacteria resident in the small intestine where enterocytes are fully capable of absorbing amino acids (Figure 1).

Impact of gut microbiota physiology on cysteine bioavailability

The bioavailability of a nutrient is the pool of this nutrient that is systemically available to be used by the whole-body cells. In the scope of this review, it is important to summarize the contribution of microbiota not only for the enrichment of cysteine in the intestinal microenvironment but also for whether this enrichment can contribute to cysteine bioavailability.

Dietary and host proteins are, upon degradation, important sources of cysteine (Figure 1). Hence, cysteine resulting from dietary and host proteins degradation could be conceptually absorbed by colonocytes, contributing to cysteine bioavailability. Nonetheless, it seems that mammalian colon mucosa is not proficient in absorbing amino acids (75), despite epithelial colonic cells expressing a representative panel of amino acid transporters (75). Unfortunately, as reviewed by van der Wielen and co-authors (75), most studies analyzing the expression of amino acid transporters were performed at the transcriptional level, not ensuring the expression of the functional protein and not allowing the evaluation of the cellular localization of these receptors. Nevertheless, if amino acids are efficiently absorbed in the colonic mucosa, meaning if they in fact enter the colonocytes and are directed to the blood, they follow a subcellular route different from that of the small intestine epithelial cells. In the small intestine, amino acid absorption occurs in the apical membrane of the cell, and their release into the bloodstream is performed through the basolateral cell membrane, while studies in pigs and horses showed that in colonocytes, the transport of lysine is only detected in the apical cell membrane without detecting how these amino acids can reach the blood (76). As indicated, in the small intestine, oligopeptides resulting from proteins that were degraded by gastric and pancreatic enzymes (pepsins and proteases) are subsequently digested by peptidases in the brush border of the intestinal wall, and free amino acids are further transported into intestinal cells, follow an intracellular circuit, exported though the basolateral membrane, and canalized into the blood circulation (77, 78). As mentioned above, butyrate can be synthesized from cysteine fermentation (63). Butyrate is mainly produced by Firmicutes species (63, 79), and interestingly, upon aging, these bacteria become less representative in gut microbiota (e.g., *Faecalibacterium prausnitzii*) (48), suggesting that aging by modulating microbiota density and representativeness can decrease the protection of colonic microenvironment against cancer since butyrate concentration decreases together with its anti-cancer effect. Furthermore, the decreased rate of butyrate production can contribute to the accumulation of cysteine in the gut lumen and consequently increase the absorption of cysteine by epithelial cells. Once again, the absorption capacity of colonocytes needs to be explored, since the majority of studies analyzed the absorption of amino acids by indirect methods, measuring preferentially the amount of absorbed nitrogen and not specifically the amino acid-derived nitrogen (80–84). This makes it difficult to determine the contribution of microbiota-released or microbiota-synthesized amino acids for systemic bioavailability, including cysteine. Furthermore, the studies dedicated to the physiological control of amino acids in the gut are antique; it is in fact a requirement to perform new studies with more sensitive and accurate methods.

Bacteria in microbiota also use free amino acids to synthesize peptides (85), which makes part of amino acid turnover pathways, but it also favors alternative ways for colonocytes to take up amino acids without depending on specific amino acid transporters. Cystine is the dipeptide of cysteine, and it seems

that most parts of cysteine may be transported across the cell membrane as cystine, mainly mediated by xCT, a glutamate/cystine antiporter. The xCT is expressed in a normal colon and may be a quite specific way of absorbing cysteine from the colonic lumen (86). Likewise, the peptide transporters (PepT) can be an alternative route to compensate for the inefficiency of colonocytes to uptake free amino acids. For instance, PepT1 is one of the most studied and is responsible for the transport of various peptides resulting from diet and putatively from microbiota metabolism (87, 88). In fact, the carrier-mediated absorption of peptides accounts for the major fraction of amino acids absorbed in the gut (89, 90). The inclusion of free amino acids in di- and tri-peptides by microbiota facilitates their import by colonocytes that are unable to transport free amino acids; this is also true for cysteine. Glutathione, a tripeptide of glutamate, cysteine, and glycine, from diet seems to be directly absorbed in the intestine (91), and it is a valued source of cysteine. Additionally, gut microbiota produces glutathione that is absorbed and exerts a great impact on the human body's antioxidant control (92). Again, as demonstrated in ovarian cancer, glutathione turnover and cysteine metabolic reliance are crucial to sustaining the adaptive capacity of cancer cells as well as chemoresistance (93–95).

Regarding human cysteine bioavailability, it is thought that the bulk of absorbed amino acids come through enterocyte absorption, in the small intestine, and amino acids in the colonic lumen will be mainly used for bacteria metabolism or may be absorbed as di- or tri-peptides but very few as free amino acids.

Cysteine metabolic circuitries favoring cancer

Cysteine occupies a core position in cancer cell metabolism (Figure 2). As described, cysteine is an important player in oxidative stress control, as a free amino acid or included in the glutathione molecule. The control of the redox cellular state is a key ability allowing the maintenance of the metabolic flow (96–99). On the one hand, the cysteine metabolic reliance provides increased glutathione levels and an efficient turnover, which permits cancer cells to cope with stressful conditions, such as hypoxia and drugs (93, 94). Hence, cysteine fitness constitutes a relevant mechanism of chemoresistance for cancer cells accounting for their capacity of escaping from the action of

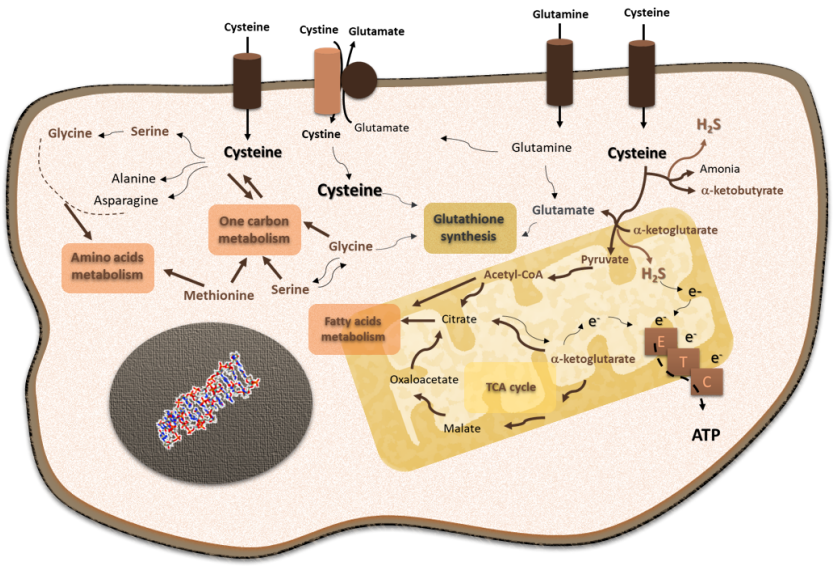

FIGURE 2
Cysteine is a core player in cellular functioning, supporting its pivotal role in cancer cell metabolism. Cysteine is imported as cystine or as cysteine. Cysteine plays a pivotal role in cancer: it is incorporated in glutathione, a reactive oxygen species (ROS) scavenger; upon degradation in cytosol or mitochondria, cysteine supplies carbon and energy metabolism through fatty acids and amino acid syntheses, tricarboxylic acid (TCA) cycle, one-carbon metabolism, and the production of ATP through the electron transport chain (ETC), and it contributes to sulfur and energy production as a generator of hydrogen sulfide (H_2S) and a donor of electrons (e–) to the ETC.

oxidative and alkylating anti-cancer drugs (93, 94, 100–106). On the other hand, cysteine is posited as valuable bioenergetics and biosynthetic source, able to replace core metabolic elements, such as glucose and glutamine. The cysteine metabolic network depends on its versatility as sulfur and as a carbon source. This network presents three main steps: 1) cysteine transport across the cell membrane, 2) cysteine catabolism, and 2) cysteine anabolism.

Cysteine import

Cysteine uptake is mediated by specific transporters, and cysteine can enter the cell as a free amino acid or as a dimer, cystine (107–111). The increased expression of xCT is described in cancer as being associated with more aggressive and chemoresistant phenotypes (100, 107, 112–116), and despite that most of these studies concern glutamate export, the role of cysteine uptake in the maintenance of those tumors can be assumed since for glutamate to leave the cell, cyst(e)ine entrance is mandatory. Although cystine is the main form taken up by cancer cells, cancer cells can also import cysteine directly (117) by overexpressing specific cysteine transporters, namely, the amino acid transporter 3 (EAAT3; SLC1A1 gene) (Nikolaos Pissimissis, Efstathia Papageorgiou, Peter Lembessis, Athanasios Armakolas, 2009; 108, 118) and the alanine-serine-cysteine-transporter 2 (ASCT2; SLC1A5 gene) (119–121). Since these transporters also mediate the transfer of other amino acids, their expression in the cancer context is not always associated with cysteine dependence. Furthermore, considering ferroptosis, a newly described cell death process, the intracellular levels of cysteine are crucial for the maintenance of glutathione to ensure the lipid peroxide scavenging. This process is catalyzed by glutathione peroxidase 4 (GPX4), which uses glutathione as a substrate. This way, xCT is associated with resistance to ferroptosis (122).

Cysteine catabolism

Cysteine degradation depends on four enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercapto-pyruvate sulfurtransferase (MpST), which acts after cysteine aminotransferase (CAT) (123). Cysteine catabolism generates H_2S and different organic compounds, such as pyruvate, serine, and α-ketoglutarate (124–129). H_2S functions as an electron donor to the electron transport chain (ETC) (124, 130, 131), and it also acts as a signaling molecule, regulating cellular processes relevant to cancer, namely, cell survival, proliferation, and angiogenesis (93, 94, 132, 133). The organic compounds generated from cysteine degradation can be canalized into different metabolic pathways, such as the tricarboxylic acid (TCA) cycle, one-carbon metabolism, amino acids, and fatty acid syntheses.

Cysteine can also be a source for pathways that are typically related to glucose, such as gluconeogenesis and the pentose phosphate pathway (PPP). Cysteine may be used to synthesize glucose through gluconeogenesis, as it originates from pyruvate and the gluconeogenic amino acid, alanine. This way, cysteine contributes to the transient pool of glucose within the cell. A very recent study showed that in fact cysteine is used to generate alanine and lactate (95), mainly synthesized from pyruvate that presents a transient permanence in the cell. Gluconeogenesis is currently receiving some attention in cancer, as a way of increasing glucose yield in the cell without depending on glucose bioavailability and transport (reviewed by 9, 10). Another glucose-dependent pathway is PPP, which can benefit from the link between glucose and cysteine metabolism by using cysteine-derived glucose. Moreover, the inhibition of the final step of gluconeogenesis prompts glucose-6-phosphate into PPP. Furthermore, cysteine contributes to glucose metabolic flow by controlling the redox state of the cell, since the pivotal enzymes of gluconeogenesis and PPP, respectively, PCK1 (phosphoenolpyruvate carboxykinase 1) and G6PD (glucose-6P-dehydrogenase), are directly regulated by Nrf2, a master regulator of redox control, which is sensitive to oxidative stress that is consequently dependent on cysteine circuitries (134, 135). In addition, PPP is also a player in redox control having cysteine-derived glutathione as an intermediate (136). Therefore, cysteine is a valuable carbon source used by cancer cells to support their energy and biomass demands.

Cysteine anabolism

Cysteine synthesis occurs through the transsulfuration pathway (TSP), and it depends on the sequential action of CBS and CSE, which are also involved in cysteine catabolism, as mentioned. The TSP is a metabolic branch that sprouted from the deviation of homo-cysteine from the methionine cycle in one-carbon metabolism (137). Homo-cysteine is condensed with serine by CBS, and the resulting cystathionine is hydrolyzed by CSE, giving rise to cysteine, ammonia, and α-ketoglutarate (138). Here, a link between cysteine metabolism and the TCA cycle can be found through α-ketoglutarate. The degradation of oxidized glutathione (GSSG), through the γ-glutamyl cycle, will allow the recycling of its three components: glutamate, cysteine, and glycine. GSSG exits the cell, and its degradation is catalyzed by γ-glutamyl transpeptidase (GGT) located at the external face of the cell membrane (139). After glutamate is released, the cysteinylglycine dipeptide can re-enter the cell through PEPT2 and be converted to cysteine and glycine upon the action of dipeptidases (140), or it can be degraded by aminopeptidase N (APN), and cysteine and glycine are again available to re-enter

the cell (141). Cysteine synthesis is linked to different amino acid metabolism; for instance, glycine and serine are glutamine-derived and are important suppliers of the folate cycle from one-carbon metabolism (as reviewed by 9).

The enzymes involved in both cysteine catabolism and anabolism, CBS and CSE, are frequently associated with malignancy and more aggressive cancer phenotypes (124, 127, 142–147), suggesting that at least one of the two pathways are relevant in cancer, and they might be working simultaneously as a way to keep on moving the metabolic cellular network. Concerning MpST, little is known about its association with cancer; however, there are some indications provided by *in vitro* assays with pharmacological inhibitors and silencing approaches, suggesting that this enzyme can be critical for cancer cell proliferation, bioenergetics, and cell signaling (125).

The metabolism of cysteine associated with its transport is composed of an endless circle moving a huge number of intermediaries that can be made available for the most varied metabolic pathways (Figure 2). In this way, cysteine provides the malignant cell with plasticity and adaptive capacity, which will benefit the progression of the disease. The enrichment of the tumor microenvironment and biological fluids in cysteine is a strong indication that this is true. Thus, the systemic bioavailability of cysteine is strictly necessary for the success of the oncological disease, and all contributions to increase these indices will contribute to the poor prognosis of the disease.

Gut microbiota affects cancer progression by controlling cysteine bioavailability, also upon aging

In the human gut, bacteria work together, and the metabolic symbioses are important components of gut biological dynamics. The metabolic expertise of different bacterial species and strains (79) keeps on the metabolic flow based on organic compounds sharing, in which some compounds are produced by certain bacteria to be used by other bacteria, contributing to the maintenance of a healthy variability and density of microbiota, ideally preserving corresponding metabolic profiles. The metabolic dynamics of gut microbiota influence human health and disease.

Metabolomics is used to assess the metabolic interplay between microbiota and host by metabolically mapping different human body fluids. Most studies on the gut microbiota metabolome are designed to investigate dysbiosis, which means disease-related metabolic profiles (148). Actually, the gut microbiota metabolome helps to define metabolic profiles that may be useful to distinguish between unhealthy and healthy individuals (reviewed by 149). Different studies have found metabolic signatures associated with inflammatory, metabolic, and neurological/neurodegenerative disorders and cancer (150–154). The studies dedicated to cancer presented promising results associating gut microbiota and metabolome with disease specificities. In colorectal cancer, associations were found with microbiome and metabolome in different disease stages (155). Genomics and metabolomics data reported that the gut microbiota regulates the immune response in hepatocellular carcinoma (156). Trials were proposed to explore the diagnostic and prognostic values of the definition of gut microbiota metabolome in breast cancer (157). Recently, Hermida etal. (158) presented a predictive study of therapy response using The Cancer Genome Atlas (TCGA) datasets from different cancer types; the authors concluded that it is possible to predict in naive biopsies, which will be the therapy outcome of tumors based on tumor microbiome RNA-seq and whole-genome sequencing analyses.

The microbiota and cancer interplay is an important connection to explore, since it encloses a possible contribution of microbiota functional network to cancer metabolic reliance, favoring systemic disease progression. We and others described that cancer patients' body fluids are enriched in cysteine, which can come from endogenous synthesis, transsulfuration pathway, and protein degradation or by increased intestinal absorption of cysteine intestinal content that originated from diet and microbiota metabolism.

Cysteine is a very important compound in cancer metabolism from different perspectives, and studies have demonstrated that cysteine is the main thiol in the biological fluids of cancer patients. In ovarian and pancreatic cancers, cysteine was shown to be a relevant carbon source, sustaining bioenergetics and biosynthesis, as well as a pivotal H_2S source needed for ATP production (93, 94, 159). Furthermore, and considering all the cancer progression journey, chemoresistant cancer cells exhibit cysteine metabolic reliance accounting for increased glutathione levels and consequently augmenting the scavenging capacity of reactive oxygen species (ROS) needed to cope with oxidative stress, which simultaneously will abrogate the cytotoxic action of most drugs conventionally used to treat cancer (8). The tumor and the systemic microenvironment are rather important in carcinogenesis and disease progression, and assuming the relevance of microbiota, we must define different scenarios since gut-located tumors will directly access organic compounds generated by microbiota, whereas tumors developed in other organs need those organic compounds to reach the bloodstream. Different contributions are needed to increase cysteine intestinal absorption mediated by membrane transporters. Since they have a regulated expression, the substrate availability, in this case cysteine-enriched gut microenvironment, is a stimulus for the expression of transporters by epithelial and cancer cells. As mentioned, little is known about the expression dynamics of cysteine transporters at the protein functional levels. This would be a prevailing step in setting up the contribution of microbiota for cysteine bioavailability in health and disease.

Considering colorectal cancer, cancer cells placed in a cysteine-rich microenvironment might benefit from cysteine without being dependent on the absorptive capacity of colonic mucosa (Figure 1). Cancer cells express different cyst(e)ine transporters that mediate its uptake directly from the colonic lumen. In colorectal cancer cells, a pivotal cyst(e)ine transporter is xCT and a mechanistic loop sustaining xCT expression involve cysteine-derived H_2S-dependent persulfidation of OTUB1, the deubiquitinase that regulates xCT stabilization, suggesting cyst(e)ine through the metabolic circuitries control the expression of its own transporter (160). Moreover, xCT is also expressed in the normal colon, but it was demonstrated that its overexpression in colorectal tumors is associated with the activation of MELK oncogene and Pi3K and RAS pathways, being xCT pharmacological blockade a way of affecting cancer cells tumorigenesis (5, 111). Actually, xCT was proposed as a biomarker for colorectal cancer recurrence (161), reinforcing the role of xCT and the need for cysteine as an important metabolic hallmark in cancer. However, other types of cancer developed in different organs present specificities that encompass the need for cysteine absorption. For sure, the pool of cysteine absorbed in the small intestine (162, 163) will benefit cancer cells with metabolic reliance on cysteine, being a cysteine pool that originated from diet and microbiota metabolism (Figure 1). Outside of the gut, the cysteine pool in the tumor microenvironment comes from the bloodstream, and the metabolic activity of cancer and non-cancer cells shares the same niche. The cysteine reliance of cancer cells implies a frequent uptake of cyst(e)ine, even when the endogenous cysteine synthesis is occurring, thereby expressing different cyst(e)ine transporters, a cancer cell can manage the import of cysteine according to its own metabolic state and needs (9).

The impact of cysteine on cancer advance is also seen in cancer patients' survival and cachexia. Cachexia is a life-threatening condition associated with different diseases and causes extreme weight loss and muscle wasting (164). Cachexia is a marker for poor cancer prognosis, occurring in about 80% of patients and accounting for at least 20% of cancer-related deaths (164–166). A study dedicated to the cachexia effects in a GI cancer cohort revealed that patients who received cysteine supplementation in parenteral nutrition had shorter overall survival as compared to those who did not receive cysteine (167). In the same study, the authors demonstrated that cyst(e)ine deprivation suppresses the growth of colorectal xenograft tumors and potentiates the oxaliplatin effect, and the mice did not lose weight (167).

In brief, cysteine interdependence of microbiota and cancer cells can be seen at least in two ways: 1) cysteine made available by microbiota can be used by cancer cells as a metabolic source, and 2) cysteine-derived compounds, such as glutathione and H_2S produced by microbiota, can be used by cancer cells as antioxidants and as important players in metabolic flow and energy production. As depicted in the review by Bonifácio etal. (10), cysteine follows different circuitries in the metabolic network, serving as a metabolic coin but also as a regulator of metabolism, accounting for cellular and body homeostasis. Cysteine versatility in cancer received recently more attention since new studies disclosed the panoply of pathways that are dependent on cysteine bioavailability, emphasizing that the cysteine metabolic map is a pivotal component of cancer cells' metabolic remodeling in order to cope with stressful conditions imposed by the tumor microenvironment and by severe disturbance of body equilibrium in advanced diseases.

Conclusions

In this review, several aspects of the intestinal microbiota and cancer duality were addressed, which together demonstrate that there is an opportunity for intervention. The impact of the GI microbiota is decisive in the bioavailability of cysteine in the human body, and this evolves with aging. Once cancer is a group of diseases mostly potentiated by aging, it is natural that cysteine and its various valences play a leading role in cancer promotion and progression.

This compilation also serves to reflect on the latest dietary practices, highly enriched in protein and low in carbohydrates. The prevailing idea that glucose is the cancer nutrient misleads the total elimination of carbohydrates in some diets recommended for cancer patients since amino acids are the main substitutes for glucose. Glutamine has long been known to be the main glucose substitute for sustaining cellular respiration, with glucose and glycolysis primarily serving biosynthesis. Currently, cysteine has also assumed a leading role in bioenergetics and biosynthesis in the metabolism of malignant cells. In addition, the reduction of carbohydrates also significantly reduces the bioavailability of butyrate and its anti-cancer protection factor.

More studies are needed to reinforce the role of the gut microbiota in the metabolic drift that accompanies aging, in which cysteine is one of the most important coins. Thus, it will be possible to establish protocols to monitor and adjust the microbiota to the aging process. A pharmacological alternative that could be tested is blocking cysteine absorption (transport of cysteine in the intestinal mucosa); considering cysteine is not an essential amino acid, the impact in normal cells would be reduced. However, this inhibition must be performed with formulations that act only at the intestinal level without being absorbed, as this could have a deleterious impact on the metabolic dynamics of the body. Therefore, different strategies can be followed in an attempt to avoid the establishment of conditions that may be more favorable to the progression of cancer.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

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

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Modulation of tumor environment in colorectal cancer – could gut microbiota be a key player?

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The treatment paradigm of neoplastic diseases has dramatically shifted with the introduction of immune checkpoint inhibitors (ICI). They induce a durable response in a wide variety of solid tumors, but this response depends on the infiltration of lymphocytes capable of recognizing and killing tumor cells. The primary predictor of intrinsic immune resistance to ICIs is the absence of lymphocytes in the tumor, the so-called "cold tumors". Colorectal cancer (CRC) remains one of the most common and challenging cancer, but it is not traditionally considered a highly immunogenic tumor. In fact, immunotherapy showed a remarkable antitumoral activity only on a small subset of CRC patients – the ones with microsatellite instability-high/deficient DNA mismatch repair (MSI-H/dMMR). Most CRCs display a molecular microsatellite stability/proficient DNA mismatch repair (MSS/pMMR) profile, so strategies to improve tumor immunogenicity are crucial. Therefore, ongoing studies investigate new approaches to convert "cold" to "hot" tumors in MSS/pMMR CRCs. In addition, it has been described that gut microbiota influences tumor development and the host immune response. Hence, the microbiota may modulate the immune response, becoming a promising biomarker to identify patients who will benefit from ICIs. Future data will help to better understand microbiota mechanisms and their role in ICI efficacy. Precision medicine in cancer treatment could involve modulation of the microbiota through different strategies to improve tumor immunogenicity. In this review, we aim to present the potential relationship between gut microbiota and the modulation of the immune system and the hypothetical implications in CRC treatment, namely ICIs.

KEYWORDS

colorectal cancer, microbiota, microbioma, dysbiosis, immunity, immunotherapy, pharmacomicrobiomics

Introduction

Colorectal cancer remains one of the most common malignancies. Although overall mortality continues to decline, it remains on the podium of cancer-related death worldwide, with 0.9 million estimated deaths worldwide in 2020 (1, 2). Moreover, notwithstanding the risk of developing CRC increases after the age of 50, it has been increasing dramatically in younger generations, and it is expected to increase by 140% by the year 2030 (3, 4).

The oncogenesis of CRC is multifaceted and encompasses both environmental and genetic factors (5).

The therapeutic approach includes localized therapies, such as endoscopic and surgical excision, radiotherapy, and systemic therapy – chemotherapy, targeted therapy, and immunotherapy, namely immune checkpoint inhibitors (ICI) (6).

ICI has changed the paradigm of cancer therapy by directing the focus to the host instead of the tumor (7). Despite promising results in both hematological and solid tumors, it has failed in most patients with advanced CRC – it only showed significant antitumoral activity in MSI-H/dMMR tumors (8). The current challenge is to overcome this poorly immunogenic profile or, as it has been described, to transform "cold" tumors into "hot" ones (9). One of the most promising areas of immune modulation toward better responses to ICI concerns the inhabitants of our own gut: the gastrointestinal tract is home to trillions of bacteria, most of them commensal. These interact with the host and the immune system, thus constituting a delicate ecosystem called the human gut microbiota (10).

In this review, we will summarize the role of human microbiota in the modulation of the immune system and immunotherapy in CRC.

Colorectal cancer

The CRC incidence and survival rates have significant disparities between developed and developing countries, making this disease a marker of socioeconomic development. Diagnosis at advanced stages is one of the determinants of these differences (1, 11). In the last ten years, the adoption of screening strategies has contributed to early detection and improved outcome (12, 13). However, statistics globally predict an increase in CRC incidence and exposure to environmental risk factors resulting from a shifting lifestyle (low physical activity, overweight and obesity, excessive consumption of red, processed meats and alcohol, and low dietary fibers) are the main reasons for this evolution (2, 14).

Several critical genes and pathways were identified as crucial factors in the initiation and progression of CRC, such as Wnt, Ras/MAPK, PI3K, TGF-β, P53, and DNA MMR pathways. Classically, investigators biologically divided CRC carcinogenesis mechanisms into two groups: those with MSI and those microsatellite stable but with chromosomal instability (CIN) (11, 15, 16). Two pathological classification systems have been proposed: The Cancer Genome Atlas project and the Consensus Molecular Subtypes. Still, more research is needed to validate their clinical application (17, 18).

The two anatomical locations of the colon have distinct embryonic origins (19, 20). We also found fundamental differences in molecular and clinical characteristics: right colon CRC is usually associated with MSI and the BRAF mutation and is more immunogenic. Left colon CRC is associated with CIN and with mutations in the *APC*, *P53*, and *SMAD4* pathways (19). It remains to be fully understood the biological mechanisms behind such differences.

The MSI tumors are identified in 2–4% of metastatic CRC (mCRC) (20). The subjacent carcinogenesis mechanism depends on the DNA MMR function that ensures the integrity and stability of genetic material by correcting mismatched bases during DNA replication. If any defect occurs in the main MMR proteins MLH1, MSH2, MSH6, and PMS2 or microsatellites, several mutations accumulate, leading to the development of tumors (21). The consequent production of multiple neoantigens induced by genomic mutations is probably one of the mechanisms by which dMMR tumors are sensitive to immunotherapy, even though a complete understanding of the mechanisms leading to improved performance of ICI in dMMR is yet to be attained (22). Furthermore, the inflammatory microenvironment in CRC is an additional feature that makes these tumors more likely to respond to ICI. Hence, evidence has reported the presence of immune cells as CD8+ and CD4+ tumor-infiltrating lymphocytes (TILS), macrophages, and natural killer (NK) cells, as well as an increase in programmed cell death 1(PD-1) and its ligand (PD-L1) in lymphocytes/tumor cells surface (23–25).

Over the last decade, the median overall survival for patients diagnosed with mCRC has doubled (26). Regarding treatment options, fluoropyrimidines alone or combined with oxaliplatin or irinotecan became a standard regimen choice. In resected stage III CRC, fluoropyrimidine alone reduces the risk of death by 10% to 15%, with an additional benefit with an oxaliplatin-based combination (27, 28). Bevacizumab, a humanized monoclonal antibody that inhibits vascular endothelial growth factor (VEGF), and cetuximab and panitumumab, both antibodies targeting the epidermal growth factor receptor (EGFR), are also approved in mCRC according to the right-sided or left-sided colon and RAS gene mutational status. In later lines, TAS-102 improved overall survival (29) and ramucirumab, ziv-aflibercept, and regorafenib are VEGF/VEGF receptor (VEGFR) inhibitors also available in refractory CRC (30, 31).

Regarding the immunotherapy advent, there was an attempt to show the efficacy of ICI in CRC. The results of three phase II studies led to FDA and EMA approval of pembrolizumab and nivolumab (± ipilimumab) for dMMR/MSI CRC previously

treated by conventional chemotherapy (31–33). Corroborating this trend, KEYNOTE-177, a phase III trial that compared pembrolizumab with chemotherapy in untreated dMMR mCRC, was responsible for decisive changes in clinical practice, with significant improvement in progression-free survival (PFS) (16.5 months vs. 8.2 months, HR 0.60; 95% CI, 0.45-0.80; *P*=0.0002). Nevertheless, about 30% of patients receiving immunotherapy had disease progression as the best response (34). The phase 2 Atezo-TRIBE trial found that the addition of atezolizumab to chemotherapy and bevacizumab improved PFS in first-line mCRC (35). Later, a significant interaction between MSI status and immunotherapy was observed, with a higher benefit in patients with MSI/dMMR. Little is known about the resistance mechanism to ICIs and tumor heterogeneity in MSI/dMMR tumors. More biomarker-based strategies are needed and a better understanding of the potential synergistic effect of immunotherapy and selective inhibitors of the Ras/BRAF/MEK/ERK pathway to improve patient selection (36).

Immunotherapy, tumor microenvironment, and patterns of immune response

The ICIs have been used in multiple solid tumors, with good outcomes and prolonged survival confirming their efficacy. Despite the proven clinical benefit, some tumors do not respond to ICI, and this is probably related to specific characteristics of the tumor and the host.

The expression of PD-L1, the constitution of the immune system around the tumor, and the tumor mutational burden (TMB) are fundamental to the success of ICI and are currently considered biomarkers predictive of response (37). The evaluation of the immune profile of patients treated with ICI showed infiltration of immune cells, mainly cytotoxic T cells, in the responders' group. On the other hand, the lack of immune cells and cytokines led to a resistance to immunotherapy, seen in non-responders' patients (38).

The tumor bed, designated by the tumor microenvironment (TME), is a complex entity constituted by a heterogeneous collection of cells, secreted factors, and an extracellular matrix. The immune infiltration of the TME can be composed of all types of immune cells (39). Interactions between immune cells and tumor cells influence the environment and produce a pro- or antitumor effect. The TILs are critical cells in the TME, with the majority being T cells (40). Some T cells are related to tumorigeneses, like regulatory T cells (Treg) or helper T cells. In contrast, others are related to the elimination of the tumor, like NK cells and cytotoxic T cells (41).

Based on the T cells infiltration, Chen and Mellman defined three types of tumors that can be correlated with response to ICI: the immune inflamed phenotype, or "hot tumor", associated with a better response, and the cold tumors, the immune-excluded and the immune-desert phenotypes (42) (Figure 1).

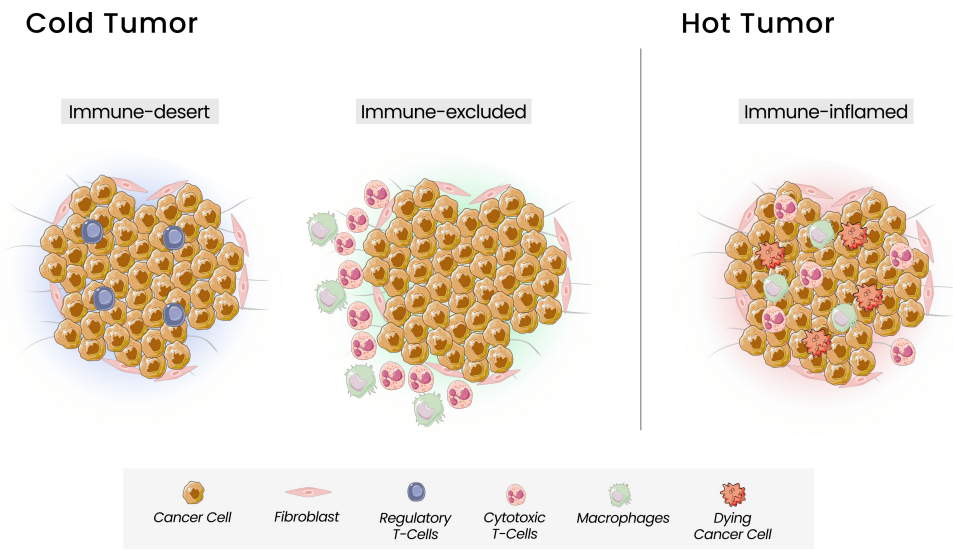

FIGURE 1
Tumor Immune Phenotypes. Three immunophenotypes are observed according to the spatial distribution of CD8 + T lymphocytes in the tumor microenvironment (TME): the immune-desert, immune-excluded and immune-inflamed phenotypes. In the immune-desert phenotype, immune cells are absent from the tumor and its periphery. In the immune-excluded phenotype, immune cells accumulate but do not efficiently infiltrate. The immune-inflamed is characterized by the infiltration of pro-inflammatory immune cells.

The immune-inflamed phenotype is characterized by the presence of immune cells, such as CD4+, CD8+ T cells, and pro-inflammatory cytokines like interferon (IFN), interleukins 12 and 23, and tumor necrosis factor (TNF)-α. In this phenotype, an antitumor immune response prevails, activating and expanding T cells (39). The immune-excluded and immune-desert phenotypes, considered "cold tumors", are characterized by a lower response to ICI and a worse prognosis. Despite the presence of immune cells in the immune-excluded phenotype, T cells are located in the stroma surrounding the tumor cells. The tumor, in this case, can promote signaling that blocks dendritic cells and other mechanisms capable of recruiting T cells to the center of the tumor. In the immune-desert phenotype, there is a lack of cytotoxic cells and a prevalence of inhibitory immune cells, like Treg (37). Beyond the paucity of immune effector cells, these last two phenotypes are characterized by a low TMB and a lack of antigen release, reinforcing their poor tumor immunogenicity. The greater the number of mutations in a tumor, the more immunogenic the tumor will be, as these mutations can provide targets for cytotoxic cells. However, some mutations can have the opposite function, acting to attenuate the immune response. Mutations that can decrease the transcription of Major Histocompatibility Complex (MHC) class I molecules will also interfere with peptide loading and presentation process, leading to a weak response (42).

Many steps can inhibit T cells priming and activation in driving immune cells into tumors, leading to a non-inflamed tumor bed. Given these different profiles of tumor behavior, more recent studies try to promote a switch in the tumor environment, turning "cold" into "hot" tumors. Several mechanisms can be used, like the stimulation of recruitment of dendritic cells, stimulation and activation of effector cells, or modification of chemokines and cytokines that can modify the cell traffic and activation (43). Epigenetic modifications, including DNA methylation and chromatin remodeling, can increase tumor immunogenicity and immune recognition, and the subsequent release of pro-inflammatory cytokines. Studies *in vitro* showed that pharmacological or genetic disruption of Treg cells might lead to the acquisition of pro-inflammatory gene signature, with increased CD4+ and CD8+ T cells recruitment to promote antitumor immunity (44). Chemotherapy, radiotherapy, oncolytic viruses, cancer vaccines, or antiangiogenic therapies are currently being studied to improve T cell infiltration. However, it is not enough to increase the number and activity of cytotoxic cells since some components of TME can inhibit their function. One proposed mechanism to convert TME into a "hotter" TME is target therapy against angiogenesis (45). Unfortunately, the clinical benefits are limited since the prolonged use of antiangiogenic therapy increase hypoxia and consequently increase the release of proangiogenic factors (45).

Human microbiota, immune system, and dysbiosis

The human gut microbiota comprises approximately 3×10^{13} bacteria and other highly diverse microorganisms, which are confined to the intestinal lumen. The microbiota is essential in regulating fundamental biological events, and this relationship has evolved into a symbiosis (10, 46, 47). The disruption of this balance, called dysbiosis, is closely related to several diseases, namely infections, autoimmune diseases, cardiovascular diseases, and cancer (48–50). The mutual interaction regulates local and systemic immune homeostasis, maintaining tolerance for commensal bacteria and allows the recognition of potentially pathogenic microorganisms.

The lamina propria beneath the epithelial cells (IECs) harbors immune cells, which encompasses the gut-associated lymphoid tissue (GALT), including antigen-presenting cells such as dendritic cells, T cells, and B cells. The mechanisms through which the microbiota regulates the immune system have been scrutinized over the last few years. Essentially, the various pattern-recognition receptors (PRRs) expressed in IECs and immune cells are thought to recognize microbe-associated molecular patterns (MAMPs) of commensal bacteria (51, 52).

The dendritic cells occupy a prominent role: they are activated by the microbes or by microbe-derived elements (e.g., metabolites, products) *via* interactions with PRRS. When activated, they travel to the mesenteric lymph nodes and orchestrate the differentiation of naïve T cells into effector T cells, mainly Tregs and helper 17 (Th17). A subset of these cells may migrate back to the intestine or enter the systemic circulation, thus locally and systemically modulating the host's immune system. The Th17 cells mediate the conversion to a pro-inflammatory and antitumor state by secreting immunostimulatory cytokines or directly activating neutrophils, versus Tregs, which release anti-inflammatory cytokines and mediate the conversion to an anti-inflammatory state (51–53). MAMPs or microbe metabolites can also stimulate the immune system through other mechanisms: stimulation of enteric neurons with the release of neurotransmitters that regulate the immune cell function; secretion of immunoglobulin (namely IgA) and their crucial role in the blockade of bacterial adherence, and activation of the innate immune response (53–55).

Gut microbiota and colorectal cancer

Many of the recognized environmental and lifestyle factors related to CRC are also linked to microbiota dysbiosis (56–58). The gut microbiota is probably at the intersection of these risk factors. As Fearon et al. proposed, the microbiota may be considered an independent driver before the transformation from adenoma to carcinoma (59). The impact of diet on microbiota was thoroughly described by O'Keefe et al., in

which a diet exchange between different populations resulted in remarkable changes in microbiota (60). It is also essential to mention the impact of consuming processed foods, as nitrate consumption, rich in processed food, can lead to the formation of N-nitroso compounds by the gut microbiota, some of which are carcinogenic (61, 62).

Dysbiosis with the unbalanced growth of certain species, including *Fusobacterium nucleatum, Bacteroides fragilis*, and *Escherichia coli*, along with a reduction in *Roseburia, Clostridia, Clostridium*, and *Clifridia*, can increase the expression of pro-inflammatory cytokines, reduce butyrate-producing bacteria along with enriching pro-inflammatory pathogens and increase the risk of oncogenesis (56, 63–65). (Figure 2) Butyrate can induce antitumor responses and help in microbiota homeostasis (66). The overgrowth of *F. nucleatum* has been associated with tumorigenesis through different mechanisms: an increase in M2 macrophages, a decrease in FOXP3+ T cells in the TME, and the presence of bacterial proteins FadA e Fap2, which activate the WNT/β catenin signaling pathway and inhibit NK cells and T cells signaling (67, 68). Other microorganisms are also linked to CRC development: fungal dysbiosis may also induce tumor cell progression (69). Oppositely, *Saccharomyces cerevisiae* could suppress the growth of tumor cells (70). The impact of the microbiota on the biological mechanisms that culminate in the differences between the right and left colon has been questioned. It has been hypothesized that there is an increased amount of pathogenic bacteria in the left colon which could explain the higher incidence of left CRC (71–73).

The microbiota is not only associated with local oncogenesis but has also been proposed as a facilitator of metastasis. Hepatic metastases are preceded by the previous formation of premetastatic niches. This is harbingered by the migration of bacteria to the liver through the portal venous system, and certain bacterial strains, such as *Escherichia coli* C17 or *Proteus mirabilis*, have been strongly associated with this mechanism (74, 75).

Gut microbiota can also improve the effectiveness of the antitumor effect of chemotherapy drugs (76). Other gut microbes might also aggravate chemotherapy-related adverse reactions *via* drugs'microbial metabolism (77).

Connection between microbiota and immunotherapy

Apart from the relationship with classic CRC chemotherapy, there is also a potential link with targeted agents and ICI. Gut microbiota is a critical modulator of TME, and it might be linked to ICI response in solid tumors. Initial findings by Vetizou et al.

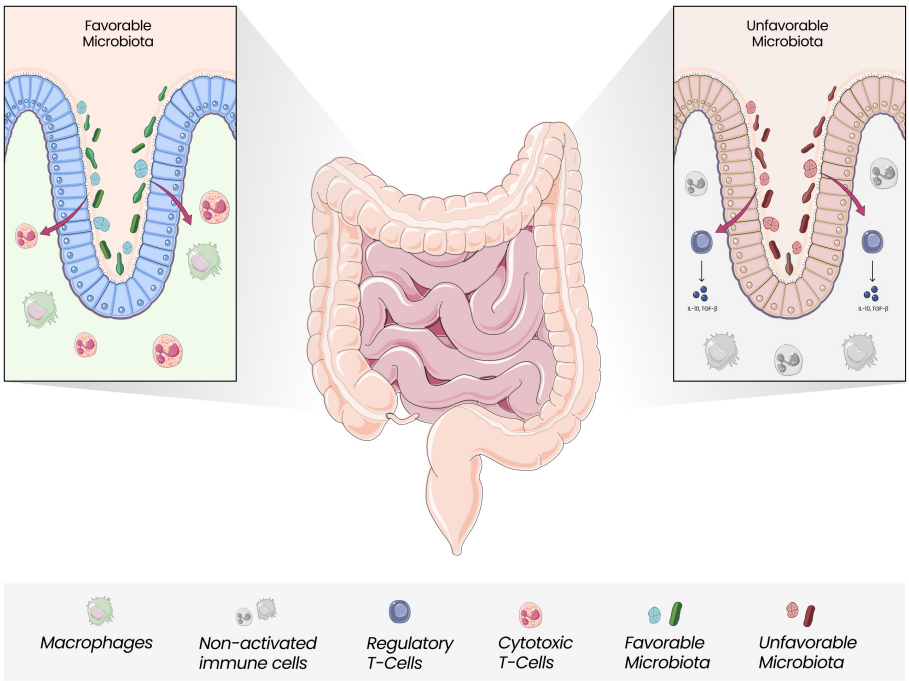

FIGURE 2
Gut Microbiota and Immune Modulation. Gut Microbiota is closely linked with the modulation of local and systemic immune responses. The unbalanced growth of unfavorable microorganisms, which seems to be more pronounced in the left colon, can mediate less efficient responses regarding antitumor activity, with Tregs releasing anti-inflammatory cytokines and promoting an anti-inflammatory state.

showed that the CTLA-4-targeting antibody ipilimumab could treat specific-pathogen-free mice but not germ-free mice (78–81). Multiple gut bacteria were found to be associated with better outcomes in patients treated with anti-PD-1 and anti-CTLA4 immunotherapy (e.g., *Akkermansia muciniphila, Bifidobacterium longem, Faecalibacterium prausnitzii*) (82, 83). It has also been described as a potential influence on immunotherapy-related adverse events (84).

Even though the mechanisms by which the gut microbiota influences immunotherapy remain under study, research appears to focus on three themes: bacteria or bacterial components that stimulate antitumor T-cell responses, molecular mimicry between bacteria and tumor epitopes, and bacterial metabolites that shape antitumor immunity (85–87). The interpretation of data linking ICI and the microbiota can be hampered by several factors: small cohorts, variable definitions of response, and the confounding factors linked to gut microbiota composition (diet, treatment, geography, ethnicity, etc.).

Given the apparent benefits in the presence of certain bacteria species, one may ask whether it will be possible to modulate the microbiota with the final aim of attuning the immune system. Microbiota modulation has been receiving widespread attention (Table 1). It can occur directly through actions of dietary components on the microbiota's composition or metabolic processes or indirectly through altering the gut physiology to change the intestinal lumen environment, thereby producing changes in the microbiota. So far, the main ways of modulating the microbiota are through diet, administration of prebiotics and probiotics, and fecal microbiota transplantation. Concerning dietary habits, data have shown a profound and beneficial metamorphosis in the microbiota composition with a high-fiber, low-fat diet (60). The administration of growth substrate (prebiotics) to induce the growth of specific strains has also shown potential in modulating the microbiota (99).

The direct introduction of bacteria, either in the form of a fecal transplant or of just a few microorganisms (specific strain

TABLE 1 Clinical studies regarding modulation of gut microbiota and cancer treatment.

Concluded Clinical Studies

	Reference	Study Population	Intervention	Results
Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer (88)	Bullman et al. Science 2017	CRC	Treatment with metronidazole	Significant decrease in *Fusobacterium* load in the tumor tissue (P = 0.002) as well as a significant reduction in tumor cell proliferation (P = 0.002).
Phage-guided modulation of the gut microbiota of mouse models of colorectal cancer augments their responses to chemotherapy (89)	Zheng et al. Nat Biomed Eng. 2019	CRC	Irinotecan-loaded nanoparticles linked to phages	Decrease in the numbers of Fusobacterium nucleatum (P = 0.01); median survival in mice increased from 20d to 42d.
Aspirin Modulation of the Colorectal Cancer-Associated Microbe Fusobacterium nucleatum (90)	Brennan et al. mBio. 2021	CRC	Administration of aspirin	Decrease in fusobacterial abundance in colon adenoma tissue.
A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients (91)	Gianotti et al. World J Gastroenterol. 2010	CRC	Administration of probiotics perioperatively	*Lactobacillus johnsonii reduces* the concentration of pathogens and modulates local immunity.
Intestinal microbiota is altered in patients with colon cancer and modified by probiotic intervention (92)	Hibberd et al. BMJ Open Gastroenterol. 2017	CRC	Administration of probiotics	Increased abundance of butyrate-producing bacteria, especially Faecalibacterium and Clostridiales spp. CRC-associated genera such as Fusobacterium and Peptostreptococcus tended to be reduced in the fecal microbiota of patients that received probiotics.
Effects of prebiotics on immunologic indicators and intestinal microbiota structure in perioperative colorectal cancer patients (93)	Xie et al. Nutrition 2019	CRC	Administration of probiotics	Preoperative period: increased serum levels of IgG; P = 0.02), IgM (P = 0.00), and transferrin (P = 0.027; all P < 0.05). Postoperative period: enhanced levels of IgG (P = 0.003), IgA (P = 0.007), suppressor/cytotoxic T cells ($CD3^+CD8^+$; P = 0.043), and total B lymphocytes ($CD19^+$; P = 0.012) Prebiotics increased the abundance of Bifidobacterium (P = 0.017) and Enterococcus (P = 0.02; both P < 0.05) but decreased the abundance of Bacteroides (P = 0.04)
Impact of the preoperative use of synbiotics in colorectal cancer patients: A prospective, randomized, double-blind, placebo-controlled study (94)	Polakowski et al. Nutrition 2018	CRC	Administration of synbiotics	Significant reductions in IL-6 levels (163.2 ± 19.5 versus 138.8 ± 12.5, P < 0.001) and CRP (10 ± 5.2 versus 7.17 ± 3.2, P < 0.001).

(Continued)

TABLE 1 Continued

Ongoing Interventional Trials

	NCT Trial No.	Phase	Study population	Intervention
Feasibility Study of Microbial Ecosystem Therapeutics (MET-4) to Evaluate Effects of Fecal Microbiome in Patients on ImmunOtherapy (MET4-IO) (95)	NCT03686202	Phase I	Solid Tumors	Microbial Ecosystem Therapeutics (MET-4) in patients on immunotherapy
A Phase I/II Open Label, Safety And Preliminary Efficacy Study of MRx0518 In Combination With Pembrolizumab In Patients With Advanced Malignancies Who Have Progressed On PD-1/PD-L1 Inhibitors (96)	NCT03637803	Phase I/II	Solid Tumors	MRx0518 in combination with pembrolizumab
Phase II, Single-arm Study of FMT Combined With Immune Checkpoint Inhibitor and TKI in the Treatment of Colorectal Cancer Patients With Advanced Stage (97)	NCT05279677	Phase II	CRC	Fecal microbiota transplantation in combination with Sintilimab and Fruquintinib
Preoperative Endoscopic Treatment With Fosfomycin and Metronidazole in Patients With Right-sided Colon Cancer and Colon Adenoma: a Clinical Proof-of-concept Intervention Study MEFO Trial (98)	NCT04312360	Phase II	CRC and Colon Adenoma	Therapeutic endoscopy with metronidazole and fosfomycin disodium

CRC, colorectal cancer; CRP, C-reactive protein; FMT, fecal microbiota transplant; IL, interleukin; Ig, immunoglobulin; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TKI, tyrosine kinase inhibitor.

or consortium) with probiotics has an undeniable role in the microbiota regulation to improve the host immunity (100, 101). Fecal microbiota transplantation is being experimentally used to treat metabolic diseases, inflammatory bowel disease, and cancer (102–106). Conversely, eradicating specific microorganisms with certain antibiotics, such as metronidazole, is also an active field of investigation (88).

Interestingly, vitamins appear to modulate microbiota as well: vitamin D is linked with anti-inflammatory and immune-modulating properties in the gut (107). Promising new research on colon-delivered vitamin B3 is associated with improving biomarkers for inflammation (108).

Conclusion

A complex tie lies between the host and gut microbiota.

In human diseases, gut microbiota mediates the immune response, modulating disease development and progression, and potentially interfering with treatment efficacy. It may play a key role also in human cancer, including the ability to modulate host immune response (109, 110). The gut microbiota may influence the anti-tumor activities by producing specific metabolites or inducing T-cell responses. On the contrary, some bacterial species improve tumor proliferation and metastasis, and understanding those interactions in the context of cancer is crucial in the quest for potential therapeutic targets. In this context, there is a shred of increasing evidence for the correlation of gut microbiota with cancer immunotherapy activity and toxicity (111).

The modulatory effect of the gut microbiota on ICI response may create new therapeutic opportunities. In MSI-H patients with intrinsic/*de novo* and acquired resistance settings, it may become essential to examining the microbiota.

Despite the advances, the underlying mechanisms, the therapeutic impact, and which specific microbes and immune cells interact with each other remain obscure. Moving forward, clinical trials will undoubtedly spur efforts to examine the influence of the immune-gut interaction on immunotherapy treatment in clinical settings.

Hopefully, this will quickly become much more than just a gut feeling.

Author contributions

ADM and DAC contributed to the conception and design of the review. ADM, RV and MV wrote the first draft of the manuscript. MS and DAC revised the manuscript. All authors read and approved the submitted version.

Conflict of interest

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

Publisher's note

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Gut microbiota in anxiety and depression: Pathogenesis and therapeutics

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Depression and anxiety disorders represent a burdensome clinical issue. Considering the unsatisfactory clinical response of some patients to antidepressant therapy, new personalized approaches are being studied. In recent years, pre-clinical and clinical studies have investigated the role of intestinal microbiota demonstrating the importance of the gut-brain axis in these diseases. Indeed, gut microbes are able to interact with the brain interfering with behavior through some mechanisms such as amino acid metabolism, short–chain fatty acids, vagus nerve, endocrine signaling and immune responses. Experiments of gut microbiota transfer from subjects with major depression to animal models corroborated the causative role of intestinal microbes in mood disorders and anxiety. Furthermore, the incidence of dysbiosis in patients with anxiety and depression suggests a potential role for gut microbiota modulators in the treatment of these disorders. In particular, several probiotics and synbiotics have been shown to be effective in improving clinical symptoms, promising results have emerged also from fecal microbiota transplantation, but the evidence is still limited. These promising results switch on the use of gut microbiota modulators as an adjunctive tool to anti-depressant therapy. Developing pharmaceutical or nutraceutical strategies to modify the composition of gut microbiota may offer novel and personalized therapeutic tools against anxiety and depression.

KEYWORDS

FMT, gut-brain axis, probiotic, prebiotics, antibiotic, synbiotic

Abbreviations: MDD, Major depressive disorders; GAD, Generalized anxiety disorder; IBS, Irritable bowel syndrome; SCFA, short chain fatty acid; BDI, Beck Depression Inventory; IBS, irritable bowel syndrome; HAM-D, Hamilton rating scale for depression; HADS, Hospital Anxiety and Depression Scale; CBT, cognitive behavioral therapy.

Introduction

Anxiety and mood disorders represent an alarming clinical issue, as well as cause of disability and mortality worldwide (1). Unfortunately, the mechanisms triggering these diseases have not yet been fully understood. Several factors such as oxidative stress (2), impaired signaling by neurotrophic factor (3) or chronic inflammation (4) have been hypothesized to be involved in the development and susceptibility of mood disorders, which presumably are caused by an interplay between genetics and environmental factors (5, 6). To date, the lack of this knowledge has a negative effect on the efficacy of common therapies, so there is a need for personalized treatment for these patients (7). In this regard, considering the pathophysiological role of the intestinal microbiome, the development of innovative therapies for these disorders can be hypothesized. Gut microbes are able to produce most neurotransmitters, influencing neurochemistry and behavior *via* the so-called "gut-brain axis" (8). Moreover, the high prevalence of stress-related psychiatric symptoms in patients with gastrointestinal disorders supports the link between gut microbiota changes and psychiatric disorders (9). The functional crosstalk among enteric microorganisms, gut and brain may occur through multiple mechanisms, including metabolic and neuroimmunological pathways. Finally, developing pharmaceutical or nutraceutical strategies to modify the composition of gut microbiota may offer novel and personalized therapeutic tools against anxiety and depression, which we will discuss below.

Gut microbiota regulates anxiety-like and depression-like behavior: Evidences from animal studies

Despite the limitations represented mainly by the difference in the composition of the human and murine microbiota, and the difficulty of translate the findings from experimental models to patients where no complete ablation of the microbiota can be achieved, studies on rodents indicate that gut microbiota influences brain function and may impact on the behavior (10). Experimental approaches used to study the microbiota-gut-brain axis included the treatment with probiotics/antibiotics, the induction of gut inflammation by injection of enteric bacterial pathogens, the use of germ-free (GF)/gnotobiotic animals and the human diseases-related fecal microbiota transplantation (FMT) (11). The main advantages of studies performed on murine experimental models are the efficacy of behavioral tests to reveal changes similar to what observed in patients affected by anxiety or depression (12), and the possibility to analyze the effects of a single bacterial phylum or species on behavior. Animal studies suggested that changes in the microbiota induced brain modifications at both molecular and behavioral level. Mice treated with a cocktail of non-absorbable antibiotics showed changes of intestinal microbiota profile (i.e., a reduction of *Shigella*, *Bacteroides* and *Klebsiella* genera and an increase of *Actinobacter* and *Lactobacillus* populations) in parallel with greater exploratory activity (13). This anxiolytic effect was accompanied by an increase of brain-derived neurotrophic factor (BDNF) levels in the hippocampus and amygdala. More importantly, the authors did not observe the same responses in animals intraperitoneally injected with the antibiotics or in germ-free mice to which the drugs were administered by gavage. Moreover, gut microbiota seems to be involved in the diet-induced brain modification. High fat diet (HFD) is a well-established experimental model able to induce changes of both insulin and leptin signaling into the brain, anxiety and memory deficits (14–16). *Soto* and colleagues demonstrated that in HFD-fed mice, oral treatment with antibiotics modified the levels of neuromodulators such as tryptophan, γ-aminobutyric acid (GABA) and BDNF, ameliorated brain insulin signaling and counteracted anxiety and depression (17). In addition, the authors documented that these effects were transferable to germ-free mice by FMT.

Indeed, a large part of these studies based on the transferability of behavioral traits from donor mice to germ-free animals *via* the intestinal microbiota. For instance, BALB/c mice have anxiety-like behavior but, when they were colonized with the microbiota from Swiss mice, they acquired a more exploratory behavior. Accordingly, germ-free Swiss mice colonized with the intestinal bacteria from BALB/c mice exhibited a more anxious behavior (13). More recently, it has been showed that mice transplanted with fecal microbiota from Irritable bowel syndrome (IBS) patients exhibited intestinal barrier dysfunction, immunological activation, and anxiety-like behavior (18). More generally, intestinal microbiota appears to influence the stress response of rodents. *Sudo* and colleagues demonstrated that plasma levels of both ACTH and corticosterone were more prone to increase upon restraint stress in GF mice than in microbiota-competent animals (19). Moreover, the colonization by *Bifidobacterium infantis* of germ free mice was able to fully reverse these effects, revealing a causative role for the gut microbiota in modulating stress responses. Accordingly, the reduced expression of inflammatory interleukins and increased the amount of BDNF in the hippocampus was obtained by oral intake of Bifidobacterium, causing anxiolytic and antidepressant effects in mice (20). Bifidobacterium administration has been also shown to offer resilience to chronic social defeat stress in mice (21). In addition, three independent studies found altered concentrations of neurotransmitters and neurotrophic factors in the brain, and reduced anxiety in GF mice (22–24). These neurochemical and behavioral findings are not actually in agreement, because enhanced hypothalamic–pituitary–adrenal (HPA) axis is usually related to increased anxiety-like behavior. *Clarke* and colleagues also reported elevated concentrations of tryptophan, the precursor

of serotonin, in the plasma and a significant increase of serotonin metabolites in the hippocampus of male GF mice compared with control animals (24). Serotonin is an excitatory neurotransmitter produced also in the gut and able to counteract anxiety and depression at central level (25). Metabolomics studies revealed elevated serum tryptophan and less serum serotonin in GF mice compared to controls (26). However, whether changes in serotonin and neurotrophic factors (e.g., BDNF) are involved in the gut microbiota-dependent modification of anxiety-like behavior remains to be elucidated.

Rodent models have provided the mechanisms by which the gut microbiota may modulate depression-like behaviors. Maternal separation is a model of early life stress that induces anxiety and depression by altering HPA axis, immune system and aminoacid metabolism along with affecting microbiota composition (27, 28). More recently, *De Palma* and colleagues demonstrated that maternal separation of GF mice did not induce depressive or anxiety behavior despite it caused increase of circulating corticosterone (29). This study suggests that gut microbiota is not required for stress-induced changes in HPA axis activity but it is necessary for development of anxiety and depression-like behaviors. Therefore, intestinal microbes appeared to regulate stress responses in the brain of animal models and this evidence stimulated the possibility of using probiotic treatments to modulate brain function in physiological and pathological conditions (30). A plethora of probiotic agents have been tested in rodent models of anxiety and depression. *Bifidobacterium* and *Lactobacillus* are the main genera that have provided beneficial effects on neurological disorders (31). *Bifidobacterium infantis* has been shown to have antidepressant effect promoting antidepressant-like performance in the forced swim test, a widely used test to evaluate the efficacy of antidepressant drugs (32). Supplementation of *Bifidobacterium infantis* also counteracted the maternal separation-induced increase of both plasma tryptophan and pro-inflammatory cytokines, which have been demonstrated to play a role in the pathophysiology of depression (33). Many studies also clarified the mechanisms underlying the effects of probiotics on brain functions. Several studies focalized the attention on the ability of probiotics to modulate the inflammatory response of the organism. *Lactobacillus rhamnosus* has been proved to inhibit *in vitro* the *Salmonella* enterica-related synthesis of pro-inflammatory interleukin-8 and tumor necrosis factor alpha (34). This bacterial strain has been also found to induce region-dependent changes in GABA receptor expression in the brain. More importantly, *Lactobacillus rhamnosus* administration reduced in mice the stress-dependent increase of corticosterone levels and counteracted the related anxiety- and depression-like behavior. Moreover, the beneficial effects of this probiotics were abolished in vagotomized animals (35). More recently, *Janik* and colleagues documented by magnetic resonance spectroscopy that chronic treatment with *Lactobacillus rhamnosus* induced significant changes in the concentration of neurotransmitters such as glutamate, N-acetyl aspartate, and GABA into the brain (36). It suggests that probiotics could affect brain activity by regulating neurochemical pathways underlying synaptic transmission and plasticity. In addition, administration of *Bifidobacterium Infantis* enhanced the expression of BDNF and N-methyl-D-aspartate receptor subunit 2a, which are molecules involved in learning and memory (19). Collectively, these studies prompt the idea that probiotics can modulate microbiome-gut-brain axis and influence brain function. Despite significant difference occurs between the human and mouse microbiomes, the evidence from experimental models suggest that changes of gut microbiota composition may affect molecular pathways involved in the onset and progression of anxiety- and depression-related behaviors Figure 1.

Dysbiosis in depression and anxiety disorders: Evidences from human studies

In recent years, some studies were conducted to investigate how the intestinal microbiota play a role in patients with anxiety and mood disorders. In particular, several data from human studies shown that fecal microbiota often has some variability between patients and healthy controls, considering microbial diversity and taxonomic compositions. Furthermore, was reported that specific bacteria were associated with metabolic or inflammatory profiles and clinical characteristics (37).

Microbial diversity is a fundamental aspect in the study of fecal microbiota that is considered a marker of health, but the reproducibility of data is strongly limited by the interference of many environmental factors (38). To date, few studies reported data about microbial diversity in humans, most of these failed to demonstrate an association between lower microbial diversity and depressive disorders (39–41), while only one study reported higher α-diversity (i.e., the number of species detectable in a microbial ecosystem) of gut microbiota in major depressive disorder (MDD) patients compared to healthy subjects (42).

Taxonomic differences are described in several studies involving MDD patients, interesting differences have been reported for the main Phyla represented. Unfortunately, the findings from human studies are often conflicting, probably due to several confounding factors. For instance, several changes in microbial composition were reported in the Phylum of *Firmicutes*, but as previous discussed, findings were often contradictory. The relative abundance of this phylum appeared to be more represented in MDD according to some studies (41, 43), however this finding it was not confirmed by farther report (42). Moreover, more differences were reported at family level considering that *Lachnospiraceae* were found increased (40–42) or decreased (39) between available studies, likewise

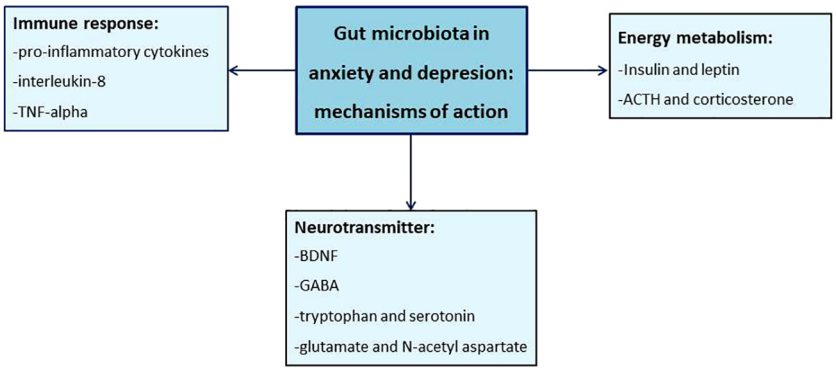

FIGURE 1
Role of the gut microbiota in the development of mood disorders. Some pathophysiological mechanisms underlying the development of anxiety and depression have been proposed, in particular the balance of immunological, neurotransmitter and hormonal mechanisms is the proposed orchestror.

Ruminococcaceae had a fluctuating representation, higher (40, 41) or lower (42) among reports. Finally, the genus level showed the most remarkable changes that were described for *Faecalibacterium* (40, 42) and *Ruminococcus* (42), these genera were decreased in subjects with depressive disorders. Similarly, changes in microbial composition were described for other phyla such as *Bacteroidetes* (39, 41–43) and *Actinobacteria* (41, 42), although with sometimes conflicting results among the various studies. Most significative differences were observed at the genus level as a reduced representation of *Bifidobacterium* (44). Furthermore, correlation between clinical characteristics of patients and microbial signature was reported. Specifically, *Fusobacteria* and *Proteabacteria* appeared to be increased or reduced, respectively in active-MDD or recovering-MDD (42).

Above, we have briefly reported the complexity and the divergences between the evidences probably due to methodological differences and environmental variability among different studies. A recent systematic review showed that about 50 bacterial taxa exhibit differences between patients with MDD and controls (45). However, the authors failed to demonstrate the prevalence of a specific bacterial taxa in the development of depression.

In the near future, meta-proteomics studies should add further elements in the understanding the association between microbiota and the development of depression. A pioneering study by *Chen* and colleagues investigated the metabolomic profile in patients with MDD and it reported several significant differences in the pathway of bacterial proteins that were mainly involved in glucose metabolism and amino acid metabolism (46).

Interesting alterations of the fecal microbiota have also been identified in patients suffering from anxiety disorder. In particular were found a reduction in microbial richness and diversity in patients with generalized anxiety disorder (GAD), associated with reduced short-chain fatty acid producing bacteria such as *Eubacterium rectale* and *Fecalibacterium*, and an increase in *Escherichia*, *Shigella*, *Fusobacterium* and *Ruminococcus* (47). More importantly, these changes were not reversed in remissive GAD. Conversely, another study failed to demonstrate any correlation between intestinal dysbiosis and anxiety in female subjects (48), confirming the variability between human studies.

Potential for therapy

Gut microbiota represents a new frontier in psychiatry. For this reason, antibiotics, probiotics, prebiotics and FMT were investigated for the treatment of anxiety (49) and depression (50). Psychobiotics define these therapeutic tools (51), in particular the main evidences on the modulation of the gut microbiota in depression and anxiety disorders were reported in the next paragraphs.

Antibiotics

Antibiotics are deep modulators of gut microbiota, and consequently they appear to change, in a positive or negative way, the nature of several gastrointestinal or extra-intestinal disorders (52). Therefore, in consideration of their known effect on behavior, they have been proposed as a therapeutic tool also in psychiatry (53). Potential and beneficial effects were described in individual with depression or anxiety related disorders.

For instance, Minocycline has been identified as a potential novel treatment for depression taking into consideration its potent anti-inflammatory and neuroprotective effects (54). In recent years, several clinical trials investigated the potential role of this drug in the scenario of depression; meta-analyses that included three RCTs reported preliminary evidence for a significant antidepressant effect of minocycline. The antidepressant effect size was found to be large (SMD − 0.78; 95%CI; 0.4–1.33; p=0.005) with moderate heterogeneity of the

pooled sample. However, the small number of published RCTs and small sample sizes were significant limitations to draw definitive conclusions (55). Furthermore, the broad-spectrum antibiotic Cycloserine was investigated for the treatment of anxiety disorders. A meta-analysis that included 21 studies that involved 1047 individuals with several psychiatrics disorders (phobia, social anxiety disorder, panic disorder, obsessive-compulsive disorder and post-traumatic stress disorder) showed that Cycloserine was associated with a small augmentation effect on exposure-based therapy and suggested that this effect was not modulated by the concurrent use of antidepressants (56). However, antibiotics have also been associated with a negative effect on mood disorders. In particular, recurrent exposure to antibiotics such as penicillins (OR 1.23; 95% CI, 1.18-1.29) or quinolones (OR 1.25; 95% CI, 1.15-1.35) appeared to be associated with increased risk for depression and anxiety (57).

Probiotics

Probiotics are defined as live microorganisms that, upon administration in adequate amounts, confer a health benefit on the host (58). To date, some studies report results on the use of probiotics in the treatment of mood disorders, albeit with some limitations as the heterogeneity of enrolled patients and the variety of the administered mixtures (59). *Miyaoka* and colleagues investigated the role of *Clostridium butyricum* (CBM588) as adjunctive therapy in patients with treatment-resistant MDD. In this study was reported a significant improvement in depression scale after 8 weeks of treatment, suggesting a potential therapeutic role for this probiotic strain in combination with antidepressant drugs (60). Another clinical trial reported that a probiotic mixture (*L. helveticus* R00052 and *B. longum* R0175) was able to ameliorate the beck depression inventory (BDI) in individuals with mild to moderate MDD compared to placebo (61). Farther, the administration of a mixture of *L. acidophilus*, *L. casei* and *B. bifidum* resulted in a significant reduction of BDI score (62). Sometimes MDD patients experienced gastrointestinal disorders and in particular IBS, in this context *Majeed* and colleagues reported significant improvement of depression and IBS symptoms in patients treated with *Bacillus Coagulans* MTCC 5856 (63). Promising results were also reported about stress and anxiety. Indeed, *Lactobacillus plantarum* DR7 appeared to be beneficial in reducing symptoms and psychological scores (64).

However, not all studies documented positive results, maybe due to probiotic strain, concurrent medications or other unexplored factors. For instance, *Romijn* and colleagues demonstrated that a probiotic mixture (*L. helveticus* R0052 and *B. longum* R0175) failed to improve depressive symptoms in individuals with low mood not currently taking psychotropic medications (65). Finally, another study clearly showed that the probiotic *B. Longum* NCCC3001 reduced depression but not anxiety scores and increased quality of life in patients with IBS (66). Furthermore, the effects were associated with changes in brain activation patterns demonstrating that this probiotic reduces limbic reactivity (66).

Prebiotics

Prebiotics are selectively fermented compounds promoting changes in both composition and activity of intestinal microbiota that offer benefits to the host (67). Few studies investigated the role of prebiotics in mood disorders. *Smith* and colleagues failed to demonstrate a significant effect of a prebiotic mixture (oligofructose enriched inulin) on mood scores in a cohort of healthy adults. However, participants reported greater well-being after consumption of inulin (68). Similarly, despite beta-glucan derived from *Saccharomyces cerevisiae* improved mood in stressed subjects, no significant differences in depression scores were observed compared to placebo (69). Moreover, another clinical trial failed to demonstrate that prebiotic supplementation improved depressive symptoms. Indeed, administration of galacto-oligosaccharides for eight weeks did not significantly modify BDI score in MDD patients compared to placebo and its effect was lower than that of probiotic mixture (61). On the other hand, prebiotics supplementation appeared to be more efficacious on psychiatric symptoms in IBS patients. Short-chain fructo-oligosaccharides (scFOS) showed beneficial effects in a population with gastrointestinal symptoms. Specifically, scFOS supplementation for four weeks resulted in a significantly improvement of depression and anxiety scores, furthermore this effect was associated to changes in microbiota composition including increase of *Bifidobacteria* in feces (70). However, another prebiotic galacto-oligosaccharide mixture (B-GOS) not improved anxiety and depression scale in individuals with functional bowel disorders, albeit some beneficial effects were reported for gastrointestinal symptoms (71).

These conflicting data confirm the need for further studies to better establish the patient cohorts and compounds more efficacious for this type of intervention.

Synbiotics

Synbiotics are defined as a synergic mixture of probiotics and prebiotics that promote beneficial effects on health, in particular prebiotics are involving in favoring the colonization of the gut by probiotics (72). A small number of clinical trials that investigated the role of synbiotics in mood disorders have been published. A first trial demonstrated that a symbiotic mixture (*Lactobacillus casaei*, *Lactobacillus acidofilus*, *Lactobacillus bulgarigus*, *Lactobacillus rhamnosus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Streptococus thermophiles*, and fructo-oligosaccharide)

was able to decrease HAM-D score and to improve depressive symptoms in patients with moderate MDD (73).

Afterwards, another clinical trial demonstrated the greater efficacy of symbiotic formulations compared to probiotics mixture alone in the treatment of mood disorder. In the clinical trial designed by *Haghighat* and colleagues (74), patients were randomly assigned to receive synbiotics (prebiotics: fructo-oligosaccharides, galacto-oligosaccharides, and inulin; probiotics: *Lactobacillus acidophilus* T16, *Bifidobacterium bifidum* BIA-6, *Bifidobacterium lactis* BIA-7, and *Bifidobacterium longum* BIA-8) or probiotics (the same mixture of synbiotics without prebiotics) or placebo for twelve weeks Table 1.

Fecal microbiota transplantation

Fecal microbiota transplantation is the infusion of a fecal suspension derived from a healthy donor into the intestine of a recipient to restore the imbalanced gut microbiota (75). Some fascinating studies on animal models have supported the idea that the transfer of "good microbes" can represent a new tool in the treatment of depression and anxiety. For example, it has been demonstrated that the transfer of healthy microbiota in an animal model of alcohol-induced anxiety and depression reduced the clinical manifestation in the animal (76). On the other hand, it was reported the "transfer of depression" trough microbiota. Indeed, germ free mice who underwent to FMT derived from MDD patients resulted in depression-like behaviors compared with colonization by microbiota derived from healthy control individuals (40). Furthermore, another study confirmed that FMT from depressed patients to microbiota-deficient rats could induce behavioral and physiological features characteristic of depression in the recipient animals, including anhedonia and anxiety-like behaviors (77). Unfortunately, the evidence for the use of FMT in humans is still limited (78, 79). A small study on 17 patients with functional gastrointestinal disorders treated with FMT reported an improvement of depression and anxiety symptoms independently of gastrointestinal symptom changes (80). A further small clinical study demonstrates that FMT in patients with IBS-D is able to reduce levels of anxiety and depression, as well as gastroenterological symptoms, in particular was associated to the

TABLE 1 Results from clinical trials on modulation of gut microbiota in anxiety and depression.

Type of drug	Drug	Effects	References
Antibiotics	Minocycline	anti-inflammatory, neuroprotective, anti depressant	(54, 55)
	Cycloserine	improves effect of conventional therapy on several psychiatric disorders	(56)
	penicillins	increased risk for depression and anxiety	(57)
	quinolones	increased risk for depression and anxiety	(57)
Probiotics	*Clostridium butyricum* (CBM588)	improves effect of conventional therapy in depression	(60)
	L. helveticus R00052 and *B. longum* R0175	Amelioration of the BDI in MDD, contrasting results by another study that failed to improve depressive symptoms	(61, 65)
	L. acidophilus, *L. casei* and *B. bifidum*	Reduction of BDI score	(62)
	Bacillus Coagulans MTCC 5856	Amelioration of depression and IBS symptoms	(63)
	Lactobacillus plantarum DR7	Amelioration in symptoms and psychological scores	(64)
	B. Longum NCCC3001	Ameliorate depression, improves quality of life, but not anxiety in IBS	(66)
Prebiotics	oligofructose enriched inulin	No significant effects on healthy subjects	(68)
	inulin	Improve weel-being in healthy subjects	(68)
	beta-glucan (derived from *Saccharomyces cerevisiae*)	No effect on depression score	(69)
	galacto-oligosaccharides	No changes on anxiety and depression scale	(61, 71)
	scFOS	Improves depression and anxiety score in IBS, correlating with the increase of Bifidobacteria	(70)
Synbiotics	*Lactobacillus casaei, Lactobacillus acidofilus, Lactobacillus bulgarigus, Lactobacillus rhamnosus, Bifidobacterium breve, Bifidobacterium longum, Streptococus thermophiles*, and fructo-oligosaccharide	Improve depressive symptoms in MDD	(73)
	fructo-oligosaccharides, galacto-oligosaccharides, and inulin*; Lactobacillus acidophilus T16, Bifidobacterium bifidum BIA-6, Bifidobacterium lactis BIA-7, and Bifidobacterium longum BIA-8*	Symbiotic mixture is superior to probiotics alone in improving depression an anxiety simptoms	(74)

The table report the main results described in human studies, however describe as within the same pharmacological class there are promising even if sometimes conflicting results.

decreased abundance of Faecalibacterium, Eubacterium and Escherichia (81). Further studies are needed to validate the procedure and to identify microbiome more efficacious for FMT.

Final remarks

The microbiota-gut–brain axis is an integrative system that involves metabolic, immunological and neuroendocrine signals, and alterations of these pathways play relevant roles in human neurological diseases. Extensive research has demonstrated that diet, drugs and stress influence both composition and function of gut microbiota, which in turn can modulate neurophysiology and behavior. Therefore, gut microbiota represents a key mechanism underlying the impact of environmental stimuli on brain function and identifying the biological pathways involved in the microbiota-gut-brain axis may be relevant to understand the pathophysiology of human mood disorders. Further, developing therapeutic strategies to modify the composition of gut microbiota may offer novel and personalized therapeutic tools (82). Indeed, several studies have reported that treatments able to modify the intestinal microbiota exerted a significant effect on the symptoms of anxiety disorders and depression in humans. More specifically, treatment with probiotics and synbiotics showed the best results in terms of symptom improvement, suggesting a potential role as adjunctive therapy. Unfortunately, the results about prebiotics alone are not satisfactory in the setting of mood disorders. Results from FMT studies in humans are fascinating but still too weak. Finally, the evidences from antibiotic studies are conflicting (83), because while some drugs such as minocycline and cycloserine have shown to have beneficial effects, other drugs of wide clinical use, as penicillins or quinolones, may increase the risk for depression and anxiety. In this review we have analyzed how some pharmacological approaches can modify the gut microbiota and promote a favorable effect on anxiety and depression. On the other hand, in recent years, "non-pharmacological" treatments are also being considered to regulate microbiota composition. It is known that diet plays a fundamental role in modulating the microbiota (84), this is true both in health and in disease. In particular, several evidences are emerging on how diet can play a role in the treatment of behavioral disorders (85). For instance, it has been shown that a diet rich in fat can favor the development or persistence of anxiety and depression, an effect sometimes reversible with probiotics (86). Furthermore, experimental models have shown how a supplementation diet with psychoactive metabolites, such as tryptophan, can have a protective role on the development of these mood disorders through the reduction of stress-induced gut barrier damage and inflammatory responses in the gut (87). Still reporting on non-pharmacological approaches, in the last year very interesting results have emerged from studies evaluating the role of cognitive behavioral therapy (CBT) in modifying the microbiota. For instance, a small study demonstrate that mindfulness CBT promote changes in gut microbiota of subjects affected by anxiety, in particular the individuals who responded better by reducing anxiety modified the microbiota making it more similar to healthy subjects and interestingly they increased the metabolism of tryptophan (88). The interpretation of these results opens up new frontiers on the modulation of the gut-brain axis, in fact it appears possible to modulate it in both directions (gut-brain and brain-gut) to obtain modifications for therapeutics.

In conclusion, drugs and non-pharmacological approaches regulating the composition of intestinal microbiota represent promising beneficial strategies against anxiety and depression. The study of the crosstalk between microbiota and brain can improve knowledge about the development of mood disorders and help to identify new therapeutic tools for the personalized medicine.

Author contributions

SB, SF, and AG contributed to conception and design of the study. SB and SF wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version

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Conflict of interest

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

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Akkermansia muciniphila: The state of the art, 18 years after its first discovery

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Akkermansia muciniphila (A. muciniphila) is an anaerobic, Gram negative and mucin-degrading bacterium of the phylum Verrucomicrobia isolated in 2004 from human feces. Although it is a common resident in the human intestinal tract, it has also been detected in other anatomical sites. Genomic studies have revealed that *A. muciniphila* can be divided into different phylogroups with distinct metabolic properties. There is growing evidence regarding its beneficial impact on human health. Indeed, *A. muciniphila* is considered as a promising next-generation probiotic for treating cancer and metabolic disorders. The large-scale production of *A. muciniphila* is, therefore, a challenge. Beside mucin-based medium, other culture strategies have enabled its isolation. The administration of both live and pasteurized forms of *A. muciniphila* has shown to be promising in animal models. Alternatively, the administration of various prebiotics has also been assessed for enhancing its abundance in the human gut. Future prospects include human clinical trials, some of which are currently ongoing. This paper provides an overview of what is currently known about *A. muciniphila's* phenotypical and genotypic traits, as well as its culture techniques and its connections to a number of human diseases and its potential application as an effective next generation probiotic.

KEYWORDS

human health, metabolic diseases, cancer, culture, probiotic, microbiota, *Akkermansia muciniphila*

Introduction

Within the human microbiome, the gut microbiota has, to date, been the most characterized, and its function and importance in maintaining the balance between human health and pathology has been widely investigated. Alteration of the composition of the gut microbiota has been linked to several diseases, including inflammatory bowel syndrome (1),

type 2 diabetes (2), and cancer (3) as well as eating disorders (4) and psychological disorders (5). Different phyla are reported in the gut, the two phyla Firmicutes and Bacteroidetes represent 90% of gut microbiota. Other reported phyla include Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (6). *A. muciniphila* is the only species in the Verrucomicrobia phylum that has been reported in the gastro-intestinal tract. Discovered and isolated from the stool of a healthy individual in 2004 by Derrien et al. (7), *A. muciniphila* relies on mucin for carbon, nitrogen and energy. Since then, it has been reported that it constitutes between 1% and 3% of the fecal microbiota and is present in more than 90% of healthy adults tested, but decreases in the elderly (8, 9). The majority of the research studies reporting the presence of *A. muciniphila* presence in the human digestive tract are based on metagenomic analysis, but only few studies have reported its isolation. The capacity of *A. muciniphila* to degrade and use mucin as a unique source of carbon and nitrogen gives it significant importance in the human gastro-intestinal tract, giving the opportunity to other bacteria to survive and grow by using the metabolites resulting from mucin degradation. These metabolites also play a role in the inflammatory status of the host (10). *A. muciniphila* was found to regulate the immune system, improve the gut barrier function and ameliorate metabolism in the case of obesity and diabetes, especially *in vitro* or in mice models (11–13). Furthermore, an association was found between high relative abundance of *A. muciniphila* and a lower incidence of obesity (14). Its abundance is found to decrease in different kind of diseases such as cancer (15–17), type 2 diabetes (18), inflammatory diseases (19) and liver diseases (20). These findings allowed the association between the presence of *A. muciniphila* and the healthy status of human beings, given that its abundance significantly decreases in many diseases. For this reason, it could be used as a marker of certain diseases with differing severity. Due to its beneficial effects on the human body, recent studies have also promoted its use as a probiotic (21, 22). To date, there are three validly published studies that reported the safety of use and the beneficial role of *A. muciniphila* in obese humans as a probiotic (12, 23, 24), while two clinical trials are in progress to evaluate the effects of the use of *A. muciniphila* in obese patients with type 2 diabetes and in hyperglycemic adults (NCT04797442/NCT05114018). The purpose of this review is to summarize what is currently known about *A. muciniphila* in terms of both the phenotypical and the genotypical characteristics, as well as its culture methods. We will also discuss its relationships with many human diseases. And most importantly, we will discuss the already established human trials and those that are still in progress focusing on and its possible use as a promising probiotic.

The *Akkermansia* genus

Since its discovery by Derrien et al. (7) in 2004, the *Akkermansia* genus, which is a part of the division Verrucomicrobia contains only two known species: *A. muciniphila* and *A. glycaniphila* (25). However, a recent study analyzing metagenome-assembled genomes of *Akkermansia* suggested the presence of two more putative species (26), while another study cited the presence of eight different species in the genus *Akkermansia* (27). *Akkermansia* spp. are Gram-negative, non-motile, non-spore forming and anaerobic bacteria. Cells are oval shaped with a mean diameter of 0.6–1.0 μm (7).

Taxonomy

A study analyzed 23 whole genome sequences of the *Akkermansia* genus and revealed that these strains formed four clades, divided into four species based on dDDH values (28), while a more recent study has divided *Akkermansia* strains into five distinct group (29). Moreover, it has been shown that single nucleotide polymorphisms (SNPs) were not evenly distributed throughout the *A. muciniphila* genomes, while genes in regions with high SNPs are found to be related to metabolism and cell wall/membrane envelope biogenesis (28).

When it comes to *A. muciniphila*, many genomic studies have been conducted in order to study its genomic diversity. *A. muciniphila* can be subdivided into three phylogroups, with high nucleotide diversity and distinct metabolic and functional profiles (30). However, a recent study has reported the presence of four different *Akkermansia* phylogroups, based on pangenome analysis (31). Another study analyzed different *A. muciniphila* strains from different phylogroups and revealed that each phylogroup has some specific phenotypes such as oxygen tolerance or sulfur assimilation. These phenotypes can influence the colonization of the gastrointestinal tract (32).

Metabolic characteristics

This genus uses mucin as its only carbon and nitrogen source, but it has been proven that it can grow in a medium containing glucose, N-acetylglucosamine and N-acetylgalactosamine, when provided with other protein sources (7, 25). The uptake of these sugars can also be enhanced by adding mucin, revealing the role of other mucin-derived components in its growth (33).

A. muciniphila has numerous candidate mucinase-encoding genes but surprisingly lacks genes coding for canonical mucus-binding domains (27). This capacity to degrade mucin might be essential to the survival of other bacteria in the human gut, as mucin degradation by *A muciniphila* provides metabolites that supports the growth of other bacteria such as *Anaerostipes caccae* by changing the transcriptional profile to induce an increase in the expression of mucin degradation genes and a reduction in the expression of ribosomal genes (34). Among the various studies aiming to identify the enzymes involved in mucin

degradation, one has succeeded in identifying a novel phospholipid-regulated β-galactosidase involved in mucin degradation (35). Further work revealed other beta galactosidases involved in the complex mucin degradation machinery (36). *A. muciniphila* can survive without the addition of vitamins to the medium. It was even proven in a recent study that some *A. muciniphila* strains were able to synthesize vitamin B12 (31).

Resistance to antimicrobial agents

A. muciniphila and *A.glycaniphila* strains have been shown to be resistant to ampicillin and vancomycin (7, 25, 37, 38). Specifically, *A. muciniphila* Muc^T was also found to be resistant to other antibiotics, including metronidazole and penicillin G, but susceptible to doxycycline, imipenem, and piperacillin/tazobactam (38). This antibiotic profile can change from one strain to another. For example, another *A. muciniphila* strain isolated in 2017 was sensitive to penicillin, imipenem, ceftriaxone and amoxicillin but resistant to ofloxacin (37). In 2015, a study aimed at assembling the genome of a strain sequenced directly from a human stool sample detected its presence, by performing an in-silico prediction of eight beta lactamase genes. Moreover, three macrolide resistance genes were detected with only one sharing 65% similarity with a known macrolide gene. Finally, resistance to vancomycin, chloramphenicol, sulfonamide, tetracycline and trimethoprim was associated with only one gene (39).

A. muciniphila distribution within the human body

Digestive tract

A. muciniphila is a common bacterial component of the human intestinal tract (9). A study by Collado et al. found that the presence of *A. muciniphila* presence increases from 16% of the samples of one-month-old infants to 90% at 12 months, while it is present in all the adult samples. Similarly, levels also increase in early life to reach levels similar to that observed in adults within a year. On the other hand, this level decreases significantly in the elderly (8).

Aiming to characterize the whole gut microbiota, Mailhe et al. collected samples from various parts of the digestive tract: the stomach, duodenum, ileum, and the left and right colon and analyzed those samples using culturomics and metagenomics. They succeeded in cultivating *Akkermansia muciniphila* in the left colon. In terms of metagenomic analysis, the Verrucomicrobia phylum, represented by the *Akkermansia* genus, was detected in the duodenum, ileum, and the right and left colon (40). Moreover, Ye et al., detected *Akkermansia*-like sequences in three out of six duodenal fluid samples (41). Another study exploring the duodenal and rectal microbiota in luminal contents and biopsy tissues in healthy volunteers found Verrucomicrobia sequences in duodenal biopsies, mucus and rectal biopsies (42). The presence of *Akkermansia* sequences was reported in the jejunal fluid, the pancreas and the bile with mean relative abundance of 0.01%, 0.05% and 0.01%, respectively, in a study exploring disturbances in the microbiome in patients undergoing pancreaticoduodenectomy (43). Analysis based on 16S rRNA genes uncovered the presence of *Akkermansia* sequences in ileocecal biopsies of patients with primary sclerosing cholangitis (PSC), ulcerative colitis and in non-inflammatory controls, with no significant differences between the different groups (44). The presence of Verrucomicrobia or *Akkermansia*-like sequences were detected much more frequently in the large intestine (45, 46) (Figure 1).

Oral cavity

There is no significant evidence of the presence of *A. muciniphila* in the oral cavity. Le et al. highlighted an absence of *A. muciniphila* in the oral cavities of 47 pediatric patients after PCR screening (47). A study performed in 2017 by Coretti et al. assessed the subgingival microbiota of smokers and non-smokers with chronic periodontitis compared to a control group. They found that the Verrucomicrobia phylum was significantly lower in people with chronic periodontitis (48). *A. muciniphila* was also detected in the saliva sample of a choledocholithiasis patient. He was the only positive patient out of six and the relative abundance was very low (41) (Figure 1). While its presence is not abundant in the oral cavity, *A. muciniphila* can be a potential therapeutic agent for periodontitis. In an experimental periodontitis mouse model, *A. muciniphila* showed protective effects by decreasing inflammatory cell infiltration and reducing alveolar bone loss (49).

Urinary tract

Although urine was long considered sterile, some studies have proved the presence of resident microorganisms by using culture and molecular based techniques (50). Few studies have detected the presence of *A. muciniphila* in the urine. Mansour et al. analyzed tissue and urine samples from patients with bladder carcinoma in order to compare the microbiota in both type of samples. Sequencing results showed that the *Akkermansia* genus was present in both type of samples but

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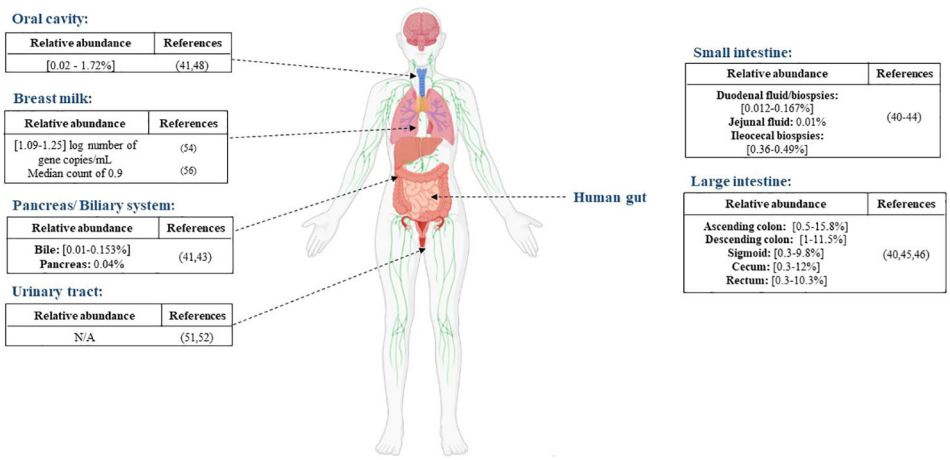

FIGURE 1
Distribution of *A. muciniphila* in different anatomical sites, the reported abundance in each site and the corresponding references.

was over-represented in the tissue samples compared to the urine samples (51). Another sequencing-based study reported a decrease in the levels of the phylum Verrucomicrobia in urine samples from an elderly Type 2-diabetes mellitus group compared with a control group (52).

Human breast milk

Human milk contains nutrients providing immunological and other health benefits to new-born babies. Studies on human milk show that it provides a source of commensal microorganisms for the new-born gut (53). As for *A. muciniphila*, its presence in human breast milk was reported for the first time in a study conducted by Collado et al. (54) The study showed that *A. muciniphila* was found in milk samples taken from women shortly after giving birth (colostrum), as well as at one and six months, with mean concentrations of 1.25, 1.09, and 1.20 log number of gene copies/mL, respectively. Moreover, they demonstrated that *A. muciniphila* was more abundant in overweight mothers than in normal weight mothers. Another study in 2014 discovered the presence of *Akkermansia*-like species using 16S rRNA sequencing in human breast tissue samples of 43 women (aged 18 to 90 years) (55). In addition, *A. muciniphila* was also found by qPCR analysis in milk colostrum samples collected from 11 women after an elective caesarean section, with a median count of 0.9 (56). Furthermore, metagenomic analysis of breast milk samples from healthy Korean mothers detected the presence of the *Akkermansia* genus (57). Finally, in a study aiming to evaluate the impact of maternal breast milk composition on children who develop coeliac disease (CD), milk samples were collected from mothers with a genetic predisposition to CD and a control group. The genus *Akkermansia* was found in milk from mothers in the CD group and the control group but was more abundant in the CD group (58). The presence of *A. muciniphila* in the human breast milk might be due to its ability to use human milk oligosaccharides (59). Although it is important to note that this ability is strain dependent (60).

A. muciniphila culture methods

The *Akkermansia* genus was isolated for the first time from a human stool sample, with *A. muciniphila* being the type species using a basal medium supplemented with 0.25% gastric mucin and 0.7% rumen fluid. The human stool was serially diluted into sterile anaerobic Ringer's solution containing 0.5 g cysteine. Each dilution was inoculated in the medium as described previously. Pure colonies were isolated using the same medium containing 0.75% agar. Since then, other studies have used the same medium in order to isolate other *Akkermansia* strains (61). Twelve years later, the same medium enabled the cultivation, from reticulated 193 python faeces (25) of *A. glycaniphila*, which depends on mucin as its only energy source for carbon and nitrogen. In a recent study focusing on distinguishing the fast and slow growing bacteria of the faecal microbiota by changing the dilution rates in mucin-supplemented media, *A. muciniphila* was isolated in low dilution rates (62). It was also suggested that the growth of *A. muciniphila* is promoted in a media rich in sugar and mucin (63). To understand how *A. muciniphila* adapts to mucin, transcriptomic and metabolomic analysis showed an upregulation of genes related to energy metabolism and cell

growth in the presence of 0.5% of mucin, correlated with smaller diameter of the cells, a sign of bacterial division, and proliferation. Moreover, enzymes such as fucosidase, beta-galactosidase and hexosaminidase were also overexpressed to degrade mucins into oligosaccharides and eventually monosaccharides to use them as a source of energy (64). Another study comparing the growth of *A. muciniphila* in static and dynamic culture simulating the physiological conditions in the colon showed that the biomass of *A.muciniphila* in dynamic culture was significantly higher after 48 hours compared to under static conditions. The same study tested the growth of *A. muciniphila* in five different culture conditions: human mucin, porcine mucin, brain heart infusion (BHI) medium only, or BHI supplemented with porcine mucin or human mucin. *A muciniphila* can grow in all the media tested, but the lowest biomass was found in BHI only, and human mucin is the most ideal for the cell growth (65).

However, some studies have proved that *A. muciniphila* can be isolated without mucin-based media culture, such as from a blood culture sample after 72 hours of subculture on Columbia agar with 5% sheep blood (37). Similarly, another strain was isolated from a stool sample after diluting it in pre-reduced phosphate-buffered saline (PBS), plating on Columbia blood agar supplemented with 5% horse blood, and subjected to two to four days of incubation at 37°C under an H_2-CO_2-N_2 (1:1:8 [vol/vol/vol]) gas mixture (66). Finally, culturomics techniques enabled the isolation of *A. muciniphila* from fresh stool samples using the following anaerobic culture conditions at 37 °C: culture bottle containing 5% sheep blood and 5% rumen fluid, YCFA medium, YCFA solid medium, reinforced clostridiales solid medium, brain heart infusion (BHI) solid medium, Columbia solid medium and, finally, De Man, Rogosa and Sharpe (MRS) solid medium (67, 68) (Table 1).

The growth of *A. muciniphila* has been proven to be pH dependent. The optimum pH was 6.5. Low pH strongly inhibits its growth, explaining its abundance in the distal colon in comparison to the proximal colon (69, 70). *A. muciniphila* also showed high tolerance to oxygen (up to 72 hours) (71). When oxygen is present at nanomolar concentrations, its growth rate and yield were increased compared to those observed in strict anaerobic conditions. This is due to the presence of cytochrome bd complex that can function as a terminal oxidase (72). *A. muciniphila* showed high tolerance to different temperatures (4°C, 22°C, and 37°C). In contrast, cell viability showed significant decrease at 44°C. In this study, its stability and tolerance to the different gastrointestinal conditions were evaluated. Interestingly, *A. muciniphila* showed stability after exposure to simulated gastrointestinal conditions. Other evaluations might be needed in order to understand the effect of stress on the metabolism and the adhesion properties of the bacterium (71).

Other studies have tested different growth conditions for *A. muciniphila*. For example, as mentioned before, a study proved that *A. muciniphila* is also able to grow on human milk *in vitro* and degrade its oligosaccharides, which is explained by proteomic analysis showing an increase in the expression of glycan degrading enzymes such as α-L-fucosidases, β-galactosidases, exo-α-sialidases and β-acetylhexosaminidases (59). *A. muciniphila* does not code for the enzyme that mediates the conversion of fructose-6-phosphate (Fru6P) to glucosamine-6-phosphate (GlcN6P), which is essential in peptidoglycan formation. This finding suggests that N-acetylglucosamine found in mucin is crucial for the growth of *A. muciniphila*, thus explaining its importance and the adaptation of *A. muciniphila* to its components (73). In contrast, bile salts were found to impede the growth of

TABLE 1 *A. muciniphila* culture methods.

Strain	Sample	Medium used/Culture conditions	Authors	Year
A. muciniphila	Stool sample	0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NaCl; 4 g $NaHCO_3$; 0.3 g NH_4Cl; 0.25 g $Na_2S.7–9H_2O$; 0.1 g $MgCl_2.6H_2O$; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 0.5 mg resazurin and 1 ml vitamin solution 0.25% gastric mucin and 0.7% rumen fluid.	Derrien et al. (7)	2004
	Blood culture sample	Incubation of blood culture sample for 96 hours Colonies isolated after 72 hours of subculture on Columbia agar with 5% sheep blood	Dubourg et al. (37)	2017
	Fecal sample	Mucin-supplemented media, low dilution rates	Adamberg et al. (62)	2018
	Intestinal microbiota samples	Bacterial growth media (containing sugars, nitrogen, vitamins, minerals, hematin, amino acids and mucin)	Yousi et al. (63)	2019
	Stool samples	Culturomics in anaerobic conditions with the following media: Culture bottle with 5% sheep blood and 5% rumen fluid/YCFA liquid medium and solid/Reinforced clostridiales solid/ Brain heart infusion solid/Colombia solid/De Man, Rogosa and Sharpe solid at 37°C	Lagier et al. (67) Diakite et al. (68)	2016/ 2020
	Stool sample	Columbia blood agar supplemented with 5% horse blood and two to four days of incubation at 37 °C under a H_2-CO_2-N_2 (1:1:8 [vol/vol/vol]) gas mixture	Ogata et al. (66)	2020

Table resuming the different studies that have succeeded in cultivating A. muciniphila, the year of publication, the origin of the sample and the media and culture conditions used in each study.

A. muciniphila, except for sodium deoxycholate which increased its growth (74).

A. muciniphila and health

Cancer

The association between cancer and changes in the gut microbiota in humans has been widely investigated. More specifically, the role of *A. muciniphila* in different types of cancer has been assessed (75). One study highlighted the decrease in the abundance of faecal *A. muciniphila* among non-small-cell lung cancer patients compared to controls (15), through metagenomic and metabolomic profiling, while an increase was detected using real time PCR on gut mucosal tissues samples of colorectal cancer patients compared to controls (16), Similarly, an abundance of *A. muciniphila* along with other bacteria was significantly increased in patients with different gastrointestinal cancer such as esophageal, gastric and colorectal cancer, compared to the control group (17) (Table 2).

A study performed on pancreatic cancer xenograft mice model showed an increase in *A. muciniphila* in the guts of mice receiving Gemcitabine treatment, as well as a decrease in tumour volume (92). In a prostate cancer mice model, the relative abundance of *A. muciniphila* in the gut was decreased. However, this decrease was reversed after receiving androgen deprivation therapy (93).

In other studies concentrating on the role of gut microbiota in the response to anti-PD1 (Programmed cell Death protein 1) immunotherapy, the presence of species such as *Bifidobacterium breve*, *Bifidobacterium longum*, *Faecalibacterium prausnitzii* and, most importantly, *A. muciniphila* in the gastro-intestinal tract of cancer patients was associated with a stronger immune response to the therapy and subsequently an extended survival of these patients (94). In another study based on anti-PD1 therapy for non-small cell lung cancer (NSCLC), two genera, *Akkermansia* and *Olsenella*, were significantly higher in the stable disease group than in the progressive disease group (95). Similarly, gastric cancer patients showed an enrichment for the genus *Akkermansia* before and after radical distal gastrectomy (96).

In epithelial tumours, metagenomic analysis of stool samples from patients receiving immune checkpoint inhibitors showed correlations between clinical responses to the treatment and the relative abundance of *A. muciniphila* (97). The same team also found an increase in *A. muciniphila* levels in patients responding favourably to immune checkpoint blockade treatment in a cohort of renal cell carcinoma patients (98).

In a study of anti-colon cancer therapy based on treatment with FOLFOX, it was demonstrated that the abundance of *A. muciniphila* significantly increased in patients receiving the treatment, which was positively correlated with the therapeutic effect (99).

In terms of colorectal cancer (CRC), it has been demonstrated that CRC tissues increase the expression of mucin2 compared to normal mucosa (100).

Finally, in a randomized trial evaluating the impact of probiotic supplementation on the outcome of gut microbiome and metastatic renal cell carcinoma (mRCC), patients who had received a treatment and had been supplemented with probiotics present a higher abundance of *A. muciniphila* in the gut (101). Furthermore, there was a positive and significant association between the presence of *A. muciniphila* and the clinical benefit of the treatment (101).

Metabolic diseases

The abundance of *A. muciniphila* is decreased in many metabolic disorders, such as inflammatory bowel diseases, appendicitis and obesity (76, 77, 79) suggesting its association with healthy intestine and normal mucosa. Eating disorders, such as binge eating disorder (78) (Table 2) have also been associated with a decrease in the levels of *A. muciniphila*.

These findings reveal the importance of *A. muciniphila* as a biomarker of health status (102). Many studies targeted treating metabolic diseases have focused on tracking the levels of *A. muciniphila* to assess the success of the therapy (103).

Liver diseases

Liver diseases are associated with changes in the gut microbiota, specifically a decrease in the levels of *A. muciniphila*. Grander et al. suggested that the decrease in levels of *A. muciniphila* in alcoholic liver disease is indirectly correlated with disease severity (20) (Table 2). In contrast, other studies have highlighted an increase in *A. muciniphila* after treatment. For example, in non-alcoholic liver disease mice models, it was reported that treatment with Bilberry anthocyanins increases the levels of *A. muciniphila* in the digestive tract, associated with the efficacy of the treatment on NAFLD (104). Similarly, another study using an alcoholic liver disease mice model showed that treatment with berberine also cause an increase in the levels of *A. muciniphila* (105).

Obesity

A. muciniphila levels are negatively correlated with obesity. Studies have shown that the abundance of *A. muciniphila* decreases significantly in overweight/obese preschool children (81), and in obese adult women (82) compared to the normal weight/lean group. Moreover, its abundance is even lower in severe obesity (80) (Table 2). The presence of *A. muciniphila* is also associated with the normal weight gain in pregnant women (106). The beneficial effects of *A. muciniphila* can also be

TABLE 2 Association between *A. muciniphila* and different clinical diseases.

Type of diseases	Pathology	Samples	Cohort	Technique	Abundance of *A. muciniphila*	Other findings	References
Metabolic disorders	**Acute appendicitis**	appendices, cecal biopsies and faecal samples	70 patients with appendicitis/400 controls	rRNA-based FISH	↓	*A. muciniphila is* inversely related to the severity of the disease.	(76)
	Inflammatory bowel disease (IBD)	Biopsies	46 IBD/20 controls	Real-time PCR	↓	*x*	(77)
	Binge eating disorder (BED)	Stool samples	101 obese patients with/without BED	Sequencing and subsequent bioinformatics	↓	x	(78)
	Ulcerative colitis (UC)	Colonic biopsies and mucus brushings	20 patients with active UC/14 with quiescent UC/20 healthy controls	Real-time PCR	↓	Inverse relationship between *A. muciniphila* and inflammation	(19)
	Alcoholic liver disease (ALD)	Fecal samples	21 patients with ALD/16 non-obese healthy controls	Quantitative PCR	↓	Decrease of faecal *A. muciniphila* indirectly correlated with hepatic disease severity	(20)
	Obesity	Fecal samples	164 participants with variable geographical origin, diet, age, and gender	Metagenomics	↓	Fecal salinity was associated with obesity and a depletion in anti-obesity *A. muciniphila*	(79)
			21 adult women with severe or moderate obesity	Metagenomics/ Quantitative PCR	↓	Significant lower *A. muciniphila* abundance in severe obesity than in moderate obesity	(80)
			20 overweight children/20 control children	Quantitative PCR	↓	*x*	(81)
			17 lean/15 obese females		↓	*x*	(82)
	Type 2 diabetes (T2D)	Fecal samples	134 Danish adults with prediabetes/134 controls	Sequencing	↓	x	(83)
			182 lean/obese individuals with T2D	Metagenomic/ Metabolomics	↓	Significant decrease of *A. muciniphila* abundance in lean individuals with T2D than without T2D, but not in the comparison of obese individuals with and without T2D.	(18)
			345 patients with T2D/nondiabetic controls	Sequencing	↑	x	(84)
		Urine samples	70 female T2DM patients/70 healthy females		↓	Decreased Akkermansia muciniphila was associated with high Fasting blood glucose and urine glucose	(85)
	Clostridioides difficile infection (CDI)	Fecal samples	50 CDI patients/50 healthy controls	Real-time Quantitative PCR	↑	x	(86)
Cancer	**Non-small cell lung cancer (NSCLC)**	Stool samples	11 NSCLC patients/ 8 controls	Metagenomics/ Metabolomics	↓	x	(15)
	colorectal cancer (CRC)	gut mucosal tissues	18 CRC patients/18 non-CRC controls	Quantitative PCR	↑	x	(16)
	Gastrointestinal cancer	Stool samples	130 gastrointestinal cancer patients/147 healthy controls	16S rRNA sequencing	↑	x	(17)

(Continued)

TABLE 2 Continued

Type of diseases	Pathology	Samples	Cohort	Technique	Abundance of *A. muciniphila*	Other findings	References
Other diseases	**Allergic asthma**	stool samples	92 children (between 3 and 8) with asthma/88 healthy children	Quantitative PCR	↓	x	(87)
	Atopic dermatitis (AD)/ Food allergy	Fecal samples	82 children with AD with absence and presence of food allergy	16S rRNA microbial analysis	↑	Fecal microbiome of children with AD and food allergy harbored relatively more *A. muciniphila* than children with AD without food allergy	(88)
	Psoriasis	Fecal samples	14 psoriasis patients/ 14 healthy controls	16S rDNA sequencing	↓	x	(89)
	CaOx dihydrate (COD) and monohydrate (COM) lithiasis	Fecal samples	24 patients diagnosed with CaOx lithiasis	Real-time PCR	↓	x	(90)
	Autism spectrum disorder (ASD)	Fecal samples	23 children with ASD/22 typically developing siblings/9 unrelated community controls	Real-time Quantitative PCR	↓	x	(91)

Table resuming the different studies that associated *A. muciniphila* with different diseases, the cohort, type of sample, and technique used in each study, as well as the change in the abundance of *A. muciniphila* and the references.
↑: Increase in abundance, ↓: Decrease in abundance.

observed in obese adults after a six-week calorie restriction period followed by a six-week weight stabilization diet. The adults included in this study had a healthier metabolic status when the abundance of *A. muciniphila* was high. Moreover, *A. muciniphila* was associated with other microbial species related to health (107).

However, another study on obese patients undergoing bariatric surgery, gastric banding or the Roux-en-Y gastric bypass procedure showed that the relative abundance of *A. muciniphila* was inversely correlated with the severity of obesity but was not associated with glucose homeostasis markers. Furthermore, a significant increase in the relative abundance of *A. muciniphila* was observed after the Roux-en-Y gastric bypass procedure but was not correlated with metabolic improvement (80).

When it comes to the mechanism of *A. muciniphila* in controlling obesity, evidence have shown that *A. muciniphila* stimulates glucagon-like peptide-1 (GLP-1) production by intestinal cells, leading overall to an improvement in insulin sensitivity, glucose tolerance and suppressing appetite (108).

Diabetes

In relation to diabetes, some studies have provided evidence revealing the association between *A. muciniphila* and the metabolism of glucose and its dysregulation. Allin et al. showed that abundance of *A. muciniphila* is decreased in individuals with prediabetes (83). One study showed that in lean individuals with T2D, the levels of *A. muciniphila* are lower compared to the control group, which is not the case with obese T2D patients (18). Another study also showed a decrease in *A. muciniphila* in T2D patients, associated with higher fasting blood glucose and urine glucose (85). However, one metagenomic study on a Chinese population found that some of the genes in *A. muciniphila* were enriched in type 2 diabetic subjects, perhaps due to differences in genes and lifestyle (84) (Table 2). In type 1 diabetes (T1D), NGS analysis of stool samples from T1D patients receiving probiotics showed an elevation of *Bifidobacterium animalis*, *A. muciniphila* and *Lactobacillus salivarius* associated with reduced fasting blood glucose levels and improvement of glycated hemoglobin levels (109). Plovier et al. recently highlighted the effect of pasteurized *A. muciniphila* to diminish fat mass development, insulin resistance, and dyslipidemia in mice. They also demonstrated that the outer membrane protein Amuc 1100 is involved in the bacterial-to-host contact through Toll-like receptor 2 signaling. Moreover, this protein partially mimics the effects of *A. muciniphila* on insulin resistance and gut barrier modification (12).

Inflammatory bowel diseases

Earley et al. quantified *A. muciniphila* in colonic biopsies and mucous swabs from patients with active ulcerative colitis and quiescent ulcerative colitis. They demonstrated that patients with active ulcerative colitis had a reduced abundance of *A. muciniphila* compared to quiescent ulcerative colitis and controls (19). Studies focusing on inflammatory bowel disease have shown that mucolytic bacteria levels increase in IBD

patients. However levels of *A. muciniphila* reduce, mainly due to the potential anti-inflammatory role of *A. muciniphila* (77). Another observational study has suggested that the relative abundance of *A. muciniphila* is inversely correlated to pain reduction in a cohort of IBS patients (110).

Other diseases

The depletion of *A. muciniphila* has also been associated with several allergic disorders, suggesting a potential educational role toward immunity. For example, decreased levels of *A. muciniphila* and *Faecalibacterium prausnitzii* in stool samples of patients with allergic asthma have been reported (87). In children with atopic dermatitis (AD), the presence of a microbial signature made it possible to differentiate between the presence and absence of food allergies. The fecal microbiome of children with AD and food allergies contains relatively less *B. breve*, *B. adolescentis*, *F. prausnitzii*, and *A. muciniphila* and more *E. coli* and *B. pseudocatenulatum* than children with AD without food allergies (88). Tan et al. also reported a decrease in the abundance of *A. muciniphila* in patients with psoriasis (89). In a study comparing the intestinal dysbiosis between CaOx dihydrate (COD) and monohydrate (COM) lithiasis, a large decrease in the mean values of the mucin-degrading *A. muciniphila* was observed, which is significantly more intense in COD than in COM lithiasis (90). Vakili et al. highlighted an increase in levels of *A. muciniphila* in patients with clostridium difficile infection (CDI) (86).

A decrease in *A. muciniphila* levels is also associated with many psychological disorders. For example, a study in children with autism showed a decrease in levels of *A. muciniphila* and *Bifidobacteria* species when compared with unaffected children (91). Another study showed that the abundance of *A.muciniphila* is reduced in ulcerative colitis patients suffering from depression, revealing a potential connection between psychological disorders and gut bacteria *via* the gut-brain axis (111) (Table 2). Finally, the protein Amuc_1100 was shown to have an antidepressant role in a chronic unpredictable mild stress (CUMS) mice model by down-regulating the brain-derived neurotrophic factor (BDNF) and inflammation in the hippocampus (112).

A. muciniphila: A new probiotic?

The development of A. muciniphila for clinical use

The consumption of certain beneficial microbes, known as probiotics, has been known to affect the gut microbiota. This is because the consumption of these organisms can trigger a variety of health benefits for the host (113). It has been noted that most of the probiotics sold on the market are microorganisms from the *Bifidobacterium* and *Lactobacillus* genera (114). They are safe to use and approved by the United States Food and Drug Administration (FDA) (115). Recently, however, new microbes identified by next generation sequencing methods are emerging and are also associated with health promotion. The safety of these microbes, called next generation probiotics (NGPs), as well as their formulation and administration are currently being processed (115). *A. muciniphila* has emerged as a potential NGPs due to its various benefits on health (116). For this purpose, an efficient and scalable workflow has been developed for the cultivation and preservation of *A. muciniphila* cells. This study resulted in viable *Akkermansia* colonies with high yields and stability, with a survival up to 97.9 ± 4.5% for one year if stored in glycerol-amended medium at -80°C (117) (Figure 2).

In recent years, there has been a lot of focus on the use of nonviable bacterial supplements (pasteurized forms) known as paraprobiotics (118) (Figure 2) as an alternative to live bacteria to lower the risk of infection. for example Druart et al. demonstrated that pasteurized *A. muciniphila* is safe to use as a food ingredient based on rat models (119). The safety of *A. muciniphila* products has also been recently reported in humans (12, 107). The pasteurized form is achieved when the bacteria suspension was heated at 70°C for 30 minutes, as described by Plovier et al. (12) By comparing the effects of live and pasteurized *A. muciniphila* on normal diet-fed mice, Ashrafian et al. showed that both forms of *A. muciniphila* could modulate lipid and immune homeostasis and improved health by modulating gut microbiota, while all these effects were dominantly observed in the pasteurized form (120). Another study conducted by Grajeda-Iglesias et al. demonstrated that pasteurized *A. muciniphila* was more efficient than the live version in elevating the intestinal concentrations of polyamines, short-chain fatty acids, 2-hydroxybutyrate, as well multiple bile acids. All these metabolites have been described to be associated with human health (121). Recent studies also started focusing on postbiotics, which refers to using inactivated cell components to promote health (122). In the case of *A.muciniphila*, many studies started focusing on the potential use of its extracellular vesicles (EVs) as postbiotics (Figure 2). For example, a study by Ghaderi et al. showed that live and pasteurized forms of *A.muciniphila* and its EVs can affect the expression of the endocannabinoid system and peroxisome proliferator-activated receptors (PPARs) genes involved in metabolic pathways, suggesting the potential possibility to use them as probiotic, parabiotic and postbiotic respectively in order to prevent metabolic diseases (123). Furthermore, *in vitro* study showed that treatment with *A. muciniphila* or its EVs could influence the expression of genes involved in the serotonin system and thus can be used as a serotonin modulation therapy (124).

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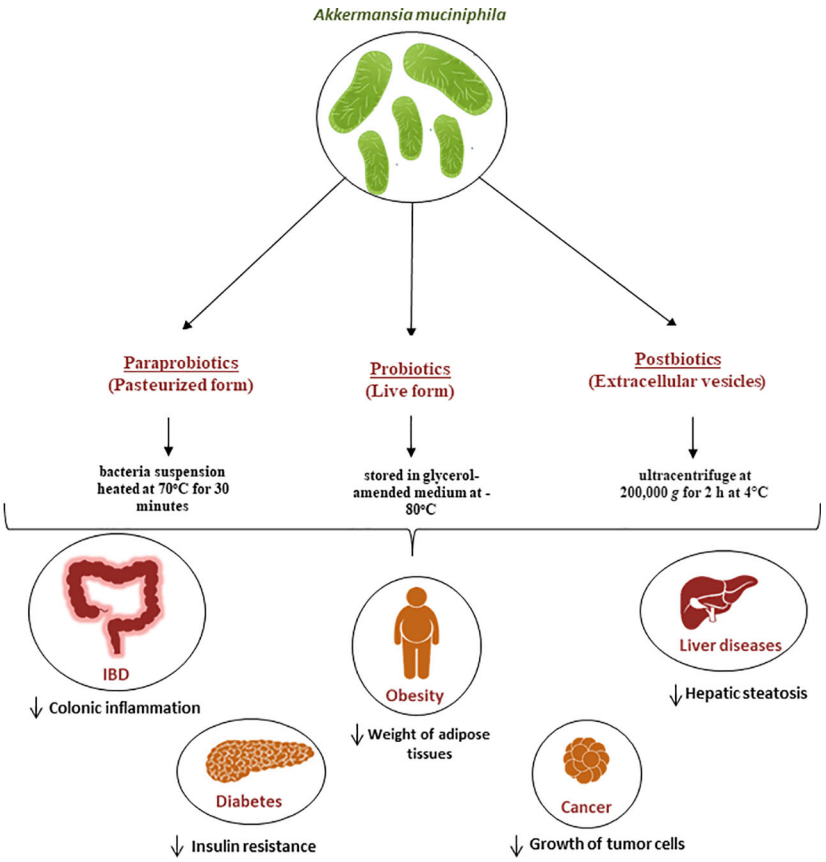

FIGURE 2
Role of *A. muciniphila* as a next generation probiotic for various metabolic diseases. Since its isolation in 2004, It has been demonstrated that *A. muciniphila* is crucial for the immune and metabolic systems regulation. It is now regarded as a "next-generation probiotic" for cancer and metabolic diseases including diabetes, liver diseases and obesity. Additionally, the pasteurized form known as parabiotic and the extracellular vesicles known as postbiotics are currently being used for the same diseases and have proven to be efficient in the treatment of these diseases.

Mice models

Many studies have focused on the causal link between *A. muciniphila* and improvements in metabolism (Figure 2). It has been shown that daily oral supplementation with live *A. muciniphila* at the onset of obesity, diabetes and gut barrier dysfunction in mice at the dose of 2.10^8 bacterial cells per day improves glucose tolerance, reduces adiposity and inflammation, therefore partly protecting against diet-induced obesity in mice (108, 125). In addition, animals receiving live *A. muciniphila* no longer exhibited insulin resistance, nor infiltration of inflammatory cells (CD11c) in the adipose tissue, which is a key characteristic of obesity and associated low-grade inflammation (108). In addition, it was noted that live *A. muciniphila* prevented the development of metabolic endotoxemia as an effect associated with the restoration of a normal mucus layer thickness (108). It is worth mentioning that all these findings have subsequently been confirmed by different groups and extended to other specific disorders such as atherosclerosis, hepatic inflammation and hypercholesterolemia (20, 126, 127). Furthermore, the administration of pasteurized *A. muciniphila* was correlated with an increase in energy expenditure in diet-induced obese mice, possibly explaining the mechanism by which administration of *A. muciniphila* can reduce body weight and fat mass gain (128).

The role and administration of *A. muciniphila* have been notably investigated in cancer. For instance, a study using prostate cancer mice model showed that the extracellular vesicles of *A. muciniphila* can be used as an immunotherapeutic agent for prostate cancer treatment, demonstrated by the decrease in tumors and the upregulation of immune cells such as tumor-killing M1 macrophages after injection of these vesicles in cancer bearing mice (129). Furthermore, the effect of the mucin degrading enzyme of *A. muciniphila* Amuc_1434* (130) was investigated in the inhibition of the proliferation of CRC tissues. The study has showed that the mucin degrading enzyme Amuc_1434* was able to inhibit the proliferation of CRC cells lines by mediating apoptosis *via* the

TRAIL pathway (131). However, Wang et al. suggested that treatment of CRC-mice with *A. muciniphila* increases the early level of inflammation and proliferation of the intestinal cells and therefore promotes the formation of tumors (132).

In liver diseases, recent studies have investigated the potential anti-fibrotic effects of heat-killed *A. muciniphila* Muc^T on the activation of hepatic stellate cell (HSC), where they demonstrated that heat-killed *A. muciniphila* Muc^T was safe and capable of improving LPS-induced HSC activation by modulating fibrosis markers (133). Moreover, oral supplementation in alcoholic steatohepatitis mice model induced a reduction in hepatic injury and steatosis, while enhancing mucus thickness and tight-junction expression (20). In an induced liver fibrosis mice model, it was revealed that treatment with live or pasteurized *A. muciniphila* or with its extracellular vesicles (EVs) can improve gut permeability, attenuating the expression of inflammatory biomarkers and subsequently preventing liver injury in treated mice (134). Another study showed that oral administration of *A. muciniphila* or its EVs could improve the anti-inflammatory responses eventually leading to a prevention from liver injury in mice (135). A recent study conducted by Rao et al. in mice explored the therapeutic effect of *A. muciniphila* in metabolic dysfunction-associated fatty liver disease (MAFLD). The study results indicated that *A. muciniphila* exhibited anti-MAFLD activity correlated with lipid oxidation and an improvement in gut-liver interactions by regulating the metabolism of L-aspartate (136).

The importance of *A. muciniphila* in maintaining good health and the negative correlation between its presence and obesity (81, 107) have initiated many studies focusing on using the mucin-degrading bacteria as a treatment. For example, studies performed on high-fat diet-fed mice models treated with *A. muciniphila* showed that this treatment prevents body weight gain, calorie intake and reduces the weight of adipose tissues, thus improving the induced metabolic disorders. In addition, it had many other beneficial effects such as improving glucose homeostasis and insulin sensitivity, inhibition of intestinal inflammation and restoration of damaged gut integrity (108, 137, 138). Administration of the pasteurized form had similar effects. A study by Ashrafian et al. showed that the pasteurized *A. muciniphila* and its EVs totally reduced the High-fat diet (HFD) induced intestinal inflammation and preserved intestinal permeability (139). Pasteurized *A. muciniphila* was shown to attenuate inflammatory response and improve intestinal barrier integrity. This is probably due to stimulating AMP-activated protein kinase (AMPK) and inhibiting Nuclear Factor-Kappa B (NF-κB) activation through the stimulation of TLR2 on intestinal epithelial cells (140). Aiming to understand the mechanisms of *A. muciniphila* involved in modulating the host metabolism, Yoon et al. identified a protein named P9 which induces glucagon-like peptide-1 (GLP-1) secretion and brown adipose tissue thermogenesis (141). Ashrafian et al. demonstrated that *A. muciniphila* or its EVs significantly reduced the body and fat weight of HFD mice and improved intestinal barrier integrity and energy balance (142).

Another study has been conducted to prove that deficiency in *A. muciniphila* is correlated with a high incidence of diabetes in a NOD mouse model. This study showed that the oral transfer of *A.muciniphila* could delay the onset of diabetes through promoting mucus production, increasing the expression of antimicrobial peptide Reg3y, lowering serum endotoxin levels and the expression of islet toll-like receptor (143). Chellakot et al. found that the administration of *A. muciniphila* EVs improved intestinal tight junction function, glucose tolerance in high-fat diet-induced diabetic mice and reduced weight gain, indicating a potential role for EVs in diabetes and thus indicating its use as a therapy (144).

The therapeutic role of *A. muciniphila* has also been studied in inflammatory bowel diseases. It has been found that treatment of ulcerative colitis dextran sulfate sodium (DSS)-induced mice with metformin alleviates the phenotype associated with an increase in the expression of mucin2 and in the abundance of *A. muciniphila* compared to the control group. Moreover, the administration of *A. muciniphila* decreases disruption of the mucus barrier and colonic inflammation (145). Similarly, another study conducted on a colitis DSS-induced mice model showed that the oral application of EVS protects against colitis phenotypes, such as body weight loss and inflammatory cell infiltration of the colon wall (146). Similar effects were also observed after treatment of the mice model with Amuc_2109, a β-acetylaminohexosidase secreted by *A. muciniphila*. Treatment with Amuc-2109 also had anti-inflammatory effects by inhibiting the expression of inflammatory cytokines (147).

It was found that the outer membrane protein Amuc_1100 of *A. muciniphila* promotes the biosynthesis of 5-HT, which is a neurotransmitter and a key signal molecule regulating the gastrointestinal tract functions and other organs (112). Wang et al. found that *A. muciniphila* or Amuc_1100 improved gastrointestinal motility function and restored gut microbiota abundance and species diversity in antibiotic-treated mice. This finding represented an important approach through which *A. muciniphila* interacts with the host and further influences 5-HT-related physiological functions (148).

The anti-inflammatory and immunoregulatory roles of *A. muciniphila* has been assessed in other diseases in mice models. It was shown that the administration of pasteurized *A. muciniphila* in a mouse model of H7N9 influenza viral infection reduced mortality, given its anti-inflammatory and immunoregulatory roles (149). Likewise, the administration of *A. muciniphila* resulted in a decrease in inflammatory cell infiltration and bone destruction in a mouse model of calvarial infection (49). Treatment with *A. muciniphila* also resulted in decreased alveolar bone and systemic inflammation loss in an experimental *Porphyromonas gingivalis* induced periodontitis model (49, 150). These findings highlight the protective effects

of *A. muciniphila* and its use as a potential therapeutic agent to various diseases. However, Lawenius et al. showed that treatment with pasteurized *A. muciniphila* in mice reduces the accumulation of fat mass but does not protect against bone loss in a model of ovariectomized mice (151).

Moreover, another study performed in mice has reported that the presence of *A. muciniphila* and its EVs in the gut promote serotonin concentration, and also has an impact on serotonin signaling/metabolism through the gut-brain axis. These results suggest that *A. muciniphila* and its EVs can be considered as a new therapy for serotonin-related disorders (152). Ding et al. demonstrated that treatment of mice with depression induced by chronic restraint stress with *A. muciniphila* can reduce the depressive-like behavior of the mice, which was correlated with the increase in β-alanyl-3-methyl-l-histidine and edaravone (153).

Human trials

Few studies on the use of *A. muciniphila* as a probiotic in humans have been conducted. The study by Plovier et al. was the first to demonstrate that the administration of live or pasteurized *A. muciniphila* is safe in humans in a cohort of 20 subjects with excess body weight. An exploratory study conducted by Depommier et al. on 32 overweight and obese insulin-resistant human volunteers also demonstrated that daily oral supplementation with either live or pasteurized *A. muciniphila* bacteria was safe and well-tolerated up for three months. Furthermore, they showed that pasteurized *A. muciniphila* improves insulin sensitivity and reduces insulinemia and plasma total cholesterol, while slightly decreasing body weight and fat mass compared to a placebo group (23). Moreover, the same team suggested that peroxisome proliferator-activated receptor alpha activation by mono-palmitoyl-glycerol might underlie some of the beneficial metabolic effects induced by A. *muciniphila* in human metabolic syndrome (24). Metabolome analysis illustrates that administration of *A. muciniphila* in prediabetic individuals leads to a decrease in some amino acids (tyrosine and phenylalanine), potentially explaining its hepato-protective role (154). Two clinical studies are ongoing to prove the efficacy of pasteurized *A. muciniphila* in improving insulin sensitivity, and to assess the weight-loss and glucose-lowering effects of *A. muciniphila* WST01 strain in overweight or obese patients with type 2 diabetes (Table 3).

Enhancing the abundance of *A. muciniphila* with prebiotics/ other probiotics

One method of favorably modulating the gut microbiota is to administer growth-promoting substrates that can be used preferentially by health-promoting bacteria to promote their growth and the production of associated desirable metabolites. The rationale of selectively enhancing beneficial microbes in the gut led to the concept of prebiotics, initially described in 1995 by Roberfroid and Gibson (158).

While some technological and regulatory hurdles may limit the use of certain strains of probiotics, it should be possible to use prebiotics and other dietary components to selectively enhance their growth in situ. The prebiotic paradigm has shifted in recent years, following the discovery of newly identified putatively beneficial gut microbiota members to target for enrichment. Through the development of new cultivation techniques and high-throughput sequencing, these studies have been able to explore the various impacts of specific fibers and products which represent untapped source of food bioactive on gut microbiota (159). For example, Anhe et al. showed that cranberry extract, rich in polyphenols, has been shown to improve diet-induced obesity and several features of metabolic syndrome (MetS) in mice, while increasing the abundance of *A. muciniphila* (160). Moreover, studies demonstrated that supplementation with grape polyphenols can promote increased intestinal abundance of *A. muciniphila* in mice fed either high-fat or low-fat diet, thus resulting in lower intestinal and systemic inflammation (161, 162). The administration of polymeric procyanidins in mice fed a high-fat/high-sucrose diet increases the proportion of *A. muciniphila* by eight times, producing beneficial effects on metabolic homeostasis (163). Another interesting fruit extract rich in polyphenols is camu-camu extract. This prebiotic can also improve the homeostasis of glucose and lipids while also increasing the abundance of *A. muciniphila* after five weeks of supplementation in HFD fed mice (164). Dietary supplementation with polysaccharides such as fucoidan decreased body weight in HFD-fed mice and also improved glucose intolerance and insulin resistance. Both fucoidans separately improved intestinal dysbiosis caused by a HFD and significantly increased the abundance of *A. muciniphila* (165). Inulin-type fructan prebiotics were found to significantly enhance the presence of *A. muciniphila*, linked to a decrease in obesity and fat mass and an improvement in insulin resistance in genetic obese and diet-induced leptin-resistant mice (166). An increase in the cecal content of *A. muciniphila* was detected by targeted qPCR following four weeks' supplementation with berberine in genetically obese mice, associated with an improvement in gut barrier function and hepatic inflammatory and oxidative stress (167). (167) Jiang et al. showed that total flavone (TFA) extracted from the flowers Abelmoschus manihot (TFA) can also enhance *A. muciniphila* in DSS-induced experimental colitis (168). Finally, dry extract of rhubarb root has also been shown to cause an increase in levels of *A. muciniphila* associated with the increased expression of *Reg3γ* in the colon, an anti-microbial peptide with an important role in the host defense system, thus protecting against metabolic disorders (169).

TABLE 3 Human studies or clinical trials on the use of *A. muciniphila* as a probiotic or enhancing its abundance through prebiotic administration.

		Prebiotic/Probiotic	Intervention	Cohort	Clinical case	Outcomes	Author/ References
Validly published studies	**Prebiotics**	Xylo-oligosaccharides (XOS)	1.4 g XOS, 2.8 g XOS or placebo taken daily	32 healthy subjects	x	↑ in *Akkermansia* sp. in those supplemented with the higher dose	(155)
		Resistant starch (RS)	Participants consumed a high (HC) or low carbohydrate (LC) diet followed by a baseline diet. *HC subjects consumed either a high RS (HRS – 66 g/d) or low RS (LRS – 4 g/d). *LC Subjects consumed either 48 g for HRS or 3 g for LRS.	39 subjects with reduced insulin sensitivity	x	↑ in the ratio of Firmicutes to Bacteroidetes. ↑ levels of *A. muciniphila*	(156)
	Probiotics/ Postbiotics	*A. muciniphila*	Oral administration of either live or pasteurized *A. muciniphila* or the membrane protein Amuc_1100* (1.5×10^8 CFU)	20 subjects with excess body weight	Obesity and type 2 diabetes	Administration of live or pasteurized *A. muciniphila* is safe in humans.	(12)
		A. muciniphila	daily oral supplementation of 10^{10} A. *muciniphila* bacteria either live or pasteurized (3 months) (10^{10} bacteria)	32 overweight/obese insulin-resistant volunteers	Obesity	1- *A. muciniphila* is safe and well tolerated. 2- Pasteurized *A. muciniphila* improved insulin sensitivity, reduced insulinemia and total plasma cholesterol. 3- Pasteurized *A. muciniphila* slightly decreased body weight and fat mass 4- *A. muciniphila* reduced the levels of markers for liver dysfunction and inflammation.	(23) (24)
		Lactobacillus plantarum, *Streptococus thermophiles*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, *Bifidobacterium longum*, and *Bifidobacterium breve*.	Oral supplementation (6 weeks)	13 individuals	Obesity	Increase in the abundance of *A. muciniphila* after the intervention	(157)
Clinical trials in progress	**Prebiotics**	oat β-glucans	5 gr of oat ß-glucan (12 weeks)	40 participants with type 2 diabetes mellitus	Type 2 diabetes	Follow up on *A. muciniphila* levels in fecal microbiota using (qPCR)	NCT04299763
		dietary fiber formulation	supplementation with 15g/day fiber powder (1 month)	20 healthy participants	x	Explore the change in *A. muciniphila* gut abundance.	NCT03785860
		Acetate	Supplementation with Acetate (Apple Cider Vinegar) (5 months).	10 patients on stable dose of antipsychotic medication for treatment of depression or anxiety.	Depression/ anxiety	Encourage the growth of *A. muciniphila*	NCT05022524

(Continued)

TABLE 3 Continued

	Prebiotic/Probiotic	Intervention	Cohort	Clinical case	Outcomes	Author/ References
	Camu Camu Capsules (CC)	2 capsules of Camu Camu daily in addition to antiretroviral therapy (12 weeks)	22 participant with HIV	HIV	1- Monitor *A. muciniphila* levels in stools. 2- Monitor gut damage and inflammation.	NCT04058392
		CC supplementation of 500 mg (3 months)	45 participants with Non-Small Cell Lung Cancer and melanoma receiving Immune Checkpoint Inhibitors	Non-Small Cell Lung Cancer and melanoma	1- Assess the safety and tolerability of CC prebiotic. 2- Discover if CC has the potential to enrich *A. muciniphila* and improve Immune Checkpoint Inhibitors efficacy.	NCT05303493
Probiotics	*Lactobacillus rhamnosus* Probio-M9	Daily oral dose (6 months)	46 patients receiving immunotherapy for liver cancer	liver cancer	Increase the abundance of *A. muciniphila*.to improve effect of immunotherapy	NCT05032014
	Lactobacillus Bifidobacterium V9		46 Non-small Cell Lung Cancer Patients receiving immunotherapy	Cell Lung Cancer		NCT05094167
	fecal microbiota capsules	x	20 participants with Advanced Lung Cancer Treated With Immunotherapy	Advanced lung cancer	1- Selection of donor of fecal microbiota based on their fecal abundance in *F. prausnitzii, B. longum*, *A. muciniphila* and *Fusobacterium* spp. 2- Manipulating the microbial populations to enhance the efficacy of immunotherapy.	NCT04924374
	DS-01 (microbial consortia consisting of 24 strains across 12 species)	2 capsules daily (12 weeks)	100 men or women with IBS with constipation	Irritable bowel syndrome	Evaluate changes of *A. muciniphila* and other species	NCT04598295
	A. muciniphila	orally given *A. muciniphila* WST01 strain powder with maximum live bacteria of 5*10^10 CFU/g (12 weeks)	60 overweight/obese and drug naïve type 2 diabetes patients	Obesity/Type 2 diabetes	Evaluate the effects of *A. muciniphila* WST01 strain in overweight or obese patients with T2D.	NCT04797442
	A. muciniphila	Daily oral dose of pasteurized *A. muciniphila* (120 days)	98 hyperglycaemic healthy adults	Dysglycaemia	demonstrate the efficacy of pasteurized *A. muciniphila* (pAKK) in improving insulin sensitivity	NCT05114018

Table resuming the different validly published studies and the clinical trials in progress that use *A. muciniphila* as a probiotic or prebiotics to enhance its abundance, the cohort, the intervention, the clinical case, the results and the references. ↑: Increase.

Clinical trials and human studies are essential when assessing the benefits of newly identified prebiotics. Of the many potential prebiotics which have been studied, only a few substrates, including Xylo-oligosaccharides (XOS) and resistant starch (RS) have been validated through human studies. Finegold et al. demonstrated that xylo-oligosaccharides promoted intestinal health by modulating the microbial community: an increase in the levels of *Faecalibacterium* sp. and *Akkermansia* sp. as well as *Bifidobacteria* was detected (155). Moreover, a randomized dietary study by Maier et al. proved that resistant starch increased the levels of *A. muciniphila* in participants who followed a high resistant starch diet (156). Other ongoing clinical studies involve the use of various prebiotics in different diseases such as T2D, cancer and other diseases in order to uncover their potential in enriching the abundance of *A. muciniphila*.

Other probiotic treatments may also increase the levels of *A. muciniphila*. For example, a fasting programme combined with laxative treatment for one week followed by a six-week probiotic intervention with a probiotic containing several different bacterial strains showed an increase in the abundance of *Akkermansia* (157). Four other clinical studies are in progress about the use of different bacteria as probiotics and their effect on modulation of the intestinal flora in cancer or IBS and, most importantly, on increasing the abundance of *A. muciniphila* (Table 3).

Other than natural components, *A. muciniphila* has been used by Payahoo et al. as a marker to assess the efficiency of a pharmaceutical agent, Oleoylethanolamide, for treatment of obese people. This study showed that abundance of *A. muciniphila* bacterium increases significantly in oleoylethanolamide group compared to the placebo group and modifies the energy balance (170).

Conclusion and perspectives

A new area of research is emerging with the study of interbacterial communication, particularly between probiotic bacteria in transit and intestinal bacteria. *A. muciniphila* has been proven to have many beneficial effects in immune and metabolic regulation which can result in stimulating host health and preventing of pathogens. Nowadays, it is considered as a next generation probiotic to treat metabolic disorders such as obesity, diabetes, inflammatory diseases, as well as cancer (Figure 2). It has been reported that *A. muciniphila* in its two forms (live and pasteurized) is safe for use in human trials and two known companies have already started producing *A. muciniphila* probiotics (A-Mansia Biotech and Pendulum). However, there is no significant evidence on the link between this bacteria and malnutrition, reason why more studies should focus on this topic. Finally, more studies and mainly human clinical trials should be carried out in order to assess mechanisms of action and long-term effects of *A. muciniphila* before using for therapeutic applications.

Author contributions

RI, RW: Writing-original draft preparation. GD: Writing, reviewing and editing. J-CL, DR: Reviewing, supervision. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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"Sociobiome": How do socioeconomic factors influence gut microbiota and enhance pathology susceptibility? - A mini-review

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The gut microbiota is becoming well recognized as a key determinant of health and disease. As a result, several studies have focused on causality and the predictive/prognostic value of the microbiota in a wide range of diseases. However, it is of greater importance to understand what sparks changes in the microbiota and how these alterations contribute to an increased susceptibility to disease. A few studies have already demonstrated that the gut microbiota could be modified by lifestyle, consequently leading to pathology. What if socioeconomic factors can also impact the gut microbiota composition and, thus, increase the susceptibility to disease? Perhaps, this is one of the factors that may have contributed to the increased inequalities between people with higher and lower socioeconomic status in terms of health. In this review, we aimed to understand more about this topic and the real impact of the "sociobiome." Furthermore, we proposed measures to mitigate the impact of these factors on the gut microbiota composition.

KEYWORDS

gut microbiome, health, disease, socioecomic status, sociobiome, gut microbiota

Background

Eradication of poverty was listed as one of the main Millennium Development Goals (MDGs) to be tackled by the WHO, especially in low-income countries (1). Thus, it indicates that health is a key determinant for increasing the socioeconomic status (SES) and, hence, can influence an individual's success throughout life. Therefore, achieving the best health odds at a young age is important.

The microbiota is intrinsically correlated with health and disease, making it promising to understand part of the pathophysiology, which, in turn, can help in the achievement of a healthier status, especially due to the therapeutic potential to modulate the composition of the microbiota.

The microbiota consists of a plethora of microorganisms, including bacteria, protozoa, archaea, viruses, and fungus, that inhabit mainly the intestines, as well as other sites of our organism, which establishes a symbiotic relationship with us. It is acquired at the moment of birth, either through vaginal or cesarean delivery, which presents as one of the first interferents of the microbiota composition, diversity, and disease susceptibility in the future (2). The establishment of a more mature, balanced, and diverse state of microbiota composition is obtained at the age of 4 years, which is divided into three main stages: 1) the developmental period (at 1 year old), where the child's microbiota is influenced by breastfeeding, geographics, maternal and/or fetal diseases, and the use of antibiotics; 2) the transitional period (at 2 years old), where exposure to the environment, such as pets, siblings, other household related-acquaintance, and chronic pathologies, among others, increases and affects the microbiota; and 3) the stable period (at 4 years old), which will remain throughout life and can be slightly modified by lifestyle and diet (2, 3).

Moreover, it is important to understand the impact of ethnicity and geographic location. One interesting study performed in Indian tribes revealed that their microbiota was dominated by *Prevotella* spp., with just slight changes at the genus and species levels mainly due to different diet nuances. Additionally, a representative microbiota core was detected, similar to that of most world populations, with *Faecalibacterium*, *Eubacterium*, *Clostridium*, *Blautia*, *Ruminococcus*, and *Roseburia* (4). Furthermore, another study, reporting on some of the tribes included in the previous one, demonstrated that the microbiota composition and respective metabolomics are shaped by ethnicity (5).

On another side of the world, specifically South America, an Amerindian tribe without previous contact with westernized people was discovered to have the most diverse and functional microbiota ever documented, indicating that exposure to westernized culture affects our collective microbiota composition (6).

Before delving further into the effects of socioeconomic features on the dynamics of the microbiota, it is important to remember that xenobiotics, including exposure to medications and environmental toxins, are a major contributor to microbiota dysbiosis. After all, westernized populations may be more exposed to these xenobiotics, leading to innumerous pathologies, especially in populations with fewer resources (7–9).

Furthermore, it appears that household exposure can be separated into household crowding and SES, especially at a young age. This may shape the individual microbiota, determining the susceptibility to disease and, hence, their chances of success in accomplishing higher SES. Therefore, the main aim of this article was to explore the importance of SES in the predisposition to pathology through its influence on the microbiota composition. The main findings are summed up in Figure 1.

Socioeconomic status *vs.* inheritability

As previously described, the microbiota is acquired at the moment of birth by transference from mother to newborn. Hence, logically, the greatest contributor to microbiota composition would be genetically determined. There are even some taxa and species that are already depicted as highly heritable. The study by Gacesa et al. (10) in a Dutch population found that some bacteria, including *Proteobacteria*, *Akkermansia muciniphila*, Bacteroidaceae species, *Parabacteroides goldsteinii*, *Bacteroides coprocola*, *Bifidobacterium longum*, *Phascolarctobacterium*, and Clostridiales, are genetically transmissible. Other studies in a Canadian population and a cohort of UK twins reported similar findings (11, 12).

Nevertheless, most studies demonstrated that cohabitation and/or SES are more important in determining the composition of the microbiota than inheritability. The Dutch study (10) showed that the intestinal microbiota of family members living separately has a lower resemblance compared to household members, even if there was no significant relationship between them. However, some bacteria of inheritability signature still contribute to the microbiota composition, to a lower degree.

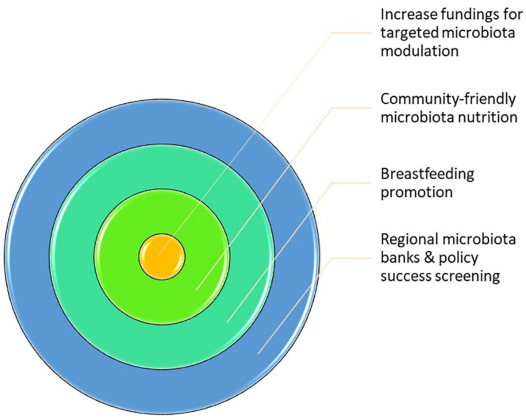

FIGURE 1
Sociobiome key points. Hereby, it is possible to get this conclusion the following conclusion: 1) Socioeconomic status (SES) has a higher contribution to microbiota composition than genetics; 2) SES is extremely important in the development of children that it also affects the adult microbiota; 3) Either individual or community SES is relevant for microbiota composition; therefore, community measures can be applied more easily and benefit even more individuals.

Moreover, another UK twin study that isolated the genetic contribution demonstrated the greater magnitude of SES in the structure of the microbiota (13).

Influence of socioeconomic status on a child's microbiota composition that reflects in adulthood

The microbiota composition, maturity, and diversity are stabilized in the fourth year of life (2), which means that all exposure during the first years will have enormous significance on the health status and, indirectly, on the SES. Thus, the Dutch study (10) demonstrated that the childhood milieu is reflected in the adult microbiota configuration through a comparison between rural and urban environments. It was described that children residing in urban environments showed lower abundance of *Bacteroides*, *Alistipes*, and *Bilophila* compared to those in rural backgrounds. Conversely, the microbiota of children in rural areas was enriched in *Prevotella copri*, *Faecalibacterium prausnitzii*, *Rothia muciliginosa*, *Bifidobacterium* spp., and *Mitsuokella* (10). All the previous bacteria from rural microbial signatures have anti-inflammatory characteristics that may enhance the resilience of the microbiota and decrease the susceptibility to disease. These findings are described in Table 1.

Furthermore, the study of Lapidot et al. (14) in Israel has shown that household crowding and SES are major contributors to the bacterial composition of young children, mainly through increasing the alpha diversity and phylogenetic variety. A lower SES was associated with a wider taxonomic range, consisting of *P. copri*, *Alistipes putredinis*, *Eubacterium biforme*, *Dialister*, *F. prausnitzii*, *Bifidobacterium*, *Oscillospira*, *Ruminococcus*, and *Sutterella*, which, in turn, are astonishingly similar to the microbiota signatures of those in rural communities (10, 14). This might be explained by the fact that individuals from villages in Israel with lower SES have decreased monthly wages, lower education levels, and are less exposed to Westernized diets, accompanied by augmented consumption of the Mediterranean diet. Moreover, household crowding showed differences in the abundance of *Alistipes onderdonkii*, *Bacteroides uniformis*, *Prevotella stercorea*, *Phascolarctobacterium*, and *A. putredinis*, with those having lower SES presenting a dominant taxon of *B. uniformis*, while a higher SES was mainly composed of *P. stercorea* and *Phascolarctobacterium* (14). The metabolic pathways were also different, with children in higher SES households showing overdeveloped secondary bile acid biosynthesis, which is important for its regulatory effect on inflammation and microbial composition (17); increased glutamate and glutamine metabolism, crucial to maintaining the intestinal barrier integrity (18, 19); and, finally, enhanced biotin metabolism, which is responsible for the metabolism of glucose, amino acids, and fatty acids (14, 20)..

Moreover, the same authors conducted a more recent study where it was observed that a lower SES showed not only significant microbiota alterations but also increased body mass index *Z*-score (BMIZ) in preadolescents (15). Children in

TABLE 1 Discriminate SES.

Geography	High SES	Low SES	Conclusions	References
Netherlands (Europe)	↓ *Bacteroides* ↓ *Alistipes* ↓ *Biophile*	↑ *P. copri* ↑ *F. prausnitzii* ↑ *R. muciliginosa* ↑ *Bifidobacterium spp.* ↑ *Mitsuokella*	Low SES children seems to have more resilient microbiota and develop less diseases	(10)
Israel (Asia)	↓ *A. Onderdonkii* ↓ *B. uniformis* ↓ *P. stercorea* ↓ *Phascolarctobacterium* ↓ *A. Putrensis* ↑ Secondary Bile Acids biosynthesis ↑ Glutamate and Glutamine metabolism ↑ Biotin metabolism	↑ *P. copri* ↑ *A. Putredinis* ↑ *E. biforme* ↑ *Dialister* ↑ *F. prausnitzii* ↑ *Bifidobacterium spp.* ↑ *Oscillospira* ↑ *Ruminococcus* ↑ *Sutterella*	Lower SES children present higher BMIZ, are more prone to obesity and had less bacterial diversity, depending in the quantity of fiber present in their diets	(14, 15)
Mexico (South America)	↑ *Saccharibacteri*	↑ *Dinococcus-Thermus* ↑ *Chloroflexi* ↑ *Elusimicrobia* ↑ *Acidobacteria* ↑ *Fibrobacter*	High SES children had increased amounts of sugar decomposing-bacteria, indicating an enhanced propensity to obesity	(16)

reduced SES households demonstrated a higher prevalence of obesity, complemented with reduced bacterial diversity due to their main diet comprising higher quantities of dietary fat without increased consumption of fibers. Therefore, the microbiota of children with lower SES is enriched in *Prevotella*, *Adlercreutzia*, *Alistipes*, and *Dorea*, which have been correlated with obesity (21) and diabetes mellitus (15, 22). These findings are displayed in Table 1.

Furthermore, a study performed in Mexico comparing the different microbiota compositions of children in westernized (higher SES) and non-westernized (lower SES) settings reported that non-westernized children had unique phyla of bacteria, namely, Deinococcus-Thermus, Chloroflexota, Elusimicrobiota, Acidobacteriota, and Fibrobacterota, more related to a vegetable-based diet. In contrast, westernized children had diminished diversity and a more representative phylum of Saccharibacteria, one of the main functions of which is the decomposition of sugar molecules. To sum up, since non-westernized children are less exposed to sugar-containing foods and eat a more diverse range of vegetables, they appear to have a more resilient microbiota that is more efficient in harvesting the energy from fibers (16).

Nevertheless, it is important to highlight the role of *Prevotella*, present in both studies, since it is still the bacterium that is vastly abundant in the human intestine. However, this genus has already been correlated with positive and negative outcomes in health. On the one hand, *Prevotella* has been implicated in glucose intolerance and insulin resistance (23). On the other hand, when a diet rich in fiber is consumed, *Prevotella* improves glucose and insulin tolerance, which points to the fact that its benefits or risks are diet-induced (24).

Socioeconomic status: Individual versus community

The composition of the microbiota is influenced by individual lifestyle, namely, diet, physical exercise, and individual SES, but is also highly dependent on neighborhood SES, which contributes to greenspace area, exposure to pollution and toxicants, stress, and the type of diet consumed, such as ultra-processed food (10, 25).

The study by Miller et al. (25) evaluated the influence of neighborhood SES on the microbiota composition in the mucosal and luminal locations of the sigmoid colon. It was noted that the alpha diversity was diminished in those in low-SES communities, which, in turn, showed higher rates of diabetes (26), cardiovascular diseases (27), asthma (28), and mortality. Moreover, an enhanced prevalence of *Bacteroides*, with a lower abundance of *Prevotella*, was reported in the microbiota of individuals belonging to higher-SES neighborhoods, probably due to better diets with increased consumption of animal products (25).

The alpha diversity, which reflects the evenness and richness of the microbiota, is a significant indicator of microbiota resilience (3). Hence, individuals with decreased alpha diversity, for example those belonging to lower-SES neighborhoods, showed less resilience, which means that they are more prone to pathologies (29).

Regarding individual SES, one study pointed out that measures of individual SES, particularly an individual's monthly wage, is a determinant of alpha diversity (13). It was demonstrated that a higher individual SES correlated with an enhanced alpha diversity, with an increased abundance of *Bacteroides* and *Prevotella*, which was in contradiction with the results of the study of Miller et al. (25), which presented a reduced abundance of *Prevotella* (13).

Sociobiome: What does the future hold?

Sociobiome can be defined as the microbiota composition of a geographic region or neighborhood as a result of exposure to similar socioeconomic factors, which determine an environment with analogous characteristics that shape the individual microbiota into great resemblance. Therefore, this sociobiome can be used to increase the success of health policies more personalized to a specific region instead of broad interventions across a territory full of diverse realities and dissimilar issues.

For instance, since the microbiota appears to interact with the development of the central nervous system, as well as the regulation of individual behavior (30), there is a possibility that not only does the SES affect a person's microbiota but also, in a reverse mode, that the microbiota composition shapes the behavior of an individual in such a way that it regulates the capacity to influence SES and to acquire habitation in specific neighborhoods (25). With this being said, it opens the possibility of modifying health disparities due to SES since there are interventions, especially those aimed at the youth, that can be fashioned to shape the microbiota of those with lower SES in order to ameliorate present and future health problems.

Hereby, we suggest some interventions that can decrease the chasm between low and high SES and equalize the health status, as reflected in Figure 2.

1. *Increase fundings for targeted microbiota modulation* (31): It is necessary to develop research on health disparities based on microbiota differences in order to obtain "antidotes" that can be used to modulate the microbiota through increasing the alpha diversity, which, in turn, will enhance the microbiota resilience and ameliorate the health status. Here, personalized therapies for microbiota modulation, such as combinations of probiotics, prebiotics, symbiotics, and antibiotics, should be developed, as well as the possibility of fecal material transplant (FMT).

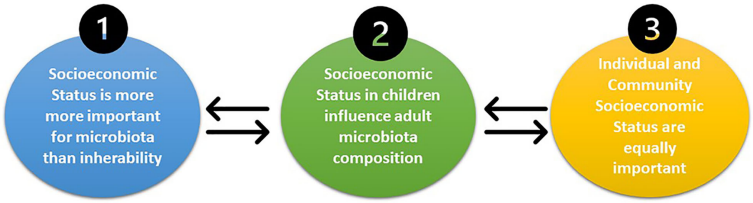

FIGURE 2
Future sociobiome interventions that might increase the quality of the community microbiota. The main interventions suggested in this manuscript are: 1) increased funding for targeted microbiota interventions; 2) community-friendly microbiota nutrition; 3) breastfeeding promotion; and 4) regional microbiota banks and policy success screening.

Furthermore, this intervention should be directed at children since it appears to have a "founder effect," with adult SES indicating a cumulative acquaintance throughout life (13).

2. *Community microbiota-friendly nutrition*: The impact of nutrition on the microbiota composition is well recognized. Hence, it would be important for the population, especially children, to have access to the best food available instead of high-fat, high-carbohydrate, and low-fiber diets. As previously shown, *Prevotella*, which has an important impact on health disparities, is highly dependent on diet, namely, fiber; hence, it is necessary to increase the fiber intake, with the aim of enhancing the beneficial effects of *Prevotella* (31). Therefore, food banks and food supplement programs should be available for children in order for them to benefit from the best diet possible. Additionally, to promote healthier nutrition, high-fiber and fresh food should have reduced taxes and/or budget supplements for low-SES families, contrarily to high-fat products that should have increased taxes.
3. *Breastfeeding promotion*: One of the first major modulators of the microbiota in youngsters is breastfeeding. However, breastfeeding is often difficult to maintain in low-SES families due to the need to work to support their families. Thus, workplaces should allow breastfeeding periods and/or receive statal support to facilitate breastfeeding (31).
4. *Regional microbiota banks and policy success screening*: Since microbiota sequencing is becoming more accessible, individual microbiota should be examined in a standardized periodicity for the optimization of health policies and evaluation of their success, along with the possibility of early detection of disease and modulation of the microbiota. Furthermore, microbiota samples could be stored under optimal conditions and, in the not-so-far future, could be transplanted in an autologous manner to restore innate microbiota homeostasis when dysbiosis is detected.

Conclusion

The microbiota has a huge impact on health and disease; subsequently, factors that can shape its composition, such as SES, have outstanding significance on the health status of an individual. Therefore, it is possible to understand that the sociobiome influences health disparities and can be targeted to reduce these inequalities. Moreover, the SES should be considered in microbiota research since it can be a crucial confounding variable that can influence the interpretation of the study outcomes.

SES appears to have a higher impact than heritability on the microbiota composition. Therefore, childhood interventions on the microbiota can increase the chances of an individual's success throughout life, along with ameliorating the country's productivity since there would be a reduction in the burden of disease. Furthermore, the sociobiome could lead to better screening of pathologies, accompanied by an enhancement in efficiency through tailored health policies specifically designed for certain neighborhoods.

To sum up, investing in personalized microbiota interventions in early life, especially in low-SES neighborhoods, could induce a win–win situation, where health disparities are attenuated alongside an increased productivity overall.

Author contributions

The present manuscript is the result of the original work by the authors. JGN and DAC: Conception and design. JGN: Writing. DAC: Revision of the manuscript. Both authors contributed to the article and approved the submitted version. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

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Gut microbiota-derived metabolite trimethylamine-N-oxide and stroke outcome: a systematic review

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Introduction: The relationship between baseline trimethylamine N-oxide (TMAO) levels and stroke outcomes remains unclear. Therefore, this systematic review aimed to summarize the existing relevant research.

Methods: We searched for studies on the association between baseline plasma levels of TMAO and stroke outcomes in the PubMed, EMBASE, Web of Science, and Scopus databases from their inception to 12 October 2022. Two researchers independently reviewed the studies for inclusion and extracted the relevant data.

Results: Seven studies were included in the qualitative analysis. Among them, six studies reported the outcome of acute ischemic stroke (AIS) and one study of intracerebral hemorrhage (ICH), respectively. Furthermore, no study reported the outcome of subarachnoid hemorrhage. Among patients with AIS, high baseline TMAO levels were associated with unfavorable functional outcomes or mortality at 3 months, as well as a high hazard ratio of mortality, recurrence, or major adverse cardiac event. Moreover, TMAO levels showed predictive utility for unfavorable functional outcomes or mortality at 3 months. Among patients with ICH, high TMAO levels were associated with unfavorable functional outcomes at 3 months, regardless of whether the TMAO value was considered a continuous or a categorical variable.

Conclusion: Limited evidence indicates that high baseline plasma levels of TMAO may be associated with poor stroke outcomes. Further studies are warranted to confirm the relationship between TMAO and stroke outcomes.

KEYWORDS

gut microbial metabolism, trimethylamine N-oxide, stroke, prognosis, systematic review

1. Introduction

In China, ~1.5–2 million newly diagnosed and recurrent cases of stroke occur annually, which makes it the leading cause of acquired disability and mortality among Chinese adults, and thus a huge burden on health resources (Liu et al., 2007, 2011). Therefore, proactive measures for the prompt assessment of risk factors affecting stroke severity and prognosis are required to improve stroke outcomes and reduce the disease burden of stroke.

Gut microbes can influence human health and disease by metabolizing substrates from the diet and host to produce bioactive compounds, including signaling compounds, biological precursors, and toxins (Clemente et al., 2012; Tremaroli and Bäckhed, 2012; Dinan and Cryan, 2017). Trimethylamine N-oxide (TMAO) is an oxidative metabolite produced by gut microbes that metabolize choline-containing lipids and carnitine-like molecules. Circulating TMAO levels are positively correlated with the risk of stroke (Zhang and Yao, 2022). However, the relationship between circulating TMAO levels and stroke outcomes remains unclear. Different studies have explored the relationship between circulating TMAO levels and stroke outcomes with varying stroke subtypes, outcome types, or treatment measures. Therefore, we aimed to conduct a systematic review to summarize the relevant literature.

2. Methods

The study protocol of this systematic review was not pre-registered; however, we strictly followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (Moher et al., 2009).

2.1. Search strategy

PubMed, EMBASE, Web of Science, and Scopus databases were searched for studies on the relationship between plasma TMAO levels and stroke outcomes from their inception to 12 October 2022. We used the following search terms: TMAO, stroke, cerebrovascular disease, ischemic infarction, ischemic infarction, ischemic brain infarction, cerebrovascular accident, intracerebral hemorrhage, intracerebral hemorrhage (ICH), subarachnoid hemorrhage, and subarachnoid hemorrhage (SAH). The detailed search strategy used in each database is provided in the Supplementary material (Supplementary Appendix S1). The reference list of each included study was also manually searched for other relevant studies.

2.2. Study selection and quality assessment

The eligibility criteria were as follows: (1) original study involving patients with stroke [acute ischemic stroke (AIS), ICH, or SAH]; (2) studies reporting on the relationship between baseline plasma levels of TMAO and stroke outcomes [3-month unfavorable functional outcome (modified Rankin Scale score ≥ 3) or mortality, hazard ratio (HR) of mortality, stroke recurrence, or major adverse cardiac event (MACE)]; (3) studies having included ≥ 100 patients; and (4) those reporting the relevant effect sizes [odds ratio (OR), HR, or area under curve (AUC)], and its corresponding 95% confidence interval (CI). For multiple studies involving the same patient source, the research team determined which study to be included in this systematic review. Retrieved articles were independently evaluated by two authors (P.Z. and Z.N.G.). Differences between the authors were settled through discussions with a third person.

The Newcastle–Ottawa Scale was used to assess the quality of the included studies, which is commonly used for case–control and cohort studies (Wells et al., 2010). It assesses eight items in three major modules: study population selection, comparability, and exposure/outcome evaluation. Two authors (P.Z. and Y.Q.) independently completed the quality evaluation process, with disagreements being resolved through discussion.

2.3. Data extraction and analysis

Four authors of the included studies were contacted for additional information. However, no responses were received. We extracted the following information from the included studies: first author, year of publication, country, number of included patients, mean age, sex ratio, treatment, characteristics of the TMAO detection methods, and covariates adjusted in the multivariable model. Two authors completed the data extraction process. First, one author (Y.Q.) independently extracted the data from the included studies, and then, the data were checked by another author (Z.N.G.). We did not perform data synthesis, given the large among-study heterogeneity and the limited number of articles available.

3. Results

3.1. Search results of the included studies

We retrieved 698 articles from PubMed, EMBASE, Web of Science, and Scopus databases. There were no relevant articles detected from other sources. After removing duplicate articles, we screened the titles and abstracts of 301 articles; among them, 289 articles were excluded at this stage [conference abstract ($n = 41$), comment ($n = 10$), review ($n = 85$), not relevant ($n = 131$), letter ($n = 8$), protocol ($n = 1$), animal study ($n = 10$), and erratum ($n = 3$)]. Of the remaining 12 articles eligible for full-text screening, 3 articles were excluded for having the same source of the study population, for reporting outcomes other than those relevant to this study ($n = 1$), and for having a sample size < 100 ($n = 1$). Finally, seven articles were included in the qualitative analysis. Figure 1 shows the detailed process of article screening. The seven included studies were published between 2019 and 2022; furthermore, six and one studies were conducted in China and Korea, respectively. Additionally, six and one studies reported outcomes of AIS and ICH, respectively, with no study reporting the outcomes of SAH. Table 1 summarizes the main characteristics of the included studies.

3.2. Quality assessment of the included studies

The Newcastle–Ottawa scale scores for six and one studies were 8 and 7, respectively (Table 2). The quality of these studies was considered to meet the requirements for inclusion in a systematic review.

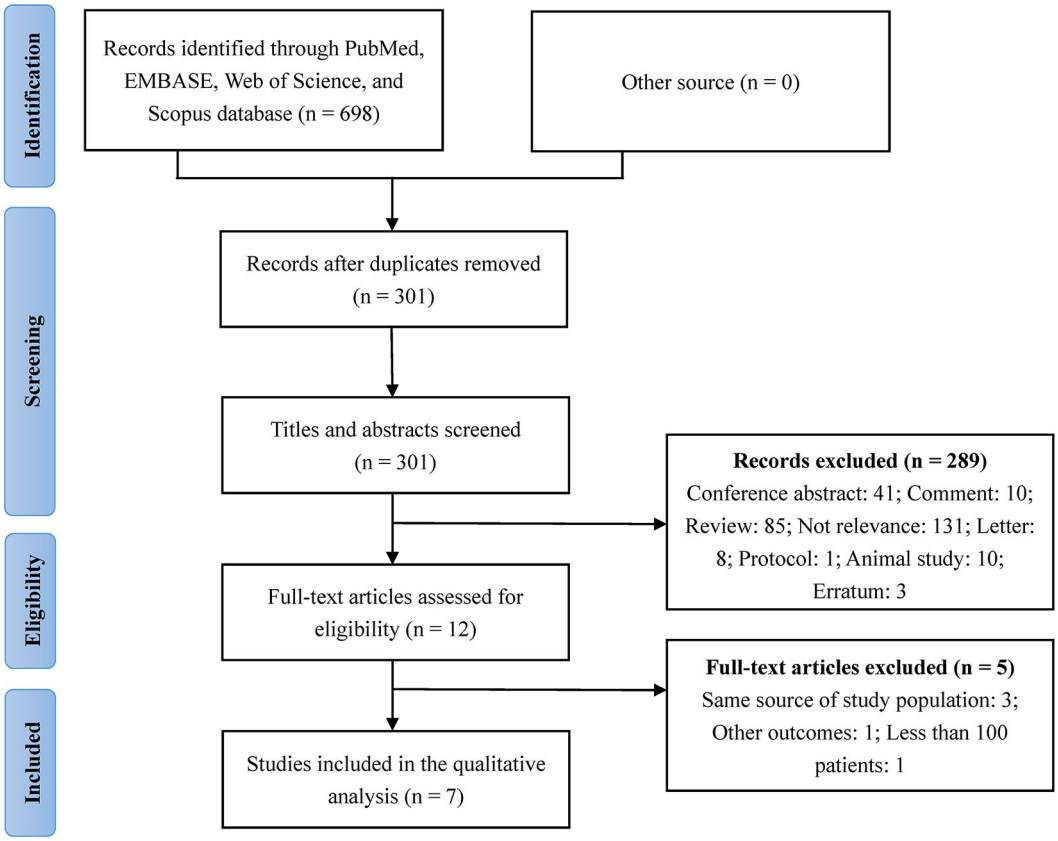

FIGURE 1
Summary of the study selection process.

3.3. Acute ischemic stroke

3.3.1. Three-month unfavorable functional outcome

Four studies examined the association between TMAO levels and unfavorable functional outcomes at 3 months after AIS; among them, three (Tan et al., 2020; Zhang et al., 2021; Chen et al., 2022) and two (Zhai et al., 2019; Zhang et al., 2021) studies treated TMAO levels as continuous and categorical variables, respectively. Furthermore, three studies reported the AUC of TMAO for predicting 3-month unfavorable functional outcomes (Zhai et al., 2019; Tan et al., 2020; Zhang et al., 2021). Notably, one study used different concentration units for TMAO (pg/ml) compared with the other studies (Chen et al., 2022), another study reported $\log_2$-transformed TMAO levels (Tan et al., 2020), and another study excluded patients who were treated with intravenous thrombolysis or endovascular therapy (Zhai et al., 2019). These studies are shown in Table 3. All four studies reported a positive association between high TMAO levels and unfavorable functional outcomes at 3 months.

3.3.2. Three-month mortality

Two studies reported the relationship between TMAO levels and the 3-month mortality after AIS; among them, one (Zhang et al., 2021) and two (Zhai et al., 2019; Zhang et al., 2021) studies treated the TMAO concentration as continuous and categorical variables, respectively. Additionally, two studies reported the AUC of TMAO for predicting 3-month mortality (Zhai et al., 2019; Zhang et al., 2021). One study excluded patients who received intravenous thrombolysis or endovascular therapy (Zhai et al., 2019). Table 3 shows detailed information. Both studies reported a positive association between high TMAO levels and the 3-month mortality.

3.3.3. HR of mortality, stroke recurrence, or MACE

One study examined the relationship between TMAO levels and the HR of mortality (Xu et al., 2021), another study examined the HR of recurrence (Xu et al., 2021), and three other studies examined the HR of MACE (Table 3) (Nam et al., 2019; Xu et al., 2021; Chen et al., 2022). These studies showed that high TMAO levels were associated with shorter survival of mortality, recurrence, and MACE.

3.4. Intracerebral hemorrhage

One study explored the association between TMAO levels and outcomes in patients with ICH. Zhai et al. (2021) reported that,

TABLE 1 Baseline characteristics of the included studies.

Study	Country	Disease	Number of patients	Mean age, y	Male, %	Treatment	Sample	Time from onset to admission	Time from admission to sample collection	Assay	Adjusted for renal function in the multivariable model
Zhai et al., 2019	China	AIS	225	68.5	55.1	Without EVT and IVT	Plasma	Within 24 h	NR	LC-MS/MS	No
Chen et al., 2022	China	AIS with LAA	291	61.68 ± 7.27	54.6	Not limited	Fasting, Plasma	Within 72 h	Within 24 h	LC-MS/MS	-
Zhang et al., 2021	China	AIS	351	66 (55, 74)	50.4	Not limited	Fasting, Plasma	Within 24 h	Within 24 h	HPLC-MS/MS	Yes
Tan et al., 2020	China	AIS	204	59 (IQR: 21)	66.7	Not limited	Fasting, Plasma	Within 72 h	Within 24 h	LC-MS/MS	Yes
Xu et al., 2021	China	IS or TIA	10027	63 (54, 70)	68.6	Not limited	Fasting, Plasma	Within 7 days	Within 24 h	LC-MS/MS	Yes
Zhai et al., 2021	China	ICH	307	66.8	57.7	Not limited	Fasting, Plasma	Within 6 h	Within 24 h	UPLC-MS/MS	No
Nam, 2019	Korea	AIS with LAA, CE or LI	357	68 (57, 75)	62	Not limited	Fasting, Plasma	NR	Within 24 h	LC-MS/MS	Yes

AIS, acute ischemic stroke; LAA, large artery atherosclerotic; IS, ischemic stroke; ICH, intracerebral hemorrhage; CE, cardioembolism; LI, lacunar infarction; NIHSS, National Institutes of Health Stroke Scale; TMAO, trimethylamine-N-oxide; NR, not report; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; HPLC-MS/MS, high-performance LC-MS/MS; UPLC-MS/MS, ultra-performance LC-MS/MS; NR, not report; IQR, interquartile range.

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TABLE 2 Newcastle–Ottawa quality assessment scale (NOS) for the included studies.

Study	Selection				Comparability of cohorts on the basis of the design or analysis	Outcome			Final score
	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study		Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow up of cohorts	
Zhai et al., 2019	*	*	*	*	*	*	*	*	8
Chen et al., 2022	*	*	*	*	-	*	*	*	7
Zhang et al., 2021	*	*	*	*	*	*	*	*	8
Tan et al., 2020	*	*	*	*	*	*	*	*	8
Xu et al., 2021	*	*	*	*	*	*	*	*	8
Zhai et al., 2021	*	*	*	*	*	*	*	*	8
Nam, 2019	*	*	*	*	*	*	*	*	8

after adjusting for potential confounders, the OR of the highest quartile to the lowest quartile of TMAO levels for unfavorable 3-month functional outcomes (mRS $\geq$ 3) was 3.65 (95% CI, 1.43–9.30; $P = 0.007$) (Zhai et al., 2021). Additionally, the TMAO level as a continuous variable was independently associated with an increased risk of unfavorable 3-month functional outcomes, with an adjusted OR of 1.26 (95% CI, 1.09–1.45; $P = 0.003$).

4. Discussion

We systematically searched for studies on the relationship between baseline TMAO levels and stroke outcomes. Although we included a limited number of studies, both unadjusted and adjusted data indicated a relationship between high TMAO levels and poor post-stroke outcomes. High TMAO levels are associated with unfavorable functional outcomes or mortality at 3 months, as well as shorter survival of mortality, recurrence, or MACE.

TMAO production is dependent on the metabolism of dietary choline and carnitine-based molecules by gut microbiota (Craciun and Balskus, 2012). First, gut microbes enzymatically produce trimethylamine (TMA) from the dietary components; subsequently, TMA enters the circulation and is oxidized to TMAO by flavin-containing monooxygenase in the liver (Wang et al., 2011). TMAO is considered a potential mediator in the pathogenesis of stroke and is closely related to the onset of stroke. Sun et al. demonstrated that elevated TMAO levels may portend an increased risk of first stroke after adjusting for important covariates (Sun et al., 2021). Another study on patients who underwent elective coronary angiography reported higher baseline TMAO levels in patients with MACE than in patients without MACE (Tang et al., 2013). When the MACE components were separately analyzed, TMAO levels showed a significant positive correlation with the risk of stroke (Tang et al., 2013). A recent meta-analysis reported that circulating TMAO levels are positively correlated with stroke risk, with stroke patients having higher levels of TMAO compared to non-stroke patients (Zhang and Yao, 2022). However, elevated levels of TMAO are presumed to be associated with stroke outcomes. Animal studies have shown that high levels of TMAO can increase the size of cerebral infarcts and lead to functional deficits, thus directly affecting the severity of stroke (Zhu et al., 2021). Recent clinical studies have also reported a relationship between elevated TMAO levels and poor prognosis in stroke patients (Zhai et al., 2019; Zhang et al., 2021). In this systematic review, we have systematically summarized the relevant literature, but the evidence remains insufficient, necessitating further research to clarify the relationship between TMAO and stroke outcomes.

TMAO is closely related to renal function. TMAO is excreted by the kidneys; accordingly, patients with poor renal function have increased plasma TMAO levels (Rhee et al., 2013; Tang et al., 2015). Circulating TMAO levels in patients with renal dysfunction are negatively correlated with renal function; moreover, abnormally high TMAO levels gradually recover after kidney transplantation (Stubbs et al., 2016). However, renal function is also associated with stroke outcomes. Studies have indicated that chronic renal failure accelerates atherosclerosis and arterial calcification even though the underlying mechanism remains unclear (Buzello et al., 2003; Massy et al., 2005). This may include increased blood levels of calcium, phosphate, and intact parathyroid hormone, as well as perturbed cholesterol metabolism and increased homocysteine levels (Massy et al., 2005; Spence et al., 2016). Recent meta-analyses

TABLE 3 Summary of the association between TMAO levels (μmol/L) and outcomes of acute ischemic stroke reported by the included studies (effect size with its 95% confidence interval).

Types of outcome	Unadjusted data		Adjusted data	
	Number of studies	Results extracted from the included studies	Number of studies	Results extracted from the included studies
3-month unfavorable functional outcome (mRS ≥ 3)				
Continuous	3	Chen et al., 2022: 1.10 (0.57–2.12)[a]	2	Zhang et al., 2021: 1.21 (1.07–1.35)
		Zhang et al., 2021: 1.35 (1.25–1.46)		Tan et al., 2020: 1.43 (1.02–2.01)[b]
		Tan et al., 2020: 1.44 (1.06–1.97)[b]		
Quartered	2	Zhai et al., 2019: Q2 vs. Q1: 1.56 (0.73–3.33)	2	Zhai et al., 2019: Q2 vs. Q1: 2.01 (0.79–5.11)
		Q3 vs. Q1: 2.78 (1.29–5.98)		Q3 vs. Q1: 2.65 (0.96–7.34)
		Q4 vs. Q1: 3.09 (1.43–6.65)		Q4 vs. Q1: 3.63 (1.34–9.82)
		Zhang et al., 2021: Q2 vs. Q1: 2.61 (1.12–6.12)		Zhang et al., 2021: Q2 vs. Q1: 1.43 (0.78–4.02)
		Q3 vs. Q1: 5.43 (2.41–12.26)		Q3 vs. Q1: 3.02 (1.34–6.12)
		Q4 vs. Q1: 12.93 (5.88–28.42)		Q4 vs. Q1: 5.65 (2.87–13.45)
AUC	3	Zhai et al., 2019: 0.63 (0.56–0.70)	0	-
		Zhang et al., 2021: 0.78 (0.72–0.83)		-
		Tan et al., 2020: 0.65 (0.54–0.71)[b]		-
3-month mortality				
Continuous	1	Zhang et al., 2021: 1.36 (1.23–1.49)	1	Zhang et al., 2021: 1.24 (1.06–1.38)
Quartered	2	Zhai et al., 2019: Q2 vs. Q1: 1.60 (0.53–4.83)	2	Zhai et al., 2019: Q2 vs. Q1: 1.43 (0.34–6.05)
		Q3 vs. Q1: 2.52 (0.88–7.20)		Q3 vs. Q1: 1.89 (0.48–7.39)
		Q4 vs. Q1: 5.64 (2.08–15.30)		Q4 vs. Q1: 4.27 (1.07–17.07)
		Zhang et al., 2021: Q2 vs. Q1: 1.73 (0.40–7.46)		Zhang et al., 2021: Q2 vs. Q1: 0.89 (0.43–3.87)
		Q3 vs. Q1: 4.15 (1.12–15.43)		Q3 vs. Q1: 2.29 (0.83–6.03)
		Q4 vs. Q1: 13.61 (3.95–46.82)		Q4 vs. Q1: 5.84 (3.05–16.12)
AUC	2	Zhai et al., 2019: 0.69 (0.60–0.77)	0	-
		Zhang et al., 2021: 0.80 (0.74–0.87)		-
HR of mortality				
Continuous	1	Xu et al., 2021: -	1	Xu et al., 2021: 1.03 (1.00–1.06)
Quartered	1	Xu et al., 2021: Q2 vs. Q1: 0.81 (0.59–1.12)	1	Xu et al., 2021: Q2 vs. Q1: 0.93 (0.67–1.30)
		Q3 vs. Q1: 0.91 (0.66–1.24)		Q3 vs. Q1: 1.12 (0.80–1.56)
		Q4 vs. Q1: 1.43 (1.08–1.90)		Q4 vs. Q1: 1.39 (1.02–1.90)
HR of recurrence				
Quartered	1	Xu et al., 2021: Q2 vs. Q1: 1.10 (0.90–1.35)	1	Xu et al., 2021: Q2 vs. Q1: 1.06 (0.87–1.30)
		Q3 vs. Q1: 1.22 (1.00–1.49)		Q3 vs. Q1: 1.20 (0.98–1.47)
		Q4 vs. Q1: 1.59 (1.32–1.91)		Q4 vs. Q1: 1.54 (1.26–1.88)

(Continued)

TABLE 3 (Continued)

Types of outcome	Unadjusted data			Adjusted data		
	Number of studies	Results extracted from the included studies		Number of studies	Results extracted from the included studies	
HR of MACE						
Continuous	1	Xu et al., 2021	-	1	Xu et al., 2021	1.02 (1.01–1.04)
Dichotomous	2	Chen et al., 2022	High vs. low: 4.16 (1.39–12.43)	2	Chen et al., 2022	High vs. low: 3.13 (1.02–9.61)
		Nam, 2019	TMAO cut off: 1.77 (1.11–2.83)		Nam, 2019	TMAO cut off: 1.69 (1.03–2.77)
Quartered	1	Xu et al., 2021	Q2 vs. Q1: 1.06 (0.88–1.27)	1	Xu et al., 2021	Q2 vs. Q1: 1.02 (0.85–1.23)
			Q3 vs. Q1: 1.16 (0.97–1.38)			Q3 vs. Q1: 1.13 (0.94–1.36)
			Q4 vs. Q1: 1.55 (1.31–1.83)			Q4 vs. Q1: 1.45 (1.21–1.74)

[a]pg/ml for baseline TMAO.
[b]log_2-transformed baseline TMAO.
TMAO, trimethylamine-N-oxide; mRS, modified Rankin Scale; AUC, area under the curve; MACE, major adverse cardiac event.

have demonstrated a relationship between renal impairment at admission with 3-month poor functional outcomes and mortality in patients with AIS treated with intravenous thrombolysis or endovascular thrombectomy (Malhotra et al., 2020; Wang et al., 2022). To summarize, baseline renal function is an important confounding factor, and assessing baseline renal function is essential to elucidating the relationship between TMAO and stroke outcomes.

One possible explanation for the effect of TMAO on stroke outcome involves the activation of the inflammatory state, which is crucially involved in the development and propagation of stroke (Wang et al., 2007; Siniscalchi et al., 2016). TMAO activates the NLRP3 inflammasome by inducing the expression of inflammatory cytokines and adhesion molecules (Seldin et al., 2016; Boini et al., 2017; Chen et al., 2017; Nam, 2019), which contributes to the disruption of the blood–brain barrier and neuronal regeneration (Yang et al., 2019). Another possible explanation is that TMAO is directly involved in platelet hyperreactivity (Zhu et al., 2016; Nam, 2019). Studies have shown that platelet hyperreactivity has adverse effects on the severity and clinical outcomes of cardiovascular diseases (Angiolillo et al., 2007; Schwammenthal et al., 2008). Thus, high TMAO levels may lead to poor outcomes in patients with stroke by modulating platelet function. At present, the mechanisms underlying the prognostic impact of TMAO on stroke have not been determined, and further research is needed.

This study has several limitations. First, we included a small number of studies, and there was high between-study heterogeneity. Therefore, caution should be applied when interpreting our findings; moreover, further studies are required to explore the relationship between TMAO and stroke outcomes. Second, all the included studies were conducted in East Asia. TMAO production is dependent on the metabolism of dietary nutrients by gut microorganisms. Individuals in different regions have different diets and may have different characteristics of gut microbiota, which may affect the circulating TMAO levels. Therefore, our findings may not be applicable in other regions. Finally, we could not determine whether publication bias affected our results.

5. Conclusion

Overall, the limited evidence indicates that high baseline plasma levels of TMAO may be associated with poor stroke outcomes. Furthermore, baseline TMAO levels have a certain predictive effect on unfavorable functional outcomes or mortality at 3 months after stroke. Further studies are warranted to determine the relationship between TMAO and stroke outcomes.

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

PZ, YQ, and Z-NG performed the literature review. PZ, RW, and YY wrote the manuscript. RW and YY helped with the outline and manuscript modification. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Mendelian randomization analysis revealed a gut microbiota–mammary axis in breast cancer

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Background: Observational epidemiological studies suggested an association between the gut microbiota and breast cancer, but it remains unclear whether the gut microbiota causally influences the risk of breast cancer. We employed two-sample Mendelian randomization (MR) analysis to investigate this association.

Methods: We used summary statistics of the gut microbiome from a genome-wide association study (GWAS) of 18,340 individuals in the MiBioGen study. GWAS summary statistics for overall breast cancer risk and hormone receptor subtype-specific analyses were obtained from the UK Biobank and FinnGen databases, totaling 400,000 individuals. The inverse variance-weighted (IVW) MR method was used to examine the causal relationship between the gut microbiome and breast cancer and its subtypes. Sensitivity analyses were conducted using maximum likelihood, MR-Egger, and MR pleiotropic residual sums and outliers methods.

Results: The IVW estimates indicated that an increased abundance of Genus_Sellimonas is causally associated with an increased risk of ER+ breast cancer [odds ratio (OR) = 1.09, p = 1.72E−04, false discovery rate (FDR) = 0.02], whereas an increased abundance of Genus_Adlercreutzia was protective against ER+ breast cancer (OR = 0.88, p = 6.62E−04, FDR = 0.04). For Her2+ breast cancer, an increased abundance of Genus_Ruminococcus2 was associated with a decreased risk (OR = 0.77, p = 4.91E−04, FDR = 0.04), whereas an increased abundance of Genus_Erysipelatoclostridium was associated with an increased risk (OR = 1.25, p = 6.58E−04, FDR = 0.04). No evidence of heterogeneity or horizontal pleiotropy was found.

Conclusion: Our study revealed a gut microbiota–mammary axis, providing important data supporting the potential use of the gut microbiome as a candidate target for breast cancer prevention, diagnosis, and treatment.

KEYWORDS

breast cancer, gut microbiota, genus, mendelian randomization, pathogenesis

1. Introduction

Breast cancer is a common malignancy affecting women worldwide, being responsible for an estimated 2 million new cases annually (Sung et al., 2021). Incidence rates are higher in developed countries, which might be attributable to lifestyle and genetic factors (Mubarik et al., 2022). Approximately 5%–10% of breast cancers are related to genetic factors, such as mutations

in genes including BRCA1 and BRCA2. Other factors, including diet, exercise, and body weight, have also been linked to the incidence of breast cancer (Brédart et al., 2021).

Although the exact mechanisms of breast cancer development are not fully understood, studies have suggested that the gut microbiota plays a role (Zhu et al., 2018; Teng et al., 2021; Zhang et al., 2021). In particular, fecal transfer experiments and studies of antibiotic use suggest that gut microbiota may be a factor in carcinogenesis (Kovács et al., 2021). Gut bacteria, comprising a community of microbes that reside in the human gut, are closely related to human health (Adak and Khan, 2019; Schoeler and Caesar, 2019). They degrade indigestible food components, releasing nutrients such as vitamins, amino acids, and short-chain fatty acids, and help maintain the balance of the intestinal microbial flora (Mei et al., 2022). However, an imbalance in the gut microbiota, characterized by an overabundance of harmful bacteria and a lack of beneficial bacteria, might contribute to breast cancer development. Certain harmful bacteria can promote inflammation, which is believed to be an important mechanism of tumor formation (Chen et al., 2019; Esposito et al., 2022). Additionally, the gut–mammary pathway, characterized by the transfer of gut bacteria by immune cells to lymph nodes and then to the breasts via blood or lymphatic circulation, has been suggested as a possible mechanism by which gut bacteria influence the development of breast cancer (Rodríguez et al., 2021).

Although the causal relationship between the gut microbiota and breast cancer is not fully understood, observational epidemiological studies have revealed an association (Okubo et al., 2020; Yoon et al., 2021). However, randomized controlled trials investigating the effects of changes in the abundance of intestinal microbes on breast cancer risk have not been conducted. To address this gap, we employed Mendelian randomization (MR), a research method that uses genetic variation as an instrumental variable to assess causality between exposure and outcome (Davey Smith and Ebrahim, 2005), to investigate the causal influence of the gut microbiota on breast cancer development.

2. Materials and methods

2.1. Study design

In this study, we conducted a rigorous MR analysis that strictly adhered to the three major assumptions of MR analysis (Davies et al., 2018). First, we ensured that the selected genetic variants were associated with the exposure, serving as a predictor of the exposure. Second, we ensured that the genetic variation was independent of any confounding factors, was assigned randomly, and was unaffected by any other factors that could influence the exposure or outcome. Lastly, we ensured that genetic variation did not influence the outcome except through the exposure. A concise summary of the overall study design is presented in Figure 1.

2.2. Sources of exposure data and selection of instrumental variables for human gut bacteria

We obtained summary statistics of gut bacteria from the genome-wide association meta-analysis of the MiBioGen study, which is currently the largest study of transgenic genetics in the human microbiome (Kurilshikov et al., 2021). The study included 18,340 samples of 16S rRNA gene sequencing data from 24 cohorts of European, African, Asian, Middle Eastern, and Hispanic ancestry. In this study, we used seven fecal DNA extraction methods to obtain transgenic taxa data, and we analyzed the microbial composition of samples by targeting three different variable regions of the 16S RNA gene (V1–V2, V3–V4, and V4). All datasets were condensed to 10,000 reads per sample, and we classified the 211 intestinal bacterial taxa into five levels (phylum, class, order, family, and genus) by the direct taxonomic box method. After excluding 15 unnamed bacterial taxa and one duplicate bacterial taxon (Zhang et al., 2022), we selected 195 gut bacterial taxa as exposures for subsequent MR analysis. We selected instrumental variables (IVs) with all-site significance $p < 1 \times 10^{-5}$ and performed clump on all IVs of each gut flora (threshold $R^2 < 0.01$, distance = 500 kb) to reduce the gap between SNPs (Sanna et al., 2019). Linkage disequilibrium (LD) among the IVs was performed to obtain more IVs. LD analysis was performed according to the European 1,000 Genomes Project (Clarke et al., 2012). Subsequently, we harmonized the exposure and outcome data. First, we removed SNPs with inconsistent directions of exposure and outcome alleles. Second, we excluded palindromic A/T or C/G alleles to avoid ambiguous or erroneous results when performing MR analysis. We used F statistic > 10 to ensure that causality was not affected by weak instrumental bias (Burgess and Thompson, 2011). The calculation formula of F statistic is as follows: $F = R^2 (n - k - 1)/ k (1 - R^2)$, where R^2 represents the variance explained by each IV of the gut microbiota, $R^2 = 2$ MAF $(1 -$ MAF$) \beta^2$, n represents the sample size of the exposure data, k represents the number of IVs, and MAF represents the minor allele frequency.

2.3. Breast cancer data sources

We obtained breast cancer genome-wide association study (GWAS) data as outcomes from two databases. The genetic influence of cancer risk for overall breast cancer and the estrogen receptor status in the UK Biobank database was obtained from a large GWAS of the Breast Cancer Association Consortium involving 228,951 participants of European ancestry, including 122,977 patients with breast cancer (69,501 ER+ breast cancers, 21,468 ER− breast cancers) and 105,974 controls (Michailidou et al., 2017). The FinnGen database included 14,000 patients with breast cancer and 149,394 controls. The number of patients with HER2− breast cancer was 12,783, and the control group comprised 149,394 subjects. The number of patients with HER2+ breast cancer was 7,729, and the control group comprised 149,279 subjects. A detailed description of the data is available on this website (data available at: https://finngen.gitbook.io/documentation/v/r8/).

2.4. Statistical analysis

In this study, we utilized the inverse variance-weighted (IVW) method as the primary analysis tool to assess the impact of the gut microbiota on breast cancer risk (Burgess et al., 2016). We also conducted sensitivity analyses using the weighted median (WM; Hartwig et al., 2017), MR-Egger regression (Bowden et al., 2015), and MR pleiotropic residual sums and outliers (MR-PRESSO; Verbanck et al., 2018) methods. The WM models yield reliable estimates provided that at least 50% of the weights were derived from valid IVs (Hartwig

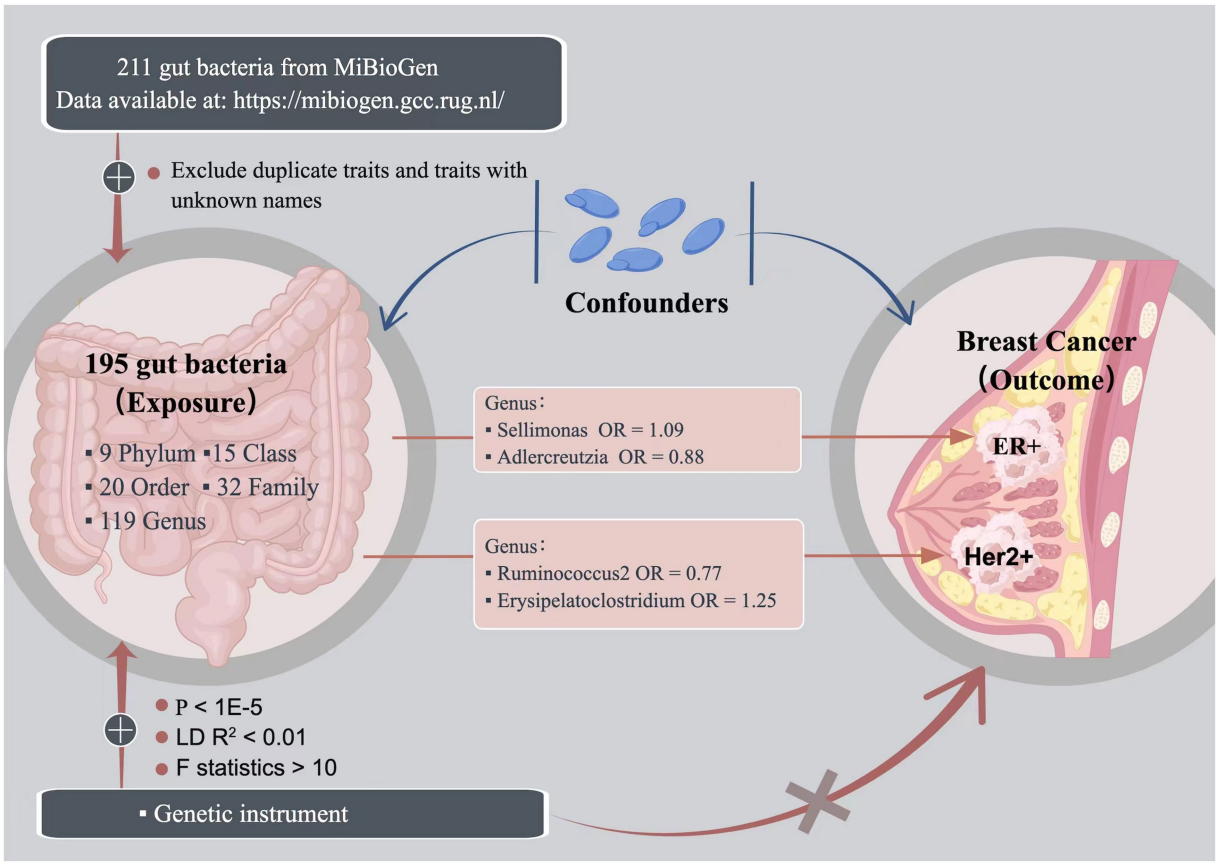

FIGURE 1
The study design of the present Mendelian randomization study of the associations of the gut microbiota and breast cancer risk.

et al., 2017). Although the MR-Egger method can account for pleiotropic effects, the obtained associations are often imprecise (Bowden et al., 2015). The MR-PRESSO approach can detect pleiotropic outliers for SNPs, and in such instances, MR analysis is repeated after eliminating these SNPs (Verbanck et al., 2018). Cochran's Q-value was used to evaluate the heterogeneity of causal inference. The intercept test of MR-Egger regression was employed to identify horizontal pleiotropic effects (Bowden et al., 2015). A value of p less than 0.05 suggested the presence of horizontal pleiotropic effects, and thus, we discarded the causal inference. To address multiple hypothesis testing, we used the Benjamini–Hochberg method and controlled for the false discovery rate (FDR; Benjamini and Yekutieli, 2001). A Benjamini–Hochberg-adjusted value of p of <0.05 was considered statistically significant. All MR analyses were conducted using the TwoSampleMR package (Hemani et al., 2018) in R software (version 4.2.1), and the circlize package was used to create circos circle diagrams (Gu et al., 2014).

3. Results

3.1. Causal inference of the relationship of the gut microbiota with breast cancer risk using the UK biobank database

In total, 195 intestinal flora were identified from the MiBioGen study and categorized into 9 phyla, 15 classes, 20 orders, 32 families, and 119 genera. In this study, we performed MR analysis of three breast cancer datasets from the UK biobank (UKB) database (total breast cancer, ER+ breast cancer, and ER− breast cancer; Supplementary Tables 1–3). Figure 2A presents the impact of changes in the abundance of 195 bacterial taxa on the risk of ER+ breast cancer based on the UKB database. Our findings suggested that an increase in the abundance of Genus_Sellimonas is associated with an elevated risk of ER+ breast cancer ($OR_{IVW} = 1.09$, $P_{IVW} = 1.72E{-}04$, $FDR_{IVW} = 0.02$; $OR_{WM} = 1.08$, $P_{WM} = 1.01E{-}02$, $FDR_{WM} = 0.82$; $OR_{MR\text{-}Egger} = 0.97$, $P_{MR\text{-}Egger} = 0.84$, $FDR_{MR\text{-}Egger} = 1.00$). Conversely, an increase in the abundance of Genus_Adlercreutzia was associated with a reduced risk of ER+ breast cancer ($OR_{IVW} = 0.88$, $P_{IVW} = 6.62E{-}04$, $FDR_{IVW} = 0.04$; $OR_{WM} = 0.90$, $P_{WM} = 4.18E{-}02$, $FDR_{WM} = 0.82$; $OR_{MR\text{-}Egger} = 0.98$, $P_{MR\text{-}Egger} = 0.92$, $FDR_{MR\text{-}Egger} = 1.00$; Table 1; Figure 3). Although the WM values of the causal inferences for the two gut genera and the risk of ER+ breast cancer were not statistically significant based on our strict FDR threshold control, the findings are consistent with the inferred direction of IVW, indicating our results are highly reliable.

3.2. Causal inference of the relationship of the gut microbiota with breast cancer risk using the FinnGen database

To deepen our understanding of the potential associations between the gut microbiota and breast cancer, we expanded our

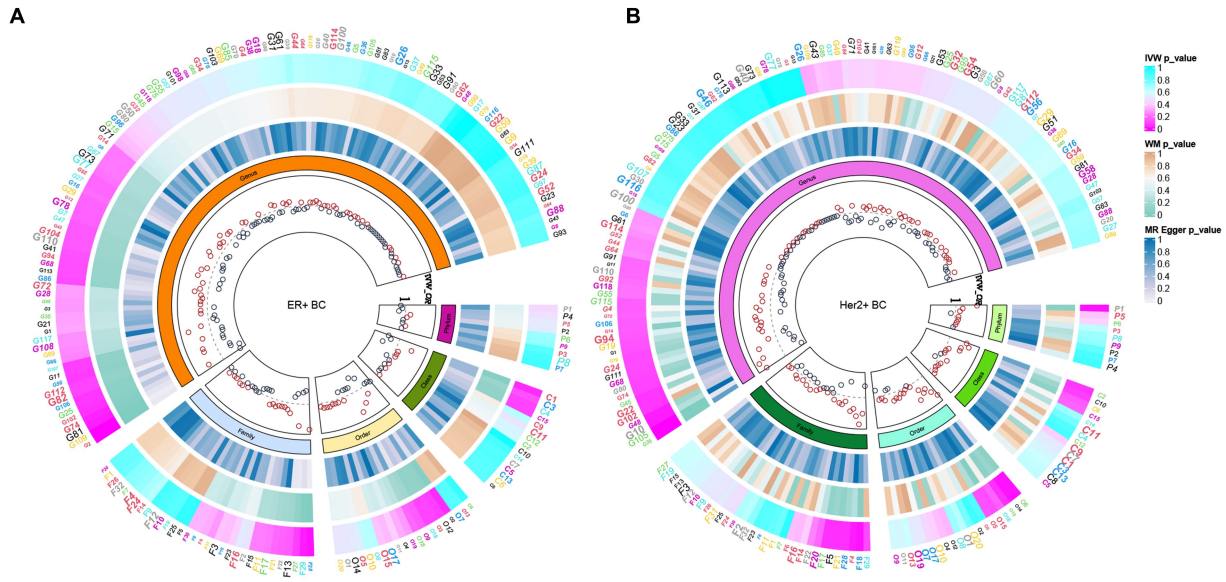

FIGURE 2
Based on the ER+ breast cancer cohort of the **(A)** UKB database and the Her2+ breast cancer cohort of the **(B)** FinnGen database, the causal relationship between intestinal bacteria and these two subtypes of breast cancer was summarized via MR analysis. The three heatmaps from the outermost circle to the inner circle represent the MR methods IVW, WM, and MR–Egger, respectively. The innermost circle represents the OR calculated by the IVW method. MR, Mendelian randomization; IVW, inverse variance-weighted; UKB, UK Biobank; OR, odds ratio; WM, weighted median; BC, breast cancer.

TABLE 1 Significant gut microbiota associated with breast cancer based on UKB.

Gut bacteria	Outcome	NSNP	Method	OR	95%CI	Value of *p*	FDR
Genus_Sellimonas	ER+ Breast cancer	10	Inverse variance weighted	1.09	1.04–1.14	1.72E-04	0.02
Genus_Sellimonas	ER+ Breast cancer	10	Weighted median	1.08	1.02–1.15	1.01E-02	0.82
Genus_Sellimonas	ER+ Breast cancer	10	MR Egger	0.97	0.75–1.25	0.84	1.00
Genus_Adlercreutzia	ER+ Breast cancer	8	Inverse variance weighted	0.88	0.81–0.95	6.62E-04	0.04
Genus_Adlercreutzia	ER+ Breast cancer	8	Weighted median	0.90	0.81–1.00	4.18E-02	0.82
Genus_Adlercreutzia	ER+ Breast cancer	8	MR Egger	0.98	0.71–1.36	0.92	1.00

MR, Mendelian randomization; NSNP, number of single nucleotide polymorphisms; UKB, UK Biobank; OR, odds ratio; CI, confidence interval; FDR, false discovery rate.

analysis to include additional subtypes of breast cancer. Specifically, we examined three distinct subtypes of breast cancer, namely total breast cancer, Her2+ breast cancer, and Her2− breast cancer, using the comprehensive FinnGen database (Supplementary Tables 4–6). Figure 2B presents the effects of changes in the abundance of 195 bacterial taxa on the risk of Her2+ breast cancer using the FinnGen database. Our analysis of this dataset from a diverse population revealed that two specific gut bacteria, Genus_Ruminococcus2 and Genus_Erysipelatoclostridium, were significantly associated with breast cancer risk. We found that an increased abundance of Genus_Ruminococcus2 was linked to a reduced risk of Her2+ breast cancer ($OR_{IVW} = 0.77$, $P_{IVW} = 4.91E{-}04$, $FDR_{IVW} = 0.04$; $OR_{WM} = 0.73$, $P_{WM} = 1.82E{-}03$, $FDR_{WM} = 0.22$; $OR_{MR\text{-}Egger} = 0.70$, $P_{MR\text{-}Egger} = 0.06$, $FDR_{MR\text{-}Egger} = 0.99$). Conversely, an increased abundance of Genus_Erysipelatoclostridium was associated with an increased risk of Her2+ breast cancer ($OR_{IVW} = 1.25$, $P_{IVW} = 6.58E{-}04$, $FDR_{IVW} = 0.04$; $OR_{WM} = 1.28$, $P_{WM} = 6.20E{-}03$, $FDR_{WM} = 0.37$; $OR_{MR\text{-}Egger} = 1.12$, $P_{MR\text{-}Egger} = 0.67$, $FDR_{MR\text{-}Egger} = 0.91$; Table 2; Figure 4). These observations highlight the potential role of specific gut microbes in the development and progression of certain subtypes of breast cancer.

3.3. Sensitivity analysis

All included variants had an F-statistic greater than 10, indicating the absence of weak instruments (min = 13.38, max = 166.56; Supplementary Table 7). To assess the robustness of the four identified causal estimates that met the FDR control, we performed a series of sensitivity analyses to test the heterogeneity of exposure to outcome. Neither Cochran's Q test nor MR-Egger revealed heterogeneity, indicating the robustness of our findings. Furthermore, the significance ($p < 0.05$) of the MR-PRESSO global test indicated the absence of horizontal pleiotropy, with no IVs identified as potential outliers. The intercepts of the MR-Egger regression did not deviate significantly from 0, and all *P*-values were greater than 0.05, indicating the absence of pleiotropy (Table 3). Leave-one-out sensitivity analysis and funnel plots confirmed the reliability and bias of the causal effects of the four identified associations (Supplementary Figures 1, 2). These results suggest a strong causal link between the identified flora and the corresponding risk of breast cancer, providing further evidence that our findings are reliable.

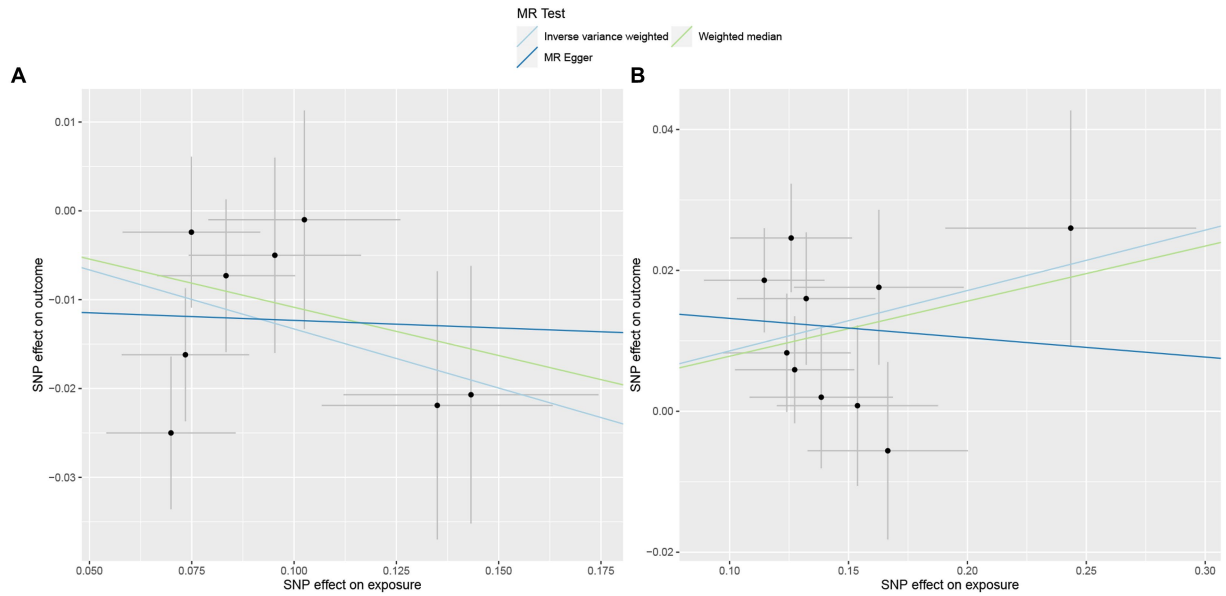

FIGURE 3
Scatterplot of three Mendelian randomization analysis methods. Causal effects of **(A)** Genus_Adlercreutzia and **(B)** Genus_Sellimonas on the risk of ER+ breast cancer.

TABLE 2 Significant gut microbes associated with breast cancer based on the FinnGen database.

Gut bacteria	Outcome	NSNP	Method	OR	95%CI	Value of *p*	FDR
Genus_Ruminococcus2	Her2+ Breast cancer	13	Inverse variance weighted	0.77	0.67–0.89	4.91E-04	0.04
Genus_Ruminococcus2	Her2+ Breast cancer	13	Weighted median	0.73	0.60–0.89	1.82E-03	0.22
Genus_Ruminococcus2	Her2+ Breast cancer	13	MR Egger	0.70	0.48–1.01	0.06	0.99
Genus_Erysipelatoclostridium	Her2+ Breast cancer	14	Inverse variance weighted	1.25	1.10–1.41	6.58E-04	0.04
Genus_Erysipelatoclostridium	Her2+ Breast cancer	14	Weighted median	1.28	1.07–1.54	6.20E-03	0.37
Genus_Erysipelatoclostridium	Her2+ Breast cancer	14	MR Egger	1.12	0.68–1.83	0.67	0.91

MR, Mendelian randomization; NSNP, number of single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval; FDR, false discovery rate.

4. Discussion

A recent study found no significant difference in the gut microbiota between Ghanaian women with and without breast cancer (Byrd et al., 2021), and another study revealed that the gut microbial composition of postmenopausal women with breast cancer and benign controls was similar (Aarnoutse et al., 2021). However, Goedert et al. reported inconsistencies in the diversity and specificity of the microbiota in patients with untreated breast cancer and healthy controls, with the former being characterized by elevated counts of Clostridiaceae, Faecalibacterium, and Ruminococcaceae (Goedert et al., 2015). Additionally, Terrisse et al. found that seven bacteria, including *Bacteroides uniformis*, *Clostridium bolteae*, and *Bilophila wadsworthia*, were associated with a worse breast cancer prognosis after comparing healthy human samples (Terrisse et al., 2021). Although the existing data primarily focus on the relationship between the gut microbiome and breast cancer, the causality remains unclear.

To explore the causal relationship between the gut microbiome and breast cancer, we conducted a study using the largest sample size to date, namely the MiBioGen study, which included 195 intestinal flora samples. We performed MR analysis by setting the gut flora as the exposure and the GWAS data of three breast cancers from the UKB and three breast cancers from the FinnGen database as the outcomes. We found that the abundance of two intestinal flora, specifically Genus_Sellimonas and Genus_Erysipelatoclostridium, increased the risk of ER+ breast cancer by 9% and that of Her2+ breast cancer by 25%. Conversely, the abundance of two other flora, Genus_Adlercreutzia and Genus_Ruminococcus2, reduced the risk of ER+ breast cancer by 12% and that of Her2+ breast cancer by 23%. Although the specific effects of the *Sellimonas*, *Erysipelatoclostridium*, and Ruminococcus2 flora on breast cancer development are unknown, *Adlercreutzia* appears to play a role in degrading isoflavones into genistein, which has been revealed to exert tumor-suppressive effects *in vivo* (Constantinou et al., 1998). Our findings are consistent with those of an animal study in which dietary modification affected the abundance of *Adlercreutzia* in feces, potentially serving as a biomarker for the efficacy of anticancer dietary supplements (Sharma et al., 2020).

Furthermore, the gut flora can affect hormone levels in the body, particularly estrogen levels, which are closely related to breast cancer development. Certain gut bacteria can boost estrogen synthesis, thereby increasing the risk of breast cancer

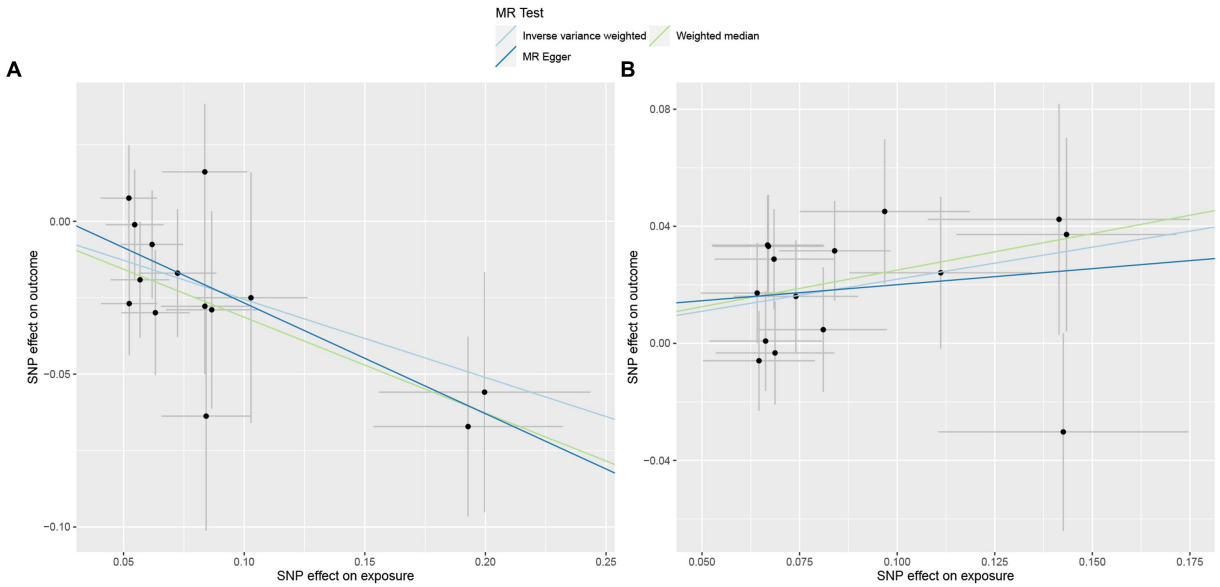

FIGURE 4
Scatterplot of three Mendelian randomization analysis methods. Causal effects of **(A)** Genus_Ruminococcus2 and **(B)** Genus_Erysipelatoclostridium on the risk of Her2+ breast cancer.

TABLE 3 Sensitivity analysis of the causal effect of gut microbes on the risk of breast cancer.

Bacterial taxa	Outcome	Heterogeneity			Pleiotropy	
		IVW Q (*p*-value)	MR Egger Q (*p*-value)	MR-PRESSO RSSobs (*p*-value)	MR Egger_ intercept	Value of *p*
Based on UKB						
Genus_Sellimonas	ER+ Breast cancer	10.21 (0.33)	9.29 (0.32)	12.66 (0.41)	0.02	0.40
Genus_Adlercreutzia	ER+ Breast cancer	6.68 (0.46)	6.16 (0.41)	8.46 (0.54)	−0.01	0.50
Based on FinnGen database						
Genus_Erysipelatoclostridium	Her2+ Breast cancer	11.51 (0.65)	11.31 (0.59)	13.24 (0.68)	0.02	0.66
Genus_Ruminococcus2	Her2+ Breast cancer	7.96 (0.85)	7.50 (0.82)	9.16 (0.87)	0.01	0.51

MR, Mendelian randomization; IVW, inverse variance-weighted; UKB, UK Biobank; MR-PRESSO, MR pleiotropy residual sum and outlier; RSSobs, observed residual sum of squares.

(Papakonstantinou et al., 2022). The gut flora can also affect the immune system, and an imbalance in the microbiota can weaken the immune system, resulting in an increased risk of breast cancer (Erdman and Poutahidis, 2015). The polymorphic microbiome is recognized as an emerging cancer hallmark, and investigating the interplay between breast tumor tissue and the gut microbiome is particularly interesting and important (Hanahan, 2022). Gut microbiome pathways can further refine breast cancer pathogenesis or complement existing risk stratification algorithms to improve their accuracy. Identifying the characteristics of gut microbes can provide valuable insights for predicting the efficacy and safety of chemotherapy in patients with breast cancer (Guan et al., 2020).

Despite our significant findings, this study had multiple limitations. Our IV selection threshold control was not sufficiently strict to achieve genome-wide statistical significance, which could lead to false-positive results. To address this, we used multiple testing correction via FDR estimation. Additionally, the number of Her2+ breast cancer cases was small, which could limit the statistical power of causal inferences for specific intestinal flora. We also did not differentiate between breast cancers according to molecular types, such as luminal A, luminal B, HER2+/−, and triple-negative breast cancer. Further research is needed to confirm these findings.

In summary, our study adds to the growing body of evidence supporting the existence of a gut microbiome–mammary axis by revealing a causal relationship between four gut microbes and the risk of breast cancer. Our study provides important scientific evidence for the potential use of the gut microbiome as a preventive, diagnostic, and therapeutic tool for breast cancer. However, further research is needed to confirm these findings and investigate the complex interplay between the gut microbiome and breast cancer. The identification of specific gut microbes and pathways involved in breast cancer pathogenesis could lead to the development of novel therapeutic interventions and refinement of existing risk stratification algorithms to

improve their accuracy. Additionally, our study highlights the importance of considering the gut microbiome as a modifiable risk factor for breast cancer and underscores the need for further research in this area.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: 211 gut bacteria from MiBioGen (data available at: https://mibiogen.gcc.rug.nl/); FinnGen database (https://finngen.gitbook.io/documentation/v/r8/).

Ethics statement

All studies for which data were disclosed were approved by the respective ethical review boards and written informed consent was provided by the participants. As this study used published data, no new ethics approval was required.

Author contributions

ZhW designed the study. SZ, WZ, HR, ZiW, and RX collected and analyzed the data. SZ and WZ drew the figures and drafted the early version of the manuscript. QL and WZ supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1193725/full#supplementary-material

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Anthracyclines and trastuzumab associated cardiotoxicity: is the gut microbiota a friend or foe? – a mini-review

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Breast cancer (BC) is one of the most prevalent cancers worldwide. Fortunately, BC treatment has taken a huge turn in the last few years. Despite these advances, one of the main issues related to systemic treatment remains the management of its side effects, including cardiotoxicity. In this regard, we highlight the irreversible dose-dependent cardiotoxicity of anthracyclines related to oxidative stress and the reversible cardiotoxicity with trastuzumab, whose mechanism is still poorly understood. Moreover, the combination of anthracyclines and trastuzumab further exacerbate the myocardial damage. More recently, altered gut microbiota composition has been linked to the long-term effects of cancer therapy, including the potential connection between treatment-related microbial changes and cardiotoxicity. *Bacteroides* spp., *Coriobacteriaceae*_UGC-002, and *Dubosiella* have already been reported as bacterial species with deleterious effects on the myocardium, mainly due to the promotion of inflammation. On the other hand, *Alloprevotella*, *Rickenellaceae*_RC9, *Raoultella planticola*, *Klebsiella pneumoniae*, and *Escherichia coli* BW25113 can induce cardioprotection, predominantly by increasing anti-inflammatory cytokines, promoting intestinal barrier integrity and early metabolization of doxorubicin. Herein, we explore the role of gut microbiota in the development of cardiotoxicity, as well as future perspectives to decrease the risk of cardiotoxicity associated with BC treatment.

KEYWORDS

gut microbiome, gut microbiota, chemotherapy, anthracyclines, doxorubicin, trastuzumab, cardiotoxicity

1 Introduction

Breast cancer (BC) is the most common cancer among women and the second most common worldwide. This type of cancer is a multifactorial disease, and several factors, such as race, ethnicity, and demographic characteristics, contribute to its incidence. It is expected that the BC mortality rate will increase by 2030, particularly in developing countries, despite all screening measures and the evolution of the therapeutic armamentarium (Adeoye and Adeoye, 2023).

Molecular BC subtypes determine prognosis and indication of specific systemic therapy, including endocrine therapy for hormone receptor-positive tumors (with some patients also requiring chemotherapy), trastuzumab-based therapy plus chemotherapy for human epidermal growth factor receptor 2 (HER2) tumors, and chemotherapy alone or combined with immunotherapy for triple-negative BC (Waks and Winer, 2019). Chemotherapy is still an essential treatment for many patients with stage I-III BC, despite the potential short- and long-term side effects (Waks and Winer, 2019).

Among the drugs most used in the treatment of BC, anthracyclines and trastuzumab stand out. Nevertheless, the toxicity of these drugs should not be underestimated. A well-known side effect is a cumulative cardiotoxicity, the main reason for dose-limited administration (Carvalho et al., 2009; Barish et al., 2019).

As such, efforts should be made to find more effective measures to overcome these drugs' toxicity while maintaining or enhancing their therapeutic efficacy. Gut microbiota dysbiosis has recently come to light as a significant player that may impact BC development, therapy, and prognosis through a various molecular mechanism. Therefore, gut dysbiosis can potentially affect the responses and toxicity profile of antineoplastic agents (Alpuim Costa et al., 2021).

Hence, the aim of this review is the exploration and clarification of the links between the influence of gut microbiota and cardiotoxicity induced by antineoplastic drugs, namely anthracyclines and trastuzumab.

2 Anthracyclines associated cardiotoxicity – mechanism of action

Doxorubicin (DOX) belongs to non-selective class I anthracycline family. Its clinical use is known for cumulative and irreversible cardiotoxicity, which leads to aberrant arrhythmias, ventricular dysfunction, and congestive heart failure, even years after chemotherapy cessation. Among all theories that have been proposed, the more accepted and defined are mitochondrial dysfunction, DNA damage, defects in iron metabolism, and higher levels of glucose consumption (McGowan et al., 2017; Henriksen, 2018).

The hallmarks of DOX-induced cardiotoxic effects have been shown to include mitochondrial damage and accumulation of dysfunctional mitochondria (Figure 1). By binding to cardiolipin, DOX accumulates in the inner mitochondrial membrane and decouples the respiratory chain complexes, which reduces ATP synthesis. Thus, DOX cardiotoxicity directly contributes to ATP deficiency by altering mitochondrial energy metabolism and bioenergetics (Henriksen, 2018).

Regarding DNA damage, the therapeutic effect of anthracyclines against cancer cells is mediated by the inhibition of topoisomerase (Top) 2α, increasing DNA breaks, and preventing DNA and RNA synthesis. In cardiomyocytes, Top 2β is also inhibited, causing double-stranded DNA breaks. The Top 2 inhibition in both cell types causes accumulation of double-stranded DNA breaks and mitochondrial dysfunction, leading to activation of cell death pathways and accumulation of reactive oxygen species (ROS) (McGowan et al., 2017; Henriksen, 2018).

Impairment of mechanisms involved in cellular iron homeostasis can occur through different mechanisms:

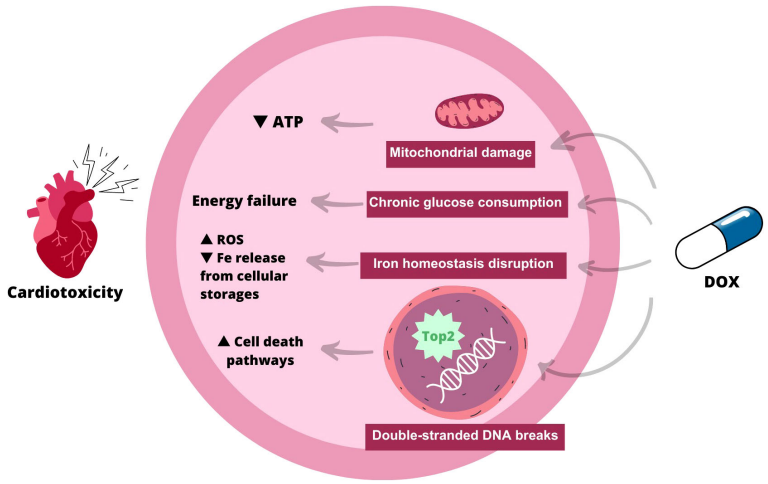

FIGURE 1
Scheme explaining the mechanism of action of doxorubicin-induced cardiotoxicity. As indicated, when doxorubicin is administered, there are several possible pathways: 1) Mitochondrial damage, leading to a decrease in ATP within myocardial cells; 2) Chronic glucose consumption, which contributes to energy failure; 3) Iron homeostasis disruption, which increases reactive species of oxygen and decreases iron from cellular storages; 4) Induction of double-stranded DNA breaks, which leads to increased apoptosis. ATP, Adenosine Tri-phosphate; ROS, Reactive Octive Species.

2.1 Formation of ROS

Within the cell, DOX is reduced to a cytotoxic semiquinone radical which is rapidly converted back to the original molecule using O_2 as an electron acceptor, leading to the formation of superoxide formation that is detoxified in H_2O_2. Secondly, the labile iron pool (LIP) - cellular pool of cheatable and redox-active iron – reacts strongly with H_2O_2, generating ROS via Fenton´s reaction. Furthermore, LIP can directly interact with DOX, creating DOX-Fe complexes that drive ROS production.

2.2 Disruption of iron localization in the cell

DOX modulates mRNA maturation of transferrin receptor and ferritin through irreversible inactivation of the RNA-binding activity of iron regulatory proteins 1 and 2. This mechanism increases iron/ferritin binding in the cytosol and reduces its release from cells storages, including mitochondria. Iron accumulation in mitochondria has been linked to ferroptosis, which is believed to play a role in DOX-induced cardiomyopathy.

Fatty acids are the primary fuel for catabolic reactions in cardiac metabolism under normal circumstances, while glycolysis is used in response to pathological events. Doxorubicin enhances serum triglyceride and blood glucose levels and at the same time triggers massive cardiac glucose uptake due to AMPK inhibition and concomitant expression of GLUT1 (normally absent in the adult heart). Chronic glucose consumption eventually becomes maladaptive and results in energy failure. Additionally, a clinical investigation found that patients with comorbidities conditions such as diabetes, dyslipidemia, and obesity are at increased risk of DOX-induced cardiotoxicity (Russo et al., 2021).

Although doxorubicin is the most studied anthracycline, in terms of chemotherapy-induced cardiotoxicity, there are a few studies exploring the same side effects in other anthracyclines, such as epirubicin, as well as anthracenedione (i.e. anthracycline analogue), namely mitoxantrone.

Apparently, even in the second generation of anthracyclines, namely with idarubicin or epirubicin, cumulative cardiotoxicity remains a relevant side effect (Minotti et al., 2004; Morelli et al., 2022). Furthermore, it was noted that also anthracyclines derivatives, such as Mitoxantrone, can induce myocardium lesions, leading to cardiac failure, however with a significant safer profile than doxorubicin (Saletan, 1987; Damiani et al., 2016; Morelli et al., 2022).

3 Trastuzumab associated cardiotoxicity – mechanism of action

Trastuzumab is a humanized monoclonal antibody that blocks the action of HER2. Its precise mode of action against cardiomyocytes is still uncertain. Nevertheless, cardiomyocyte growth and proliferation are significantly influenced by HER2 and the ErbB family of tyrosine kinase receptors. Thus, blocking of downstream intracellular signaling by trastuzumab may affect cellular metabolism, leading to sarcomere disruption and impaired cell proliferation. In addition, DOX and trastuzumab can have a synergic effect, as the former initiates an oxidative damage process and the latter blocks HER2 downstream signaling, essential to cellular repair (Barish et al., 2019).

The main mechanism of action of trastuzumab stands for binding to HER2 receptors, leading to the blockade of their dimerization and, subsequently, their downstream signaling. However, it was recently unveiled a new side mechanism that involves the immune system. Exactly after the connection of antibody-receptor, it occurs an interaction between the Fc receptor of anti-Her2 antibody and FC receptors of immune cells, such as neutrophils, NK cells, γδ T cells and macrophages. As a consequence, it activates the immune system, conducting to an enhanced presence of tumor antigens in the tumor microenvironment, which leads to increased cytotoxicity, with the aim to augment the efficacy of antigen-presenting cells (APC). Moreover, NK cells in one way, improve the cytotoxicity of CD8+ T cells, through dendritic cells priming, as well as induce a Th1 phenotype and, in another way, NK cells promote a pro-tumoral cytokine production, recruiting even more T and myeloid cells (Bianchini and Gianni, 2014).

4 Effect of gut microbiota on cancer treatment-associated cardiotoxicity

4.1 Anthracyclines - Doxorubicin

Although the mechanism of DOX-induced cardiotoxicity is becoming clearer, there are still some gaps that might be fulfilled by microbiota research. Recently, a considerable number of studies have shifted the focus from chemotherapy-induced cardiotoxicity to a possible cause related to gut dysbiosis. Indeed, it has been observed that after DOX treatment, there are three main impacts on the intestinal microbiota (Table 1): 1) Direct lesion of the intestinal mucosa; 2) Structural/composition changes; 3) Metabolomic modifications.

4.1.1 Direct lesion of the intestinal mucosa

It has been reported that patients undergoing chemotherapy frequently develop acute intestinal mucositis (Bianchini and Gianni, 2014), and so An L. et al. explored DOX-induced damage to the intestinal barrier. As a result, after DOX administration, via intraperitoneal injection, in a dosage of 5 mg/Kg, there was infiltration of lymphocytes clusters, gut ulcers, and loss of goblet cells, which gave rise to endotoxemia (Szeto et al., 2018; An et al., 2021).

Furthermore, the concentrations of tight-junction proteins, like claudins, occludins and, particularly, zonulin (ZO-1), whose function is to stabilize the intestinal membrane, were reduced in the DOX group. When a fecal microbiota transplant (FMT) was

TABLE 1 Microbiota effects in doxorubicin-induced cardiotoxicity.

Bacteria/Metabolite	Mechanism	Type of study	Ref
1. Direct lesion on intestinal barrier			
	Cluster of lymphocytes, gut ulcers and loss of goblet cells	Animal	(Chelakkot et al., 2018; An et al., 2021)
	Increase concentration of endotoxins	Animal	(Chelakkot et al., 2018; An et al., 2021)
	Decrease of tight-junction proteins, such as ZO-1	Animal	(Chelakkot et al., 2018; Huang et al., 2022)
2. Microbiota Composition Changes			
***Bacteroides* spp.**	Modification of gut microbiota network	Animal	(Rocha and Smith, 2013; Huang et al., 2022)
Actinobacteria* - *Coriobacteriaceae	Modification of colonic macrophages into M1-like pro-inflammatory macrophages with increased levels of TNF-α and IL-1β	Animal	(Liu et al., 2020; Lin et al., 2021)
Coriobacteriaceae_UGC-002	Increase myocardial enzymes, which means increased myocardial damage	Animal	(Liu et al., 2020)
Dubosiella	Increase myocardial enzymes	Animal	(Guo et al., 2018; Liu et al., 2020)
Alloprevotella	Increase intestinal barrier integrity, prevent pathogen proliferation and substrate for colonocytes	Animal	(Dubin et al., 2016; Huang et al., 2022)
Rikenellaceae_RC9	Same as previous effects. Attenuate colitis through CTLA-4 disruption by increasing the regulatory T-cells differentiation	Human	(Dubin et al., 2016; Yan et al., 2018)
Enterobacteriaceae* – *Raoultella planticola	Metabolize doxorubicin into 7- deoxydoxorubicinol and 7-deoxydoxorubicinolone, which are inactive metabolites	Human	(Alpuim Costa et al., 2021; Mamic et al., 2021)
***Klebsiella pneumoniae* & *E. coli* BW25113**	Inactivate doxorubicin through molybdopterin-dependent enzymes	Human	(Alpuim Costa et al., 2021; Mamic et al., 2021)
3. Metabolome modifications			
Secondary bile acids	Increase triglyceride gathering and inflammation, leading to myocardial apoptosis and fibrosis	Animal	(Romano et al., 2015; Liu et al., 2020)
TMAO	Increase inflammation through induction of NLRP3 inflammasome, leading to cardiac fibrosis. Also, induces differentiation of monocytes into macrophages and foam cells, contributing to atherosclerosis development	Animal/ Human	(Brown and Hazen, 2018; Li et al., 2019; Liu et al., 2020; Xiong et al., 2022)
SCFA	Anti-inflammatory effects. Decrease ROS.	Animal	(Carvalho et al., 2009; Liu et al., 2020)

Green color corresponds to beneficial effects and red is associated with deleterious effects. It is divided in 3 parts: 1. Direct lesion of the intestinal barrier; 2. Microbiota composition changes; 3. Metabolomics modifications. ROS, Reactive oxygen species; TMAO, Trimethylamine N-oxide; SCFA, Short-chain Fatty Acids; ; NLRP3, NOD-, LRR- and pyrin domain -containing protein 3; CTLA-4, Cytotoxic T-Lymphocyte- associated protein 4; ZO-1, Zonula occludens 1; IL, Interleukin; TNF, Tumour Necrosis Factor.

performed, ZO-1 levels were restored and the concentration of endotoxins was decreased, which means that this chemotherapy induces damage to the the intestinal wall and, one of the possible approaches to reduce this side effect, could be the FMT (Chelakkot et al., 2018; An et al., 2021).

4.1.2 Microbiota structural/composition changes

First, a significant decrease in alpha diversity was observed (Junpaparp et al., 2013). Moreover, it was possible to correlate some bacterial modifications with cardiotoxicity outcomes.

Starting with bacteria with deleterious effects, in terms of cardiotoxicity, *Bacteroides* spp. was identified as a relevant harmful microorganism that influences the entire gut microbiota network. This bacterium is a gram-negative and obligate anaerobic, with the characteristic of being an opportunistic pathogen in infections (Rocha and Smith, 2013; Huang et al., 2022).

Furthermore, the phylum *Actinobacteriota* was also increased in mice under DOX treatment (by intraperitoneal injection, with a cumulative dosage of 20 mg/Kg), suggesting a negative impact on cardiovascular disease, in Liu et al. study, it was even observed that a

family from the abovementioned phylum, named *Coriobacteriaceae*, was actually increased and contributed to modifying colonic macrophages into a pro-inflammatory M1 macrophag, which amplified the concentrations of TNF-α and IL-1β, pro-inflammatory cytokines that promote cardiotoxicity (Liu et al., 2020; Huang et al., 2022). Additionally, one genus of this family, *Coriobacteriaceae*_UCG-002, had a particularly higher concentration compared to other genes and was associated with serum myocardial enzymes, pointing to a potential increase in DOX-induced cardiac lesion (Huang et al., 2022). Alongside with this cardiac condition, another genus was correlated with increased myocardial enzymes, namely *Dubosiella* (Huang et al., 2022). In fact, *Dubosiella* was reduced in a study that revealed beneficial outcomes from yellow wine compounds, demonstrating that their absence resulted in enhanced effects. In this study, mice were submitted to a DOX treatment, via intravenous tail injections, with a dosage of 4 mg/Kg, one time per week, for 4 weeks straight (Lin et al., 2021).

In another perspective, the intestinal microbiota can also protect against DOX-induced cardiotoxicity, through different mechanisms. One of them comprises the strengthening of the intestinal barrier by increasing the levels of *Alloprevotella* and *Rikenellaceae*_RC9 and decreasing of *Prevotellaceae*_UCG-001. All referred bacteria are producers of short chain fatty acids (SCFA), namely acetate, propionate, butyrate and valeric acids, using mucin as a substrate. These SCFAs are important because they can prevent pathogenic proliferation, uphold the intestinal barrier, and provide food for colonocytes (Guo et al., 2018; An et al., 2021). *Rikenellaceae*_RC9 also has the ability to decrease colitis symptoms through anti-inflammatory mechanisms and CTLA-4 disruption by increasing the differentiation of regulatory T-cell (Dubin et al., 2016; An et al., 2021).

On the other hand, the other beneficial mechanism is the early metabolization of DOX. *Raoultella planticola*, under specific anaerobic conditions, can inactivate DOX through reductive deglycosylation, originating 7-deoxydoxorubicinol and 7-deoxydoxorubicinolone, inactive metabolites and thus reducing the bioavailability and toxicity of DOX. Furthermore, some bacteria, such as *Klebsiella pneumoniae* and *Escherichia coli* BW25113, can metabolize DOX into inactive metabolites, with a different mechanism using molybdopterin-dependent enzymes (Yan et al., 2018; Alpuim Costa et al., 2021).

4.1.3 Metabolomic modifications

Doxorubicin has been shown to modify metabolic pathways such as the biosynthesis of glycans, amino acid, lipids and other complimentary metabolites. Moreover, even the microbial metabolites are altered after administration of this chemotherapeutic agent, such as SCFA, trimethylamine N-oxide (TMAO) and other aminoacidic chemicals. and therefore, may contribute to DOX-induced cardiotoxicity (Mamic et al., 2021).

Some bacterial metabolites have beneficial effects on the mechanism of cardiotoxicity, like SCFA, but, on the other hand, some contribute and even exacerbate the deleterious consequences of DOX use, such as TMAO and secondary bile acids.

Secondary bile acids consist of primary bile acids converted by bacterial enzymes, such as 7α dihydroxylase, which can be found in the small intestine, and create more hydrophobic bile acids (Witkowski et al., 2020; Alpuim Costa et al., 2021). The main problem of these secondary bile acids is the interaction with farmesoid X receptor (FXR) and G Protein-coupled membrane receptor 5 (TGR5), which in turn contribute to triglyceride accumulation and inflammation, leading to myocardial apoptosis, fibrosis, and subsequently cardiac damage (Calkin and Tontonoz, 2012; Huang et al., 2022).

Furthermore, TMAO, another deleterious bacterial metabolite, originated by the breakdown of some proteins like lecithin, choline and carnitine, by specific bacteria of the phyla *Firmicutes* and *Actinobacteria* (Romano et al., 2015; Huang et al., 2022). The TMAO is still not fully understood, as it can be protective or aggressive in the context of cardiovascular disease. However, the most studied mechanism of deleterious effects is the ability to interact with monocytes, causing their differentiation into macrophages and foam cells (Brown and Hazen, 2018), as well as the induction of NLRP3 inflammasome, promoting atherosclerosis and cardiac fibrosis and, therefore, increasing cardiovascular risk (Li et al., 2019; Huang et al., 2022).

Finally, SCFAs, which are carboxylic acids originating from bacterial metabolism through fermentation of fibers and non-digestible carbohydrates, produced mainly in cecum and colon (Xiong et al., 2022), are associated with numerous beneficial actions, predominantly related to anti-inflammatory characteristics. One of the main protective actions against cardiotoxicity is that some SCFAs, such as butyrate, can reduce the amount of ROS, thereby, reducing cardiac damage (Russo et al., 2019; Huang et al., 2022). This decrease of oxidative stress happens by inhibiting histone deacetylase (HDAC), which in turn, can activate Nrf2- Keap1 pathway (Nuclear erythroid-related factor 2 – Kelch-like ECH-associated protein 1). If the cell is inert, Keap 1 is blocking Nrf2, promoting ubiquitination and subsequent degradation of cells. However, if an increase in oxidative stress is detected, there is an accumulation of Nrf2 synthesis in the nucleus of the cell that, accompanied by Maf proteins, interacts with antioxidant response element (ARE), at the promoter region of antioxidative genes, originating antioxidant enzymes (González-Bosch et al., 2021). With less oxidative stress, myocardial cells apoptosis diminishes, which means a reduction in cardiac lesions.

5 Anti-Her2 monoclonal antibodies – trastuzumab

Di Monica M. et al. demonstrated the importance of microbiota in trastuzumab efficacy, in a mice model, as well as suggested the existence of a gut microbiota/immune mediated trastuzumab activity axis. Summing up, mice were treated with broad-spectrum antibiotics (streptomycin and vancomycin), which resulted in the diminution of *Actinobacteria*, specially *Coriobacteriaceae*, *Clostridiales*, namely *Lachnospiraceae*, *Turicibacteraceae* and *Bacteroidetes*, particularly *Prevotellaceae*,

mainly SCFA-producing bacteria. Subsequently, it was noted a significant degradation of the intestinal barrier that, in turn, lead to decreased efficacy of APCs with reduced innate immune system activation. Additionally, it was observed a reduction in the concentration of IL12p70.

Afterwards, these antibiotic-treated mice were submitted to faecal material transplant (FMT) from non-antibiotic treated mice that resulted in re-establishment of intestinal microbiota homeostasis, accompanied by an augmented activation of the innate immune system, recovery of the intestinal barrier and a significant increase of IL12p70 (Di Modica et al., 2021; Di Modica et al., 2022).

Moreover, this study revealed the importance of IL12p70, a cytokine liberated by dendritic cells in response to microbiota signal, which acts through the activation of APC cells, that induce T and NK cells, originating an increase of trastuzumab efficacy (Vignali and Kuchroo, 2012).

Furthermore, the same research group evaluated the microbiota of women that do not respond to trastuzumab and, remarkably, there was an akin composition to antibiotic-treated mice, with the same phyla and taxonomic family's changes in composition, as described above. Furthermore, this lack of responsiveness to trastuzumab was settled after a FMT from the non-responsive women to mice (Di Modica et al., 2021; Di Modica et al., 2022).

6 How can cardiotoxicity associated with cancer treatment be improved through the microbiota?

Cardiotoxicity is a relevant drawback both for the cancer therapy efficacy, since there is a need to postpone, adapt or interrupt the treatment regimen (Tran et al., 2020), and for the quality-of-life of cancer patients.

Therefore, it is crucial to find a way to significantly reduce cardiotoxicity. In fact, there are some measures, namely low dosages, since in some cases this cardiotoxicity is dose-dependent (e.g., DOX) (Carvalho et al., 2009), increased surveillance with regular echocardiography (Chung et al., 2013) and, even, the administration of regular drugs used in the treatment of cardiovascular pathology (Mir et al., 2023), such as statins, beta-blockers, among others.

However, these preventive measures against cardiotoxicity are still insufficient and, often compromise the well-being of disease-free patients in the future (Swain et al., 2003).

Thus, the intestinal microbiota has become an important ally in this battle against the side effects of anthracyclines and other chemotherapy drugs. Here, we propose some microbiota-based ideas that might be tested with the hope of reducing cancer treatment-induced cardiotoxicity:

1. Adjuvant therapy administration of symbiotics composed of mucins, as prebiotics, a multi-strain probiotic with *Alloprevotella*, *Rikenellaceae_RC9*, *Raoultella planticola* and *E. coli* BW25113, and a postbiotic, particularly SCFA to reduce the pro-inflammatory and oxidative microenvironment, improves the intestinal barrier integrity and increases DOX metabolism (Dubin et al., 2016; An et al., 2021; Huang et al., 2022);
2. Use of nanotechnology drug delivery to get SCFA directly into the myocardium for protection against inflammation and ROS;
3. Reproduction of the same enzyme mechanism as *Raoultella planticola* and *E. coli* BW25113 DOX metabolization (Alpuim Costa et al., 2021). Afterwards, administration of these enzymes a few hours after DOX treatment;
4. Personalized nutrition to obtain a more favorable intestinal microbiota, rich in SCFA-producing bacteria and other beneficial ones, to increase the anti-inflammatory and anti-oxidant activity (Yan et al., 2018; Huang et al., 2022);
5. Application of concurrent hyperbaric oxygen therapy with DOX treatment for increased cardioprotection (Karagoz et al., 2008).

Since there is no other manuscript or on-going clinical trial that can possibly explain the role of microbiota in the cardiotoxicity associated to trastuzumab, we hypothesize that the use of certain SCFA-producing bacteria, namely from the family's *Coriobacteriaceae*, *Lachnospiraceae*, *Turicibacteraceae* and *Prevotellaceae* or even co-administer SCFA concomitant with trastuzumab may reduce the side effects, particularly cardiotoxicity, due to the fact that we could use lower drugs.

7 Conclusion

Cancer treatment is a fast-moving and dynamic field with a promising future. However, most of these therapies are still limited by their side effects, which can compromise the quality of life of cancer patients and even the quality of cancer treatment. Focusing on BC, the most used pharmacotherapy comprises anthracyclines and anti-HER-2 drugs, mainly trastuzumab, which have a well-known associated cardiotoxicity, that remains one of the main issues in BC treatment.

Recently, the microbiota is becoming more important as a potential influence on the side effects of chemotherapy on the organism. Hereby, several microbiota mechanisms that positively or negatively influence DOX-induced cardiotoxicity was described. Therefore, gut microbiota homeostasis appears to be a relevant pathway to decrease cardiotoxicity induced by cancer treatment and it relevant to support this line of research, with the goal to develop safer and more effective therapies against cancer.

Author contributions

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The potential links between human gut microbiota and cardiovascular health and disease - is there a gut-cardiovascular axis?

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The gut-heart axis is an emerging concept highlighting the crucial link between gut microbiota and cardiovascular diseases (CVDs). Recent studies have demonstrated that gut microbiota is pivotal in regulating host metabolism, inflammation, and immune function, critical drivers of CVD pathophysiology. Despite a strong link between gut microbiota and CVDs, this ecosystem's complexity still needs to be fully understood. The short-chain fatty acids, trimethylamine N-oxide, bile acids, and polyamines are directly or indirectly involved in the development and prognosis of CVDs. This review explores the relationship between gut microbiota metabolites and CVDs, focusing on atherosclerosis and hypertension, and analyzes personalized microbiota-based modulation interventions, such as physical activity, diet, probiotics, prebiotics, and fecal microbiota transplantation, as a promising strategy for CVD prevention and treatment.

KEYWORDS

gut microbiota, gut-heart axis, dysbiosis, cardiovascular diseases, atherosclerosis, hypertension, TMAO, SCFAs

1 Introduction

Humans have a diverse and dense ecosystem of microorganisms called the human microbiota, which has been known for almost a century. However, we are only now starting to grasp many of these microorganisms' functions in human health and development (1).

The human microbiota comprises more than 100 trillion microbial species, within which bacteria, fungi, viruses, and protozoa are distinguished (1, 2). These microorganisms, together with their genes (microbiome), form a dynamic microbial community that inhabits different areas of the human body, playing a vital role in the host's health (1). The site of the human body that hosts the most significant number and diversity of microorganisms is the gastrointestinal tract, more precisely the gut, having a significant impact on human homeostatic processes such as nutrient metabolism, maintenance of intestinal mucosal barrier integrity, regulation of satiety, defense against pathogens either by pH modification or secretions of antimicrobial peptides or changes in cell signaling pathways, and development of the immune system (3, 4). These microorganisms coexist in harmony with their host, demonstrating a symbiotic relationship. Although a balance between the microbiota and its host must be observed to optimize metabolic and immunological functions, there is no ideal composition because each person has a unique microbiota (5). Thus, considering gut microbiota characteristics such as high diversity, stability, and resilience, and the symbiotic interactions with the host, we can define it as a superorganism (6, 7). Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, are the four major phyla in the gut microbiota, and in healthy adult individuals, the first two prevail (8, 9). Its composition remains stable over time, but the microbiota is characterized by some volatility, demonstrated by a diverse set of genetic and environmental factors like dietary composition, social interactions, infections, and antibiotic exposure, that can shape its composition (10, 11).

Most studies prove that the balance between the microbial species in the gut microbiota is fundamental for maintaining the body's homeostasis (11). The term dysbiosis refers to an imbalance in the microbiota composition with a consequent change in its functions, whereby its normal beneficial state changes to a possible harmful state for human health, with pro-inflammatory effects and immune dysregulation associated with several disorders (12). Increasing evidence points to the possibility of using variations in the F/B ratio, the ratio of the microbial communities Firmicutes and Bacteroidetes, as a biomarker for pathological disorders (13). However, a growing body of proof suggests that gut microbiota impacts intestinal disorders and numerous extra-intestinal disorders such as neurological disorders, cardiovascular diseases (CVDs), cancer, and many others (14). Understanding the cause or consequence of this situation and how to maintain or restore the composition of the gut microbiota will be very helpful in developing new therapeutic interventions (15).

In the past decade, CVDs have emerged as the leading cause of death worldwide, taking an estimated 17.9 million lives yearly (16, 17). Besides genetic factors, environmental factors and intestinal microbiota were also acknowledged as one of the main factors for the development of CVD. Also, diabetes, obesity, and metabolic syndrome, three major risk factors for CVDs, have been linked to intestinal dysbiosis as a risk factor for development (18, 19). One example of the potential link between gut microbiota and CVD is the production of trimethylamine N-oxide (TMAO), a compound that has been linked to CVD, with high plasma TMAO levels having a close association with the risk of developing atherosclerosis (16, 19, 20).

The purpose of the present review is to explore the role of the gut microbiota concerning the development of CVD, focusing on our previous works (20), and the most current evidence regarding TMAO as a biomarker for CVD and the effects of its precursors, choline, and carnitine, on TMAO formation and the associated high CVD risk, as well as the beneficial effects of short-chain fatty acids, bile acids and polyamines in CVD development (21–23).

2 Gut microbiota ecology and its implication on cardiovascular diseases

2.1 Bacterial microbiota

Emerging evidence suggests that the gut microbiota may be an essential contributor to the development of CVDs, such as atherosclerosis, hypertension, coronary artery disease, and stroke. Many researchers have reported a connection between CVD phenotypes and changes in the relative abundance of specific microbial taxa or the richness or variety of the bacteria in the gut (24, 25).

The gut barrier is a complex system that separates the intestinal lumen from the rest of the body (26). It plays a critical role in maintaining the health and integrity of the body by preventing the translocation of harmful substances and microorganisms from the gut into the rest of the systemic circulation (26, 27). In a healthy individual, the intestinal barrier is intact and functions appropriately, being maintained by physical factors like tight junctions between epithelial cells, mucus production, and mucosal immunity (28, 29). The barrier comprises several layers, including the mucus layer, the epithelial cell layer, and the underlying immune system (26, 30). The mucus layer is a thin layer that coats the gut's surface and acts as a physical barrier to prevent the adherence of pathogens and harmful substances. The epithelial cell layer is composed of a single layer of cells that forms the outermost layer of the gut and acts as a selective barrier allowing the passage of nutrients and water into the body while preventing the translocation of harmful substances. The underlying immune system also plays a role in maintaining the integrity of the intestinal barrier, helping to prevent the invasion of pathogens and other harmful substances by producing antibodies and other immune cells that can target and neutralize them (26, 27). According to the leaky gut theory, decreased gut barrier function has been linked to health problems, causing bacterial compounds to enter the bloodstream of the host, which causes an inflammatory response (31). Numerous studies demonstrate altered intestinal

integrity in heart failure patients, and higher blood levels of pro-inflammatory cytokines are associated with more severe symptoms and worse outcomes. These situations have also been reported in conditions like inflammatory bowel disease, food allergies, autoimmune disorders, and CVD (31, 32).

Some evidence suggests that lipopolysaccharides (LPS) and leaky gut may be related (33). The LPS are large molecules found in the outer membrane of gram-negative bacteria, also known as endotoxins. They are released when gram-negative bacteria die and lyse, releasing their content into the surrounding environment. Therefore, LPS can cause acute and chronic inflammatory reactions when they enter the bloodstream, as the immune system recognizes them as foreign invaders and mounts a range of physiological responses with toll-like receptors (TLR)-4 being the key interlocutor and determine cytokine cascade and caspase activation (33, 34). Recently, studies have been shown to increase intestinal permeability in animals, with some describing that individuals with leaky gut have higher levels of LPS in their bloodstream and are more predisposed to developing CVD (35, 36). However, the relationship between LPS and leaky gut still needs to be fully understood, and more research is needed to confirm these findings. Another example is the pathogenic gram-negative bacteria *Salmonella* spp. which can breach the intestinal epithelium and alter tight junctions, causing diarrhea via water and electrolyte loss into the intestinal lumen (26). There, inflammation brought on by bacterial translocation to the gut mucosa because of gastroenteritis might worsen gut barrier failure and create a vicious cycle (37, 38).

One way the bacteria from gut microbiota may affect cardiovascular health is through its impact on inflammation (39). Inflammation is a normal immune response to injury or infection; however, chronic low-grade inflammation is a critical factor in the development of CVDs, and gut microbiota dysbiosis has been shown to lead to inflammation through the production of various signaling molecules and the activation of immune cells (40). This may be due to certain types of bacteria that can produce substances that can stimulate an inflammatory response, like pro-inflammatory cytokines that can stimulate acute-phase reactants and contribute to atherosclerosis (41). In addition, the gut microbiota may also influence CVD through its effects on metabolism by affecting lipids and glucose and leading to dyslipidemia and insulin resistance, known risk factors for CVD (39). There is also evidence that the gut microbiota may be involved in developing arterial stiffness, a key predictor of CVD. This may be due to the influence of the gut microbiota on the production of SCFAs, which have been shown to affect arterial stiffness in animal models (42).

Altogether, the evidence suggests that bacteria from the gut microbiota play a significant role in the development of CVDs. However, further research is needed to understand the mechanisms underlying this relationship and how this information can prevent or treat CVD.

2.2 Viral microbiota

The viral microbiota refers to the DNA and RNA viruses, including eukaryotic viruses, bacteriophages, retroviruses and archaeal viruses, living in and on the human body, which are highly heterogeneous across populations (43, 44). These viruses can significantly impact the overall microbiota (44). Phages are classified as either lytic or lysogenic; lytic phages reproduce by infecting and killing their host cells, while lysogenic phages integrate their genetic material into the host cell's genome and replicate. Some phages are thought to have a symbiotic relationship with their host cells, while others may cause harm (45). The virome is a relatively new area of research, and much is still unknown about the types and roles of phages in the human body. It is thought to be highly diverse, with thousands of different types of phages present in the body. Recent studies have characterized the virome at several body sites, including the skin, mouth, gut, and respiratory tract. Some phages are thought to play a role in maintaining the microbiota's balance and protecting against infection by harmful bacteria (46).

There is evidence suggesting that the viral microbiota may be related to CVD. Some studies have found that individuals with CVD have a different virome profile than those without and that specific phages may be associated with an increased risk of CVD. De Jonge PA et al. study has provided us with the knowledge that viromes from individuals with metabolic syndrome, a well-known risk factor for CVD, have less richness and relative abundance than those belonging to healthy controls (47). This study identified increased viral clusters associated with Bacteroidaceae in the metabolic syndrome population. Moreover, Bacteroides prophages may influence bacterial metabolism, hence modifying microbiota composition in the gut. Additionally, the authors discovered a potential new viral biomarker of metabolic syndrome, VC_818_0, a phage from Roseburia/Blautia bacteria belonging to the Candidatus Heliusviridae phage family. Since the abovementioned bacteria are usually found in healthy microbiota compositions, VC_818_0 phage, which contains genes with metabolic expression, may change the metabolic behavior of these bacteria (already described in marine environments) (48, 49), promoting a deleterious modification of their virulence, hence, enhancing metabolic syndrome (47).

Furthermore, evidence sheds light on the effect of the Microviridae family on coronary heart disease (CHD). First, it was observed that CHD patients had an increased quantity of Virgaviridae and lower amounts of enteric viruses than healthy controls, perhaps due to the type of diet or even the medical therapy (50). Afterward, it was noted that the virome from normal gut individuals was dominated by phages from Siphoviridae, Podoviridae, and Myoviridae with lower quantities of Microviridae. In contrast, CHD viromes are mainly dominated by Microviridae and Virgaviridae, with fewer Siphoviridae, Podoviridae, and Myoviridae (51, 52). So, this study found no causal correlation between CHD patients and their viromes (53).

2.3 Fungi microbiota

Fungi are a diverse group of microorganisms found in various body sites. Like bacteria, fungi are an essential part of the microbiota and play multiple roles in human health. Several fungi

types are considered standard parts of the human microbiota, including yeasts, such as Candida and molds (54). These fungi are typically harmless when present in small amounts, but when they grow out of control, they can have harmful effects on human health. For example, some fungi in the gut produce enzymes that help to break down food, while others may have a role in regulating the immune system (55). Some evidence suggests that the mycobiome may be related to CVD, a condition affecting the heart and blood vessels. One example is Candida, which is more prevalent in individuals with CVD than those without (56, 57). Candida has been shown to produce toxins that can damage blood vessels and promote inflammation, which may contribute to the development of CVD (57). Other fungi, such as Aspergillus, have also been linked to an increased risk of CVD (58).

The CVD does not happen randomly. Indeed, some risk factors are already identified, such as atherosclerosis, and hypertension, among others (19, 59). With that being said, a new study explored the role of mycobiome in the physiopathology of the abovementioned risk factors. Atherosclerosis is a significant risk factor involved in CVD, extensively analyzed, and is related to the acute and chronic expression of CVD. It was demonstrated that some fungus species might be correlated with atherosclerosis. Mucor spp., from the family Mucoraceae and phylum Zygomycota, is associated with decreased carotid intima-media thickness (cIMT). Moreover, individuals with obesity, when the mentioned fungus is detected, had the same risk as non-obese individuals. With further exploration of Mucor spp., it was possible to demonstrate that Mucor racemosus can be used as a cardiovascular risk biomarker since it was related to a decreased risk on Framingham Risk Score and cIMT (60).

A more relevant risk factor for CVD is hypertension. Mycobiome has a relevant influence on hypertension development. It was observed that individuals in a state of pre-hypertension share the same bacterial and fungal microbiota modifications as individuals with diagnosed hypertension. Interestingly, bacterial richness and diversity reduce when an unhealthy state is reached, while fungal diversity is increased precisely when a pathology, like hypertension, is present. Moreover, some fungi can be used as potential biomarkers for hypertension, such as the increased quantity of Malassezia spp., which is known to promote pro-inflammatory states, as well as diminished concentrations of Mortierella, which can be found in healthy individuals, with an apparent probiotic effect in the bacterial species (61). However, the relationship between the fungi microbiota and CVD still needs to be fully understood; more research is required in order to confirm these findings and determine the exact role of fungi in the development and progression of CVD. In the meantime, maintaining a healthy lifestyle, including following a healthy diet and regular exercise, is crucial to reducing the risk of CVD and other health problems.

3 Gut microbiota metabolites

Gut microbiota can modulate human metabolism by producing small molecules, such as the transformation of dietary components into hormone-like signals or physiologically active metabolites, that play vital roles in inflammatory signaling and interact directly and indirectly with host immune cells. These metabolites can have a variety of effects on the body, both positive and negative (62). Some metabolites, such as SCFAs, have been shown to have several beneficial effects on the body, while others, like TMAO, have been linked to an increased risk of certain diseases (63, 64). The role of gut microbiota metabolites in health and disease is an active area of research, but it still needs to be fully understood how these metabolites influence the body. However, understanding the role of gut microbiota metabolites may help researchers develop strategies to prevent or treat several conditions.

3.1 Short chain fatty acids

The SCFAs are carboxylic acids with less than six carbons, produced by the fermentation of dietary fibers and non-digestible carbohydrates, that evade digestion by host enzymes in the upper gut and are metabolized by bacteria in the cecum and colon, with decline concentrations from proximal to the distal colon as the substrates used for fermentation are exhausted gradually (63). These compounds are essential for maintaining gut health and have been shown to have several beneficial effects on the body, including reducing inflammation, improving insulin sensitivity, regulation of gene expression, and regulating the immune system (62, 65). Diet composition directly influences the production of SCFAs; specifically, Bacteroidetes and Firmicutes can ferment indigestible fibers in the gut to produce acetate, propionate, and butyrate, respectively, that can be absorbed and used as an energy source (63, 66).

Acetate is the most abundant SCFA and is thought to have diverse beneficial effects on the body, including reducing inflammation, preventing the overgrowth of harmful bacteria, regulating pH, and improving gut barrier function. Propionate is also thought to have anti-inflammatory effects, limiting the growth of dangerous bacteria. It has been shown to improve insulin sensitivity in animal studies, which may be beneficial for people with diabetes or at risk of developing diabetes. Moreover, its potential role in appetite control has been studied, suggesting that propionate may help reduce food intake and promote weight loss, affecting the release of hormones involved in appetite regulation, like ghrelin (67). Butyrate is considered the primary energy source for colonic epithelial cells, and its deficiency has been associated with the development of colitis and cancer (68). Furthermore, it plays a role in maintaining gut barrier integrity by strengthening the tight junctions between epithelial cells that control intracellular molecular pathways between the lumen and the hepatic portal system, reducing gut permeability, preventing toxins from entering the bloodstream, and causing systemic inflammation (19, 69). Thus, butyrate has been shown to have anti-inflammatory and anti-cancer effects, persuading apoptosis of colon cancer cells and regulating gene expression by histone deacetylase inhibition (5).

Some studies have found that acetate and propionate are associated with weight loss and improved insulin sensitivity, possibly by reducing the absorption of carbohydrates in the gut, so it has been studied as a potential treatment for conditions such as

obesity, type 2 diabetes, and certain types of cancer (68). On the other hand, studies have also found that butyrate may have neuroprotective effects and may benefit people with multiple sclerosis and Alzheimer's disease (70, 71).

Recent research has suggested that SCFAs have a beneficial effect on cardiovascular health. Several studies have found that consuming a diet high in dietary fibers, which promote the production of SCFAs in the gut, is associated with a lower risk of CVD. One of the ways in which SCFAs may protect against CVD is by reducing inflammation, a known risk factor for CVD, and suppressing the inflammatory response. The SCFAs may also help improve lipid metabolism, essential for cardiovascular health (19). Some studies have found that consuming SCFAs can improve lipid profiles, such as lower low-density lipoprotein (LDL) and higher high-density lipoprotein (HDL) levels (72). Chen et al. treated Caco-2 cells with SCFAs to see whether they affected the genes' expression in cholesterol absorption. Butyrate was shown to inhibit NPC1L1 and to increase ABCG5/G8 gene expression in a dose-dependent manner while increasing the transcriptional activity of liver X receptors in these cells, suggesting that butyrate protects against the development of atherosclerosis (73). Moreover, SCFAs have been found to play a role in regulating glucose metabolism, which is vital for preventing type 2 diabetes, a risk factor for CVD, with studies finding that consuming SCFAs can lead to improvements in insulin sensitivity, lowering blood sugar levels and reduce the risk of developing diabetes (74, 75).

Inhibiting the growth of dangerous pathogens such as *Salmonella* spp. and *Escherichia coli* while promoting the growth of good bacteria like *Lactobacillus* and *Bifidobacteria* are also effects of high concentrations of SCFAs in the gut lumen (72). Additionally, they may help improve the endothelial cells' function that lines the blood vessels, helping to reduce the risk of atherosclerosis and other cardiovascular problems.

Once SCFAs are absorbed into the bloodstream through the walls of the large intestine by a process known as passive diffusion, they are transported to the liver via the portal vein and then distributed to several tissues, where they can interact with specific receptors such as G protein-coupled receptors (GPRs) and influence gene expression, cellular metabolism and immune response (66, 76). Acetate and butyrate will mainly participate in lipid biosynthesis, and propionate will mainly participate in gluconeogenesis (66).

Overall, SCFAs and gut microbiota are closely interlinked. Maintaining a healthy balance of gut microbiota and sufficient intake of dietary fibers and non-digestible carbohydrates can support the production of SCFAs and help promote overall gut health, positively affecting cardiovascular health. However, more research is needed to fully understand the effects of SCFAs on CVD, and more human studies are needed to confirm the findings.

3.2 Trimethylamine N-oxide

The gut microbiota plays a crucial role in TMAO formation, as different types of bacteria have different abilities to break down and produce trimethylamine (TMA) and TMAO. Some studies have shown that certain types of bacteria, such as those from the Prevotella and Bacteroides genera, are more efficient at producing TMA and TMAO than others (3, 16). The TMAO is a metabolite produced by certain gut bacteria when they break down foods containing choline, lecithin, and carnitine, commonly found in red meat, eggs, fish, and dairy products. It depends on the initial formation of the TMA compound by the microbiota present, especially in the first portion of the colon, which is absorbed and transported to the liver by the portal circulation, where it is metabolized by hepatic flavin-containing monooxygenase 3 (FMO3) to form TMAO (21). Then, the liver can release TMAO, which will be taken up by extra-hepatic tissues or eliminated by perspiration or urine (Figure 1). However, this compound can also be absorbed by macrophages during the formation of atherosclerotic plaque, with TMAO molecules binding to specific receptors on the surface of the macrophages, which triggers a series of signaling events inside the cell that activate specific pathways that induce the expression of genes involved in cholesterol metabolism, inflammation, and oxidative stress (77, 78). This can lead to the accumulation of cholesterol in macrophages and the formation of foam cells, a type of fat-filled cells that can accumulate in the arteries walls and contribute to the development of atherosclerosis (78). In addition, TMAO can regulate the differentiation of monocytes into macrophages and foam cells, influencing pro-fibrotic processes in the heart and kidney through growth factors (79); it can also facilitate the release of calcium ions due to the stimulation of platelet activity, which will activate the prothrombotic pathways (78), and also impairs reverse cholesterol transport, in which the cholesterol is removed from peripheral tissues and transported back to the liver for excretion (80). So, TMAO can play an essential role in regulating inflammation and result in protective or causative effects, stimulating or attenuating the production of inflammatory cytokines that can attract more immune cells, forming a vicious circle that leads to foam cells formation and atherosclerosis development (81).

Some researchers have suggested that TMAO may have a few different functions. It may play a role in regulating gut function, affecting gut motility and modulating the gut barrier function and immune response. Also, it has a role in regulating energy metabolism by modulating the activity of enzymes involved in fatty acid oxidation (82). Nevertheless, TMAO play a role in regulating cardiovascular function, by modulating the function of blood vessels and platelets, which may contribute to the development of CVDs (21). Therefore, studies have shown that high levels of TMAO in the blood may be associated with an increased risk of heart attack and stroke, and high levels of TMAO can cause platelet hyper-responsiveness to various agonists in both humans and animals, which increases vascular inflammation and has a prothrombotic direct effect (21, 83). These effects are likely related to the pathophysiology of type 2 diabetes, obesity, and CVDs (84). Nevertheless, TMAO has also positively affected diabetic peripheral neuropathy, glucose tolerance, and arterial hypertension (85).

The mechanisms by which TMAO promotes atherosclerosis are not fully understood; however, several potential mechanisms have been proposed. One of the most outstanding is related to the activation of NLRP3 inflammasome, a crucial mediator of

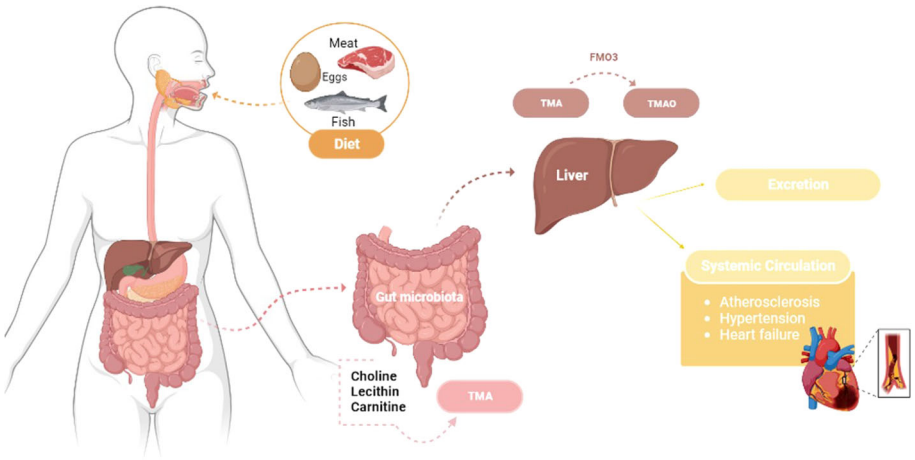

FIGURE 1
Trimethylamine N-oxide pathway: from food intake to CVDs development – transformation of dietary choline, lecithin, and carnitine into TMAO through gut microbiota metabolism and hepatic oxidation by the hepatic flavin-containing monooxygenase 3 (FMO3), which can be absorbed by extrahepatic tissues or excreted in urine. Atherosclerosis, hypertension and heart failure are all impacts of TMAO that can lead to CVDs. TMA - Trimethylamine; TMAO - Trimethylamine N-oxide.

inflammation, and a significant contributor to the development of atherosclerosis, along with the modulation of the gut microbiota, leading to the production of other harmful metabolites (16, 86). Heianza et al. aimed to evaluate the relationship between gut microbiota metabolites and the risk of major adverse CVD events and death, and after analyzing 19 prospective studies, the authors found that higher levels of TMAO and its precursors were associated with a higher risk of major adverse cardiovascular events and all-cause mortality (87). This may be a potential biomarker for predicting CVD risk, and further research is needed to understand the mechanisms underlying the association between gut microbiota metabolites and CVDs.

Overall, TMAO is recognized as one of the most promising metabolites that may be an independent risk factor for CVDs. A potential therapeutic target for CVDs measuring TMAO levels in blood or urine may help identify individuals at high risk for CVD (59, 88). Research on TMAO and how it contributes to the onset of atherosclerosis is still emerging, but TMAO may be a significant factor in this condition. More research is required to establish the most effective approaches to prevent or treat CVDs and better understand the mechanisms underlying this connection. Therefore, this complex process involves multiple steps and signaling pathways, and understanding this is essential to develop new strategies to prevent or treat atherosclerosis and other related conditions.

3.3 Bile acids

Traditionally, bile acids (BAs) were known only for their relevance in lipid metabolism. They are essential molecules produced in the liver and secreted into the small intestine, influencing dietary fats' breakdown and absorption (89). However, recently, BA has been associated, directly or indirectly, with immune signaling, metabolism, differentiation, and microbiota modulation (90, 91).

The substrate for BAs is cholesterol, and then, through the enterohepatic circulation, these BAs are deposited in the gallbladder. The BAs can be divided into primary or secondary. The most common primary BAs, produced in the liver are cholic acid (CA) and chenodeoxycholic acid (CDCA). After the conjugation with bile salts, glycolic acid (GCA), taurocholic acid (TCA), glycochenodeoxcholic acid (GDCA), taurochenodeoxycholic acid (TDCA), and ursodeoxycholic acid (UDCA) are obtained. The secondary BAs result from the synthesis of the bacterial portion of microbiota, through 7α dihydroxylation, in the small intestine and is deoxycholic acid (DCA) and lithocholic acid (LCA), the latter being the most hydrophobic (24, 91). One of the recently discovered functions of BAs is the fact that they can be used as hormones, mainly for farnesoid X receptor (FXR) and G Protein-coupled membrane receptor 5 (TGR5), to decrease fatty acid oxidation, triglyceride accumulation, and NF-kB inactivation in the aorta (23, 92).

These compounds can affect the diversity of the gut microbiota by altering the growth and survival of certain bacterial species, as they can act as signaling molecules that regulate the expression of genes involved in bacterial metabolism, virulence, and antibiotic resistance, which could result in alterations to the gut microbiota, having both favorable and unfavorable consequences on human health. Recently An et al. demonstrated that depending on the type of microbial strain and particular BA, they can have marked antibacterial effects against the gut microbiota, both *in vitro* and *in vivo*, and according to the findings of this investigation, colonic microorganisms are more vulnerable to BAs than cecal microbes (93). Also, Quinn et al. established the ability of the gut microbiota to conjugate BAs with different molecules like amino acids, producing phenylalanocholic acid, tyrosochilic acid, and lithocholic acid, which are found in humans and enriched in patients with inflammatory bowel disease or cystic fibrosis (94). In addition to their impact on bacterial diversity, BAs can serve as growth substrates for specific bacterial species, including those that produce SCFAs. They can also stimulate the gut epithelium's

production of antimicrobial peptides, which helps protect against pathogenic bacteria (95). Also, BAs can alter the function of the gut barrier by controlling the production of tight junction proteins, which are crucial for preserving intestinal integrity and limiting the translocation of bacteria and toxins across the gut epithelium (96).

Nevertheless, the intestinal microbiota can impact the metabolism of BAs. Some types of gut bacteria can convert primary BAs into secondary BAs, which are different from primary BAs and have other purposes. For instance, studies have demonstrated secondary BAs' anti-inflammatory and anti-cancer properties (97, 98).

While the specific mechanisms underlying these effects are still being elucidated, it is clear that BAs play a crucial role in maintaining the health of the gut ecosystem, and their interaction is dynamic and complex. In summary, elevated secondary BAs and increased ratios of secondary BAs: primary BAs are more associated with CVD (23, 99). One of the best research pathways to understand the impact of microbiota in cardiovascular disease is focusing on BAs metabolism, particularly secondary BA, its effects, and its relevance in CVD physiopathology (24). Additional research may lead to new therapeutic approaches for the treatment of gut-related disorders like inflammatory bowel disease, obesity, type 2 diabetes, and CVDs, and is essential for developing a comprehensive understanding of human health and disease.

3.4 Polyamines – cadaverine, putrescine and spermidine

Cadaverine and putrescine are polyamines synthesized by bacteria. Bacteria often produce them during the decomposition of animal or plant tissue, contributing to unpleasant odors associated with decay and putrefaction. Cadaverine originated from L-lysine through lysine decarboxylase LdcC or acid-inducible CadA (100, 101). Moreover, putrescine arises from synthesizing the substrate L-carnitine, by SpeC or SpeF or the substrate L-arginine, through SpeA and SpeB (102, 103). Spermidine originated from the substrate S-adenosine-L-methionine decarboxylated and putrescine through SpeE (104, 105). Cadaverine and putrescine are later degraded, by the lysine degradation pathway, to succinate (106, 107). Spermidine is only degraded to N-acetylspermidine through SpeG (108, 109). All these polyamines have modulatory effects on the microbiota to promote cardiovascular protection (22, 110).

However, the causal relationship between polyamines and cardiovascular benefits is still in the beginning. Liu S. et al. exploited the effect of one of the polyamines, spermidine, in a mouse model of abdominal aortic aneurysms (AAAs). First, AAAs are associated with a remarkable microbiota dysbiosis, with diminished alpha and beta diversity, accompanied by a shift in bacterial composition, namely increased *Bacteroides* spp., which are pro-inflammatory species, and lower concentrations of *Oscillospira* spp. and *Ruminococcus* spp., species with anti-inflammatory properties. Moreover, the described microbiota dysbiosis upheld functional modifications, especially in polyamines. Furthermore, when spermidine was administered, the intestinal microbiota was modulated with increased concentrations of *Prevotella* and *Desulfovibrionaceae* and decreased wholes of *Parabacteroides* (111). In this study, it was observed that the protective effect of spermidine seems to be associated with a modulation of gut microbiota composition into a more anti-inflammatory one, as well as in the increment of *Desulfovibrionacea* species that can improve polyamine metabolism and promote a more resilient intestinal barrier (111, 112).

Moreover, spermidine is essential for a better heart failure prognosis. This polyamine can act by two different pathways: 1) Direct pathway, where spermidine can avoid cardiac hypertrophy, diminish systolic blood pressure, improve echocardiographic parameters, decrease fibrosis, and, therefore, postpone the progression of heart failure; 2) Indirect pathway, where spermidine can modify intestinal microbiota, decreasing F/B ratio and raising the levels of *Muribaculaceae* spp., therefore ameliorating the intestinal microenvironment *Muribaculaceae* spp. are Gram-negative bacteria found in mice intestines (113), especially after acarbose treatments, since these bacteria produce propionate, a SCFA with anti-inflammatory properties, which is associated with increased longevity in mice (114).

Both cadaverine and putrescine are toxic to humans and animals in large quantities, and they can cause a range of adverse health effects, including nausea, vomiting, and respiratory problems (115). However, polyamines' relationship with cardiovascular benefits are important since they might have implications for the promotion of improved cardiovascular health.

4 Interactions between the gut microbiota and cardiovascular diseases

4.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory condition in which the arteries become narrowed and hardened, with an accumulation of lipids and cells, such as white blood cells, endothelial cells, and foam cells in the membranes, resulting in the formation of plaques in the arteries (116, 117). In this condition, innate and acquired immunity are involved, and inflammation of vessel walls is an essential feature of atherosclerosis, contributing to plaque instability and thrombotic occlusion of arteries (118, 119). This process can lead to serious health problems, like heart attacks, strokes, and acute CHD (117).

Recent research has highlighted the potential role of gut microbiota in the development of atherosclerosis by promoting inflammation and altering lipid metabolism (41). In fact, by describing a case of bacterial translocation from the gut to the heart and the discovery of gut bacterial DNA in atherosclerotic plaques, recent studies have established the gut as a potential reservoir of pathogenic microorganisms and with TMAO shown to be involved in the development of the disease (39). One way gut microbiota can promote inflammation is by producing pro-inflammatory compounds such as LPS that can activate immune cells and promote the recruitment of inflammatory cells to the

arterial wall (120). Additionally, gut microbiota can also modulate the production of other pro-inflammatory molecules, such as TNF-α, IL-1β, and IL-6, which can contribute to the development of atherosclerosis (120, 121). Another way is by altering lipid metabolism, converting dietary components such as choline, lecithin, and carnitine into TMAO which can increase the uptake of lipids by cells in the blood vessel walls and promote the formation of plaques (122). Moreover, gut microbiota can also affect the host's insulin resistance and glucose metabolism and the levels of certain hormones such as leptin and ghrelin, which can lead to increased inflammation or regulate appetite, leading to the development of atherosclerosis (123).

This disease develops gradually over time; one of the critical pathways involved in its development is the independent-metabolism pathway, characterized by the accumulation of lipids, particularly cholesterol, in the endothelial cells lining the blood vessels (39, 124). The process begins with injury to the endothelial cells, which can be caused by several factors, such as hypertension, smoking, and diabetes (119). Once the endothelial cells are damaged, they become more permeable, allowing lipids to accumulate in the blood vessels tunica intima, the innermost layer of the arteries. This accumulation triggers an inflammatory response which results in the recruitment of monocytes to the injury site, converting them into foam cells, which are characterized by their high content of lipids, resulting in foam cells and other inflammatory cells, along with extracellular matrix components and smooth muscle cells, to form a plaque on the inner wall of the vessels (124). As the plaque grows, it can block blood flow through the vessel, and if a blood clot forms or a rupture occurs, it can cause serious complications such as heart attack or stroke (125, 126). So, the independent-metabolism pathway is a pivotal contributor to the development of atherosclerosis and its associated complications (121).

The metabolism-dependent pathway is another mechanism that contributes to the development of atherosclerosis. By changing the production of different metabolites, dysbiosis can also have pro-atherosclerotic effects. The TMAO is one of the primary metabolites that play a significant role in atherosclerosis progression, as mentioned above (41, 121, 122). This pathway is also characterized by the accumulation of lipids, particularly triglycerides, in the liver and adipose tissue, and the process begins with the overconsumption of calories and/or a diet high in saturated and trans fats, which leads to an increase in the production of very low-density lipoprotein (VLDL) particles in the liver. These particles are rich in triglycerides and are transported to adipose tissue, where they are taken up by adipocytes and converted into triglyceride-rich lipoproteins (TRLs). Their accumulation in adipose tissue leads to insulin resistance and inflammation, both of which contribute to the development of atherosclerosis as insulin resistance progresses, the adipose tissue secretes higher levels of adipokines, signaling molecules that promote inflammation and increase the risk of atherosclerosis. Additionally, the accumulation of TRLs in the liver produces more extensive and denser LDL particles, which are more prone to sticking to the blood vessel walls and contribute to developing plaques (124). It is important to note that, like the independent-metabolism pathway, the dependent-metabolism pathway is not the only mechanism that contributes to the development of atherosclerosis, and it may act together with multiple pathways to contribute to the disease (122).

It is important to note that the relationship between gut microbiota and atherosclerosis is complex and still not fully understood, so more research is needed to determine the specific mechanisms by which gut bacteria contribute to the development of this disease and how to exploit this information to develop new therapeutic strategies. Therefore, controlling the risk factors, such as maintaining a healthy diet, regular physical activity, and avoiding smoking, can help lower the risk of developing atherosclerosis.

4.2 Hypertension

One of the most critical public health issues is hypertension, which increases the risk of pathological strokes, CHD, kidney failure, and early mortality, estimated to affect around one-third of adults worldwide (127). Genome-wide association analyses reveal that only 5% of hypertension occurrence can be explained by genetics, being assumed to be fueled by a combination of genetics and lifestyle variables (128). Environmental elements like dietary salt intake, alcohol use, and inactivity are also linked to increased blood pressure (59, 127).

The exact mechanism by which gut microbiota influence hypertension is not fully understood, but their link has recently been the subject of numerous animal and human studies (129, 130). Hypertension occurrence is often accompanied by gut microbiota imbalance, including decreased diversity, altered enterotype distribution, and variation in bacterial populations, and it is thought that certain types of gut bacteria may produce substances that can affect blood pressure; for example, some bacteria may produce SCFAs that have anti-inflammatory effects, while others may produce substances that increase inflammation and contribute to the development of hypertension (130). Additionally, gut microbiota may influence hypertension by affecting how the body processes and metabolizes nutrients, such as sodium and potassium, given that these nutrients play a crucial role in regulating blood pressure, and an imbalance can lead to high blood pressure (131).

Dysbiosis can accelerate the development of hypertension, described as a slight reduction in the artery lumen that raises peripheral vascular resistance and leads to high blood pressure and atherosclerosis (132). Although the direct connection between hypertension and TMAO has not yet been fully established, it is known that it prolongs the hypertensive effect of angiotensin II and determines an increase of vascular inflammation and a direct prothrombotic effect by the promotion of platelet hyper-responsiveness to multiple agonists both in humans and rodents (133, 134). Blood pressure regulation is generally linked to the renin-angiotensin system, which involves the angiotensin-converting enzyme (ECA) (83). Studies have also found that individuals with higher levels of TMAO in their blood tend to have higher blood pressure compared to those with lower levels, and reducing TMAO levels through dietary interventions, such as decreasing the intake of animal-based protein and fat, has been shown to lower blood pressure in some individuals (84).

To sustain host immunity and gut microbiota homeostasis, SCFAs are essential. Kang et al. demonstrated that SCFAs produced by gut microbiota are involved in modulating blood pressure and can potentially affect the secretion of renin and blood pressure by stimulating host G-protein-coupled receptor (GPR) pathways (135). Yang et al. demonstrated in two rat models that hypertension was associated with gut microbiota dysbiosis, characterized by an increased F/B ratio, a sharp decline in acetate and butyrate-producing bacteria, and an accumulation of lactate-producing bacteria (13). Li et al. demonstrated that hypertension is associated with an increase in the populations of *Klebsiella, Prevotella, Coprobacillus, and Enterobacter* and a decrease in the populations of *Anaerotruncus, Coprococcus, Ruminococcus, Clostridium, Roseburia, Blautia and Bifidobacterium*, correlated with a reduction of F/B ratio and in the production of SCFAs (136). Also, in a review by Verhaar et al. these results were discussed (137). In animal studies, acetate and propionate were also associated with lowering blood pressure and had cardiovascular preventive effects (130).

Animal models of hypertension, such as Dahl-sensitive rats, spontaneously hypertensive rats, angiotensin-II-induced hypertensive rats, and deoxycorticosterone acetate-salted mice, exhibit different gut microbiota compositions from wild-type animals, like a lower abundance of SCFAs-producing bacteria and Bacteroidetes, and higher abundance of lactate-producing bacteria, Proteobacteria and Cyanobacteria (129, 138, 139). Overall, animal models of hypertension help study the disease's underlying mechanisms and test potential treatments; however, it should be noted that the results obtained from animal models may not always translate to humans.

Therefore, the relationship between gut microbiota and hypertension is complex and not fully understood, but gut microbiota may contribute to developing and managing high blood pressure. Further research is needed to fully understand this relationship and determine the best ways to manipulate the gut microbiota, reduce TMAO levels, and improve cardiovascular health.

5 Therapeutical interventions

Therapeutic interventions on gut microbiota use several strategies to manipulate the composition and function of the gut microbiota to improve health. They can include a variety of strategies, such as probiotics, prebiotics, antibiotics, diet, physical activity, and fecal microbiota transplantation (140). These have been used to treat a variety of conditions, and studies have also suggested that gut microbiota modulation could have the potential to not only improve gut health but also reduce the risk of developing CVDs and improve overall health and well-being (6, 141). To restore gut barrier integrity, treatments like probiotics or drugs are probably doomed to failure if used alone. Instead, lifestyle adjustments that consider factors like exercise, sunlight exposure and vitamin D levels, circadian rhythm modulation, and stress management are more likely to produce favorable outcomes (26).

5.1 Probiotics, prebiotics, and symbiotics

Some studies suggest that therapeutic interventions aiming at the gut microbiota are effective in treating and preventing CVDs, and they mainly involve probiotics, prebiotics, or symbiotics (142, 143). Probiotics and prebiotics have a critical role in nutrition, sickness, and health, which has boosted their importance in research and commercial circles worldwide. Their use has been studied concerning CVDs, including atherosclerosis, hypertension, diabetes, and metabolic syndrome, with promising results (144–146), as observed in Table 1.

Probiotics are 'live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host', so strictly selected strains can have this potential and only in adequate amounts, as higher doses doesn't offer the same benefit (154). Probiotics' positive impact on human health or their ability to prevent disease is mainly brought on by their ability to compete with pathogenic microorganisms, antagonize pathogens, modulate gut microbiota composition, alter pH, or regulate the host's immune response (146). *Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus*, and *Enterococcus* are among the lactic acid bacteria that make up most of these. Their effects on CVDs are strain-specific and depend on the dose, duration, and specific population studied (144, 145). Several studies have suggested that probiotics can have a beneficial effect on CVDs by reducing inflammatory mediators and blood glucose levels, ameliorating the epithelial barrier function, and competing against pathogens with nutrients and adhesion sites, with some probiotic strains being found to lower blood pressure, and regulating cholesterol levels (26, 145).

Prebiotics are 'non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host heath' (155, 156). Just like probiotics, one way in which prebiotics may be beneficial to CVDs is through their effects on gut microbiota, helping improve gut barrier function and reducing inflammation. Some prebiotics like fructooligosaccharides (FOS) and galactooligosaccharides (GOS) have been found to lower cholesterol levels by reducing the absorption of cholesterol from the gut and by increasing the production of BAs (157). Intestinal enzymes can break down neither oligosaccharides nor polysaccharides. Hence, the gut microbiota transports prebiotics to the colon, where they are fermented, and consequently, their adverse effects are caused mainly by their osmotic properties (155). Besides that, prebiotics are believed to have no severe or potentially fatal adverse effects (144, 158).

Symbiotics are a combination of probiotics and prebiotics; the idea behind it is that by combining the two, the probiotic will have

better survival and colonization in the gut, leading to a more significant beneficial effect on the host (158). These have been studied for various health benefits, including improving gut health, boosting the immune system, and reducing the risk of certain diseases such as allergies, obesity, and diabetes. Also, they have been studied for their potential in treating certain gastrointestinal disorders such as inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS) (159, 160). These supplements could help restore the normal gut microbiota, encourage the growth of good bacteria, and stop the spread of pathogens. By focusing on the gut microbiota and preserving immune homeostasis in the body, probiotics, prebiotics, and symbiotics may be considered promising intervention strategies to prevent or improve CVDs. However, it is essential to note that while health benefits are observed, they should not be relied upon solely to treat or prevent CVD.

Antibiotics are a class of drugs used to treat bacterial infections; however, they can also have unintended consequences on the gut microbiota (161). When antibiotics are taken, they target the pathogenic bacteria causing the infection and the beneficial bacteria that comprise the gut microbiota. The high intake of antibiotics disrupts the delicate balance of the gut microbiota, causing an imbalance and favoring systemic diseases (162). One of the most common effects is diarrhea, caused by the overgrowth of pathogenic bacteria such as *Clostridium difficile* (163). Antibiotics can also increase the risk of other infections and contribute to the development of antibiotic resistance. Additionally, antibiotics can have long-term effects on the human microbiota. Studies have shown that they can alter the gut microbiota composition for up to a year after the treatment, leading to a decrease in the diversity of bacteria present and an overgrowth of potentially harmful bacteria (162). This disruption could lead to an increase of pro-

TABLE 1 Animal studies and clinical trials using probiotics and prebiotics in several cardiovascular diseases as a therapeutic approach.

Study	Year	Disease	Treatment	Type of study	Via	Outcome	Reference
Sun et al.	2016	Ischemic stroke	*C. butyricum*	No mention	Animal study	Protective effects against ischemic stroke; attenuate neurological deficit, ameliorate histopathological changes alleviate oxidative stress and inhabit apoptosis.	(147)
Tenorio-Jiménez et al.	2018	Metabolic Syndrome	*Lactobacillus reuteri* V3401	5 × 109 CFU/mL	Clinical trial	2-week administration of L. reuteri V3401 in capsules was associated with lower levels of inflammation biomarkers, such as TNF-, IL-6, IL-8, and sICAM-1, and a reduced risk of CVD in obese adults with metabolic syndrome.	(148)
Raygan et al.	2018	Type 2 diabetic patients with Coronary Heart Disease (CHD)	*L. acidophilus, L. reuteri, L. fermentum, Bifidobacterium bifidum* and Selenium	200 µg/day selenium + 8×109 CFU/day probiotic	Clinical trial	Probiotic and selenium co-supplementation reduce inflammatory factors and oxidative damage through producing short chain fatty acids in the gut and the decreasing production of free radicals, and due to blocking activation of nuclear factor-kB through modulating selenoprotein genes expression and inhibiting production of reactive oxygen species.	(149)
Hassan et al.	2020	Atherosclerosis	*Lactobacillus plantarum* ATCC 14917	0.2 mL (109 CFU)	Animal study	L. plantarum ATCC 14917 supplementation decreases the progression of atherosclerotic lesion formation by alleviating the inflammatory process and lowering oxidative stress.	(150)
Mähler et al.	2020	Hypertension	*L. paracasei, L. plantarum, L. acidophilus, and L. delbrueckii; Bifidobacteria longum, B. infantis, and B. breve; Streptococcus thermophilus*	9 × 1011 CFU	Clinical trial	Probiotic can convert dietary components into active metabolites that cause a reduction of pro-inflammatory immune cell function and promote a BP-lowering effect.	(151)
Li et al.	2021	Heart Stroke	*Bacillus licheniformis* CMCC 63516	1 × 108 CFU/mL	Animal study	Preventive effects on heat stroke in rats by sustaining intestinal barrier function, such as increasing tight junctions and decreasing intestinal injury and modulating gut microbiota by increasing the ratio of *Lactobacillus* and *Lactococcus.*	(152)
Wang et al.	2023	Hypertension	*Clostridium butyricum*-pMTL007-GLP-1	109 CFU/mL	Animal study	CB-GLP-1 had markedly reduced blood pressure and improved cardiac marker ACE2, AT2R, AT1R, ANP, BNP, β-MHC, α-SMA and activating AMPK/mTOR/p70S6K/4EBP1 signaling pathway.	(153)

ACE-2 -Angiotensin-converting enzyme type 2; ANR/BNR - Atrial/brain natriuretic receptor; AT1R/2R - angiotensin-II receptor type 1/2; BP - blood pressure; BCKADC - Branched-chain alpha-keto acid dehydrogenase complex; CFU - colony-forming unit; CVD - cardiovascular disease; F/B - Firmicutes-Bacteroides; FMT - Faecal Microbiota Transplant; GLP -1 - Glucagon-lyke peptide type 1; IL - interleukin; MHC - major histocompatibility complex; SCFA - Short chain fatty acids; SMA - spine muscular atrophy; TMAO - Trimethylamine N-oxide; TNF - tumour necrosis factor.

inflammatory cytokines production, oxidative stress, and impaired endothelial function, which could trigger systemic inflammation, insulin resistance, and endothelial dysfunction, all of which contribute to the pathogenesis of CVDs (164–166). In fact, some studies demonstrated an increased risk of CVDs, such as myocardial infarction and stroke, in patients who received specific classes of antibiotics, like macrolides or fluoroquinolones (167–169). Beyond all, this can lead to other health problems such as allergies, obesity, inflammatory bowel disease, and mental health disorders (170, 171). Therefore, by introducing probiotics and/or prebiotics during or after antibiotic treatments, the balance of the gut microbiota can be restored, through eliminating harmful bacteria and enhancing the gut barrier function, contributing to reduce risk of CVDs and others (172, 173).

Thus, probiotics, prebiotics, and symbiotics all play a role in maintaining digestive and overall health, including cardiovascular health, especially when antibiotics are in question; however, while these supplements can be helpful, they should not replace a balanced diet, exercise, and medical advice in the prevention and treatment of CVDs.

5.2 Nutrition and physical activity

Various factors can influence gut microbiota composition, including age, genetics, and lifestyle (141, 174). In addition to probiotics and prebiotics, dietary and lifestyle changes can also be effective in restoring balance to the gut microbiota and improving cardiovascular health, including increasing the intake of fruits, vegetables, and whole grains and reducing the intake of processed and sugar foods (175). Exercise, stress management, and getting enough sleep are essential to maintaining healthy gut microbiota and preventing CVD, even in high-fat diet situations (176, 177).

As mentioned above, gut microbiota composition can be influenced by various factors, including diet, age, genetics, and lifestyle. Diet plays a significant role in shaping the composition and function of the gut microbiota, and its modulation is one way to improve the gut microbiota and promote overall health (175, 178). The types and amounts of nutrients that are consumed can have a direct impact on the growth and survival of different microbial species. A diet high in processed foods, refined sugars, and saturated fats has been linked to an increase in harmful bacteria, like Proteobacteria and *Bacteroides fragilis*, and a decrease in beneficial bacteria in the gut, which can contribute to the development of CVD, such as hypertension, high cholesterol, and obesity by the production of pro-inflammatory compounds. On the other hand, a diet rich in plant-based fiber, fruits, vegetables, and whole grains can help promote the growth of beneficial bacteria in the gut, such as *Bifidobacteria* and *Lactobacilli*, and reduce the risk of these diseases. These bacteria can ferment dietary fibers and produce SCFAs linked to health benefits, like improving gut barrier integrity, increasing mucus production, antimicrobial proteins, and Treg cells, and affecting tight junction assembly (179, 180). According to multiple clinical trials, the Mediterranean diet, rich in fruits, vegetables, and whole grains, which are all good sources of fiber, has been associated with a reduced risk of CVD and other chronic diseases, as it promotes the growth of beneficial bacteria and blood pressure reduction, as well as promotes protective effects on coronary events, strokes, and heart failure (81, 84). Some studies have also shown that certain dietary fats, such as omega-3 fatty acids, can benefit the gut microbiota and improve CVD (181). Additionally, research has shown that different diets can lead to distinct gut microbiota, and some have suggested that switching to another diet can rapidly change its composition (182). For instance, some studies have shown that switching from a Western diet to a Mediterranean diet can rapidly alter the gut microbiota, with beneficial effects attributed to the high proportion of fibers, mono- and poly-unsaturated fatty acids, antioxidants, and polyphenols (183, 184).

Besides diet, physical activity has won much praise for its capacity to control metabolism, insulin sensitivity, weight, and other aspects of health. However, the importance of exercise in controlling the human gut microbiota is becoming increasingly supported by research. Regular physical activity is part of a healthy lifestyle and helps reduce the risk of developing CVD. Exercise can improve cardiovascular health by reducing blood pressure and cholesterol levels, improving blood flow and reducing the risk of blood clots, strengthening the heart muscle and improving its functions, and controlling weight which will reduce the risk of obesity (12, 185). A critical study by Matsumoto et al. discovered that five weeks of exercise training in rats led to an increase in the production of SCFA-butyrate, which is a metabolite from dietary fiber fermentation by bacteria like *Bifidobacteria*, and this shift was also associated with improved endothelial function and a reduction in the development of CVDs (186). In another study, Monda et al. described that even with a high-fat diet, exercise could reduce inflammatory infiltration and protect gut morphology and integrity (176). However, it is essential to note that while exercise can have many beneficial effects, it is not a substitute for a healthy diet.

5.3 Fecal microbiota transplantation

The FMT is a medical procedure involving transferring healthy gut bacteria from a donor to a recipient. The idea behind FMT is to restore a healthy balance of gut bacteria in individuals with an imbalance or lack of beneficial bacteria, a condition known as dysbiosis. In individuals with this condition, the balance of gut bacteria is disrupted, leading to a reduction in the diversity and abundance of beneficial bacteria, resulting in a variety of symptoms, such as diarrhea, abdominal pain, and weight loss, as well as an increased risk of developing chronic diseases like inflammatory IBD, Clostridium difficile infection, and metabolic disorders (187). So far, FMT has had a resoundingly positive clinical impact on recurrent *Clostridium difficile* infection. Recently, ulcerative colitis has been extensively studied in other microbiota-related disorders like CVDs (188, 189). This procedure is typically performed by administering a stool sample from a healthy donor, usually via a colonoscopy, sigmoidoscopy, enema, or orally, to the recipient, aiming to repopulate the recipient's gut with a diverse and balanced community of bacteria that can improve the overall health of the gut

microbiota, and in turn, improve the overall health of the individual (190, 191).

Despite the evidence surrounding CVDs and gut microbiota, few studies have explored the potential effect of FMT on these diseases. Hu et al. centered on the question of whether FMT could be helpful in myocarditis treatment, with a murine model of experimental autoimmune myocarditis, resulting in reduced inflammatory infiltration, improved functions of the blood vessels, and gut microbiota rebalance, proposing a potential therapeutical strategy (192). In another study, Toral et al. demonstrated that transplanting healthy feces into spontaneously hypertensive rats reduces blood pressure by modifying sympathetic nerve activity associated with increased levels of SCFAs (193). Kim et al. also studied FMT impact on CVDs, observing that when hypertensive donors' feces were transferred to germ-free mice, the recipient mice's blood pressure rose compared to germ-free mice that received healthy FMT (194). A recent study by Hatahet et al. demonstrated that gut microbiota modulation with FMT associated with butyrate treatment, could alleviate systolic and diastolic function in obese mice (195). On the other hand, Gregory et al. discussed the transmission of atherosclerosis susceptibility using FMT in an animal model, proving that not only positive effects can come from FMT procedure (196). In Table 2 we resume the findings of some animal studies and clinical trials from the last years.

Altogether, these findings point to a significant role of the gut microbiota in the development of CVDs; nevertheless, more human data and clinical trials are required to support the use of FMT in CVD before it can be applied broadly.

While some researchers considered FMT as a safe and effective treatment option for various conditions, with success rates that are often higher than traditional medical treatments, others are still suspicious of the procedure's benefits. Therefore, it's still considered an experimental treatment and not yet widely available or approved by regulatory agencies worldwide (187, 198).

6 Conclusions

In conclusion, the gut microbiota is a complex and dynamic community of microorganisms that plays a critical role in human health and disease. Emerging evidence suggests that the gut microbiota may be linked to the development of CVD, such as atherosclerosis, hypertension, diabetes, and others. Recent studies have highlighted the importance of the gut-heart axis in the pathogenesis of CVDs, with an increasing body of evidence linking gut dysbiosis its development. Despite the promising results from animal models and some human studies, further research is needed to better understand the mechanisms by which gut microbiota influence the cardiovascular system and to determine the safety and efficacy of these interventions in clinical settings. The potential prophylactic and therapeutic implications of this research are exciting and we look forward to continued advancement of scientific knowledge in this field.

TABLE 2 Fecal microbiota transplantation results in animal studies and clinical trials in various cardiovascular diseases.

Study	Year	Disease	Treatment	Type of study	Via	Outcome	Reference
Hu et al.	2019	Myocarditis	FMT	Animal study	Oral gavage	Reduced inflammatory infiltration, improved functions of the blood vessels, and gut microbiota rebalance with an increase in microbial richness and diversity. Increase F/B ratio.	(192)
Toral et al.	2019	Hypertension	FMT	Animal study	Oral gavage	Reduced blood pressure by modifying sympathetic nerve activity associated with increased levels of SCFAs.	(193)
Kim et al.	2017	Hypertension and Myocarditis	FMT	Animal study	Oral gavage	Hypertensive donors' feces were transferred to germ-free mice and the recipient mice's blood pressure rose compared to germ-free mice that received healthy FMT. Also, obese mice receiving FMTs from healthy resveratrol-fed mice have improved glucose homeostasis, and decreased inflammation and myocarditis	(194)
Gregory et al.	2015	Atherosclerosis	FMT	Animal study	Oral gavage	Atherosclerosis susceptibility was transmitted with FMT	(196)
Hatahet et al.	2023	Heart failure	FMT	Animal study	Oral gavage	Improvement systolic and diastolic early dysfunction following FMT. Both FMT and butyrate plays a significant role in reducing the level of inactive p-BCKDH in the heart.	(195)
Smits et al.	2018	Metabolic Syndrome	FMT	Randomized Controlled Trial	Nasoduodenal infusion	Single lean vegan-donor FMT in metabolic syndrome patients resulted in detectable changes in intestinal microbiota composition but failed to elicit changes in TMAO production capacity or parameters related to vascular inflammation.	(197)

Author contributions

Conceptualization: CA, JN, and PB, Writing – original draft preparation: CA and JN. Writing – review and editing: CA, JN, DC and PB. Supervision: DC and PB. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

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

The author(s) DC and PB 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

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Gut microbiota: key facilitator in metastasis of colorectal cancer

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Colorectal cancer (CRC) ranks third in terms of incidence among all kinds of cancer. The main cause of death is metastasis. Recent studies have shown that the gut microbiota could facilitate cancer metastasis by promoting cancer cells proliferation, invasion, dissemination, and survival. Multiple mechanisms have been implicated, such as RNA-mediated targeting effects, activation of tumor signaling cascades, secretion of microbiota-derived functional substances, regulation of mRNA methylation, facilitated immune evasion, increased intravasation of cancer cells, and remodeling of tumor microenvironment (TME). The understanding of CRC metastasis was further deepened by the mechanisms mentioned above. In this review, the mechanisms by which the gut microbiota participates in the process of CRC metastasis were reviewed as followed based on recent studies.

KEYWORDS

gut microbiota, colorectal cancer, metastasis, tumor progression, immune evasion

1 Introduction

Colorectal cancer (CRC) has the third highest incidence rate among all types of cancers globally (1). The main cause of death of CRC patients is metastasis, which is also a clinical challenge (2, 3). Metastasis is a multi-step and multi-factor process including the separation of tumor cells from each other, invasion into surrounding tissues, adhesion to endothelial cells, and migration from the primary site to secondary site. Several mechanisms have been implicated, such as epithelial-mesenchymal transition (EMT) (4, 5), changes in expression of intercellular adhesion molecules (6), loss of structural integrity of the basement membrane (7), remodeling of the pre-metastatic niche (8), and induction of angiogenesis (9). Nonetheless, it is worth noting that current understanding of CRC couldn't fully illuminate the role of systematic factors like exercise, diet and aging in CRC metastasis.

Gut microbiota located within the intestinal tract comprises a large and diverse community including bacteria, yeasts, fungi viruses and parasites, which are referred to as the second gene pool of the human body (10). As one of the earliest encountered foreign antigens in the human body, gut microbiota plays essential roles in various physiological and pathological processes. Previously, the main roles attributed to gut microbiota were the synthesis of essential amino acids and vitamins, the digestion of polysaccharides that are

difficult to assimilate, and contribution to human metabolic processes (11). Additionally, gut microbiota provides essential signals for the development and functioning of immune system (12). In recent years, numerous studies have suggested that the gut microbiota also participates in oncogenesis and progression of cancer, particularly in the process of metastasis (13–15). On the one hand, the gut microbiota secretes various metabolites or virulence factors that damage host DNA (16) and contributes to a pro-inflammatory environment (17), leading to pre-cancerous lesions. On the other hand, the gut microbiota directly interacts with cancer cells, thereby increasing invasion and proliferation of cancer cells (18). Furthermore, several studies have indicated that the gut microbiota may facilitate metastasis by affecting the recruitment of immune cells and remodeling the tumor microenvironment (TME) (19–23). The mechanisms by which the gut microbiota participates in the process of CRC metastasis were reviewed as followed based on recent studies.

2 Gut microbiota promotes the proliferation and invasion of CRC cells

2.1 Gut microbiota promotes the proliferation of CRC cells

The progression of CRC involves multiple signaling pathways (24, 25). The disruption of cell cycle and the acquisition of unlimited proliferative capacity are key steps in cancer progression. Researches have indicated that Fusobacterium, a specific type of bacteria, has a significantly higher relative abundance in CRC tissue compared to normal one (26–28). The quantity of Fusobacterium also exhibits statistical differences between different stages of cancer progression. Furthermore, during the transition from adenoma to malignant tumor, the abundance of Fusobacterium gradually increases (29). Recent studies have demonstrated that the gut microbiota may promote cancer cell proliferation through mechanisms as follows.

2.1.1 Modulating RNA-mediated targeting effects

RNA-mediated targeting effects are important mechanisms of epigenetic regulation (30), including the synthesis of various non-coding RNAs and their impact on downstream genes (31, 32). MicroRNAs (miRNAs) are one of the key players in this process, regulating various biological processes such as tumorigenesis. Recent studies have indicated that the gut microbiota is involved in RNA-mediated targeting effects that regulate cancer cell proliferation.

Fusobacterium not only facilitated the proliferation and invasiveness of co-cultured CRC cell lines but also promoted tumor formation in APCMin/+ mice. Fusobacterium activated the TLR4/MYD88 receptors on the surface of cancer cells, leading to the activation of NFκB. NFκB then binds to the upstream region of the transcription start site (TSS) of miR21, upregulating its expression. MiR21, in turn, bound to the 3' end binding site of RASA1, inhibiting its expression (33). RASA1 is a member of the RAS GTPase-activating protein (RAS-GAP) family, and its binding to the well-known oncogenic protein RAS can inhibit RAS activity (34). Some studies have suggested that mutations or loss of function in RASA1 in CRC leads to activation of the RAS-MAPK cascade (35–37). The MAPK pathway is reported to induce the synthesis of cycling D1, promoting cell division (38). The MAPK pathway has also been shown to participate in the proliferation of cancer cells in multiple studies (39, 40).

Peptostreptococcus micros (P. micros) is an opportunistic pathogen found in the oral cavity that is closely associated with periodontitis (41). It can also cause suppurative infections in various organs throughout the body (41). Chang et al. found that P. micros could significantly foster the proliferation of LoVo and HT-29 cell lines *in vitro* (42). To unveil the underlying mechanism, they constructed xenograft models. It came out that tumors derived from cancer cells co-cultured with P. micros had larger volume and weight (42). Further investigations revealed that P. micros suppressed the expression of protein tyrosine phosphatase receptor R (PTPRR) by upregulating miR-218-5p, ultimately activating the Ras/ERK/cFos signaling pathway (42). The Ras/ERK signaling pathway is part of MAPK pathway and also participates in the proliferation of CRC cells (43).

Significantly associated with inflammatory bowel disease (IBD) and CRC, Enterotoxigenic Bacteroides fragilis (ETBF) is a molecular subtype of Bacteroides fragilis (44, 45). ETBF could downregulate the expression of miR-149-3p in cancer cell lines and influences the selective splicing of the KAT2A gene through PHF5A. Ultimately, KAT2A directly binds to the promoter region of SOD2, activating the SOD2 gene (46). SOD2 has been shown to modulate energy metabolism and promote proliferation of CRC (47).

2.1.2 Activating the cascades of cancer signaling

The Wnt/β-catenin signaling pathway plays a crucial role in physiological processes such as cell proliferation and differentiation, stem cell renewal, embryonic development, and tissue homeostasis (48). Dysregulation of this pathway is widely considered a key oncogenic signal and is of significant importance in the development of different kinds of cancers (49). Certain bacteria, such as Fusobacterium nucleatum (F. nucleatum), could facilitate cancer cell proliferation through the Wnt/β-catenin pathway (18, 50). For example, F. nucleatum produces a virulence factor called FadA (51), which binds to the E-cadherin domain EC5 on the surface of CRC cells. This interaction leads to the dephosphorylation of β-catenin, accumulation of β-catenin in the cytoplasm, and translocation of β-catenin to the cell nucleus. Subsequently, the expression of transcription factors lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF), NFκB, and oncogenes such as Myc and Cyclin D1 is upregulated, promoting CRC cell proliferation (18). Additionally, FadA could promote the expression of chk2 through the E-cadherin/β-catenin pathway, leading to increased DNA damage and elevated proliferative capacity in CRC cells (50). Furthermore, some studies have reported that probiotics have the ability to inhibit cancer cell proliferation and promote apoptosis (52–55). qPCR and western blot results have shown that during this process, the gene expression and protein content of β-catenin in CRC decrease, suggesting that

probiotics may inhibit CRC cell proliferation by regulating β-catenin-related pathways (52). Nonetheless, the underlying mechanisms of these effects are still need to be explored.

The PI3K-Akt pathway is widely activated in various tumors and is closely associated with tumor development (56–59). Gram-positive anaerobic bacteria, such as Peptostreptococcus anaerobius (P. anaerobius), present in the oral cavity and intestines (60), could bind to integrin α2β1 on the surface of CRC cells through its surface protein called putative cell wall binding repeat 2 (PCWBR2). This interaction activates the PI3K-Akt signaling pathway through Focal Adhesion Kinase 9 (FAK9), ultimately promoting cancer cell proliferation (61).

The MAPK-ERK pathway, a cell proliferation signaling pathway located on the cell surface and extended to the nucleus, plays a crucial role in cell proliferation (62). Activation of the MAPK-ERK pathway is increasingly implicated in the occurrence and progression of CRC (63). The oral pathogenic bacterium Porphyromonas gingivalis (P. gingivalis), once colonizing the colon, can selectively invade CRC cells and activate the MAPK-ERK pathway, thereby promoting tumor proliferation (64).

Not only individual bacterial species but also the overall balance of the gut microbiota is crucial in regulating cancer proliferation. Bai et al. have found that smoking induced gut microbiota dysbiosis altered gut metabolites and impaired gut barrier function, ultimately activating the oncogenic MAPK-ERK signaling and enhancing cancer cell proliferation (65). Portulaca oleracea, a medicinal plant and a member of the Portulacaceae family, is well-known for its resistance against microbiota, inflammation, and cancer (66). Portulaca oleracea extract (POE) has been found to reduce tumor quantity and improve survival rate in carcinogen-induced mouse models through restoring the balance of gut microbiota. Further results have shown that POE upregulates the expression of TP53, inhibits the Wnt/β-catenin signaling pathway and reduces the expression of c-Myc and Cyclin D1, ultimately suppressing cancer cell proliferation (67).

2.2 The gut microbiota promotes the invasiveness of cancer cells

In addition to unlimited proliferative capacity, invasive growth into surrounding tissues is another characteristic of malignant tumors. Breaking through the basement membrane is the first step for distant metastasis (68). It has been shown that a positive correlation between the gut microbiota and tumor progression stages exists (29). Since one of the defining criteria for tumor progression stages is the depth of tumor infiltration (69), the gut microbiota has the potential to regulate the invasive properties of cancer cells.

2.2.1 Secreting microbiota-derived functional substances

The metabolic products derived from microorganisms, such as l-2-hydroxyglutarate, succinate, fumarate, d-2-hydroxyglutarate, and lactate, can accumulate in tumor lesions and exacerbate the malignancy of the tumor (70). Furthermore, some metabolites could hijack signaling pathways related to tumor metastasis through gene regulation (71). Formate, a major metabolic product of F. nucleatum, can activate the AhR signaling pathway in CRC, enhancing its cancer stem cell properties and increasing the invasiveness of CRC, ultimately promoting cancer metastasis (72).

EMT is a cellular biological process (73, 74) that endows cancer cells with invasive and anti-apoptotic capabilities (75, 76). EMT triggers the process of dissemination and invasion, ultimately leading the formation of metastases (77, 78). Certain strains of Escherichia coli can produce a virulence protein called cytotoxic necrotizing factor 1 (CNF1) (79). CNF1 induces the recruitment of mTOR to lysosomes, consequently increasing invasiveness of CRC cell lines and inducing the expression of EMT markers (80). These findings suggest that gut microbiota has the potential to induce EMT in cancer cells.

Hydrogen sulfide (H_2S) has been identified as the third gasotransmitter after nitric oxide (NO) and carbon monoxide (CO) and participates in a variety of biological processes (81). There are two sources of luminal H_2S: the inorganic and organic metabolism of intestinal bacteria (82) and endogenously synthesized in the mammal cells (83). Endogenous H_2S fosters metastasis, partly through induction of ATP citrate lyase (ACLY) to facilitate EMT (84, 85). Since the luminal H_2S mainly originate from bacterial metabolism and directly contact with intestinal epithelial cells (86), the intestinal flora has the potential to facilitate CRC metastasis through modulating endogenous H_2S synthesis and related pathways.

2.2.2 Regulating mRNA methylation

The presence of the microbiota has been shown to induce epigenetic changes in mouse tissues at transcriptional level (87, 88). N6-methyladenosine (m6A), one of the epigenetic modification mechanisms of mRNA, could influence various fundamental biological processes (89). METTL3, the main m6A methyltransferase, is involved in the progression of several types of cancers, including acute myeloid leukemia (90), hepatocellular carcinoma (91), and lung cancer (92). In CRC, F. nucleatum has been shown to inhibit the Hippo pathway and activate the YAP signaling, leading to the suppression of METTL3 expression through the transcription factor FOXD3. The inhibition of METTL3 resulted in decreased m6A methylation of KIF26B mRNA, a gene associated with cell-cell adhesion and important for cancer cell invasion. Consequently, the expression of KIF26B were promoted, leading to enhanced tumor cell invasiveness. Therefore, F. nucleatum could induce epigenetic modifications in the KIF26B gene at transcriptional level through the YAP/FOXD3/METTL3 axis, ultimately facilitating the invasiveness of cancer cells (93).

In conclusion, gut microbiota is capable of promoting proliferation and invasiveness of CRC cells via multiple mechanisms (see Figure 1 for details).

3 Gut microbiome promotes dissemination and survival of cancer cells

Most kinds of cancers rely on blood vessels, lymphatic vessels, and other channels for metastasis. Survival pressure like anoikis,

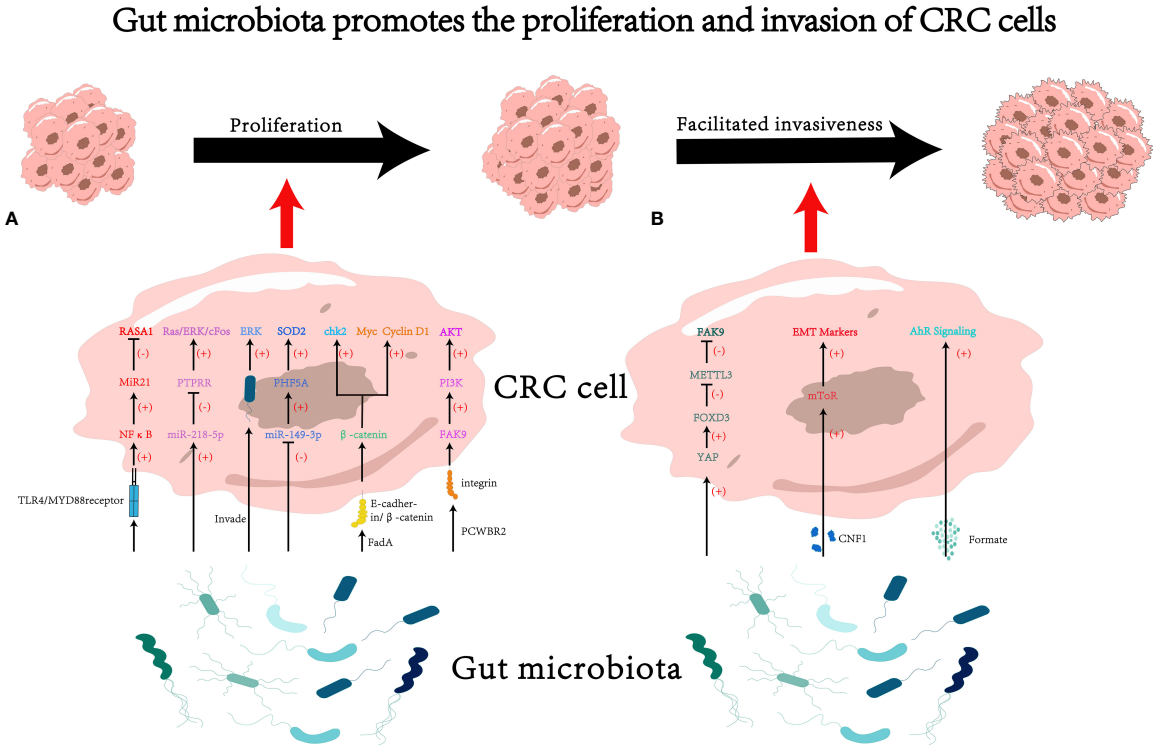

FIGURE 1
The impact of gut microbiota on proliferation and invasion of colorectal cancer (CRC) cells. **(A)** Gut microbiota may influence cancer cell proliferation through various pathways, such as modulating RNA-mediated targeting effects (33, 42, 46) and activating the cascades of cancer signaling (18, 50, 60, 64). **(B)** Gut microbiota may influence cancer cell invasiveness through various pathways, such as secreting microbiota-derived functional substances (70, 79), and regulating mRNA methylation (93). PCWBR2, putative cell wall binding repeat 2. CNF1, cytotoxic necrotizing factor 1.

shear forces, and immune attacks are exerted on cancer cells once they enter the circulatory system (68). Therefore, dissemination and survival are crucial prerequisites for cancer cells to complete metastasis. In recent years, studies have found that gut microbiota not only promotes cancer cell proliferation and increases their invasiveness but also facilitates cancer cell dissemination and survival (94–96). The mechanisms behind this include regulating intravasation of cancer cells to facilitate dissemination, participating in immune evasion to promote cancer cell survival, and modulating the tumor microenvironment to facilitate the formation of metastatic lesions.

3.1 Regulating intravasation to foster the dissemination of CRC cells

Structural and functional disruptions of vascular basement membrane (97), as well as tumor cell reprogramming (98), are two important processes involved in hematogenous metastasis of tumors. The former provides a physical basis for cancer cells to breach blood vessels and enter the bloodstream, while the latter enhances the intravasation and migration capabilities of tumor cells.

Under a high-fat diet, elevated levels of deoxycholic acid (DCA) in the host gut was detected, which enhanced vasculogenic mimicry in tumor tissues (99) —— the formation of structures that lack endothelial cells but possess normal vascular functions (100). This study suggests that the gut microbiota's regulation of host metabolism may contribute to vasculogenic mimicry and promote tumor metastasis. However, the direct association between bile salt-hydrolyzing bacteria and intestinal DCA levels requires further investigation. Therefore, the mechanisms by which the gut microbiota regulates tumor vasculogenic mimicry through DCA still need to be further validated.

The adhesion of circulating tumor cells to endothelial cells and extravasation into pre-metastatic sites is an important process in tumor metastasis (101). Intercellular adhesion molecule 1 (ICAM1), a member of the immunoglobulin superfamily, has been shown to promote tumor cell adhesion to endothelial cells and facilitate metastasis (102). Its expression levels also positively correlate with tumor progression and metastasis in clinical settings (103). F. nucleatum could activate the NF-κB pathway by acting on the pattern recognition receptor ALPK1 on cells, thereby upregulating ICAM1 expression and promoting CRC cell adhesion to endothelial cells (96), ultimately facilitating metastasis of CRC.

3.2 Participating in immune evasion to promote the survival of CRC cells

Immune surveillance imposes strong selective pressure on cancer cells (104). The gut microbiota can directly or indirectly

inhibit the function of immune cells, thus mediating immune evasion of tumor cells (61, 105–107).

The TIGIT (T cell immunoglobulin and ITIM domain) receptor is expressed on all NK cells and some other types of lymphocytes (108). F. nucleatum can directly interact with the TIGIT receptor through its surface virulence protein Fap2, thereby inhibiting the cytotoxicity of NK cells against cancer cells and ultimately inducing immune evasion of tumor cells (105). In addition to affecting the host's innate immunity, the gut microbiota also regulates host adaptive immunity. Research by Jiang et al. has shown that succinate produced by F. nucleatum could inhibit the cGAS-IFNβ pathway, leading to reduced levels of chemokines CCL5 and CXCL10 in the tumor, thereby limiting the migration of CD8+ T cells to TME and suppressing the anti-tumor response of CD8+ T cells (109).

Myeloid-derived suppressor cells (MDSCs) from the bone marrow exert immunosuppressive effects through the depletion of amino acids and the expression of TGFβ and PD-L1 (110). Certain specific pathogens such as F. nucleatum and P. anaerobius can induce tumor-derived chemokine CXCL1 to recruit the MDSCs, thereby suppressing anti-tumor immunity (61, 107). The gut microbiota can also activate the TLR-calcineurin-NFAT-IL-6 signaling cascade on MDSCs, leading to the STAT3-dependent induction of the inhibitory protein B7H3/4, resulting in functional inhibition of cytotoxic T cells and ultimately promoting tumor immune evasion (111).

It's worth noting that the effects imposed on the anti-tumor immunity by gut microbiota is a double-edged sword. A consortium of 11 bacterial strains was found to induce a strong CD8+ T cell response that boosted the efficacy of immune checkpoint blockade in mice (112). Other species such as Enterococus hirae could facilitate anti-tumor immunity in mice by enhancing CD8+ T cell anti-tumor responses when used in combination with cyclophosphamide chemotherapy (113). Bachem et al. discovered that butyrate, a microbiota-derived short-chain fatty acid (SCFA), enhances CD8+ T cell metabolism and promotes their differentiation into memory T cells (114). Similarly, microbiome-derived inosine could facilitate the differentiation of T_H1 cells in an adenosine 2A receptor-dependent manner and consequently improve the antitumor effect induced by the ICB therapy (115). Since the adenosine 2A receptor has been demonstrated to inhibit T_H1 differentiation *in vitro* as well as antitumor immunity *in vivo* (116–119) and only a few has reported that adenosine 2A receptor signaling can sustain T_H1 and antitumor immunity (120, 121), the crosstalk between microbiota-derived metabolites, adenosine 2A receptor signaling and host immunity needs to be further investigated. In terms of clinical practice, a phase I clinical trial enrolling 20 patients have shown that fecal microbiota transportation (FMT) in combination with anti PD-1therapy could lead to a promoted immune status in patients with melanoma (122). These researches indicate that the correlation between gut microbiota and host immunity could be far more complicated and worth further investigation.

Besides regulating the anti-tumor immunity, the gut microbiota plays an important role in the development and maturation of the host immune function. Germ-free mice are unable to develop mature isolated lymphoid follicles (123). Additionally, the gut microbiota can regulate the function of different types of immune cells such as Treg cells, DC cells, and T cells, thereby establishing a normal intestinal immune homeostasis during early host development by balancing local pro-inflammatory and anti-inflammatory responses (124–126). Similarly, changes in the functional status of the immune system can change the composition of the gut microbiota. Activation of the AhR pathway in Th17/Th22 cells can induce the production of IL-22 and IL-17, which in turn can stimulate intestinal epithelial cells to secrete antimicrobial peptides, ultimately limiting the proliferation of pathogenic microbial communities (125). Individuals with immune deficiencies are more prone to dysbiosis of the gut microbiota, leading to various chronic inflammations (127).

These facts indicate that the gut microbiota-immunity axis is a complex bidirectional process. During the occurrence and development of tumors, changes in the gut microbiota are accompanied by immune dysregulation. The aforementioned studies have revealed various mechanisms by which the gut microbiota participates in immune evasion, providing a new perspective for a deeper understanding of the correlation between gut microbiota and the host immunity.

3.3 Modulating TME to facilitate colonization of CRC cells

TME consists of various cell components (128, 129), and its complexity has made it a tendency to view the TME as an organ itself (128). In certain situations, these components can produce bioactive factors and release them into the TME, thereby promoting tumor angiogenesis, invasion, and metastasis (130–133). Recent studies have found that there could be multiple kinds of bacteria with regulation effect in the TME besides the cell component. For instance, Xu and colleagues found that F. nucleatum could facilitate tumor metastasis in a CCL20-dependent manner (94). Although the only known receptor for CCL20—CCR6 is mainly expressed in immature dendritic cells, innate lymphoid cells, regulatory CD4 T cells, Th17 cells and B cells (134), a positive correlation between F. nucleatum-induced CCL20 expression and F4/80+ CCR6+ macrophage in lung metastasis tissues was observed (94). And they also found that F. nucleatum could directly promote the polarization of M2 macrophages in tumor tissues (94). Current researches have shown that M2 macrophages play important roles in immune suppression, tumor angiogenesis, and EMT (135–137). There are also studies showing that several bacteria such as segmented filamentous bacterium (SFB; Candidatus Savagella), ETBF, Bifidobacterium spp., F. nucleatum could modify the polarization of $CD4^+$T cells into T_H17 cells (138–141). T_H17 cells has been indicated to foster an inflamed and tolerogenic TME (142, 143), providing a potential mechanism by which gut microbiota facilitates the future process of metastasis.

Not only is the microenvironment of the primary tumor important for tumor metastasis, but also the remodeling of the microenvironment in the metastatic foci plays a crucial role in the process of tumor metastasis. According to the "seed and soil" hypothesis, certain tumor cells can selectively settle in organs with suitable growth environments (144). Since the microenvironments of different organs vary, a particular type of tumor cells tends to preferentially colonize a specific organ (145). This preference may originate from the selective remodeling of the target organ by the primary tumor before metastasis occurs (146). Proteus mirabilis (P. mirabilis) and Bacteroides vulgatus (B. vulgatus) can modulate the hepatic immune niche by regulating the proliferation of Kupffer cells and inhibiting their phagocytic ability, ultimately fostering liver metastasis of CRC (19).

In conclusion, gut microbiota can facilitate the dissemination and survival of CRC cells via different ways(see Figure 2 for details).

4 Conclusion

Studies on the correlation between gut microbiota and CRC can be traced back to 1951. Subsequent advancements in techniques such as 16S rRNA sequencing and metagenomic sequencing have made it possible to identify gut microbiota that are significantly associated with CRC. Multiple mechanisms have been proposed regarding how the gut microbiota regulates anti-tumor effects and participates in pathological processes especially metastasis of CRC in the following years (see Table 1 for details). Consequently, CRC is a suitable model disease to investigate novel strategies for early cancer detection. Stool-based screening such as 16s rRNA sequencing is considered as a promising, non-invasive approach compared with colonoscopies (155). For bacteria widely participated in the initiation and progression of CRC, high-specific therapy strategies such as targeted antibiotic (156) and bioinorganic hybrid bacteriophage (157) has presented an attractive prospect for prevention and curation.

Recent studies have implicated that oncogenesis and progression of cancers could be consequences of the dysregulated immunologic function. Mechanisms like immune checkpoint shed a light on the complicated networks between cancer and immunity. Still, such theories cannot fully illuminate the role of systematic factors, such as exercise, diet and aging, in crosstalk between cancer and immunity. As one of the earliest encountered environmental antigens in the human body, gut microbiota could facilitate cancer metastasis and modulate immune response through mechanisms mentioned afore, which may explain the role of systematic factors in cancer and immunologic function. Nonetheless, more efforts should be dedicated to further unveil the mechanisms by which systematic

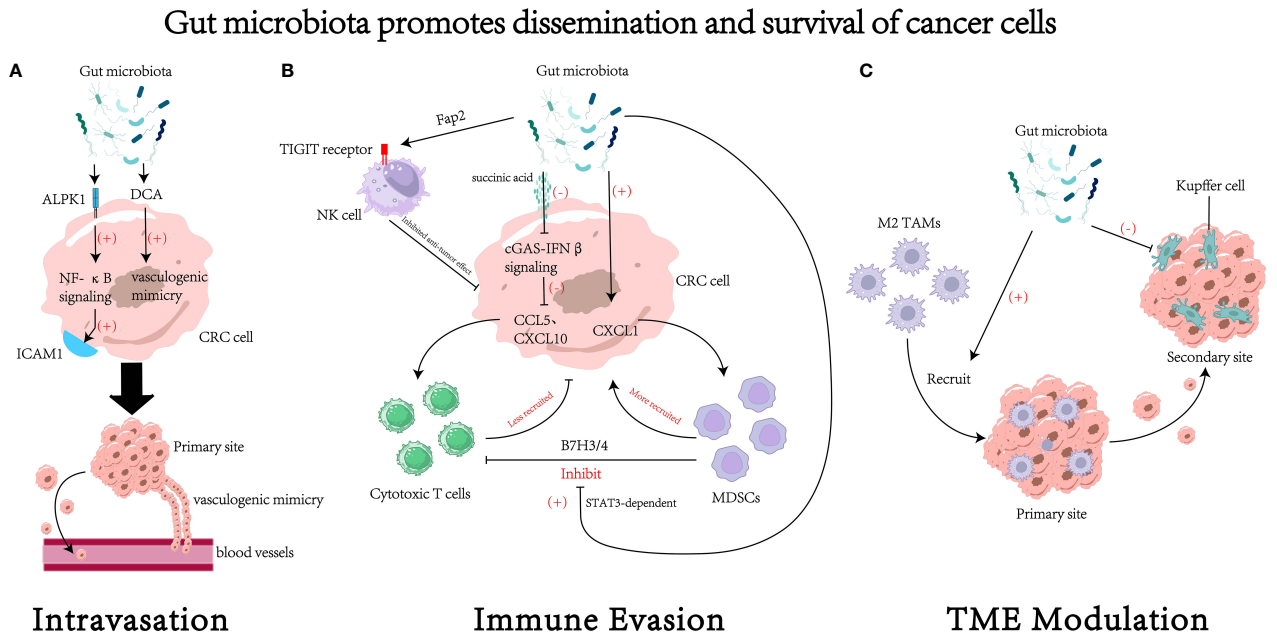

FIGURE 2
The impact of gut microbiota on the dissemination and survival of colorectal cancer (CRC) cell. **(A)** Gut microbiota could regulate the process of intravasation through various mechanisms, such as increasing the expression of intercellular adhesion molecule 1 (96) and promoting the formation of vasculogenic mimicry (99). **(B)** Gut microbiota could regulate survival of cancer cells by modulating tumor immune evasion through various pathways. For example, gut microbiota can inhibit the cytotoxic activity of natural killer (NK) cells through receptor-ligand interactions (105), reduce the recruitment of cytotoxic T cells (109), increase the recruitment of myeloid-derived suppressor cells (MDSCs) (61, 107), and foster the suppressive effects of MDSCs on cytotoxic T cells (111). **(C)** Gut microbiota may remodel the tumor microenvironment (TME) to facilitate the process of dissemination and colonization. For instance, it could recruit M2 tumor associated macrophages (M2 TAMs) to the primary site (94) and inhibit the function of Kupffer cells in the secondary site (19). DCA, deoxycholic acid. ICAM1, intercellular adhesion molecule 1. MDSCs, myeloid-derived suppressor cells. TAMs, tumor associated macrophages.

TABLE 1 Overview of milestones unveiling the correlation between gut microbiota and CRC.

Significance	Studies	Year
➢ Exploring the association between gut microbiota and CRC for the first time.	• Enterococcal endocarditis associated with carcinoma of the sigmoid; report of a case (147).	1951
➢ Identification and characterization of gut microbiota associated with CRC using RNA sequencing technology.	• Genomic analysis identifies association of Fusobacterium with colorectal carcinoma (148).	2012
➢ Elucidation of the mechanisms by which gut microbiota promote the occurrence and development of CRC for the first time.	• Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin (18).	2013
➢ The association between gut microbiota and radiotherapy as well as chemotherapy.	• Enterococcus hirae and Barnesiella intestinihominis facilitate cyclophosphamide-induced therapeutic immunomodulatory effects (79).	2016
	• Gut microbiota modulates dendritic cell antigen presentation and radiotherapy induced antitumor immune response (149).	2020
➢ Cross-cohort studies of gut microbiota and its association with colorectal cancer using metagenomic sequencing technology.	• Meta-analysis of fecal metagenomes reveals global microbial signatures that are specific for colorectal cancer (150).	2019
	• Metagenomic analysis of colorectal cancer datasets identifies cross-cohort microbial diagnostic signatures and a link with choline degradation (151).	2019
➢ Gut microbiota and tumor immunotherapy.	• Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota (152).	2015
	• Fecal microbiota transplant overcomes resistance to anti-PD-1 therapy in melanoma patients (153).	2021
	• Dietary fiber and probiotic s influence the gut microbiome and melanoma immunotherapy response (154).	2021

CRC, colorectal cancer; PD-1, Programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte-associated antigen-4.

factors such as gut microbiota regulate the process of cancer oncogenesis and progression.

Author contributions

SY: Writing – original draft. SW: Writing – review & editing. BX: Writing – review & editing. CP: 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

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Fecal microbiota transplantation—could stool donors' and receptors' diet be the key to future success?

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Fecal microbiota transplantation (FMT) is indicated in many countries for patients with multiple recurrences of *Clostridioides difficile* infection (CDI) for whom appropriate antibiotic treatments have failed. Donor selection is a demanding and rigorous process in view of the implementation of FMT programs worldwide. One of the most noteworthy factors that has been shown to affect FMT outcomes is the microbial diversity of the stool donor. A detailed assessment of the donor's microbiota is crucial, as the microbiota is complex, dynamic, and resilient, and a healthy microbiota has several dimensions in addition to the absence of pathogens. Diet is one of the most important factors that modulates the composition and function of the gut microbiome (GM) and has a critical role in orchestrating the host–microbiota crosstalk throughout life. The diversity of the human GM seems to be related to variations in dietary patterns. Currently, the dietary patterns of stool donors and receptors are not taken into consideration in any way for FMT. In this study, we reflect on the importance of including this type of assessment in the stool donor screening process and knowing the impact of diet on the GM, as well as the importance of monitoring receptors' diet to ensure the engraftment of the transplanted microbiota.

KEYWORDS

diet, fecal microbiota transplantation, FMT receptors, gut microbiota, stool donors

Introduction

The gut microbiome encodes over 3 million genes, whereas the human genome consists of approximately 23,000 genes (1). Therefore, the metabolic capacity of the gut microbiome greatly exceeds the metabolic capacity of human cells (2). The gut microbiota (GM) has a crucial role in the maintenance of health, with protective, structural, and metabolic

functions (3). An imbalance in its composition and function (dysbiosis) has been associated with many disorders (4), including *Clostridioides difficile* infections (CDIs).

Fecal microbiota transplantation (FMT) was first described in the fourth century by the traditional Chinese medicine doctor Ge Hong (5), but it was only in 1983 that Schwan et al. published the first report of a successful treatment with FMT for CDI, through retention enema (6). Currently, FMT is an established treatment for recurrent CDI (7, 8), but it also seems promising as a therapy for many other disorders (9).

Fecal microbiota transplantation

FMT is a procedure in which the fecal microbial content from a healthy donor is administered into another patient's intestinal tract, with the aim of treating a certain disease linked with the alteration of the GM (10). FMT can be performed through the upper gastrointestinal tract (GIT), via a duodenal tube or capsules taken orally, or through the lower GIT, via colonoscopy or an enema (9) (Figure 1).

FMT is indicated in many countries (7, 8) for patients with multiple recurrences of CDI for whom appropriate antibiotic treatments have failed (7), and it has cure rates of 80%–90% (13). In addition, it seems promising as a treatment for many other conditions (9). FMT has been studied in inflammatory bowel disease (14), obesity, and metabolic syndrome (15). FMT also seems promising in oncology (16), it might be useful in the prevention and treatment of psychiatric illnesses (17), and has the potential to treat Alzheimer's and Parkinson's diseases (18). More recently, it has been proposed as a potential treatment for COVID-19 (19).

Stool donor screening and stool receptor follow-up

Donor selection is a demanding and rigorous process in view of the implementation of FMT programs worldwide (20). In fact, choosing the right donor could be challenging in clinical practice because of the absence of a clear definition of a healthy GM, and because of the complexity of the host response (such as the immune response) and dietary habits (2).

Potential stool donors should undergo a detailed questionnaire including medical history, infectious diseases, intestinal health, and risk behaviors (21). They should also undergo blood and stool tests to prevent the direct transmission of infectious diseases and avoid transferring an adverse microbiota profile that could possibly increase the risk of the receptor developing other diseases related to an abnormal GM (21, 22).In addition, the US Food and Drug Administration (FDA) has recommended, since March 2020, the screening of stool and stool donors for the presence of SARS-CoV-2 infection (23).

In recent years, the debate about stool donor screening has become deeper. In fact, donors whose stool results in substantially more successful FMT outcomes than the stool of other donors have been described as "super-donors" (24). One of the most noteworthy factors that has been shown to affect FMT outcomes is the microbial diversity of the stool donor (25).

In addition, the stool receptors' follow-up is focused on the side effects or complications of FMT in the short term (10, 21), and their long-term follow-up includes only the documentation of clinical details and relevant clinical results beyond the first 24 h (21). It does not take into consideration what the receptor should do to keep the transplanted microbiota in balance.

The importance of the donor's microbiota

The gut microbiome is complex, dynamic, and resilient, as any biological system (26), and a healthy microbiota has several dimensions in addition to the absence of pathogens. So it is hard to define what a healthy human GM at an exact taxonomic level (27). However, a high level of taxa diversity, a high level of microbial gene richness, and stable microbiome functional cores indicate healthy GM communities (28).

It has been demonstrated that low levels of bacterial gene richness leads to increased adiposity, insulin resistance and dyslipidemia, and a further pronounced inflammatory phenotype (2), indicating the impact of GM on metabolic processes. Besides, an increasing number of diseases are linked with intestinal dysbiosis, such as metabolic, cardiovascular, and neurologic diseases (4), and pathologies such as inflammatory bowel (29) and autoimmune diseases (30). Individuals with these pathologies should not be stool donors, to prevent the transmission of a dysbiotic microbiota that could itself cause the disease in the receptor. Furthermore, donors are excluded based on disease, as we assume that because they have the disease they have an altered GM, but a question remains unanswered: what about those who already have an altered GM and do not have the pathology yet? We propose that the exclusion of these types of donors start to be considered.

Impact of diet on the gut microbiota

Diet is one of the most important factors that modulates the composition and function of the GM and has a crucial role in orchestrating the host–microbiota crosstalk throughout life (31).

What we eat is a key factor in the composition of the GM, as diet is thought to explain about 20% of microbial structural variations in humans, indicating the ability of dietary approaches to aid in disease management through GM modulation (32). The integration between the GM, food groups, and short-chain fatty acid (SCFA)-producing bacteria is promising in the quest to further upgrade and transform dietary habits (33).

A diverse diet, especially in the number of different types of plant foods eaten, has been linked with greater microbial alpha-diversity, and is thought to enhance the diversity of substrates for the proliferation of numerous taxa (32). The interactions between diet and the GM are described in Figure 2.

The diversity of the human GM seems to be related to variations in dietary patterns (39). In fact, several studies have demonstrated

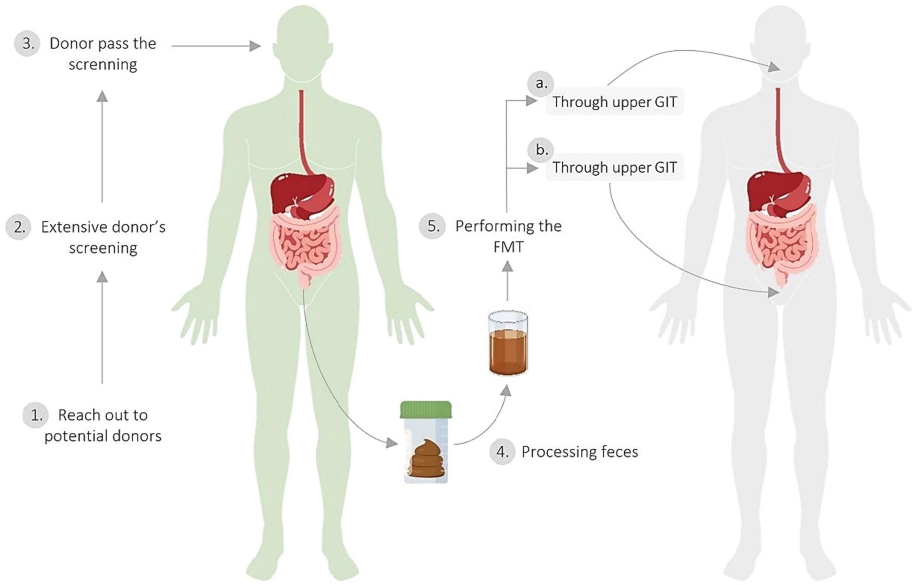

FIGURE 1
Fecal microbiota transplantation process. 1. Recruitment of potential healthy donors through media and advertising. 2. Extensive donor screening for medical history, infectious diseases, intestinal health, and risk behaviors; blood and stool testing; and screening of stool and stool donors for the presence of SARS-CoV-2 infection. 3. Donor passes the screening. 4. Processing feces through dilution (with 0.9% NaCl) and filtration to obtain microbiota. 5. Performing the FMT **(A)** through the upper GIT, through a duodenal tube or capsules taken orally or **(B)** through the lower GIT, through colonoscopy or an enema. The image is an adaptation of Bou Zerdan M. *et al.* (11), Alabdaljabar et al. (12), and Ooijevaar et al. (9).

the ability of the Mediterranean diet (MD) to modulate the GM and host's health (40–42). The MD is characterized by a high level of polyphenol-rich product content (extra-virgin olive oil, red wine, vegetables, grains, legumes, whole-grain cereals, and nuts), a positive fatty acid profile [high levels of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), and low levels of saturated fatty acids (SFAs)], and a low intake of processed meat and refined sugars (43). Adherence to the MD was found to be related to increased levels of SCFAs (acetate, propionate, butyrate, and lactate) (3, 42), *Prevotella*, and fiber-degrading *Firmicutes* (44, 45). It is worth mentioning that SCFAs are used as energy sources and participate in numerous metabolic pathways, including gluconeogenesis and lipogenesis, hence contributing to whole-body energy homeostasis (2). The MD has also been linked to improvements in the diversity and richness of the GM (35). Conversely, the Western diet, high in total fat, animal proteins, processed food, refined sugars, and food additives, leads to a dysbiosis in GM composition and is connected with obesity and other metabolic disorders (35).

Discussion

Future prospects for dietary screening of stool donors and receptors' follow-up

Currently, the dietary patterns of stool donors and receptors are not taken into consideration in any guidance for FMT. In addition, no clinical practice recommendations are available to provide receptors or stool donors with dietary advice for FMT (46). Clancy et al. reported that, overall, health professionals and researchers who work with FMT reported that diet was a significant consideration for FMT receptors and donors, and that it would affect the outcomes of the FMT (46). Although they did not usually advise patients to see a dietitian/nutritionist before or after the FMT, and did not feel certain in giving dietary guidance, or that there was enough evidence to provide dietary counsel (46). Owing to the great contribution of diet to the composition and modulation of the GM (43), we consider it crucial to include this step in stool donor screening protocols, in order to guarantee the better quality of the transplanted microbiota and consequent benefits to the host. Moreover, the dietary follow-up of the stool receptors should also be taken into consideration, in order to guarantee the long-term efficiency of the FMT. This assumes a greater importance when FMT is intended to treat metabolic diseases. A study conducted by our group (data not published) showed—through the application of the Mediterranean Diet Adherence Screener (MEDAS) (47), a validated questionnaire to assess adherence to the MD—that only 55.6% (25 out of 45) of the potential stool donors had a level of high adherence (≥ 10) to the MD. These data suggest that the absence of chronic diseases may not be a suitable criterion for donor selection and that diet, as well as other lifestyle factors, should be evaluated to increase FMT efficiency and applicability in health. In addition, recent studies have demonstrated the importance of dietary habits, in particular fiber intake, in optimizing the success of FMT in the treatment of metabolic diseases (48–50). One study used autologous FMT to prolong the beneficial effect of a modified MD on weight regain. The findings of this study provided a provocative

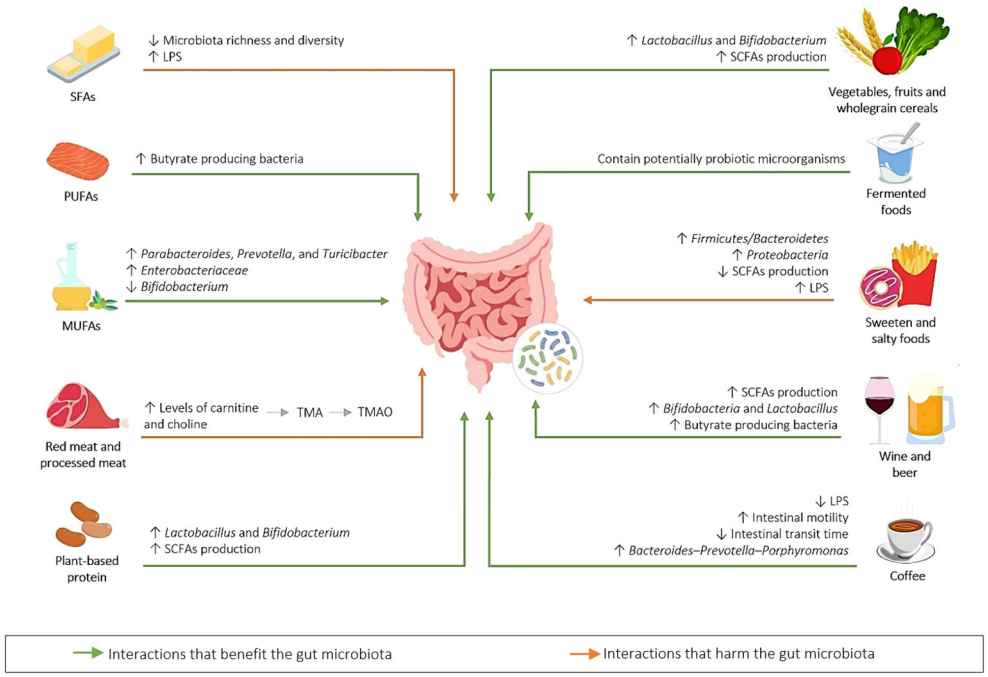

FIGURE 2
Gut microbiota–diet interactions. Common dietary components are metabolized by the GM to produce metabolites (for example, red and processed meat, containing high levels of carnitine and choline, both of which are precursors that the gut bacteria use to produce trimethylamine (TMA), which is converted by the enzyme flavin-containing monooxygenase 3 (FMO3) into trimethylamine *N*-oxide (TMAO) (34), that has been associated with atherosclerosis). A diet rich in saturated fatty acids (SFAs), sweet and salty foods modify the GM, causing elevated levels of lipopolysaccharides (LPSs) in the circulation, leading to a pro-inflammatory state (metabolic endotoxemia) (35). Some foods have a positive effect on the GM, for example, those that elevate short-chain fatty acid (SCFA) production and the abundance of *Lactobacillus* and *Bifidobacterium* (34), and those that are included in the Mediterranean diet, such as olive oil (35). Fermented foods (36), wine and beer (37), and coffee (38) consumption also have a positive effect on the GM composition. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LPS, lipopolysaccharide; SCFA, short-chain fatty acid; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide.

perspective where the co-supplementation of low-fermentable fiber may increase the potency of FMT (50). Mocanu et al. also provided a proof of concept for the use of a single-dose oral FMT combined with daily low-fermentable fiber supplementation to improve insulin sensitivity in patients with severe obesity and metabolic syndrome (49). Considering these data, would it not be important to evaluate and, if necessary, modify the dietary habits of the FMT receptor in future protocols?

Concluding remarks and perspectives

Knowing the impact of diet on the GM, we propose that potential stool donors undergo dietary screening, to increase the probability of a beneficial and functional fecal microbiota being transplanted. We also believe that specific guidelines for stool receptors should be developed, mainly in the treatment of diseases other than CDI.

In addition, besides the inclusion of a dietary screening tool for the stool donor candidates it could be interesting to add nutrition counseling a few months prior to the stool donation in order to improve the quality of the stool donated, if necessary. Nutritionists could also enhance long-term FMT success by giving nutrition counseling services as part of multidisciplinary health care groups (51), as a healthy diet provides the commensal microbes with the substrates necessary for their proliferation and survival (24).

We hope to stimulate future research so that further information about dietary habits of FMT receptors and stool donors can be collected and thus make it possible to better understand the relationship between diet and FMT results. With this information, it would be possible to create a score for stool donors, where diet and other factors that modulate the GM are included. In fact, a validation study of a score of this kind would be interesting to assess whether or not those with the highest scores are, in fact, better donors.

The increased application of FMT in clinical practice will notably have a key impact on public health, as the prevalence of chronic diseases continues to increase. Therefore, the development of FMT protocols that honor this scientific evidence and promote the creation of more detailed screening and follow-up processes are of the utmost importance.

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

RS: Writing – original draft. LD: Writing – review & editing. AP: Writing – review & editing. LN: Writing – review & editing. CC: Writing – review & editing. DP: Writing – review & editing. CM: Writing – review & editing.

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Conflict of interest

CM, DP, CC, and LN are co-founders of YourBiome®, a spin-off of Universidade NOVA de Lisboa. LD and AP were YourBiome® employees under the project ALT20-03-02B7-FEDER-069744.

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

Publisher's note

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Diversity of the microbiota communities found in the various regions of the intestinal tract in healthy individuals and inflammatory bowel diseases

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The severe and chronic inflammatory bowel diseases (IBD), Crohn disease and ulcerative colitis, are characterized by persistent inflammation and gut damage. There is an increasing recognition that the gut microbiota plays a pivotal role in IBD development and progression. However, studies of the complete microbiota composition (bacteria, fungi, viruses) from precise locations within the gut remain limited. In particular, studies have focused primarily on the bacteriome, with available methods limiting evaluation of the mycobiome (fungi) and virome (virus). Furthermore, while the different segments of the small and large intestine display different functions (*e.g.*, digestion, absorption, fermentation) and varying microenvironment features (*e.g.*, pH, metabolites), little is known about the biogeography of the microbiota in different segments of the intestinal tract or how this differs in IBD. Here, we highlight evidence of the differing microbiota communities of the intestinal sub-organs in healthy and IBD, along with method summaries to improve future studies.

KEYWORDS

IBD, microbiome, dysbiosis, bacteriome, mycobiome, virome

Introduction

Dysbiosis (altered abundance and diversity of microbiota; bacteria, fungi, and viruses) is a known hallmark of the inflammatory bowel diseases (IBD), Crohn disease (CD) and ulcerative colitis (UC) (1). However, the precise definition of a healthy and diseased microbiome remains poorly defined (2). It is well recognized that this is in part due to the significant inter- and intra-individual heterogeneity of the microbiome (microbiota,

microenvironment, interactions with the host), along with limitations in sampling and processing techniques (3, 4). Yet, one factor that remains largely overlooked is the significant diversity of the microbiome in the various subsections of the intestinal tract, with many studies describing sample collection from only the "small intestine" or "large intestine". Here we summarize what is currently known about the variations in the composition of the microbiota communities identified at specific sites along the gastrointestinal (GI) tract in healthy individuals and patients living with IBD.

The functions of the subsections of the small and large bowels

Food substrates, host cells, and luminal and mucosal gut microbiota come into close contact throughout the intestinal tract, generating a microenvironment rich in microbe–microbe and host–microbe interactions, which are closely linked to health and disease outcomes (5, 6). It is important to recognize the segments of the intestinal tract include the duodenum, jejunum, and ileum, which make up the small intestine; while the cecum, ascending colon (ASC), transverse colon, descending colon, sigmoid colon, and rectum make up the large intestine. These organs serve diverse roles from digestion, to absorption of nutrients and water, to microbial fermentation of proteins and fibers (Figure 1) (7, 8). Previous review articles have highlighted the in-depth physiology of these organs (7, 8) however, here we will briefly highlight their roles to better support discussion of the diverse microbiota within the segments of the small and large intestines.

Small intestine

The duodenum is the first and shortest portion of the small intestine, which plays a crucial role in the digestion of food contents exiting the stomach with assistance from pancreatic secretions containing digestive enzymes (Figure 1A) (9). Both the duodenum and jejunum (the mid-segment of the small intestine) are responsible for the bulk of nutrient absorption and assimilation (10). Further, the jejunum is also responsible for the absorption and digestion of most dietary lipids (11). The most distal segment of the small intestine, known as the ileum, is involved in the absorption of bile acids and simple sugars (6, 12). The ileum also contains the collection of lymphoid follicles located in the mucus membrane known as Peyer's patches, which are master immune regulators of the intestine where interactions occur between antigens and microbiota with immune cells. These interactions are mediated by both nucleotide-binding oligomerization domain two (NOD2), a pattern recognition cytosolic protein highly expressed in the ileal Paneth cells with its loss of function linked to CD, and other pathogen recognition receptors that can also be altered in IBD, resulting in abnormal responses targeting commensal microbiota (13–15).

Large intestine

The ileocecal valve, which joins the small and large intestines, shields the opening of the ileum into the cecum (8). While the bulk of digestion and absorption of food occurs in the small intestine, the large intestine aids in final water absorption and waste removal (Figure 1B) (8). The proximal parts of the colon (cecum, ASC, and transverse colon) are responsible for carbohydrate fermentation by microbiota, producing short-chain fatty acids (SCFA)s (16). Protein fermentation producing branched-chain fatty acids typically occurs in the distal descending and sigmoid segments of the colon (16). The colon is also responsible for obtaining key vitamins, such as cobalamin (B_{12}) found in animal products, yeast, and algae, along with minerals such as calcium (17). Further, the ASC is responsible for the absorption of sodium (Na^+) via electroneutral sodium-chloride transport, and the descending colon has been reported to be associated with amiloride-insensitive Na^+ absorption (17).

Microbiota profiles of the small intestine

Due to sampling challenges, including the inability to easily access the small intestine via endoscopy (proximal duodenum) or colonoscopy (terminal ileum), limited research on the microbiota of the small intestine has been performed (18). Hence, there is often a reliance on animal models which do not completely reflect human intestinal microbiota (19, 20). The microenvironment of the small intestine is less favorable for microbial growth than the colon due to the lower pH, increased concentration of oxygen, and antimicrobial peptides produced by host cells of the epithelial lining of the small intestine such as α- defensins, C-type lectins interfacing as a shield against pathogenic microbes (21, 22). As such, most microbes in the small intestine are fast-growing, facultative anaerobes (21). Generally, microbial abundance increases significantly after exiting the duodenum (10^1-10^3) CFU/ml) and continuing to the jejunum (10^4-10^7 CFU/ml) and ileum (10^3–10^8 CFU/ml) (23). Below we discuss the key microbial species identified in healthy sections of the small intestine and the changes reflected in IBD (Figure 2).

Duodenum

The duodenum is located between the acid-secreting stomach and the nutrient-absorbing jejunum, therefore participating in continued digestion and nutrient absorption, displaying a lower overall abundance of microbes compared to the rest of the intestinal tract, yet greater diversity (by phyla) than the rectum (24). The bacteriome (16S rRNA) of healthy adults profiled in biopsy tissues shows the duodenum is chiefly dominated by phyla Pseudomonadota (formerly Proteobacteria), Bacteroidota (formerly Bacteroidetes), Actinomycetota (formerly Actinobacteria), and

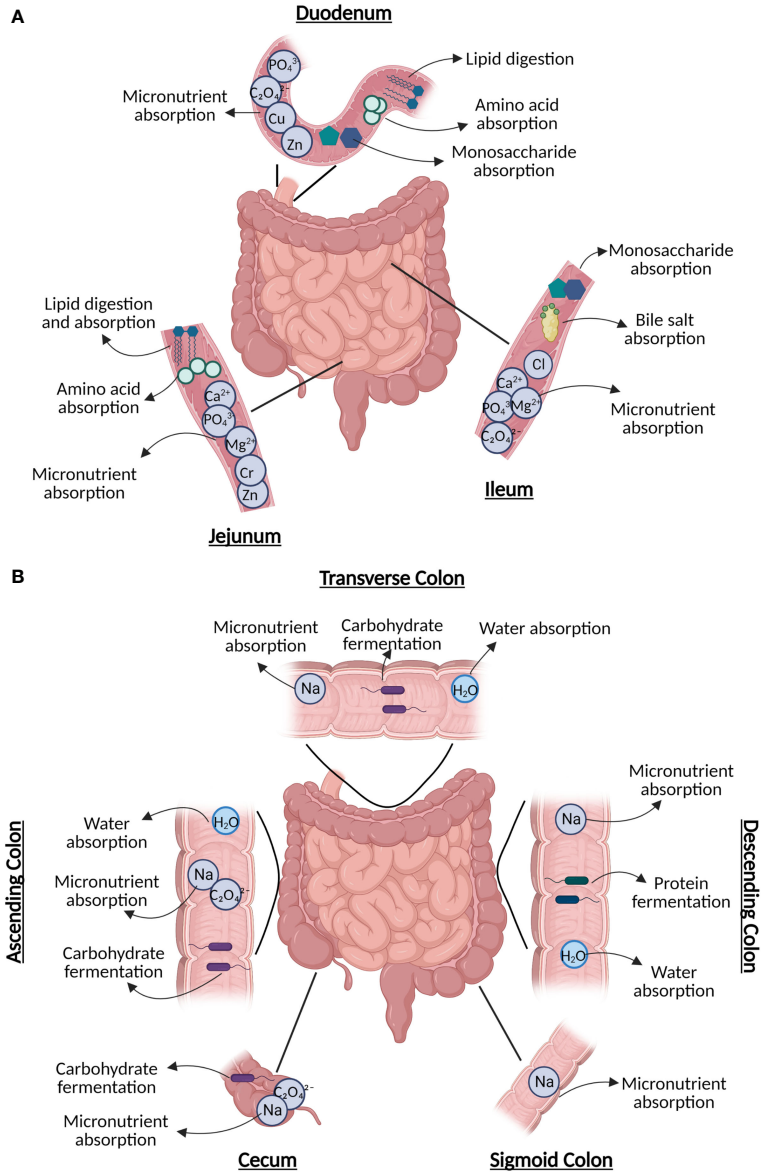

FIGURE 1
The diverse roles of subsections of the **(A)** small intestine, including digestion (duodenum), nutrient absorption (duodenum/jejunum), lipid digestion (jejunum), and sugar absorption (ileum); and **(B)** large intestine, including water absorption (every section), carbohydrate fermentation, and remaining nutrient absorption. Figure created in BioRender.

Bacillota (formerly Firmicutes), along with the genera *Acinetobacter, Bacteroides, Prevotella*, *Bifidobacterium, Escherichia*, and *Lactobacillus* (24–26). Luminal (mucus) duodenum samples predominantly house genera *Stenotrophomonas* and *Streptococcus* (24). Interestingly, one small study (9 participants) using Chinese healthy volunteers identified several rare bacterial phyla (OP10, SR1, Mycoplasmatota [formerly Tenericutes], Thermotogota [formerly Thermotogae], Deferribacterota, and Spirochaetes), and noted that the microbial samples they collected from biopsies were more conserved than luminal mucosal samples (24). Some of the reasons underlying this variable microbial profile (biopsies vs mucosal) may include the rapid transit time of luminal contents, low pH, and high concentrations of bile acids, digestive enzymes, host-defense peptides (HDPs), and immunoglobulins (21, 27). This microenvironment reduces microbial colonization, while certain phyla of fungi, such as Ascomycota, Basidiomycota, Mucoromycota and Zoopagomycota (formerly Zygomycota), can thrive in these low pH conditions (27, 28). There are currently no studies on the virome (eukaryotic viruses or bacteriophages) in the duodenum of healthy individuals.

In contrast, IBD patients have a lower abundance of mucosal duodenal bacteria (25). Beneficial genera of bacteria *Bifidobacterium* and *Lactobacillus* are notably decreased in IBD, whereas the populations of *Bacteroides* and *Escherichia* genera are increased (25). Furthermore, F. Sjöberg et al. performed a novel pilot study where luminal fluids were sampled from treatment-naïve children who were suspected of having IBD, highlighting a low richness and a reduced prevalence of Actinomycetota (formerly Actinobacteria), Bacteroidota (formerly Bacteroidetes), and

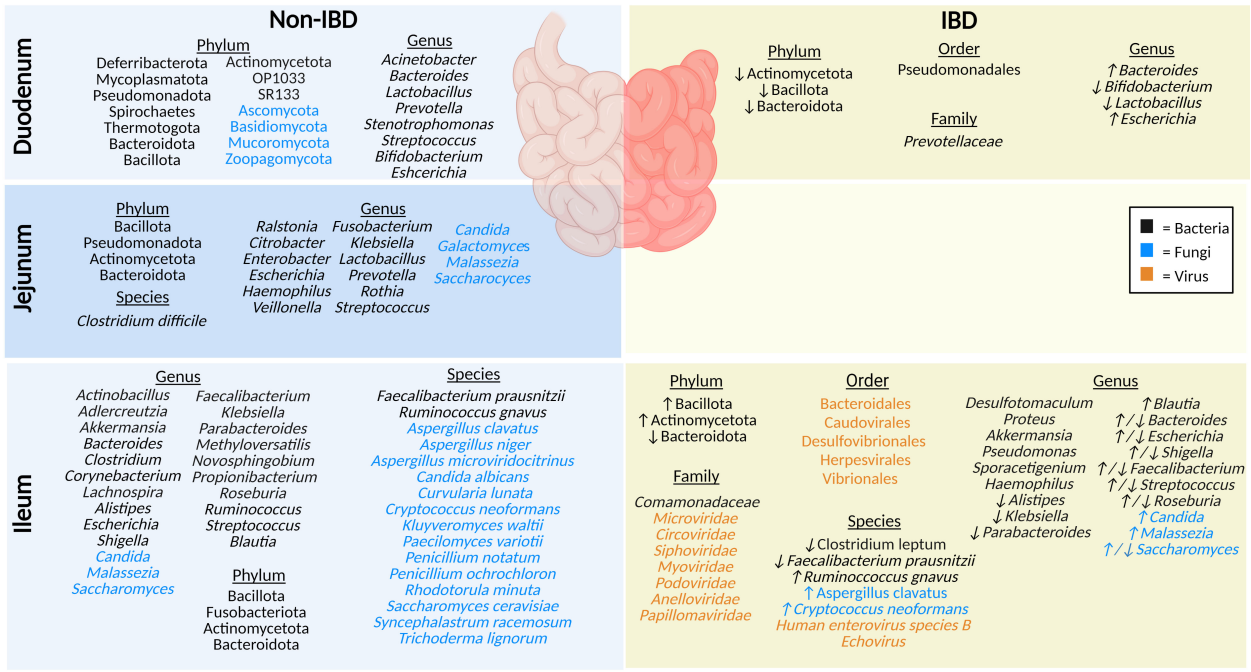

FIGURE 2
The microbiota populations previously identified within the different sections of the small intestine in non-IBD (left) and IBD (right) patients. Black text (bacteria), blue text (fungi), orange text (viruses). Figure created in BioRender.

Bacillota (formerly Firmicutes) phyla (26). Limited studies highlight key differences in the duodenal microbiota compared to other segments of the intestinal tract, and in IBD compared to non-IBD. The duodenal mycobiome and virome (eukaryotic viruses or bacteriophages) in individuals with IBD has not been defined.

Jejunum

The jejunum is a structurally and functionally distinct region of the small intestine, involved in nutrient absorption such as magnesium and phosphate, along with absorption and digestion of most dietary lipids (10, 11). In healthy individuals, the microbiota plays a crucial role in lactate production, which is an important energy source for stem cells in the small intestine (11). Unfortunately, sampling difficulties account for one of the reasons why the jejunal microbiota is understudied. The jejunal microbiota of healthy humans includes a high abundance of members of phyla Bacillota (formerly Firmicutes), Pseudomonadota (formerly Proteobacteria), Actinomycetota (formerly Actinobacteria), and Bacteroidota (formerly Bacteroidetes) (23). To a lesser extent, other detected genera include *Enterobacter, Escherichia, Lactobacillus, Streptococcus, Klebsiella, Veillonella, Fusobacterium, Rothia, Prevotella, Ralstonia*, *Haemophilus* and *Citrobacter*, and the species *Clostridium difficile* (29–31). In addition, a recent review article highlighted fungal genera including *Malassezia, Candida, Saccharomyces*, and *Galactomyces* in the jejunum of healthy individuals (32). While the jejunal microbiota has not been defined in IBD patients, damage and inflammation during active disease result in the reduction of the epithelial barrier of the jejunum, allowing entry of microbial lipopolysaccharides, demonstrating links between the microbiota and IBD (1, 11). In addition, the jejunal mycobiome in IBD patients and the virome (eukaryotic viruses or bacteriophages) in both healthy and IBD individuals have not been described thus far.

Ileum

Although any part of the GI tract may be affected by CD, the terminal ileum is the most commonly affected area in CD pathogenesis (33). In a healthy individual, the ileum plays a significant role in the absorption of simple sugars and bile acids, which is significantly altered in ileal CD and may have a significant impact on luminal bacteria and fungi in particular (12, 34). Phyla identified using 16S sequencing of ileal mucosa in healthy adults include Actinomycetota (formerly Actinobacteria), Bacteroidota (formerly Bacteroidetes), Bacillota (formerly Firmicutes), and Fusobacteriota (35). The healthy ileal bacteriome is dominated by a high abundance of the genera *Clostridioides, Streptococcus, Bacteroides*, and *Corynebacterium* (35, 36). Other bacteria identified in the ileal mucosa include the genera *Alistipes*, *Blautia*, *Escherichia, Shigella, Faecalibacterium, Klebsiella, Parabacteroides, Actinobacillus*, *Novosphingobium*, *Methyloversatilis*, *Akkermansia*, *Propionibacterium*, *Ruminococcus*, *Aldercreutzia*, *Lachnospira*, and *Roseburia;* in particular the species *Ruminococcus gnavus* and *Faecalibacterium prausnitzii* (35, 37). The ileal lumen of healthy individuals also houses fungi from the genera *Saccharomyces,*

Malassezia, and *Candida*, along with the species *Saccharomyces cerevisiae, Aspergillus clavatus, Aspergillus niger, Candida albicans, Curvularia lunata, Penicillium notatum, Penicillium ochrochloron, Kluyveromyces waltii*, and a smaller percentage of species including *Paecilomyces variotii, Aspergillus microviridocitrinus, Rhodotorula minuta, Trichoderma lignorum, Syncephalastrum racemosum* and *Cryptococcus neoformans* (32, 38–41). The ileal virome was examined in healthy control stool but has not been precisely examined in the ileum using appropriate sampling techniques to date (42).

The microbiota composition of the ileum is notably different in individuals with IBD. This includes an increase in Actinomycetota (formerly Actinobacteria) and Bacillota (formerly Firmicutes), and a reduction in Bacteroidota (formerly Bacteroidetes) in ileal mucosa (35). At the family level, ileal bacteria *Comamonadaceae* have been described, and the IBD mucosa include the genera *Proteus* and *Desulfotomaculum* (37). Many other genera have been specifically identified in IBD only; for example, *Akkermansia* were only found in IBD ileal mucosal samples and the genera *Pseudomonas, Haemophilus*, and *Sporacetigenium* were only found in UC ileal mucosal samples (35). The genera *Alistipes, Klebsiella*, and *Parabacteroides* were decreased in IBD patient ileal mucosal samples, and *Blautia* and *Roseburia* were increased (35). In contrast, another study found a reduction in IBD mucosal genera *Roseburia* (39). Meanwhile, the *Bacteroides* genera was decreased in UC mucosal samples but increased in CD, and *Shigella, Escherichia, Faecalibacterium*, and *Streptococcus* were decreased in CD mucosal samples but increased in UC samples (35, 37). At the species level, in ileal IBD samples there was elevated mucosal species *Ruminococcus gnavus* along with reduced mucosal species *Clostridium leptum*, and reduced luminal *Faecalibacterium prausnitzii* (39, 41). The mycobiome is significantly different between CD and non-IBD ileal biopsy samples; a high abundance of the genus *Saccharomyces* and the species *Aspergillus clavatus*, and *Cryptococcus neoformans* was identified (39). In contrast, another study found a decrease in the abundance of *Saccharomyces* and an increase of *Malassezia* and *Candida* in CD patient biopsies compared to healthy controls (40). Comparing virome results of CD biopsies against that of healthy control stool samples, demonstrated that ileal biopsies from active CD patients had a high abundance of bacteriophages and eukaryotic viruses from the order Caudovirales, Bacteroidales, Herpesvirales, Vibrionales, and Desulfovibrionales, and the families *Microviridae*, *Circoviridae*, *Anelloviridae*, *Papillomaviridae*, among other unidentified viruses (42). A study examined bacteriophages present in biopsies and gut wash samples from pediatric CD patients and identified bacteriophages from the Caudovirales order (*Myoviridae, Siphoviridae*, *Podoviridae*) in the ileum (43). At the species level, *Human Enterovirus* species B and *Echovirus* were also identified in ileal biopsies collected from advanced ileocecal CD patients (44). Sampling difficulties have led many studies to compare and contrast the small intestine as a whole between healthy and IBD patients, when the sample likely represents the terminal ileum collected during colonoscopy (5, 45). Furthermore, differences in sampling techniques and sites (*e.g.*, biopsies, gut washes, gut brushings) have resulted in conflicting results across studies, particularly when compared to stool from healthy controls (42, 46). While these studies are excellent examples of the variation that occurs between the intestinal sub-organs, and in IBD patients, improved sampling techniques, highlighted by Tang et al. (47), are sure to broaden our understanding of the precise role of the duodenal, jejunal, and ileal microbiota in IBD in future.

Microbiota profiles of the large intestine

Regional differences are particularly noticeable when comparing the segments of the colon because microbial diversity progressively increases from the proximal to the distal colon (23, 48). The colon is a more conducive habitat for microbiota growth compared to the small intestine because it has a longer transit time and higher pH, a lower cell turnover, a lower redox potential, and fewer antimicrobials (21, 49). In this microenvironment, many bacteria in the colon are fermentative, polysaccharide-degrading anaerobes (21). Interestingly, the colonic mucosal mycobiome displays an overall increased fungal load in IBD during disease flare, compared to healthy individuals (50, 51). IBD fecal samples generally display an increased Basidiomycota:Ascomycota ratio, increased *C. albicans* species, and decreased *S. cerevisiae* species, although discrepancies exist between studies (50, 51). Below we discuss the key microbial species identified to be abundant in healthy sections of the colon and the changes reflected in the IBD colon (Figure 3).

Cecum

The cecum absorbs large volumes of water and electrolytes, and the microbes present here typically ferment carbohydrates (52). Studies of the healthy luminal microbiota of the cecum show that it is home to prevalent bacteria from the phylum Actinomycetota (formerly Actinobacteria), Bacteroidota (formerly Bacteroidetes), Bacillota (formerly Firmicutes), and Pseudomonadota (formerly Proteobacteria) (35, 53). Bacteria from the family *Baceteroidaceae, Rhodocyclaceae, Pasteurellaceae, Aeromonodaceae, Carnobacteriaceae, Prevotellaceae, Flavobacteriaceae, Enterococcaceae, Erythrobacteraceae, Sphingomonadaceae*, and *Alcaligenaceae* have been identified in healthy luminal cecum samples (35, 53–55). At the genera level, bacteria such as *Parabacteroides, Shigella, Dorea, Coprococcus, Blautia, Bacteroides, Alistipes, Lactobacillus*, *Bifidobacterium, Fusobacteria, Lachnospira, Enterococcus, Faecalibacterium, Roseburia*, *Escherichia, Prevotella*, and *Chryseobacterium*, along with a smaller populations of *Eubacterium, Clostridium*, and *Ruminococcus* were identified in the cecum (35, 53–55). Many of these microbes play a key role in the fermentation of non-digestible carbohydrates (resistant-starch and fiber) (21, 54). A preprint article looking into the mycobiome, using eukaryotic rRNA operon internal transcribed spacer-2 sequencing (ITS), in the colon of non-IBD individuals identified cecal fungal species, including members of the *Malasseziale* order, along with species

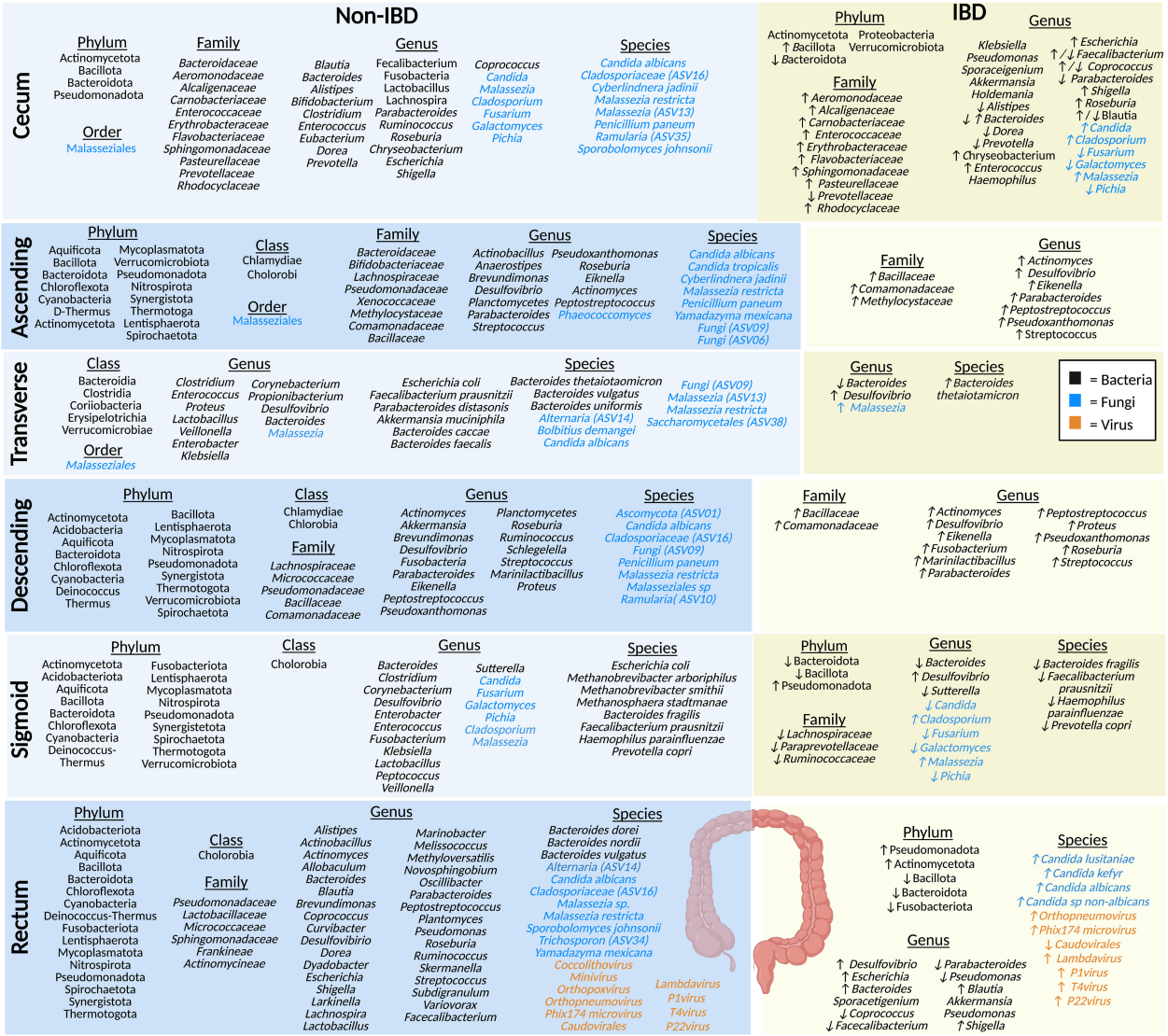

FIGURE 3
The microbiota populations previously identified within the different sections of the large intestine in non-IBD (left) and IBD (right) patients. Black text (bacteria), blue text (fungi), orange text (viruses). Figure created in BioRender.

Malassezia restricta, Malassezia (ASV13), Cladosporiaceae (ASV16), Ramularia (ASV35), Penicillium paneum, Sporobolomyces johnsonii, C. albicans, and *Cyberlindnera jadinii*, with a lower abundance of *C. albicans* in the cecum and ASC compared to other sections of the large intestine noted (56). Another study, also using ITS sequencing detected *Malassezia, Candida*, and, *Cladosporium* and found a higher abundance of the genera *Pichia*, *Fusarium*, and *Galactomyces*, compared to individuals with IBD (57). No studies of the cecal virome (eukaryotic viruses or bacteriophages) have been published in humans to date to the best of our knowledge.

Compared to the cecal mucosa of healthy controls, IBD patients have a decrease in bacteria from the Bacteroidota (formerly Bacteroidetes) phyla and an increase in Bacillota (formerly Firmicutes) (35). The Actinomycetota (formerly Actinobacteria), Proteobacteria, and Verrucomicrobiota phylum were found in IBD patient samples and supposedly not in healthy controls (35). A study looking at the cecal bacterial community in the mucosa of Chinese IBD patients found a higher abundance of the families *Rhodocyclaceae, Pasteurellaceae, Aeromonadaceae, Carnobacteriaceae, Flavobacteriaceae, Enterococcaceae, Erythrobacteraceae, Sphingomonadaceae*, and *Alcaligenaceae*, and a lower abundance of *Prevotellaceae* in CD patients; at the genus level *Prevotella, Coprococcus*, and *Blautia*, were decreased and *Chryseobacterium* and *Enterococcus* were increased in CD, compared to healthy controls (55). Another study found a decrease in the genera *Alistipes, Bacteroides, Dorea*, and *Parabacteroides*, and an increase in *Roseburia*, *Escherichia, Shigella*, and, interestingly, *Blautia* in IBD mucosal samples (35). In CD samples there was a decrease in *Coprococcus* and *Faecalibacterium* compared to healthy controls, but these were increased in UC samples (35). Other bacterial genera found the cecal mucosa of IBD patients have been identified including *Haemophilus, Klebsiella, Pseudomonas,*

Sporacetigenium, *Akkermansia*, and *Holdemania* in UC samples, compared to healthy controls (35). In comparison to the mycobiome of healthy controls, the genus *Malassezia* is the predominant fungi identified in the mucosal samples of patients with CD, along with *Candida* and *Cladosporium* (57, 58). In addition, there is also a reduced population of *Pichia*, *Fusarium*, and *Galactomyces* in CD compared to healthy individuals (57). Interestingly, the cecum is also the site where the appendix, a thin tube-like independent extension, attaches to the intestinal tract (59). The appendix is thought to serve as a reservoir for beneficial microbes in healthy individuals (60) or possibly pathobiont microbiota in IBD patients (59). Therefore, as the cecum is in closest proximity to the appendix, the microbiota may be significantly influenced by the appendiceal microbiota.

Ascending colon

Water and any remaining indigestible materials are further absorbed by the ASC, which solidifies food particles to form stool (61). ASC biopsies revealed the presence of bacteria from the phyla Thermotoga (formerly Thermotogae), Actinomycetota (formerly Actinobacteria), Bacillota (formerly Firmicutes), Pseudomonadota (formerly Proteobacteria), Bacteroidota (formerly Bacteroidetes), Mycoplasmatota (formerly Tenericutes), Cyanobacteria, Synergistota, Verrucomicrobiota, Deinococcus-Thermus, Aquificota, Lentisphaerota, Nitrospirota, Spirochaetota and Chloroflexota, along with the class Chlamydiae and Chlorobia, and the genera *Planctomycetes* and SRB (*Desulfovibrio*) (62, 63). A study by Chindi et al. sampled mucosal brushings from the ASC of male volunteers for the analysis of mucosa-associated microbiota using 16S sequencing (64). They found that the family level in the healthy ASC included bacteria *Bacteroidaceae, Bifidobacteriaceae*, and *Lachnospiraceae* which play an essential role in non-digestible carbohydrate fermentation and production of SCFAs (64). Mucosal samples of healthy individuals were also predominated by *Pseudomonadaceae*, *Xenococcaceae, Methylocytaceae, Bacillaceae*, and *Commonadaceae* families and the genera *Brevundimonas*, *Actinobacillus*, *Anaerostipes*, *Actinomyces, Peptostreptococcus, Parabacteroides, Pseudoxanthomonas, Eiknella, Streptococcus*, and *Roseburia* (37). A study looking at the mycobiome in the ASC mucosa of non-IBD individuals (preprint) found species from the order Malasseziales, and the genus *Phaeococcomyces*, along with species such as *M. restricta, Fungi (ASV09), Fungi (ASV06), P. paneum, Yamadazyma mexicana, C. tropicalis, C. albicans*, and *C. jadinii* (56). Currently, there are no published studies of the ASC virome.

In the ASC of CD patients, there is an increase in pathobiont bacteria at the family level, including *Methylocystaceae* and *Comamonadaceae*, along with the genera *Actinomyces*, *Peptostreptococcus*, *Parabacteroides*, with a lesser increase in the family *Bacillaceae*, along with the genus *Pseudoxanthomonas*, *Eikenella*, and *Streptococcus* (37). Crypt mucosal biopsies have also shown an increase in SRB (*Desulfovibrio*) in patients with UC compared to healthy individuals (63). *Desulfovibrio* are typically considered resident commensals in the microbiota of healthy individuals however, they can transition into opportunistic pathobionts and increase in abundance within a dysbiotic microenvironment, such as UC (65). However, no studies have been performed on the ASC mycobiome or virome (eukaryotic viruses or bacteriophages) in IBD patients.

Transverse colon

In addition to the absorption of water and nutrients, the main function of the transverse colon is sodium absorption (66). A study of mucosal biopsies from healthy Swedish volunteers displayed a high abundance of the classes Clostridia, Bacteroidia, Erysipelotrichia, along with the species *Bacteroides thetaiotaomicron*, *Bacteroides faecalis*, *Bacteroides uniformis*, and *Bacteroides caccae* in healthy individuals (67). In contrast, a smaller abundance of the classes Verrucomicrobiae and Coriobacteriia, and the genera *Desulfovibrio* and *Bacteroides* were also found in the healthy transverse colon (37, 67). In addition, carotenoid biosynthesis, which displays a protective role in the gut by regulating the intestinal immune responses, was found to be enhanced due to the presence of bacterial species *Bacteroides vulgatus*, *Akkermansia muciniphila*, *F. prausnitzii*, and *Parabacteroides distasonis* (67). Healthy mucosal biopsies display a high prevalence of other bacterial genera including *Clostridium, Enterococcus, Propionibacterium, Veillonella, Corynebacterium*, *Enterobacter, Klebsiella, Lactobacillus, Proteus*, and the species *E. coli* (68). A study by Kourkoumpetis et al. (preprint), mentioned earlier, also looked at the mycobiome community within the transverse colon of non-IBD individuals and found species from the order Malasseziales and species such as *Malassezia restricta, C. albicans, Malassezia (ASV13), Alternaria (ASV14), Bolbitius demangei, Saccharomycetales (ASV38)*, and *Fungi (ASV09)* (56). Another study also found the genera *Malassezia* in a healthy British cohort (69). There are no reports about the virome (eukaryotic viruses or bacteriophages) of healthy individuals in the transverse colon.

The *Bacteroides* genera are known to produce enzymes involved in tryptophan (Trp) metabolism which is reduced in IBD (67, 70). This suggests a potential depletion of *Bacteroides* in IBD patients (71). However, one species, *Bacteroides thetaiotaomicron* was found to be increased in mucosal transverse colon biopsies from CD patients (69). As seen in other segments of the colon, there is an increase in sulfur reducing bacteria (SRB; *e.g.*, *Desulfovibrio*) in transverse colon mucosal biopsies from patients with UC, compared to healthy controls (37). Another study showed an increase in the *Malassezia* genus in CD mucosa of British and Dutch cohorts compared to healthy controls (69). However, studies on the transverse colon bacteriome and mycobiome remain limited in IBD and there are no studies that have investigated the virome (eukaryotic viruses or bacteriophages) in the transverse colon of humans to date.

Descending colon

The descending colon serves as a conduit which holds feces until it is discharged into the rectum (61). In healthy individuals, the descending colon is thought to be dominated by beneficial microbes such as the *Lachnospiraceae* family members, which are essential for protein fermentation (72). The healthy descending colon contains the phyla Bacillota (formerly Firmicutes), Pseudomonadota (formerly Proteobacteria), Bacteroidota (formerly Bacteroidetes), Actinomycetota (formerly Actinobacteria), Mycoplasmatota (formerly Tenericutes), Thermotogota (formerly Thermotogae), Synergistota, Deinococcus-Thermus, Chloroflexota, Lentisphaerota, Nitrospirota Aquificota, Verrucomicrobiota, Acidobacteria, Spirochaetota, and Cyanobacteria (62). Furthermore, members of the class Chlamydiae and Chlorobia have been identified in healthy descending colon biopsies (37, 62). At the family level, bacteria identified in the mucosa of the descending colon include *Micrococcaceae, Bacillaceae*, and *Commonadaceae*, along with lesser abundant families such as *Pseudomonadaceae* (37, 62).

At the genus level, *Actinomyces*, *Roseburia*, *Akkermansia* and *Streptococcus* are commonly identified in the healthy descending colon mucosa, while *Parabacteroides, Shigella, Brevundimonas and Ruminococcus* are found in lesser abundance (37). Furthermore, the genera *Planctomycetes*, SRB (*e.g.*, *Desulfovibrio*), *Fusobacteria*, *Eiknella, Peptostreptococcus, Marinilactibacillus, Proteus*, and *Pseudoxanthomonas* have been identified in biopsies from healthy descending colon (37, 62). The descending colon mycobiome of non-IBD individuals includes a high abundance of *M. restricta, C. albicans, Fungi (ASV09), P. paneum, Cladosporiaceae (ASV16), Ascomycota (ASV01)* and, to a smaller extent, *Ramularia (SV10)* and *Malasseziales* sp (56). No studies have been published characterizing the descending colon virome (eukaryotic viruses or bacteriophages) in healthy individuals to date.

In IBD patients, there is an increase in bacteria from the family *Bacillaceae* along with genera SRB (*e.g.*, *Desulfovibrio*), *Eikenella*, *Streptococcus*, *Peptostreptococcus*, *Marinilactibacillus*, *Proteus*, *Parabacteroides*, and *Ralstonia* in CD patients as evidenced by sequencing of mucosal samples (37, 63). Similarly, there is also a slightly increased abundance of the family *Comamonadaceae* as well as the genera *Actinomyces*, *Fusobacteria*, and *Pseudoxanthomonas* (63). Again, to the best of our knowledge, no studies currently exist on the virome (eukaryotic viruses or bacteriophages) and mycobiome of the descending colon in IBD patients (73).

Sigmoid colon

The sigmoid colon is responsible for the transfer of stool into the rectum (61). Bacteria identified in healthy sigmoid colon biopsies were from the phyla Bacillota (formerly Firmicutes), Pseudomonadota (formerly Proteobacteria), Bacteroidota (formerly Bacteroidetes), Actinomycetota (formerly Actinobacteria), Mycoplasmatota (formerly Tenericutes), Thermotogorta (formerly Thermotogae) Synergistetota, Deinococcus-Thermus, Chloroflexota, Lentisphaerota, Nitrospirota, Aquificota, Acidobacteriota, Spirochaetota, Cyanobacteria, Verrucomicrobiota and Fusobacteriota, along with the class Chlorobia, and the genera SRB (e.g., *Desulfovibrio*) (62, 63). The sigmoid colon has a greater abundance of the genus *Bacteroides* compared to other sections of the colon (48, 74, 75). Other genera including *Veillonella, Clostridium, Corynebacterium, Sutterella*, *Lactobacillus, Klebsiella, Peptococcus* and *Enterobacter* were also found in high abundance in the mucosal analysis of non-IBD patients, along with other bacteria of smaller abundance such as *Enterococcus* and *Fusobacterium* (74–76). At the species level, high abundance of *E. coli, B. fragilis, F. prausnitzi*, *Haemophilus parainfluenzae*, and *Prevotella copri* were uncovered in the mucosal analysis of non-IBD patients (74–76). At the species level, *Methanobrevibacter arboriphilus, Methanobrevibacter smithii*, and *Methanosphaera stadtmanae* are found in high abundance in healthy individuals (77). In examining the healthy sigmoid mycobiome, *Candida*, *Pichia*, *Fusarium*, *Galactomyces, Malassezia*, and *Cladosporium* have been identified in the sigmoid colon mucosa (57). Again, no information could be found on the virome (eukaryotic viruses or bacteriophages) of the sigmoid colon in healthy individuals.

In IBD patients, there is a decrease in microbiota α-diversity [within each biopsy sample; Shannon index (4.25 vs 3.45) and Chao1 index (156.29 vs 98.67)] and a clear separation based on β-diversity analysis [between the biopsy samples; weighted and unweighted UniFrac with a PERMANOVA test ($p = 0.001$ for both)] in sigmoid colon mucosal biopsies, compared to healthy controls (76). Specifically, the inflamed mucosa in IBD patients was found to have a decrease in Bacteroidota (formerly Bacteroidetes) and Bacillota (formerly Firmicutes) and an increase in Pseudomonadota phyla, compared to healthy controls (76). When comparing IBD inflamed mucosa in flare to IBD patients not in flare, there was a decrease in *Ruminococcaceae*, *Lachnospiraceae*, and *Paraprevotellaceae* families, along with a decreased in genera *Bacteroides* and *Sutterella*, and species *B. fragilis, F. prausnitzii, H. parainfluenzae*, and *P. copri* (76). There is also an increase in the genera SRB (e.g., *Desulfovibrio*) in IBD biopsies (63). A study by Limon et al., analyzed the mucosal mycobiome and found that fungi belonging to the genera *Malassezia* and *Cladosporium* are found in higher abundance in patients with CD, compared to healthy controls (57). However, *Pichia*, *Fusarium*, and *Galactomyces* were found to be decreased in CD, compared to non-IBD along with a slight decrease in *Candida* as well (57). No studies have been conducted on the virome (eukaryotic viruses or bacteriophages) in the sigmoid colon to date.

Rectum

The main role of the rectum is to store feces until it is expelled by defecation (8). Healthy rectal biopsies and swabs contain phyla Fusobacteriota, Bacillota (formerly Firmicutes), Pseudomonadota (formerly Proteobacteria), Bacteroidota (formerly Bacteroidetes), Actinomycetota (formerly Actinobacteria), Mycoplasmatota (formerly Tenericutes), Thermotogota (formerly Thermotogae) Synergistota, Deinococcus-Thermus, Chlorflexota, Lentisphaerota, Nitrospirota, Aquificota, Acidobacteriota, Spirochaetota, and Cyanobacteria (35, 62). At the class level, the rectum contains bacteria from Cholorobia (62, 63). The rectal mucosa of healthy

individuals displays a predominance of families *Frankiaceae* and *Actinomycineae*, along with the presence of *Pseudomonadaceae, Spingomonadaceae, Lactobacillaceae*, and *Micrococcaceae* (35, 37, 62). Many bacterial genera have been identified in the rectal mucosa including *Dyadobacter, Curvibacter, Melissococcus, Variovorax, Larkinella*, *Actinomyces, Peptostrepococcus, Streptococcus, Marinobacter, Actinobacillus, Brevundimonas, Roseburia, Ruminococcus, Lachnospira, Lactobacillus, Allobaculum, Planctomyces, Novosphingobium, Methyloverstatilis, Skermenlla, Alistipes*, *Bacteroides*, *Blastia*, *Coprococcus*, *Dorea*, *Shigella*, *Oscillibacter*, *Parabacteroides*, *Pseudomonas*, *Subdigranulum, Desulfovibrio, Escherichia*, and *Faecalibacterium* (35, 37, 60, 62, 63, 67). Species specifically identified in the healthy rectal mucosa include *B. vulgatus, B. dorei*, and *B. nordii* (60, 67). The mycobiome of healthy individuals is thought to include a high abundance of *M. restricta, Malasseziales sp, C. albicans, Trichosporon* (*ASV34*), and *Cladosporiaceae* (*ASV16*) and to a smaller extent *Yamadazyma mexicana, Sporobolomyces johnsonii*, and *Alternaria* (*ASV14*) (Preprint data) (56). The healthy rectal mucosa includes eukaryotic viruses and bacteriophages such as *Coccolithovirus*, *Minivirus*, *Orthopoxvirus, Phix174microvirus, P1virus, T4virus, P22virus*, *Orthopneumovirus*, *Lambdavirus*, and *Caudovirales* (78). In contrast, in the IBD there is an increase in the phyla Pseudomonadota (formerly Proteobacteria) and Actinomycetota (formerly Actinobacteria) and a decrease in Bacteroidota (formerly Bacteroidetes), Bacillota (formerly Firmicutes), and Fusobacteriota in the rectal biopsies compared to healthy controls (35). At the genus level, *Pseudomonas* was found to be highest in the rectum, compared to the ileum, cecum, and mid-colon in CD and UC patients (35). Compared to healthy rectal samples, there was an increase in the genera *Blautia*, *Shigella*, and *Escherichia* in CD and UC patient samples, *Bacteroides* in CD patient samples only (35), and *Desulfovibrio* in UC biopsies (63). The genera *Akkermansia* and *Sporacetigenium* were only found in UC rectal biopsies (35). Furthermore, there was a decrease in *Coprococcus*, *Faecalibacterium*, *Parabacteroides*, and *Pseudomonas* in IBD (35). Further, swab cultures from IBD patients confirmed the presence of fungal species such as *C. albicans, Candida* sp. non-*albicans, C. lusitaniae*, and *Candida kefyr* (79). Another study using enrichment of virus-like particles of Chinese individuals showed that patients with UC have an increase in the abundance of bacteriopahges and eurkaryotic viruses from the genus *Phix174microvirus, P1virus, Lambdavirus, T4virus, P22virus*, and *Orthopneumovirus* in their rectum but a decrease in mucosa *Caudovirales* phage diversity and richness compared with healthy controls (78).

The strengths and weaknesses of commonly utilized methods of investigating gut microbiota

Currently, different techniques, ranging from traditional culturing methods to the most recent advanced metagenomic sequencing or next-generation sequencing (NGS) technologies, have been used to examine the microbiome in health and disease (80). However, much remains to be uncovered for a variety of reasons. Firstly, sampling issues not only cause difficulty in obtaining mucosal and luminal microbiota from select regions of the intestinal tract, but differences in collection and sample processing can also lead to variable results (3). Heterogeneity of the microbiome between and within patient's also results in variable findings with most studies ignoring these factors when publishing their results (*e.g.*, time of day, age, sex, stress, diet, host factors, and environmental factors) (3). The vast majority of studies publishing microbiota data have a higher proportion of Caucasian male individuals while ignoring most other factors including diet, which is largely why there is such discrepancy in the literature when describing what a healthy microbiome is. This includes the preparation protocols that patients undergo prior to colonoscopy and endoscopy, which can have significant effects on the microbiota profiles (4). In addition, the vast majority are obligate anaerobes, which poses a challenge during specimen collection, transport, and storage (81).

16S rRNA sequencing, 18S rRNA sequencing, whole genome shotgun metagenomics, and internal transcribed spacer (ITS)-next generation sequencing (NGS)-based amplicon sequencing have been utilized to explore uncultivated gut microbial communities (82). Many research studies have relied on 16S rRNA amplicon sequencing only, which, while more affordable and accessible, offers little to no functional information (46). While many studies claim to have examined the "microbiota" using this technique, it identifies only 16S ribosome containing bacteria and limited fungi, entirely ignoring the gut virome (46). As such, while the gut contains an abundance of viruses (primarily bacteriophages) and there is a well-recognized role of bacteriophages, eukaryotic viruses, and viral stage (*i.e.*, lytic or lysogenic) in UC (Caudovirales class and families *Virgaviridae*, *Anelloviridae*, *Circoviridae*, *Picobirnaviridae*) and CD (Caudiovirales class and families *Siphoviridae*, *Myoviridae*, *Podoviridae*), the profile of the virome is not well defined for the specific sub-organs of the intestine (83–87). This is important as bacteriophages drive horizontal gene transfer between bacteria in the gut, and likely contribute to shaping the microbiome and immune responses in IBD (85, 88, 89). Meta-genomics and meta-transcriptomics on regionally gathered samples may provide novel information due to their ability to provide more in-depth sequencing and functional information (90). However, these techniques require higher sample biomass and are more prone to human DNA and transcript contamination (particularly in biopsy samples), which can typically be overcome through the removal of host DNA prior to sequencing (23, 90).

Meta-genomic analysis of stool samples is more common for analysis of gut microbiota compared to mucosal microbiota samples because stool allows for easier longitudinal investigations of study participants by non-invasive sample collection (91). Whereas mucosal intestinal brushings and washes are more difficult to obtain as longitudinal sample collection is reliant on follow-up endoscopy, which could require the participants to undergo non-essential surgical procedures (91). Furthermore, mucosal microbiota samples are collected following endoscopy preparation which has significant impacts on the microbiota composition; therefore, while mucosal samples can better reflect the precise microbiota of a defined intestinal location, the stool (luminal) microbiota reflects a more natural

microbiota sample (92). Conversely, while much information about the human gut microbiota originates from analyses of stool samples, the stool microbiota is mixed with food residues and ingested microbial contamination, shedding intestinal mucosa, inhibitors that may impair PCR amplification/NGS procedures, and passing microbes (93). While the stool microbiota is easily accessible, it does not reflect the microbiota at the region-specific sites of the digestive tract, however it does represent the unique luminal microbiota community (94). Mucosa-associated communities are sampled either through mucosal washes/brushings or within biopsies (95, 96). Biopsy samples collected during endoscopy represent a mix of loose and strongly adherent mucosal layers (97). These samples may not fully represent the overall mucosa-associated microbiota, especially in patchy diseases like CD. Biopsy collection is also invasive and may contain high proportions of human DNA, which can interfere with microbial DNA analysis, limiting these samples to use of 16s rRNA methods primarily (98). Researchers have explored alternative methods for sampling low microbial biomass in the GI tract. One proposed approach involves using intestinal "lavage" samples or gut washes/brushings, which include fluid remaining in the bowel after bowel preparation (99). These gut wash samples contain a mix of luminal and loosely adherent mucosal communities. Gut washes are collected by flushing the mucosal surface with sterile saline and aspirating the resulting mixture of mucus, allowing for sampling of both the loose mucus layer interface (MLI) and the adherent mucosal layer (98, 100). MLI sampling has shown promise in providing sufficient material for multi-omic experiments and identifying novel taxa relevant to IBD (98, 101, 102). However, as mentioned earlier, colonoscopy preparation is known to impact gut microbiota composition (92) and significant differences have been noted between mucosal microbiota, biopsy microbiota, and stool microbiota composition (96, 103, 104). Another source of concern is the need for consistency of sample handling, often at the discretion of the study participants, which kits (e.g., OMNIgene and BIOME-Preserve) attempt to help researchers overcome (93). Evaluation of the traditional stool collection method versus OMNIgene GUT kit revealed a significant influence on microbiota composition, although the reliability of these kits is not yet fully confirmed and confirmation should be performed by users prior to proceeding with study recruitment (93). Moreover, the overall outcome of microbial samples, such as the genetic composition of gut microbes, is influenced by collection and storage conditions (105). For example, the composition of Bacillota:Bacteroidota (formerly Firmicutes:Bacteroidetes) phyla in fecal samples is significantly affected by storage temperature (106). Traditional at-home stool collection requires patients to freeze stool, although there is no way to accurately record patient adherence to appropriate collection methods. Recent studies have demonstrated that this can possibly be overcome with OMNIgene GUT kit as it claims to keep samples safe for up to 60 days at room temperature (107). While much progress is being made among studies when it comes to sample collection, handling, and microbiota identification methods, there remains considerable divergence of opinion on the optimal scientific strategy for examining the microbiome and the sub-biomes (bacteriome, mycobiome, virome) (108, 109). Findings of investigations employing different approaches are much more inconsistent for mycobiome and virome than studies of the bacteriome, for example (51).

Lastly, while identification methods have provided vast amounts of information about the microbiota to date, microbiome exploration is further hampered by live-model flaws (110). For example, it is difficult to recapitulate the precise microenvironment of the gut for the live culture of microbiota communities (111). Researchers utilize variable culture conditions such as aerobic culture versus anaerobic culture, different culture media that do not entirely represent the gut microenvironment, and culture methods which lack mechanical microenvironment factors such as fluid flow, villi architecture, and peristalsis (111). Many microbes are difficult or arguably impossible to culture in a laboratory setting, and some select microbe species are well known to outcompete their community members, producing a culture unlike that of the sample source (112). As a result, simulating the entire activities of the human digestive system and real-time observations of interaction dynamics are difficult (113). While mouse models are the traditional animal of choice in many research studies, the pig shares clear microbiome similarities over other non-primate models in digestive tract anatomy, physiology, and immune response when compared to humans (114, 115). In addition, pigs and humans share more non-redundant genes in their microbiome than other model organisms, such as mice (116). While of course, humanized axenic mouse models present another opportunity to investigate the impacts of the gut microbiome in health and disease (117).

Currently, there is a lack of published literature regarding both the mycobiome and virome (50, 118). Initially recovering the fungal DNA is troubled by the thick cell wall (119). Further, sequencing technologies have not been well-adapted to identify species in the mycobiome, with different fungal extraction methods from fecal samples potentially driving the variation in results between studies (119). As well, the ITS, which are the preferred method for identifying fungi, vary in length between species and quality reference databases are lacking, leading to a lack of confidence in identification (119). The gut virome is a relatively new field of study and most of the studies to date have been limited to fecal samples (84, 120). Further, there are limited complete viral genome sequences, including sequences for bacteriophages, compared to bacterial genomes, troubled by viruses lacking an evolutionary conserved marker (*e.g.*, 16S rRNA), leading to a significant volume of unidentified species during bioinformatics analysis of sequenced datasets (82, 84, 86, 120, 121).

Common considerations for microbiota research moving forward

The growing need to understand the regional composition of gut microbial communities as well as their significance to health and disease is an important step to enhance our understanding of the precise role of the gut microbiome in these settings. Recognizing the variability in microbiota communities housed in the various sub-organs of the intestinal tract, described in this review, future research should emphasize sampling different segments of the intestine and greater care should be taken with regard to communicating the precise location that samples (such as biopsy,

gut brushings and gut washes) were collected in published manuscripts. The growing need to bridge the gap in healthcare requires collaboration among medical laboratory personnel, clinicians, and researchers studying the gut microbiome. However, the invasive nature of sampling techniques poses challenges in recruiting participants and obtaining a large variety of clinical samples from each participant (122). As a result, low sample sizes can impact the statistical power and generalizability of research findings (123). To overcome these obstacles, careful research planning, collaboration with experts, and clear communication with participants are essential (124).

In conclusion, this review highlights some of the key differences identified to date in the communities of microbes that take residence in the various segments of the intestinal tract in both healthy individuals and patients living with IBD. With rising incidence rates of IBD globally and significant recognition of the role of the microbiome in IBD, it is more imperative than ever that we improve our understanding of the microbiome in health and disease through improved sample collection, processing, research techniques, and reporting in peer-reviewed manuscripts (125).

Author contributions

HA and SL conceived, developed, and coordinated the project. SL, AV, MB-J, HO, and HA drafted the manuscript. AV, HO, SL, and HA were responsible for the figure preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Elucidating the causal association between gut microbiota and intrahepatic cholangiocarcinoma through Mendelian randomization analysis

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Background: Intrahepatic cholangiocarcinoma (ICC) is an aggressive liver cancer with poor prognosis. The gut microbiota has been linked to ICC, but evidence for causality is lacking. Elucidating causal gut microbiota-ICC links could inform prevention and treatment strategies.

Materials and methods: We performed a bidirectional two-sample Mendelian randomization (MR) study to investigate causal associations between gut microbiota and ICC risk. Genome-wide significant single nucleotide polymorphisms (SNPs) associated with gut microbiota abundances were utilized as instrumental variables (IVs). Multiple methods assessed causality and sensitivity analyses evaluated result robustness. Bioinformatics analysis of genetic loci linked to gut microbiota and ICC examined potential mechanisms.

Results: Genetically predicted increases in *Veillonellaceae*, *Alistipes*, *Enterobacteriales*, and *Firmicutes* were suggestively associated with higher ICC risk, while increases in *Anaerostipes*, *Paraprevotella*, *Parasutterella*, and *Verrucomicrobia* appeared protective. Bioinformatics analysis revealed differentially expressed genes near gut microbiota-associated loci may influence ICC through regulating pathways and tumor immune microenvironment.

Conclusion: Our findings provide suggestive evidence for causal links between specific gut microbiota and ICC risk.

KEYWORDS

intrahepatic cholangiocarcinoma, Mendelian randomization, gut microbiota, bioinformatics analysis, tumor immune microenvironment

Introduction

Intrahepatic cholangiocarcinoma (ICC) is an aggressive cancer arising from biliary differentiation (Valle et al., 2021). Globally, it ranks as the second most prevalent form of liver cancer, accounting for roughly 15% of all primary liver malignancies (Massarweh and El-Serag, 2017). In 2022, approximately 30,000 lives are expected to be claimed by liver cancer, with ICC contributing to approximately 20% of these deaths and displaying a discouraging 5-year survival rate of less than 20% (Wang et al., 2022). ICC commonly arises in the context of persistent inflammation, which leads to cholestasis and damage to cholangiocytes. Established risk factors for ICC include hepatolithiasis, sclerosing cholangitis, viral hepatitis, obesity-related steatohepatitis (Khan et al., 2019; Labib et al., 2019). Surgery remains the only potentially curative option for ICC patients, although it is suitable for only 20–30% of individuals, and there is a high rate of tumor recurrence (Moris et al., 2023). Currently, comprehensive treatment strategies for ICC have extremely limited efficacy. This underscores an urgent need to elucidate novel mechanisms underlying ICC pathogenesis and progression, in order to develop more effective therapies against this aggressive malignancy.

Recent studies have revealed close associations between human commensal microbes and complex diseases such as mental disorders, cardiovascular diseases, and cancer (Xiao et al., 2022; Bendriss et al., 2023; Chen et al., 2023). Modulating the gut microbiota and enhancing gut barrier function has emerged as a promising new approach for preventing and treating certain diseases (Koning et al., 2023). The direct anatomical connection between the bile duct and intestinal tract via bile secretion pathways suggests potential relevance in elucidating specific associations between biliary tract disease and the gut microbiota. Cholangiocytes are continuously exposed to a wide range of commensal microbes and microbe-associated molecules that can profoundly influence the homeostasis of the cholangiocyte microenvironment. Several clinical studies have reported an increased presence of *Helicobacter species* in stool samples from patients infected with *Opisthorchis viverrini*. Specifically, they have observed the overexpression of *Helicobacter* genes, CagA and CagE. The proteins produced by these genes traverse the plasma membrane, initiating the phosphorylation of sarcoma family kinases, which may act as signaling molecules, thereby promoting fibrosis and inflammation of the bile ducts. *In vitro* experiments have demonstrated that co-culturing cholangiocarcinoma (CCA) cells with CagA-positive *Helicobacter species* leads to higher expression levels of the antiapoptotic factor Bcl-2 and the activation of mitogen-activated protein kinase and nuclear factor-kappa B (NF-κB) signaling pathways, resulting in the further proliferation of bile duct cancer cells. Additionally, numerous investigations have underscored the pivotal role of gut microbiota in upholding the integrity of the intestinal mucosal barrier and fostering the evolution and maturation of the immune system (Nagashima et al., 2023). However, the extent of research into the intricate interplay between gut microbiota and ICC is still rather limited. Consequently, a comprehensive understanding of the intricate mechanisms through which the gut microbiota exerts its influence on the genesis and therapeutic avenues of ICC warrant an in-depth exploration.

Increasing evidence highlights the interrelation between gut microbiota and ICC, however, establishing a definitive cause-and-effect relationship remains elusive. Further research is warranted to establish causal relationships and elucidate the underlying mechanisms by which the gut microbiota may influence disease, in order to provide novel insights into potential gut microbiota-targeted therapeutic strategies. The Mendelian randomization (MR) employs genetic variants derived from genome-wide association studies (GWAS) as instrumental variables (IVs) to infer the causal implications of environmental exposure on the observed outcomes. Since an individual's genotype is established at conception and remains fixed throughout their life, there is no potential for reverse causation or confounding bias to influence the relationship between genotype and disease (Bowden and Holmes, 2019). This unique characteristic of genetic makeup ensures that any observed associations between specific genetic variants and diseases are less susceptible to the issues of causality being misinterpreted or distorted by external factors. In this current investigation, a two samples MR analysis was conducted with the aim of probing the inherent causal connections between gut microbiota and the occurrence of ICC.

Materials and methods

Data sources

The GWAS data for ICC was sourced from a large-scale meta-analysis conducted by Jiang et al. (2021)[1] in a European population, which included 456,348 individuals, 11,842,647 variants and 2,989 binary traits. The analysis of the gut microbiota was performed by the Microbiome Genome (MiBioGen)[2] Consortium, encompassing a cohort of 18, 473 individuals (24 cohorts) from various countries with 122,110 loci of variation (Kurilshikov et al., 2021). The majority of participants exhibited European ancestry, with a total of 13,266 individuals (72.3%) included in this group. A comprehensive tally of 211 taxa was systematically classified across five distinct biological categories, encompassing 9 *phyla*, 16 *classes*, 20 *orders*, 35 *families*, and 131 *genera*. Notably, 15 unidentified taxa lacking definitive taxonomic classification were excluded from the analysis, as these ambiguous groups cannot provide meaningful biological insights into potential causal relationships with disease outcomes. Ultimately, this resulted in the inclusion of 196 well-defined taxonomic units (comprising 9 *phyla*, 16 *classes*, 20 *orders*, 32 *families*, and 119 *genera*) in the present study. The details of the data sources in present MR study are shown in **Table 1**.

Abbreviations: ICC, intrahepatic cholangiocarcinoma; SNPs, single-nucleotide polymorphisms; MR, Mendelian randomization; IVW, inverse variance weighted; MRE, MR egger; WMed, weighted median; WMod, weighted mode; SMod, simple mode; CI, confident interval; IVs, instrumental variables; LD, linkage disequilibrium; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

1 https://www.ebi.ac.uk/gwas/efotraits/EFO_1001961

2 https://mibiogen.gcc.rug.nl

Selection of IVs

Firstly, single nucleotide polymorphisms (SNPs) meeting the locus-wide significance criterion of $P < 1 \times 10^{-05}$ were meticulously chosen as prospective IVs associated with the gut microbiota. Secondly, to procure independent IVs from distinct loci, a linkage disequilibrium (LD) threshold of $R^2 < 0.001$ and a clumping distance of 10,000 kb were employed in the analysis of 1000 Genomes EUR dataset. Thirdly, strict adherence was maintained to the principle of selecting SNPs with consistent allele effects on both the exposure and outcome variables. Accordingly, palindromic SNPs devoid of A/T or C/G polymorphisms were deliberately excluded from the pool of IVs. Finally, we extracted the summary data of the IVs on the health indicator under study and used the F statistics ($F = \text{beta}^2/\text{se}^2$) to assess the strength of the IVs (Burgess and Thompson, 2011). A value greater than 10 was considered indicative of a powerful instrument.

Sensitivity analysis

The detection of heterogeneity between the two samples was carried out using Cochran's *Q*-test (Bowden et al., 2018), applying both the Inverse Variance Weighted (IVW) and MR Egger (MRE) methods (Burgess and Thompson, 2017). A significance level of $p < 0.05$ was considered as indicative of the presence of heterogeneity. The application of MR-PRESSO aimed to mitigate the influence of horizontal pleiotropy through identification and elimination of potential outliers (Verbanck et al., 2018). Furthermore, a leave-one-out sensitivity analysis was conducted to affirm the robustness of the findings, systematically excluding individual SNPs with each iteration. Scatter plots and funnel plots were generated to provide a visual interpretation of the outcomes derived from the MR analyses and to discern any potential outliers within the data.

MR analysis

A comprehensive MR analysis was conducted to ascertain the potential causal association between the gut microbiota and the susceptibility to ICC. This investigation encompassed a range of statistical methods, including the IVW, MRE, Weighted Median (WMed), Weighted Mode (WMod), and Simple Mode (SMod) methods. Consistent causal effects across multiple methods strengthen confidence in the results and conclusions. Contrasting methods also help pinpoint outliers and biases. Employing a range of methods enables assessing the robustness of the findings and ensures invalid instruments or pleiotropy do not lead to spurious conclusions. The entirety of data analyses was executed utilizing RStudio (Version: 2023.06.1 + 524) in conjunction with the Two Sample MR package (version 0.5.7). MR-PRESSO analysis was conducted employing the R package "MRPRESSO" (version 1.0).

Reverse MR

In this study, we will perform a reverse MR analysis employing a set of gut microbiota that have been established as causally related to ICC. The objective is to mitigate the potential influence of reverse causality, thereby enhancing the trustworthiness of our research findings.

Bioinformatics analysis

To explore the potential mechanisms underlying the role of gut microbiota in the development of ICC, we performed a bioinformatics analysis using RStudio and utilized online databases to identify genes enriched with strongly correlated genetic loci that are shared between gut microbiota and ICC. First, we utilized the NCBI database[3] to identify putative candidate genes corresponding to the SNPs found to be shared between gut microbiota traits and ICC. We conducted a comparative analysis of miRNA expression between ICC and non-cancerous tissues utilizing the GEPIA online database,[4] which is based on data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) project. In order to comprehensively investigate the role of differentially expressed genes in ICC, we utilized the STRING[5] database to analyze genes that are correlated with these differentially expressed genes for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. GSCA,[6] an integrated platform for genomic, pharmacogenomic, and immunogenomic gene set cancer analysis, offers a comprehensive resource. By merging clinical data and information about small molecule drugs, researchers can identify potential biomarkers and promising therapeutic agents, facilitating enhanced experimental planning and subsequent clinical trials. In our study, GSCA was instrumental in revealing the correlation between drug sensitivity and immune cell interactions associated with genes enriched in strongly correlated genetic loci shared between gut microbiota and ICC.

Results

Identification of IVs for MR analysis

The directed acyclic graph of the present study is depicted in **Figure 1**. Initially, a comprehensive set of 2591 SNPs, corresponding to 196 distinct taxonomic units of the gut microbiota, were extracted. These selections were made based on the stipulated threshold for locus-wide statistical significance ($P < 1 \times 10^{-5}$) and the LD threshold ($R^2 < 0.001$, with a clumping distance of 10,000 kb). It was observed that all IVs exhibited an F-statistic surpassing the threshold of 10, signifying the absence of substantial indications of weak instrument bias. Comprehensive details regarding the IVs across various categories of gut microbiota are meticulously presented in **Supplementary Table 1**.

3 https://www.ncbi.nlm.nih.gov/
4 http://gepia.cancer-pku.cn/
5 https://string-db.org/
6 http://bioinfo.life.hust.edu.cn/GSCA/#/

TABLE 1 Details of the genome-wide association studies and datasets used in our analyses.

Exposure or outcome	Sample size	Ancestry	Links for data download	PMID
Human gut microbiome	18,340 participants	Mixed (72.3% European)	https://www.ebi.ac.uk/gwas/	33462485
Intrahepatic Cholangiocarcinoma	456348 participants	European	https://www.ebi.ac.uk/gwas/	34737426

PMID, PubMed Identifier.

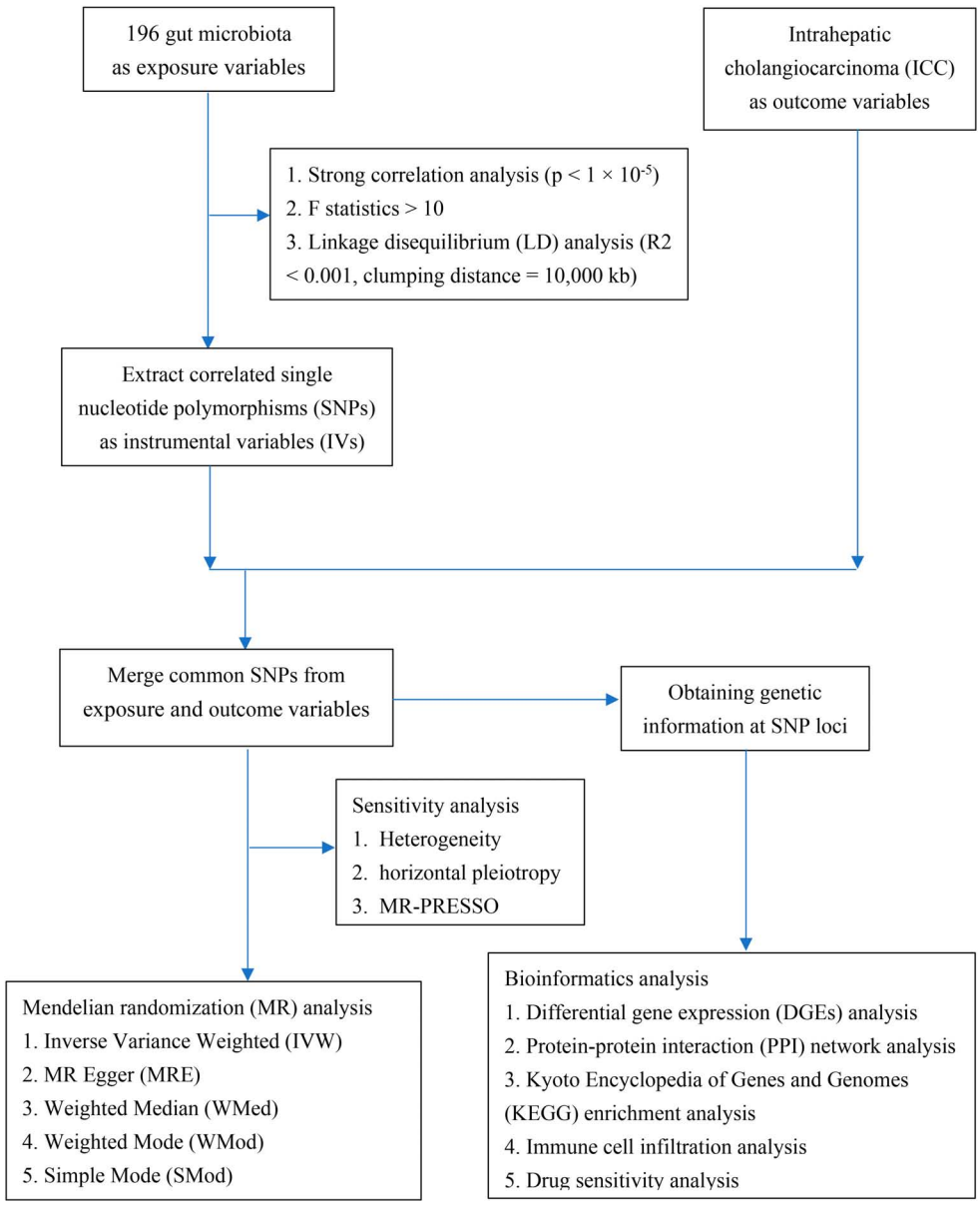

FIGURE 1
The directed acyclic graph of the present study.

Causal influence of gut microbiota on ICC

A two-sample MR analysis was systematically executed to assess the potential causal linkage between individual categories of gut microbiota and the occurrence of ICC. Using the IVW method, we found suggestive evidence for a causal association between genetically predicted increases in *Veillonellaceae* (OR = 3.582; 95% CI: 1.292–9.929; P = 0.014), *Alistipes* (OR = 5.648; 95% CI: 1.316–24.245; P = 0.020), *Enterobacteriales/Enterobacteriaceae* (OR = 5.632; 95% CI: 1.156–27.429; P = 0.032), and *Firmicutes* (OR = 3.545; 95% CI: 1.025–12.258; P = 0.046) and higher risk of ICC, while genetically predicted increases in *Anaerostipes* (OR = 0.135; 95% CI: 0.033–0.564; P = 0.006), *Paraprevotella*

(OR = 0.268; 95% CI: 0.107–0.672; P = 0.005), *Parasutterella* (OR = 0.323; 95% CI: 0.115–0.907; P = 0.032) and *Verrucomicrobia* (OR = 0.168; 95% CI: 0.048–0.588; P = 0.005) appeared to confer protective effects against ICC (Figure 2). Among the traits mentioned earlier, the *Enterobacteriales* and the *Enterobacteriaceae* were observed to belong to the same bacterial category, with identical IVs. Moreover, the MRE, WMed, SMod, and WMod yielded causal effect estimates exhibiting comparable magnitudes and directions to those obtained from the previously mentioned IVW method (Supplementary Table 2 and Figure 3). In our analysis, we have observed that while some p-values are below the conventional significance threshold of 0.05, the corresponding false discovery rate (FDR) values are above this threshold. Our rationale for choosing to interpret the results based on p-values stems from the specific context of our study. We recognize the importance of FDR correction in controlling for multiple comparisons, but believe that in this particular context, p-values remain a relevant and informative metric, especially when the assessment of significance requires a more conservative approach.

Sensitivity analysis

We applied Cochran's Q statistics utilizing IVW and MRE methodologies to assess heterogeneity. The outcomes revealed no significant heterogeneity among the IVs (all p-values > 0.05, Table 2). Additionally, both the MR-Egger intercept and the MR-PRESSO global test substantiated the absence of statistically significant directional horizontal pleiotropy (all p-values > 0.05, Table 2). Moreover, the leave-one-out analysis demonstrated the absence of influential IVs that would yield a noteworthy impact on the outcome if retained (Figure 4). These conducted sensitivity analyses, encompassing Cochran's Q statistics, MR-Egger intercept, MR-PRESSO global test, and leave-one-out analysis, collectively showcased the robustness of the two samples MR findings. Furthermore, the funnel plot and forest plots are presented to visualize a symmetrical pattern, indicating the reliability of the results (Supplementary Figures 1, 2).

The result of reverse MR analysis

Finally, we assessed the possibility of reverse associations between these bacterial traits and ICC through reverse MR analyses. A total of 15 IVs were identified based on the specified threshold for locus-wide statistical significance ($P < 1 \times 10^{-5}$), the LD threshold ($R^2 < 0.001$, with a clumping distance of 10,000 kb), and an F-statistic exceeding the threshold of 10 (Supplementary Table 3). Using the IVW method, we did not uncover statistically significant associations between ICC and any of these bacterial traits (Supplementary Table 4).

Differential gene expression analysis of near genetic loci

To acquire a more profound comprehension of the association between gut microbiota and ICC, we carried out an extensive analysis of the genetic loci linked to both gut microbiota and ICC (Supplementary Table 5). Analysis of RNA-seq data encompassing 36 ICC samples and corresponding paraneoplastic tissues sourced from TCGA and GTEx databases unveiled notable disparities in the expression of 17 genes. Among these genes, nine were associated with gut microbiota that promotes ICC, including *Veillonellaceae* (TECPR2), *Alistipes* (TOP1MT and CAPZB), *Enterobacteriales* (KCNQ1), and *Firmicutes* (AMBP, NID2, CAB39, SPEF2, and FRMD4A). Conversely, the remaining eight genes were linked to gut microbiota with inhibitory effects on ICC, including *Anaerostipes* (SOS1, PALLD, and REEP6), *Paraprevotella* (WWTR1), *Parasutterella* (CC2D2A), and *Verrucomicrobia* (CENPN, MTTP, and DST). All of these genes exhibited statistically significant differences (Figure 5). These differentially expressed genes may play a significant role in the development of ICC.

Protein-protein interaction (PPI) network and KEGG pathway analysis

To explore the differences in functionality and pathways between these two groups of genes, we conducted a comparative analysis involving the PPI network and KEGG pathway analysis. The PPI network of the differentially expressed genes is depicted in Figures 6A, B, and we further investigated the top 20 genes with the strongest interactions among them. These genes are divided into three clusters based on their functional associations. KEGG pathway analysis was also performed to predict the altered pathways linked to two groups of genes. The KEGG pathway analysis revealed that genes associated with gut microbiota promoting ICC formation were primarily enriched in pathways related to Gastric acid secretion, AMPK signaling pathway, and mTOR signaling pathway (Figure 6C). In contrast, genes linked to gut microbiota inhibiting ICC formation were predominantly enriched in pathways such as ErbB signaling pathway, Endocrine resistance, and EGFR tyrosine kinase inhibitor resistance, as indicated by our KEGG pathway analysis (Figure 6D).

Immune cell infiltration analysis and drug sensitivity analysis

To explore the role of differentially expressed genes in shaping the tumor immune microenvironment during tumor progression, we conducted an analysis of immune cell infiltration in ICC as outlined in scholarly literature. In the genome of gut microbiota that promotes ICC development, AMBP exhibits a significant positive correlation with Infiltration Score, significantly promoting the infiltration of Monocytes and Th17 cells in ICC tissues (Figure 7A). Conversely, SPEF2 displays a significant negative correlation with Infiltration Score and significantly inhibits the infiltration of Macrophages in ICC tissues (Figure 7A). In the genomic context of gut microbiota that inhibits ICC development, both REEP6 and MTTP exhibit a significant positive correlation with Infiltration Score, significantly promoting the infiltration of Mucosal-Associated Invariant T cells (MAIT), Macrophages, as well as Natural Killer (NK) cells, and Follicular Helper T

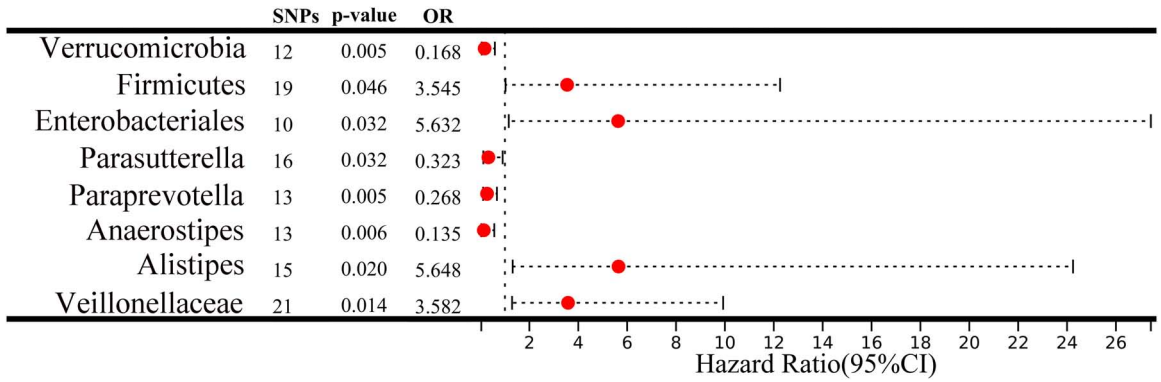

FIGURE 2
A forest plot depicts the associations between genetically predicted increases in 8 bacterial taxa and intrahepatic cholangiocarcinoma (ICC) risk. CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

TABLE 2 The heterogeneity and pleiotropy analysis of the MR study on gut microbiota and ICC.

Exposure	Outcome	Method	Heterogeneity		Horizontal pleiotropy		MR-PRESSO
			Q	Q_*p*-value	Egger intercept	*p*-value	*p*-value
Verrucomicrobia	ICC	MRE	4.795	0.904	−0.048	0.762	0.951
		IVW	4.892	0.936			
Firmicutes	ICC	MRE	16.151	0.513	−0.117	0.342	0.527
		IVW	17.108	0.516			
Enterobacteriales	ICC	MRE	5.600	0.692	0.231	0.433	0.725
		IVW	6.282	0.711			
Parasutterella	ICC	MRE	9.901	0.769	−0.062	0.638	0.805
		IVW	10.133	0.811			
Paraprevotella	ICC	MRE	10.402	0.495	−0.057	0.740	0.619
		IVW	10.518	0.571			
Anaerostipes	ICC	MRE	9.312	0.593	−0.095	0.526	0.679
		IVW	9.740	0.639			
Alistipes	ICC	MRE	8.587	0.803	0.203	0.373	0.808
		IVW	9.437	0.802			
Veillonellaceae	ICC	MRE	13.399	0.818	0.037	0.672	0.850
		IVW	13.584	0.851			

MR, Mendelian randomization; ICC, intrahepatic cholangiocarcinoma; Q, Cochran's *Q*-test; MRE, MR egger; IVW, inverse variance weighted.

(Tfh) cells in ICC tissues (Figure 7B). Conversely, CC2D2A and WWTR1 display a significant negative correlation with Infiltration Score and significantly inhibit the infiltration of Macrophages and NK cells in ICC tissues (Figure 7B). In order to investigate the drug sensitivity of genetic loci associated with both gut microbiota and ICC, we performed a drug sensitivity analysis utilizing the GDSC database (Figures 7C, D). In genes associated with gut microbiota that promote ICC, we found a positive correlation between KCNQ1 expression and sensitivity to YM155, QL-VIII-58, and Docetaxel; CAB39 showed a negative correlation with AT-7519; AMBP displayed a positive correlation with YM155, QL-VIII-58, THZ-2-102-1, Docetaxel, ZG-10, and AT-7519, while it exhibited a negative correlation with Erlotinib, Lapatinib, EHT 1864, FH535, and Pazopanib; NID2 had a significant negative correlation with Docetaxel and Pazopanib; TECPR2 showed a negative correlation with QL-VIII-58, Docetaxel, and ZG-10. TOP1MT displayed a negative correlation with THZ-2-102-1, ZG-10, AT-7519, EHT 1864, and FH535. In genes associated with gut microbiota that inhibit ICC, the expression of WWTR1, REEP6, CC2D2A, DST, and PALLD is positively correlated with sensitivity to AT-7519, Tubastatin A, AR-42, BHG712, BMS345541, BX-912, CAY10603, CP466722, GSK1070916, I-BET-762, JW-7-24-1, KIN001-260, Methotrexate, NG-25, NPK76-II-72-1, Navitoclax, PHA-793887, PIK-93, QL-XI-92, TG101348, THZ-2-102-1, TL-1-85, TPCA-1, Vorinostat, and XMD13-2, while it is negatively correlated with

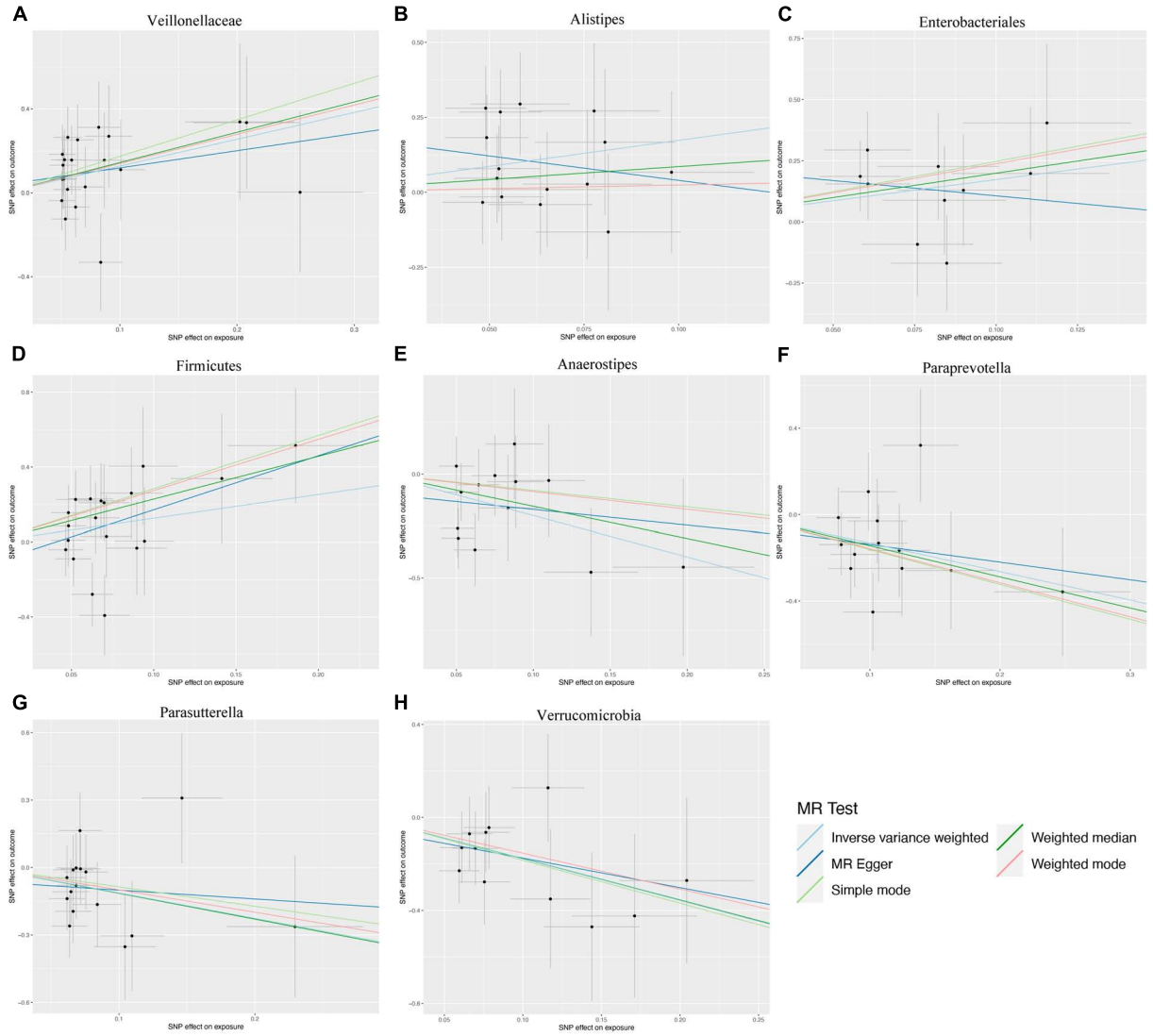

FIGURE 3
Scatter plots illustrating the genetic associations with eight bacterial taxa and intrahepatic cholangiocarcinoma (ICC). **(A)** Veillonellaceae, **(B)** Alistipes, **(C)** Enterobacteriales, **(D)** Firmicutes, **(E)** Anaerostipes, **(F)** Paraprevotella, **(G)** Parasutterella, **(H)** Verrucomicrobia.

17-AAG and Docetaxel. However, the results for SOS1 are the opposite.

Discussion

Intrahepatic cholangiocarcinoma is an insidious form of liver cancer that has been exhibiting a rising incidence globally (Clements et al., 2020). Despite accounting for only 15% of primary liver malignancies, ICC arising from the biliary epithelium represent a major and growing threat to public health worldwide (Beal et al., 2018; Banales et al., 2019; Khan et al., 2019). The human intestine harbors a complex gut microbiota comprising bacteria, fungi, archaea, viruses, and protozoa that plays a vital role in maintaining human health (Jandhyala et al., 2015). This gut microbiota exists in symbiosis with the gut mucosa and provides critical immunologic, metabolic, and gastrointestinal protective functions in healthy individuals (Wang Q. et al., 2023). A reduction in microbial biodiversity within the gut microbiota could elevate susceptibility to diverse diseases, including the development of malignancies such as cancers (Lee et al., 2023; Rajapakse et al., 2023). Similarly, there has been substantial research concerning the role of the gut microbiota in the occurrence and progression of ICC, as well as its implications for diagnosis and treatment strategies (Zhang et al., 2022; Pomyen et al., 2023). Nonetheless, a comprehensive causal relationship analysis concerning the interplay between gut microbiota and ICC remains lacking in the current literature.

Consequently, our study first conducted a two-sample MR analysis, utilizing summary statistics from GWAS, to investigate the potential causal link between gut microbiota and ICC. This analytical approach not only holds promise for effective ICC prevention and intervention strategies but also provides innovative insights into ICC pathogenesis through the perspective of gut microbiota.

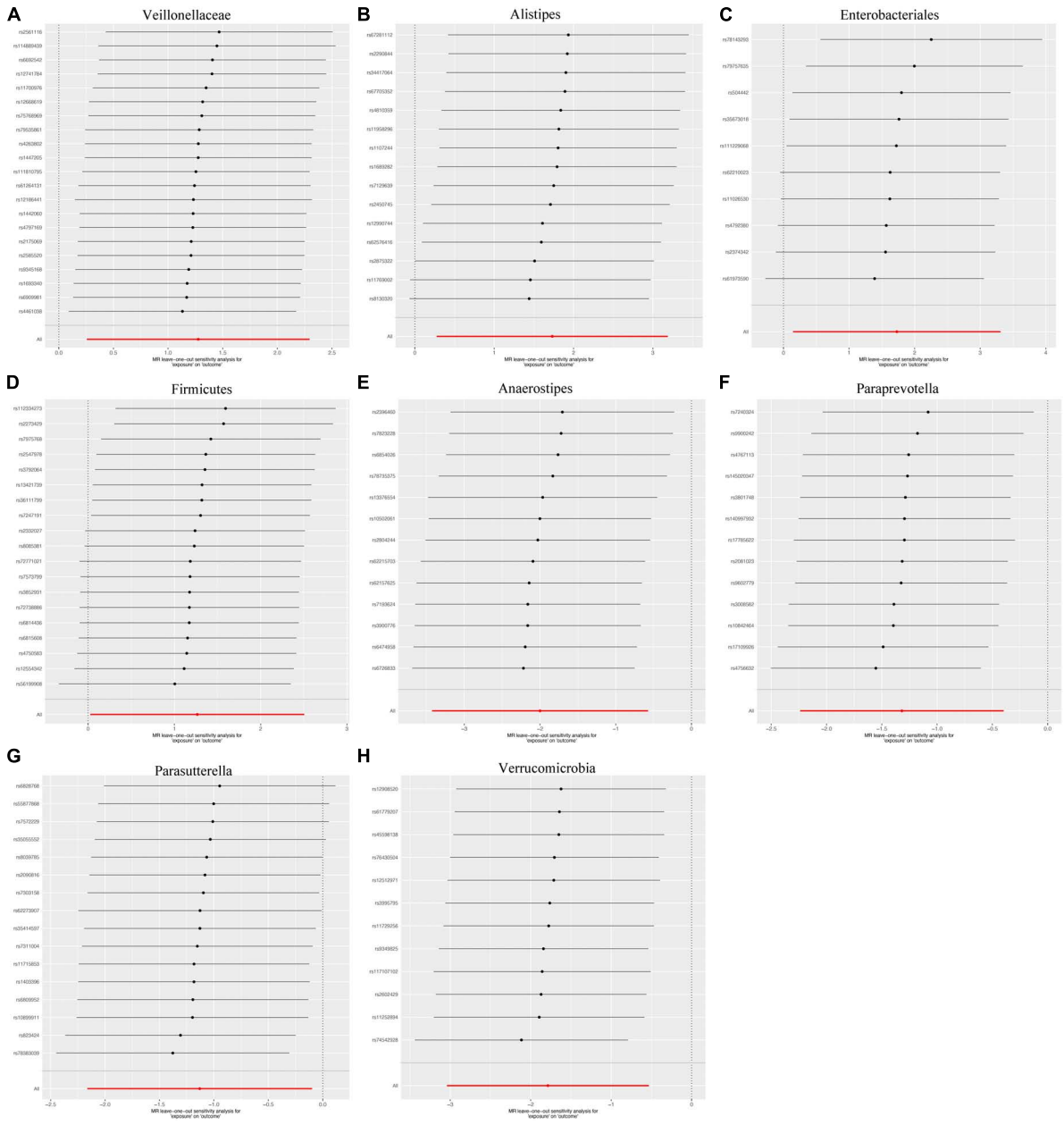

FIGURE 4
Leave-one-out the sensitivity analysis plot-the causal effect of eight bacterial taxa on intrahepatic cholangiocarcinoma (ICC). **(A)** Veillonellaceae, **(B)** Alistipes, **(C)** Enterobacteriales, **(D)** Firmicutes, **(E)** Anaerostipes, **(F)** Paraprevotella, **(G)** Parasutterella, **(H)** Verrucomicrobia.

Anatomically and physiologically, the hepatobiliary duct and the gastrointestinal tract are intricately interconnected, forming a 'gut-liver axis' that plays a pivotal role in regulating liver pathology and influencing both intrahepatic and systemic immune responses (Gopalakrishnan et al., 2018). Consequently, the gut microbiota assumes a significant role in modulating anti-tumor immune mechanisms. Impaired intestinal barrier function, disturbances in the intestinal environment, and reduced microbial diversity along the mucosal lining have been reported in various hepatobiliary duct disorders (Li et al., 2023; Rajapakse et al., 2023). Previous research has indicated a substantial increase in *Candida albicans* abundance in ICC cases, with alterations in its composition becoming more prominent as the TNM stage of ICC advances (Zhang et al., 2022). *Candida albicans* has been shown to expedite the progression of gastrointestinal cancer through the upregulation of matrix metalloproteinases synthesis, oncometabolite production, activation of pro-tumor signaling pathways, as well as the enhancement of prognostic marker genes associated with metastatic occurrences (Talapko et al., 2023; Wang X. et al., 2023). On the other hand, *Saccharomyces cerevisiae* has been identified as a microbial population that exerts a protective role against liver injury (Lai et al., 2009; Sivignon et al., 2015). It has demonstrated the potential to impede the progression of colorectal tumor growth by facilitating epithelial cell apoptosis, modulating intestinal immunity, and altering gut microbial composition (Li et al., 2020). However, it is noteworthy that *Saccharomyces*

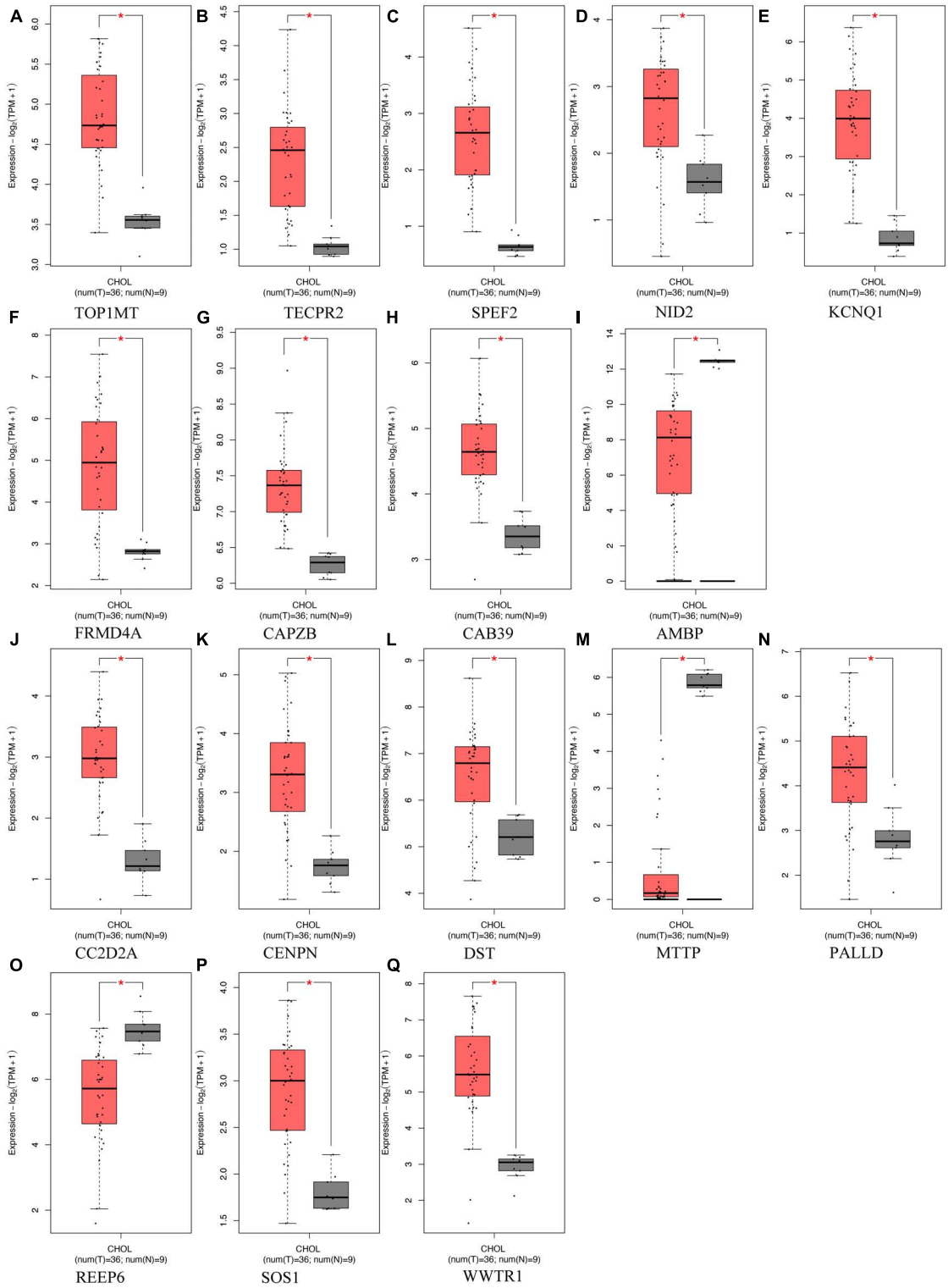

FIGURE 5
Differentially expressed genes located in proximity to genetic loci associated with both gut microbiota and intrahepatic cholangiocarcinoma (ICC) expression, comparing their expression in ICC tumor tissues (depicted in red) vs. normal tissues (depicted in gray). **(A-I)** Genes associated with gut microbiota promoting ICC formation, **(J–Q)** Genes linked to gut microbiota inhibiting ICC formation. *Indicates statistical significance at $p < 0.05$.

cerevisiae is notably diminished in patients with ICC (Zhang et al., 2022).

In the present two-sample MR study, we detected suggestive causal associations between eight specific bacterial genera and the risk of ICC. Our findings provide suggestive evidence for causal associations between genetically predicted increases in the abundances of *Veillonellaceae*, *Alistipes*, *Enterobacteriales*, and *Firmicutes* and an elevated risk of ICC. In contrast,

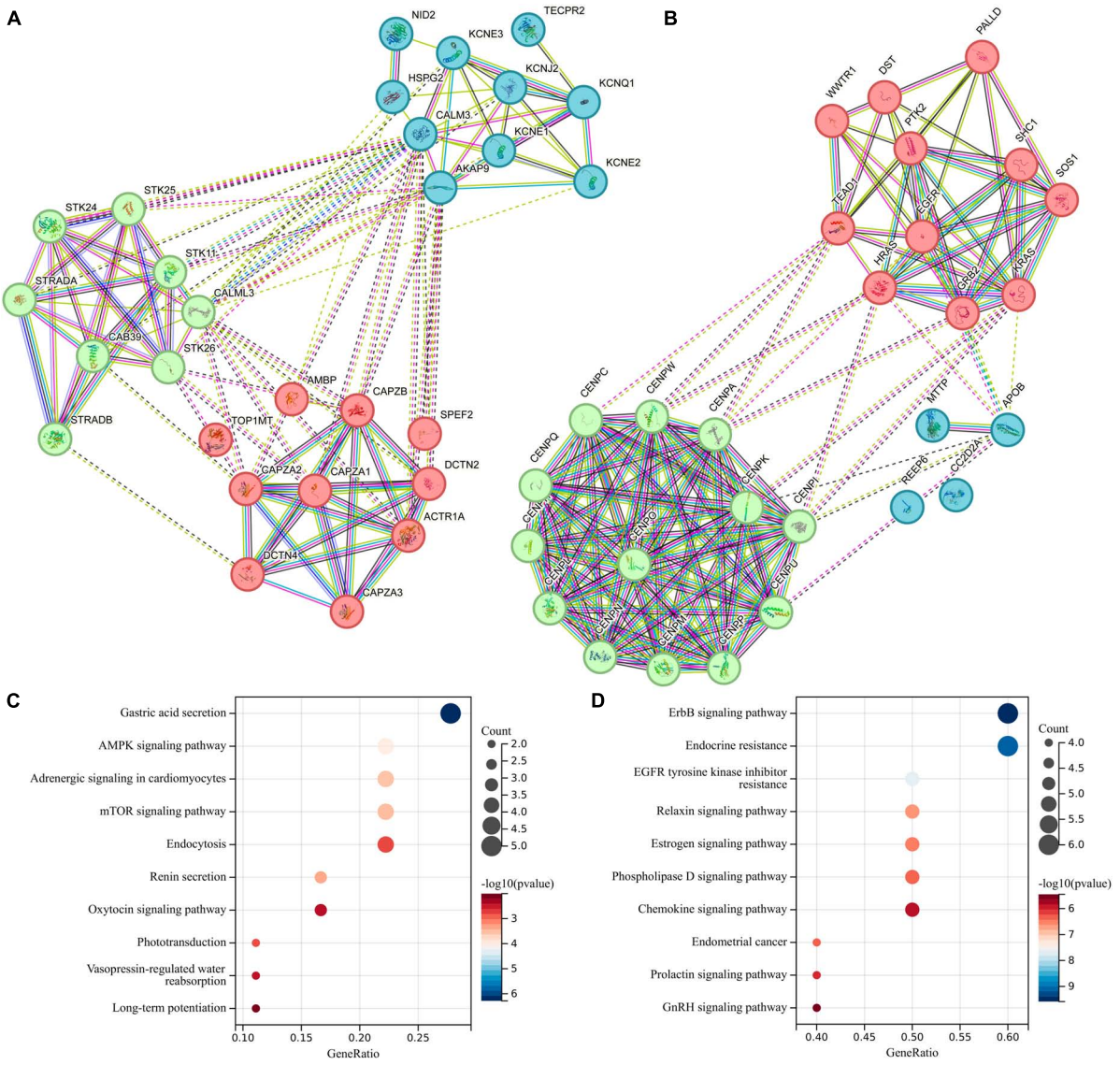

FIGURE 6
Protein-Protein Interaction (PPI) network and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for differentially expressed genes located in proximity to genetic loci associated with both gut microbiota and intrahepatic cholangiocarcinoma (ICC). **(A)** PPI network for genes associated with gut microbiota promoting ICC formation. **(B)** PPI network for genes linked to gut microbiota inhibiting ICC formation. **(C)** KEGG for genes associated with gut microbiota promoting ICC formation. **(D)** KEGG for genes linked to gut microbiota inhibiting ICC formation.

genetically predicted increases in the levels of *Anaerostipes*, *Paraprevotella*, *Parasutterella*, and *Verrucomicrobia* appeared to confer protective effects against ICC. We further constructed a schematic diagram illustrating the potential mechanisms by which these gut microbiota influences the formation of ICC (**Figure 8**).

Elevated abundances of *Veillonellaceae* have been identified in the intestinal microbiota of liver cancer patients (Ponziani et al., 2019; Ren et al., 2019). Increased Veillonellaceae may accelerate hepatic steatosis by producing carbohydrates and short-chain fatty acids. However, it can also generate carbon monoxide and hydrogen sulfide, which have toxic effects on both normal hepatocytes and bile duct cells, potentially contributing to the development of liver cancer (Fukui, 2019; Demir et al., 2020; Lee et al., 2023). Although *Alistipes* is predominantly found in the intestinal tract of healthy humans, it has also been isolated from the bloodstream, appendix, and abdominal regions, highlighting its potential opportunistic pathogenic role in human diseases (Shkoporov et al., 2015; Parker et al., 2020). The study also discovered that *Alistipes* could promote the development of colorectal cancer through activation of the interleukin-6/signal transducer and activator of transcription 3 signaling pathway (Feng et al., 2015). The levels of *Enterobacteriales* have been notably elevated in the intestines of patients with bacterial liver abscess, indicating a close association with its occurrence (Chen et al., 2018). A significant enrichment of *Enterobacteriales* was discovered to be associated with extended survival in cervical cancer patients undergoing chemoradiation (Sims et al., 2021). This enrichment could potentially augment the tumor infiltration of CD4 + lymphocytes, alongside activated subsets of CD4 cells expressing KI67 + and CD69 + markers throughout the course

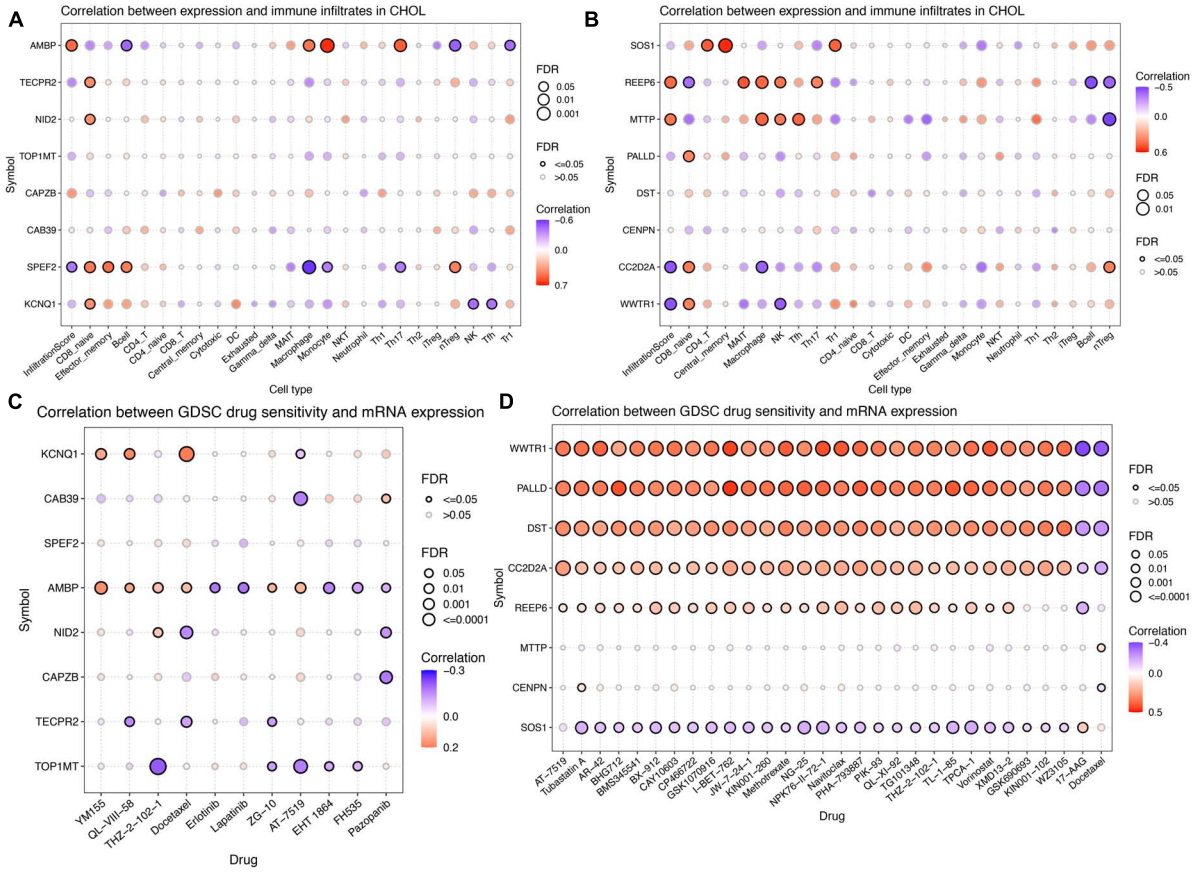

FIGURE 7
Immune cell infiltration analysis and drug sensitivity analysis for differentially expressed genes located in proximity to genetic loci associated with both gut microbiota and intrahepatic cholangiocarcinoma (ICC). **(A)** Immune cell infiltration analysis for genes associated with gut microbiota promoting ICC formation. **(B)** Immune cell infiltration analysis for genes linked to gut microbiota inhibiting ICC formation. **(C)** Drug sensitivity analysis for genes associated with gut microbiota promoting ICC formation. **(D)** Drug sensitivity analysis for genes linked to gut microbiota inhibiting ICC formation.

of radiation therapy (Sims et al., 2021). Yan et al. (2023) revealed dysbiosis of Firmicutes in patients with Hepatitis B Virus-Related Chronic Liver Disease (HBV-CLD), which was linked to negative regulation of liver function and T cell immune responses.

Recent studies have suggested that the gut bacterium *Anaerostipes* may confer protective effects against specific types of cancer. Notably, in murine models, a negative association between the abundance of *Anaerostipes* and the incidence of colorectal cancer is notably observed, and these protective mechanisms are believed to be linked to the production of butyrate and the enhancement of intestinal barrier function (Singh et al., 2022). This aligns with our research findings, as we have also discovered that *Anaerostipes* exerts an inhibitory role in the formation of ICC. An elevated prevalence of *Paraprevotella*, negatively correlated with hepatocellular carcinoma, may be attributed to potential mechanisms such as its anti-inflammatory properties and the inhibition of pro-carcinogenic microorganisms (Chen et al., 2015; Routy et al., 2018). Additionally, Pi et al. (2020) identified a notable presence of *Parasutterella* in colorectal cancer patients undergoing PD-1 treatment. Consequently, they propose that the PD-1/PD-L1 signaling pathway may modulate the metabolic activity of intestinal flora, including *Parasutterella*, thus enhancing immune surveillance against tumors (Pi et al., 2020). In a prior study conducted by Su et al. (2023) it was similarly observed that *Verrucomicrobia* maintained a robust negative correlation with ICC.

Our research has further revealed that *Veillonellaceae*, *Alistipes*, *Enterobacteriales*, and *Firmicutes* can promote the formation of ICC through the regulation of the AMPK signaling pathway and the mTOR signaling pathway. Meng et al. (2023) have similarly reported that alterations in the AMPK- mTOR signaling pathway can exacerbate the progression of disrupted energy metabolism, chronic inflammation, hypoxia, and cellular aging within the tumor microenvironment. These factors collectively promote the transformation of fatty liver into liver cancer. Our investigation has also unveiled that *Anaerostipes*, *Paraprevotella*, *Parasutterella*, and *Verrucomicrobia* inhibit the development of ICC through the regulation of the ErbB signaling pathway and the resistance to EGFR tyrosine kinase inhibitors. EGFR and ErbB belong to a family of cell membrane protein receptors capable of receiving external stimuli and initiating downstream signaling cascades, thus instigating a range of regulatory processes relevant to both physiological functions and pathological conditions. Studies have demonstrated that inhibiting EGFR can effectively impede hepatocellular carcinoma cell survival, migration, and invasion (Jin et al., 2021).

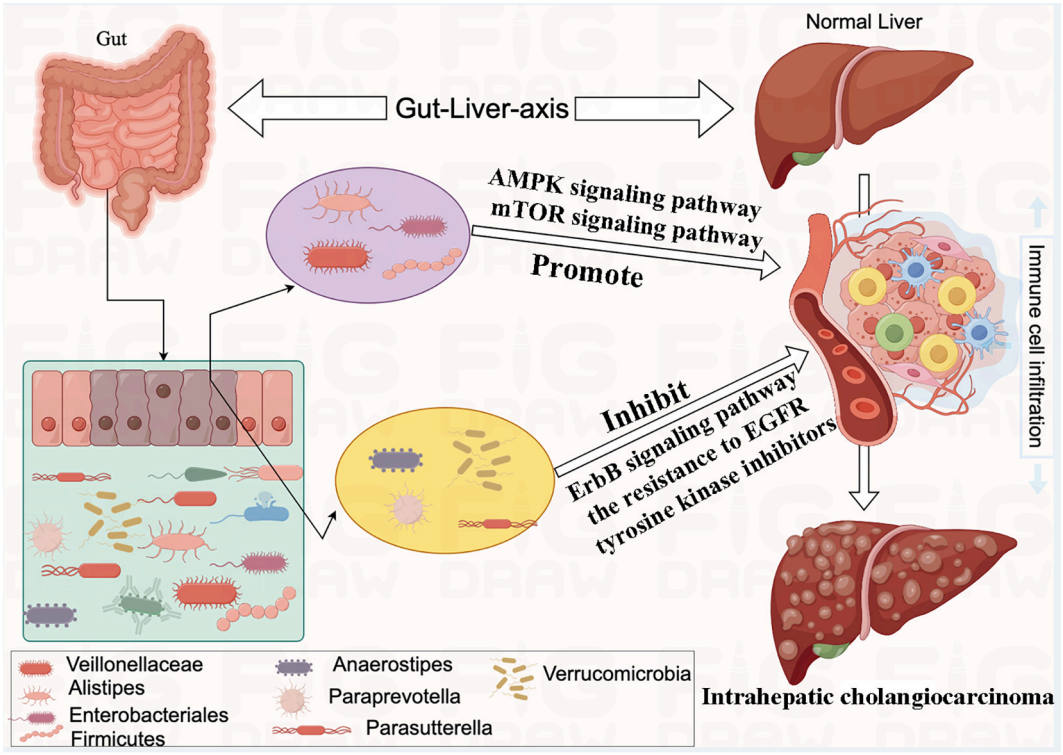

FIGURE 8
The mechanism of eight bacterial taxa and influencing the formation of intrahepatic cholangiocarcinoma (ICC).

The role of the immune system in immunosurveillance and its control over tumor growth is now firmly established. Tumor growth and progression are frequently linked to an impaired or fatigued anti-tumor immune response (Dumauthioz et al., 2018). Previous evidence suggests distinct gut microbiota can enhance systemic and antitumor immune response (Gopalakrishnan et al., 2018). In present study, we also investigated the influence of genetic loci on the gut microbiota regarding tumor immune cell infiltration. However, the underlying mechanisms of this phenomenon require further exploration.

In terms of ICC treatment, we also explored further. We identified a gene, SOS1 (rs6726833), located near a genetic locus associated with the gut bacterium *Anaerostipes*, which showed a negative correlation with ICC. The expression of SOS1 was positively correlated with sensitivity to 17-AAG. The HSP90 inhibitor 17-AAG effectively suppressed cell growth, leading to G2/M cell cycle arrest and the induction of apoptosis in cholangiocarcinoma cells. Zhang et.al demonstrated that the inhibition of HSP90 function by 17-AAG may offer a promising therapeutic approach for treating human cholangiocarcinoma (Zhang et al., 2013). We also identified WWTR1 (rs140997932), REEP6 (rs78735375), CC2D2A (rs35414597), DST (rs9349825), and PALLD (rs6854026), which are located near genetic loci associated with *Paraprevotella*, *Anaerostipes*, *Parasutterella*, *Verrucomicrobia*, and *Anaerostipes*. CAY10603 is a highly selective acetylcholinesterase inhibitor (AChE), and its sensitivity shows a significant positive correlation with the expression levels of these genes. CAY10603, through its selective inhibition of AChE and subsequent elevation of acetylcholine levels, activates surface receptors on cholangiocarcinoma cells, inducing apoptosis in tumor cells (Khorsandi et al., 2021).

While this study provides novel suggestive evidence for causal links between specific gut microbiota and ICC risk, an important limitation is that the GWAS data utilized was primarily from populations of European ancestry. Both the GWAS data on ICC risk and the gut microbiota GWAS data had samples that were over 70% European. Thus, the results may not be fully generalizable to non-European populations. Further research in more diverse populations is needed to determine if similar microbiota-ICC associations are present across different ethnicities. Additionally, environmental and lifestyle factors that influence the gut microbiota likely vary across populations, so replication in non-European cohorts is important. In summary, although this MR analysis provides initial evidence for potential microbiota-based prevention and treatment opportunities for ICC, confirmation in multi-ethnic studies is needed before translating findings to clinical practice globally.

Conclusion

The present two-sample MR study provides suggestive evidence for causal associations between specific gut microbiota and risk of ICC. Genetically predicted increases in *Veillonellaceae*, *Alistipes*, *Enterobacteriales*, and *Firmicutes* were associated with higher ICC risk, while increases in *Anaerostipes*, *Paraprevotella*, *Parasutterella*, and *Verrucomicrobia* appeared protective against ICC. Bioinformatics analysis revealed gut microbiota may influence

ICC development through regulating pathways like AMPK, mTOR, EGFR and tumor immune microenvironment. Further research is warranted to confirm the causality and elucidate mechanisms underlying the gut microbiota-ICC link, to inform potential microbiome-targeted prevention and therapeutic strategies.

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

ZC: Data curation, Investigation, Methodology, Software, Writing – original draft. WS: Investigation, Methodology, Software, Writing – original draft, Writing – review and editing. KC: Data curation, Software, Writing – original draft. CL: Data curation, Formal analysis, Software, Writing – original draft. XL: Methodology, Software, Supervision, Writing – review and editing. QL: Formal analysis, Project administration, Writing – review and editing.

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Conflict of interest

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

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Supplementary material

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SUPPLEMENTARY FIGURE 1
Forest plot of the causal effects of gut microbiota associated single nucleotide polymorphisms (SNPs) on intrahepatic cholangiocarcinoma (ICC). **(A)** Veillonellaceae, **(B)** Alistipes, **(C)** Enterobacteriales, **(D)** Firmicutes, **(E)** Anaerostipes, **(F)** Paraprevotella, **(G)** Parasutterella, **(H)** Verrucomicrobia.

SUPPLEMENTARY FIGURE 2
Funnel plot showing the relationship between the cause-effect of gut microbiota and intrahepatic cholangiocarcinoma (ICC). **(A)** Veillonellaceae, **(B)** Alistipes, **(C)** Enterobacteriales, **(D)** Firmicutes, **(E)** Anaerostipes, **(F)** Paraprevotella, **(G)** Parasutterella, **(H)** Verrucomicrobia.

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The effect of an exopolysaccharide probiotic molecule from *Bacillus subtilis* on breast cancer cells

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Introduction: Many well-known risk factors for breast cancer are associated with dysbiosis (an aberrant microbiome). However, how bacterial products modulate cancer are poorly understood. In this study, we investigated the effect of an exopolysaccharide (EPS) produced by the commensal bacterium *Bacillus subtilis* on breast cancer phenotypes. Although *B. subtilis* is commonly included in probiotic preparations and its EPS protects against inflammatory diseases, it was virtually unknown whether *B. subtilis*-derived EPS affects cancer.

Methods: This work investigated effects of EPS on phenotypes of breast cancer cells as a cancer model. The phenotypes included proliferation, mammosphere formation, cell migration, and tumor growth in two immune compromised mouse models. RNA sequencing was performed on RNA from four breast cancer cells treated with PBS or EPS. IKKβ or STAT1 signaling was assessed using pharmacologic or RNAi-mediated knock down approaches.

Results: Short-term treatment with EPS inhibited proliferation of certain breast cancer cells (T47D, MDA-MB-468, HCC1428, MDA-MB-453) while having little effect on others (MCF-7, MDA-MB-231, BT549, ZR-75-30). EPS induced G1/G0 cell cycle arrest of T47D cells while increasing apoptosis of MDA-MB-468 cells. EPS also enhanced aggressive phenotypes in T47D cells including cell migration and cancer stem cell survival. Long-term treatment with EPS (months) led to resistance *in vitro* and promoted tumor growth in immunocompromised mice. RNA-sequence analysis showed that EPS increased expression of pro-inflammatory pathways including STAT1 and NF-κB. IKKβ and/or STAT1 signaling was necessary for EPS to modulate phenotypes of EPS sensitive breast cancer cells.

Discussion: These results demonstrate a multifaceted role for an EPS molecule secreted by the probiotic bacterium *B. subtilis* on breast cancer cell phenotypes. These results warrant future studies in immune competent mice and different cancer models to fully understand potential benefits and/or side effects of long-term use of probiotics.

KEYWORDS

probiotics, breast cancer, exopolysaccharide, commensal bacteria, IKK beta, STAT1

Introduction

Breast cancer is the most common malignancy worldwide and is the second-leading cause of cancer-related death in the U.S (1, 2). Recent development suggests that microbial dysbiosis, an abnormal stage or maladaptation of the microbiome due to disturbances, may play a pathologic role in breast cancer (3). Numerous epidemiological studies in humans and mice both associated antibiotic use with increased breast cancer risk (4–11) while consumption of probiotics or prebiotics were associated with decreased breast cancer risk (12–17). In addition, well-known risk factors for breast cancer including age, high level of circulating estrogen, alcohol consumption, obesity, low physical activity, early menarche, high breast density, and periodontal disease have all been associated with changes in the microbiome (3, 18–23). Changes in microbial communities were observed in breast tissues, breast tumors, milk ducts, distal gut and the urinary tract (3, 19, 22, 24–28). The breast microbiome was altered in the presence of a benign or invasive breast tumor, presence of distant metastases, or treatment with chemotherapy (29). Specific microbial signatures further correlate with breast cancer subtypes as well as clinical outcome (30). Together, these data suggest that dysbiosis induced by various causes may contribute to breast cancer development and/or progression. Thus, it is not surprising that the microbiome has now been recognized as a part of the tumor microenvironment, believed to play important roles in immune suppression and/or supporting tumor growth (31).

Bacillus subtilis is a ubiquitous Gram-positive bacterium commonly included in commercial probiotic preparations. *B. subtilis* is also used to ferment a variety of non-dairy, traditional foods in many parts of Asia (32, 33). Although *B. subtilis* has not been studied in the context of breast cancer, it is known to secrete a variety of bioactive molecules, including antimicrobial peptides, polyketides, and bacteriocins (34). On the contrary, *B. subtilis* is the primary producer of the serine protease subtilisin, which depletes tumor suppressor proteins Deleted in Colorectal Cancer (DCC) and Neogenin in breast cancer cells, leading to enhanced migration and cancer development (35, 36).

B. subtilis can also form robust biofilms, which are an assembly of tightly associated bacteria encapsulated in a self-produced extracellular matrix (37). Exopolysaccharide (EPS), whether secreted into the extracellular matrix or remain bound to the cell surface, provides structural support to the extracellular matrix and is an important component in biofilm formation (38). The Knight laboratory has purified and studied exclusively EPS from *B. subtilis*. On western blots, EPS appeared as a single band at approximately 300 kDa, suggesting that EPS may be one large structure with structural analysis currently underway (39). EPS was found to have profound immunomodulatory properties via modulation of TLR4 signaling on myeloid cells (39–41). Systemic administration of EPS was found to be protective against a number of T-cell mediated inflammatory disease, including *C. rodentium* induced acute colitis, systemic *S. aureus* infection, house dust mite (HDM)-induced allergic eosinophilia, and acute Graft-versus-Host Disease (39–45).

Although a number of exopolysaccharides produced by various bacteria were tested for their anti-tumor activities *in vitro*, the majority of EPS studied were from probiotic lactic acid-producing bacteria (46–49). This study was the first to investigate effects of EPS treatment on breast cancer cells *in vitro* and *in vivo* across multiple cell lines and cancer-associated phenotypes. Our results demonstrate the complexity of EPS effects on breast cancer phenotypes from inhibiting bulk cell proliferation in the short-term to enhancing aggressive tumor phenotypes, leading to a pro-tumorigenic effect on cell-derived xenografts. Thus, bacterial molecules may influence growth properties of some types of breast cancer cells in a multifaceted manner, necessitating further studies to optimize the microbiome to benefit breast cancer prevention and treatment.

Materials and methods

Cell lines and culture conditions

MCF-7, T47D, MDA-MB-231, MDA-MB-453, MDA-MB-468, ZR-75-30, HCC1428, and BT549 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were grown in antibiotic-free Roswell Park Memorial Institute Medium (RPMI-1640, Thermo Fisher Scientific, Waltham, MA). RPMI-1640 was supplemented with 10% Fetal Bovine Serum (FBS, Gemini Bio Products, Sacramento, CA), 2mM L-glutamine (Thermo Fisher Scientific, Waltham, MA), 100μM non-essential amino acids (Invitrogen, Carlsbad, CA), and 1mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA). T47D cells were maintained in above RPMI media supplemented with penicillin (50 U/mL, Hyclone, Cat#SV30010) and streptomycin (50 μg/mL, Hyclone, Cat#SV30010) when culturing cells for long-term EPS treatments or injection in mice. All cell lines were authenticated by short tandem repeat allelic profiling (ATCC, Manassas, VA) and maintained at below 20 passages. All cells were regularly tested for mycoplasma contamination using the MycoSensor QPCR assay kit (Agilent Technologies, Santa Clara, CA). Cells were maintained in a 37°C incubation chamber at 95% O_2 and 5% CO_2.

Preparation of exopolysaccharide derived from *B. subtilis*

EPS was isolated from the *B. subtilis* DK7019 strain, provided by Dr. Daniel B. Kearns of Indiana University. This strain of *B. subtilis* was genetically modified (sinR::cat tasA::cat ΔpsgB Physpank-eps) to overproduce and secrete EPS under isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible conditions while lacking gamma-polyglutamic acid (γPGA). *B. subtilis* bacteria were cultured in 1.5% Luria Bertani broth (LB, Miller formulation) to stationary phase (OD=0.6 – 0.7), then grown for 4 hours on 1.5% Luria Bertani agar plates (LB, Miller formulation) with 0.1M IPTG. Bacterial supernatant was collected in a digest solution (0.45% NaCl, 50 μg/mL DNase and 30 μg/mL RNase) and centrifuged at 9000 x g at 20° C for 20 min, twice. Supernatant was incubated in 37°C water bath for 15mins, following by digestion with 40μg/mL proteinase K at 56°C overnight. EPS was precipitated with 3-4 volume of cold

ethanol at -20°C for at least 4 hours. The precipitate was pelleted by centrifugation at 13,700 x g at 4°C for 30 min, resuspended in an appropriate volume of water, and boiled at 95°C for 10 min. EPS was then purified by gel filtration on Sephacryl S-500 column (GE Healthcare). Carbohydrate-positive fractions were identified using a modified phenol sulfuric acid assay (50, 51). EPS-containing fractions were pooled and centrifuged through a Vivaspin column (Millipore, Germany) to isolate molecules larger than 30,000 kDa. Finally, EPS was dialyzed using a 10K MWCO Slide-A-Lyzer (Thermo Fisher Scientific, Waltham, MA) for 3 days, and filter sterilized using a 0.22μm PES syringe filter (Millipore, Germany). All EPS preparations were quantified for total carbohydrate concentration using a modified phenol sulfuric acid assay, assessed for the lack of protein and nucleic acid content by spectrometry, and tested for the ability to inhibit T47D proliferation prior to use.

Drugs, antibodies and reagents

Cerdulatinib and TPCA-1 were purchased from Selleck Chemicals (Houston, TX) and suspended in 100% DMSO to a stock concentration of 1mM and stored at -80°C. Stock solutions were diluted in medium to a working concentration of 1μM. Recombinant human IFNγ protein was obtained from CellGenix (Cat# 1425-050). Matrigel Basement Membrane Matrix was purchased from Corning (Tewksbury, MA, Cat# 354234) for mice experiments. Antibodies used for flow cytometry included: PE anti-human TLR4 antibody (Biolegend, Cat# 312805), PE mouse IgG2a Kappa isotype control (Biolegend, Cat# 400211), biotin anti-mouse IgG2a antibody (Biolegend, Cat# 407103), PE Streptavidin (Biolegend, Cat# 405203). Live/Dead Fixable Aqua Stain Kit was used purchased from Invitrogen (Cat# L34957). Western antibodies STAT1 (#9172), Phosphorylated STAT1 (Tyr701, #7649), STAT3 (#9132), Phosphorylated STAT3 (Tyr705, #9131), P38 (#9212), Phosphorylated P38 (Thr180/Tyr182, #4511), P65 (#4764), Phosphorylated P65 (Ser536, # 3033) Phosphorylated IkBα (Ser32, #2859), Phosphorylated IKKα/ß (Ser176/180, #2697), and RelB (#4922) were purchased from Cell Signaling Technologies (Danavers, MA). Loading control β-Actin (A5441) was purchased from Sigma Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibodies, including anti-rabbit (#7074) and anti-mouse (#7076) were purchased from Cell Signaling Technologies.

RNA interference and transfection

A pool of four siRNAs was purchased from Dharmacon GE Life Sciences (Lafayette, CO) for each of the following genes: IKK-beta (ON-TARGETplus SMART pool Cat# L-003503-00-0005) and P65 (ON-TARGETplus SMART pool Cat# L-003533-00-0005). Non-targeting scrambled control siRNA (SCBi) was purchased from Qiagen (Germantown, MD). The siRNAs were reconstituted in siRNA Diluent Buffer (10mM Tris-HCl, pH 8.0, 20mM NaCl, 1mM EDTA) at 10μM working solution and stored at -20°C. The transfection reagent Lipofectamine RNAiMAX (Cat# 13778150) was purchased from Thermo Fisher Scientific (Waltham, MA) and used at a ratio of 1:1 with 50nM of appropriate siRNA according to the manufacturer's protocol. 1.2 million T47D cells were plated in a 10-cm^2 tissue culture overnight. The iMAX solution was prepared by adding 60μL of RNAiMAX to 940μL of Opti-MEM (per transfection) in a 2.0 mL eppendorf tube. In parallel, 60μL of siRNA was added to 940μL of Opti-MEM per transfection in separate tubes. Solutions were incubated for 5 minutes at room temperature. After incubation, 1000μL of iMAX solution was then added to each siRNA condition and allowed to incubate for 20 minutes at room temperature. The adherent cells were then washed with PBS 2X and 9mL of RPMI was added to each plate followed by 2000μL of the siRNA + iMAX solution in a drop-wise fashion. Plates were gently swirled to mix the solution and incubated at 37°C for 48 hours before splitting into experimental groups.

Proliferation assays

Cells at a density of 50,000 were seeded in triplicate in a 6-well tissue culture plate and allowed to adhere overnight. Cells were treated with either 5μg/mL of EPS or equivalent volume of sterile PBS, and media was changed every other day. Separate wells were plated to count the number of live cells following treatment on day 2, 4 and 6. Briefly, cells in each well were trypsinized, individualized and 10μl of this cell mixture was added to 10uL of trypan blue. Live cells were counted using the Invitrogen Countess Automated Cell Counter (Hampton, NH).

XTT survival assay

Cells at a density of 2,500 were plated into a flat-bottom 96-well tissue culture plate to adhere overnight. Cells were treated with either PBS or increasing concentrations of EPS (0 – 10,000 ng/mL), with n=6 wells per treatment. Media was changed every other day. On day 6, media was aspirated and 150uL of working XTT solution containing 0.5 mg/mL XTT (Goldbio, Cat# X-200-100) and 3.75 μg/mL Phenazine methosulfate (Sigma, Cat # P9625-1G) in phenol-red free RPMI. Plate was covered in aluminum foil and incubated at 37° C for 2h. Absorbances at 450nm (A450) and 690nm (A690) were measured using a plate reader. To calculate corrected absorbance, we subtracted (A450 - A690) of each sample with that of a blank well containing XTT solution only. Percent proliferation was calculated as [(Corrected absorbance of EPS sample/Corrected absorbance of PBS sample)*100]. Data were graphed as log(EPS concentration) versus Percent Proliferation. The log(inhibitor) vs response – Variable slope (four parameters) model on GraphPad Prism (San Diego, CA) was used to determine the IC50 (inhibitory concentration at 50%).

Cell cycle analysis

Cells at a density of 100,000 were plated in triplicate in a 12-well tissue culture plate to adhere overnight. Cells were pretreated with stated concentrations of inhibitors or DMSO for 30min if applicable, following by treatment with either 5μg/mL of EPS or equivalent volume of sterile PBS for 24h. Cells, media, PBS wash, and trypsin solution were collected into a flow-activated cell sorting (FACS) tube and centrifuged at 500g for 5mins. The cell pellet was washed in 1mL cold PBS, centrifuged, and resuspended in 400uL of ice-cold PBS. To fix cells, 800uL of ice-cold 100% ethanol was added drop-wise under slow vortexing. Cells were stored at -20°C for at least 2 hours. On the day of analysis, cells were allowed to equilibrate to room temperature, resuspended and centrifuged at 500g at 4°C for 5min. Cells were washed once in 1mL cold PBS, and resuspended in 150μL of staining solution containing 50g/mL of propidium iodide (Sigma-Aldrich) and 10μg/mL of RNAse A in PBS. Tubes were covered with aluminum foiled and incubated for 1h at 37°C. Cell cycle analysis was conducted using LSRFortessa or FACSCantoII flow cytometers (BD Biosciences) according to the manufacturer's instructions (Cell Signaling Technology. Data was analyzed using the Cell Cycle model on FlowJo V10 (BD Biosciences).

Cell death analysis

Cells at a density of 100,000 were plated in triplicate in a 6-well tissue culture plate to adhere overnight. Cells were pretreated with either 5μg/mL of EPS or equivalent volume of sterile PBS for 3 days with no media change. When cells reached 80-90% confluency on the day of analysis, cells along with media, PBS wash, and trypsin solution were collected into a flow-activated cell sorting (FACS) tube and centrifuged at 1200 RPM at room temperature for 5mins. Cells were washed with cold PBS twice, and resuspended in 1mL of 1X binding buffer (10mM Hepes/NaOH, pH7.4, 140 mM NaCl, 2.5 mM CaCl2, 556454, BD biosciences, San Jose, CA). Live cells were counted using trypan blue exclusion and the Countess Cell Counter. Cells at a density of 100,000 were transferred to a new FACS tube, centrifuged and resuspended in 100μL of 1X binding buffer (BD Biosciences) containing 5μL of FITC-Annexin V (Cat# 556420, BD Biosciences, San Jose, CA) and 5μL of 7-AAD (BD Pharmingen, Cat#51-68-98E). Cells were incubated in the dark at room temperature for 15min, followed by addition of 400μL of 1X binding buffer (BD Biosciences). Cells were analyzed within 1 hour on the LSRFortessa or FACSCantoII flow cytometers (BD Biosciences) according to the manufacturer's instructions (BD Biosciences). Data was analyzed with gating strategies to exclude debris on FlowJo V10 (BD Biosciences).

Wound-healing migration scratch assay

Cells at a density of 200,000 were plated in triplicate in a 12-well tissue culture plate to adhere overnight. Cells were pretreated with stated concentrations of inhibitors or DMSO for 30min if applicable, following by treatment with either 5μg/mL of EPS or equivalent volume of sterile PBS for 2 days until confluent. Then cells were starved in media containing 3% FBS and drug treatments overnight. Media was aspirated and 3mL of PBS added to the well. Then a 10μL pipette tip was used to scratch the confluent monolayer of cells, creating a cross shape in the well. The scratches were immediately imaged at 2 locations of the cross at 10X objective under the microscope (0 h). Media was changed to contain 3% FBS with continued treatment of either EPS or PBS. At 24h and 48h post-scratch, media was changed and scratches were imaged at the same location relative to the cross shape. Migration rate was quantified as open gap area using ImageJ according to Venter and Niesler protocol (52). Percent wound closure was calculated as [100 - (Gap area at 24h or 48h/Gap area at 0h)*100].

Xenograft tumor growth

All animal study protocols were approved by Loyola University's Institutional Animal Care and Use Committee. T47D cells were expanded in 150cm^2 tissue culture treated flasks and treated with 5μg/mL EPS or equal volume of PBS for 5 days. Then 40 million EPS or PBS-treated T47D cells were transferred to a Nunc Cell Factory System (Thermo Scientific, Cat# 140004TS) with continued treatment for another 3 days. On collection day, cells were trypsinized and resuspended in Matrigel® Matrix Basement Membrane Phenol-Red Free (Cat# 356237, Corning, Bedford MA) to a concentration of 4 million live cells per 100μL of Matrigel. For EPS-treated cells, EPS was also added to the Matrigel : Cell suspension to an estimated concentration of 300μg/mL. Then 100μL of Matrigel : Cell suspension was injected bilaterally into the fourth mammary fat pads of 9-10 weeks old, female, ovariectomized Foxn1 nu/nu athymic nude mice (Envigo, IN). Mice were also implanted with a 0.3cm silastic capsule containing 17β-estradiol for a constant release of 83-100pg/mL as previously described (53). The estrogen capsule was replaced after 8 weeks. Each mouse monitored by tagging the ear with a number. Four mice per group were implanted with EPS or PBS-pretreated cells followed by intraperitoneal injection with respective 50μg EPS or 100μl PBS 3 times/week. Tumor area (length x width) was measured weekly using Vernier calipers. Mice were euthanized on day 94 and tumors were imaged, weighed, and frozen at -80 °C. Tumor growth as tumor weight and tumor volume ($V=0.5\times L\times W^2$) were calculated and graphed.

For the experiment with NOD.SCID mice, 100 million T47D cells per condition were grown and pretreated *in vitro* with PBS or EPS for 8 days as above. On collection day, EPS-treated cells were resuspended in Matrigel with EPS added to a concentration of 80μg/mL. Four million cells were injected bilaterally into the fourth mammary fat pads of 9-10 weeks old, female, ovariectomized NOD.SCID mice (Envigo, IN). Five mice were used for PBS group and seven mice for EPS group. Each mouse was injected (i.p.) with 25μg EPS or 100μl PBS 3 times/week and tumor area

(length x width) was measured weekly using Vernier calipers. Mice were euthanized on day 87 to assess tumor burden.

RNA sequencing and pathway analysis

T47D cells ($4x10^5$), MCF-7 cells ($1x10^6$), MDA-MB-231 cells ($2x10^5$), or MDA-MB-468 cells ($8x10^5$) were plated in $10cm^2$ dishes overnight. The following day, cells at <70% confluence were treated with either 5µg/mL EPS or equal volume of PBS and incubated at 37°C for 24 hours. Each condition was performed in 3 biological replicates. Total RNA was extracted using the RNeasy mini Kit (Qiagen, Germantown, MD) and sent to Novogene for RNA-library preparation and RNA-sequencing. Novogene performed the initial analysis. Additional analysis was conducted on differentially regulated genes using the Metascape pathway analysis software (https://metascape.org), with pathway enrichment being plotted by p-value for the number of genes in a given Gene Ontology (GO) pathway.

Statistical analysis

Experiments were conducted in triplicate and repeated at least three independent times, with results reported as Mean ± SEM. Statistical analysis was performed and figures were generated using Prism Version 9 (GraphPad Software). A two-sided Student's *T*-test was used to compare 2 groups, and P-values <0.05 were considered statistically significant. An ANOVA with a post- hoc Tukey's test was used to compare multiple groups. For mice studies, tumor volumes were calculated as $[(LxW^2)/2]$. Linear regression analysis was performed and the slope of tumor growth over time for each treatment group was used to compare the growth rates between treatment groups.

Lysate preparation and western blot analysis

Mammosphere forming assay

Reverse transcription and real-time polymerase chain reaction

See Supplementary Materials and Methods for full descriptions.

Results

The effect of EPS on proliferation of breast cancer cells

Various exopolysaccharides produced by bacteria display anti-cancer activities *in vitro* (54–57). EPS produced by *B. subtilis* acts on myeloid cells to inhibit T-cell proliferation (40, 42, 44, 45). Thus, we hypothesized that EPS would inhibit the proliferation of breast cancer cells. We measured proliferation of a panel of breast cancer cells representing different subtypes (ER+PR+, HER2+, ER-HER2-, ER+HER2+) in response to PBS or 5µg/mL EPS in a time-dependent manner. Of the eight cell lines tested, four were inhibited by EPS (T47D, HCC1428, MDA-MB-453, and MDA-MB-468) (Figure 1A), while the rest were unresponsive (MCF-7, ZR-75-30, MDA-MB-231, and BT549) (Figure 1B). The sensitivity to EPS seemed to be independent of breast cancer subtypes at least based on these cell lines. To determine if sensitivity to EPS was concentration dependent, cells (T47D, MDA-MB-468, and MCF-7) were treated with increasing concentrations of EPS for 6 days, and we found that the proliferation of both T47D and MDA-MB-468 cell lines was inhibited in a concentration-dependent manner, while the MCF-7 cell line was unaffected (Figure 2A). Previous studies showed that TLR4 was required for biological effects of EPS on immune cells (39–41). To investigate the role of TLR4 on EPS-

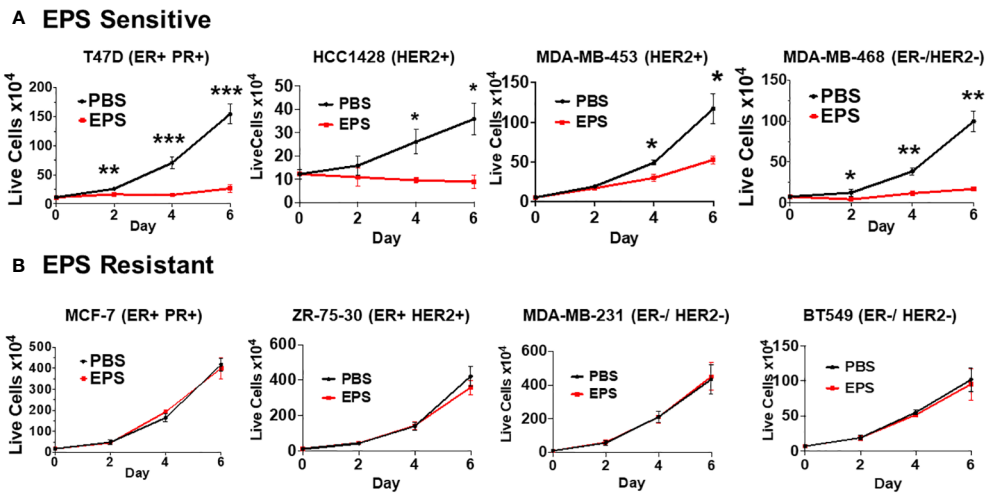

FIGURE 1
Sensitivity of different breast cancer cell lines to EPS. The proliferation rates for 8 breast cancer cell lines were measured by treating cells with PBS or 5 µg/mL EPS everyday for 6 days. **(A)** T47D, HCC1428, MDA-MB-453, and MDA-MB-468 cells were treated with PBS or EPS for 6 days. Live cells were counted and plated at day 0, and then following treatment at day 2, 4, and 6. **(B)** MCF-7, ZR-75-30, MDA-MB-231, and BT549 cells were treated and live cells counted as described in **(A)**. Data are mean values ± SEM of 3 independent experiments performed in triplicate. Statistical significance was calculated using a Student's T-test. * P ≤ 0.05, **P ≤ 0.01 *** ≤ 0.001.

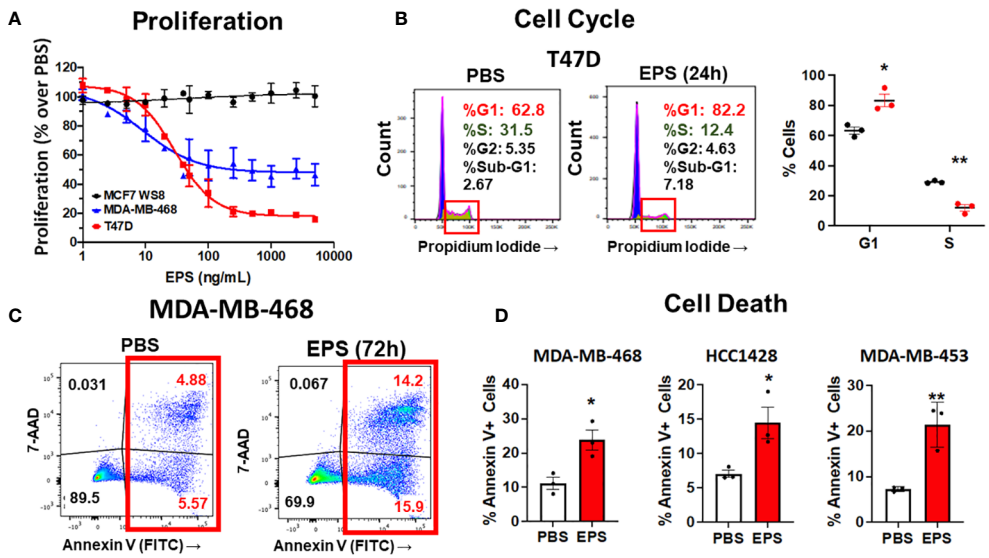

FIGURE 2
Analysis of cell cycle arrest and cell death in EPS-treated cells. **(A)** Proliferation of three breast cancer cell lines grown in medium containing PBS or increasing concentrations of EPS for 6 days. Proliferation with the PBS-treated group set at 100%. Data mean values ± SEM of 3 independent experiments. **(B)** Cells were treated with PBS or 5μg/mL EPS for 24h, stained with propidium iodide, and cell cycle analysis was performed. Representative flow cytometry plots (left) with graphical summary of 3 independent experiments performed in triplicate (right): Data are mean values ± SEM. Statistical significance was calculated using a Student's *T*-test * $P \leq 0.05$, ** ≤ 0.01. **(C)** Flow cytometric analysis of cells treated with PBS or 5μg/mL EPS for 3 days and stained with Annexin V and 7-AAD. **(D)** Percent Annexin V^+ cells as mean ± SEM of 3 independent experiments performed in duplicate for MDA-MD-468 and HCC1428 cells and as mean ± SD of only one experiment performed in triplicate for MDA-MB-453 cells. Statistical significance was calculated using a Student's *T*-test. * $P \leq 0.05$, ** $P \leq 0.01$.

mediated growth inhibition of breast cancer cells, we utilized a CRISPR/Cas9 knockout approach to delete TLR4 in T47D cells. Flow cytometry showed that TLR4 is undetectable in T47D wild type, Cas9, or knockout cells (Supplementary Figure 1A). DNA sequencing confirmed that both alleles of the TLR4 gene had an insertion or a deletion (Supplementary Figure 1B), and yet EPS decreased proliferation of both wild type and TLR4 knockout cells (Supplementary Figure 1C). These results suggest that EPS-mediated inhibition of breast cancer proliferation is independent of TLR4.

Cell cycle progression and cell death

Since EPS inhibited cell proliferation of some types of breast cancer cells, we hypothesized that EPS induced cell death and/or cell cycle arrest in the responsive breast cancer cells. To test these possibilities, each of the four responsive cell lines (T47D, MDA-MB-468, HCC1428, and MDA-MB-453) was treated with PBS or 5μg/mL EPS for 24 hours and assessed for cell cycle progression and cell death. EPS increased the percentage of T47D cells in the G1/G0 phase and decreased cells in the S phase (Figure 2B), but had little effect on cell death (Supplementary Figure 2). The other three cell lines (MDA-MB-468, HCC1428, MDA-MB-453), displayed minimal change in cell cycle progression in response to EPS (Supplementary Figures 3A, 4A, 5A). However, EPS increased Annexin V+ MDA-MB-468 cells by 2 to 3-fold (Figure 2C) and similar results were observed for HCC1428, MDA-MB-453, and MDA-MB-453 cells (Figure 2D and Supplementary Figures 4B, 5B). Because of the heterogeneity of breast cancer cell lines, it was not surprising that EPS induced cell cycle arrest in some cell lines and cell death in others.

Survival of breast cancer stem cells and cell migration in response to EPS

A thorough investigation of any new cancer agent should include assessment not only of proliferation, but also of other cancer-associated phenotypes, including survival of cancer stem cells and cell migration. We tested if EPS affected breast cancer stem cells (BCSCs), or tumor-initiating cells, a small population of cells within bulk tumors displaying stem-cell properties. These cells are capable of self-renewal, differentiation along mammary epithelial lineages, proliferation, and clonal nonadherent spherical clusters (mammosphere formation) (58, 59). Due to these stem-like characteristics, BCSCs are thought to be responsible for treatment resistance, recurrence and metastasis (60–68). We utilized the mammosphere formation assay, which assesses BCSCs based on their ability to survive and proliferate in a 3D culture, and tested if EPS altered the survival of BCSCs. Surprisingly, pretreatment of bulk T47D cells with EPS increased mammosphere forming efficiency by nearly 2 fold compared to control PBS-treated cells (Figure 3A).

The wound-healing scratch assay was performed on T47D cells to measure their migration capacity in response to EPS, and these cells showed increased cell migration compared to PBS-treated cells (Figure 3B). These results suggest that although EPS induces G0/G1

cell cycle arrest of T47D cells, it paradoxically enhanced survival of BCSCs and increased their rate of migration.

Effect of EPS on growth of T47D tumor xenografts in athymic, nude and NOD/SCID mice

To determine the physiological role and implication of long-term EPS treatment on breast tumor growth, we first utilized an orthotopic xenograft model in which ER+ T47D human breast cancer cells were injected into the mammary fat pads of female athymic, nude mice. Mice from each group (N=4) were treated with PBS or 50μg EPS via intraperitoneal (i.p) injection thrice weekly. EPS treatment significantly increased the rate of tumor growth in nude mice, although it did not significantly increase the mass of tumors (Figure 3C). In numerous other studies, EPS has been shown to induce an anti-inflammatory state, and we considered the possibility that EPS indirectly promotes tumor growth by inducing a tolerogenic immune state. Although nude mice lack a functional thymus, they have a functional innate immune compartment as well as extrathymic T cell development. As EPS is known to impact myeloid cells (39–41), we tested the effect of EPS on tumor growth using a more immunocompromised mouse model, NOD/SCID that lacks innate immune function. In experiments similar to those with the athymic, nude mice, EPS treatment increased both the rate of tumor growth and tumor mass (Figure 3D). These data suggest that increased tumor growth of T47D cells following long-term and frequent exposure to EPS is possibly due to intrinsic effects of EPS on breast cancer cells.

Global gene expression profiling and pathway analysis

We employed an unbiased approach to discover mechanisms by which EPS modulates phenotypes of breast cancer cells. We aimed to identify genes and pathways altered by EPS in sensitive cells, but not in resistant cells. RNA-sequence analysis (RNA-SEQ) was performed on two sensitive cell lines (T47D and MDA-MB-468) and two resistant cell lines (MCF-7 and MDA-MB-231) treated 20 hr with PBS or EPS. Volcano plots for 3 biological replicates showed that EPS induced expression of more genes in EPS-sensitive cells than EPS-resistant cells (Figure 4A). KEGG pathway analysis of RNA-SEQ data showed that the top pathways altered in EPS-treated T47D cells were DNA replication and G1 transition, in agreement with the G1 cell cycle arrest induced by EPS. In addition, pathways related to bacterial/viral infection and immune responses were among the top pathways altered by EPS, including interferon and TNF signaling (Figure 4B). We hypothesized that EPS activates critical pathways leading to observed phenotypes and identified 290 genes that were upregulated by EPS in the sensitive but not resistant cell lines. Gene enrichment analysis was performed on this set of genes using the Metascape pathway analysis software. The canonical NF-κB was the top transcriptional regulator of these genes (Figure 4C). Together, these data suggest that EPS activates an

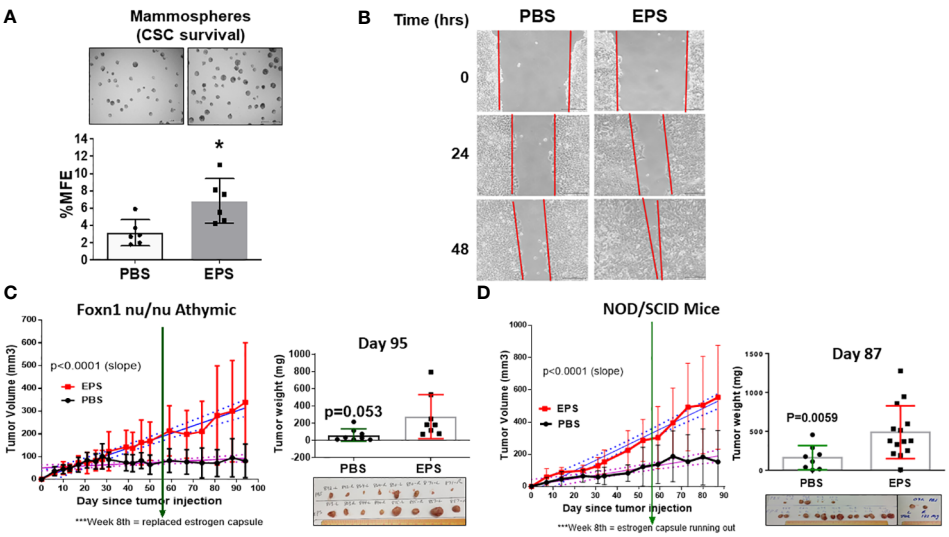

FIGURE 3
Effect of EPS on survival of cancer stem cells, migration, and tumor growth of T47D cancer cells. **(A)** Representative images (4X magnification) of mammospheres larger than 100μm and percent mammosphere forming efficiency (%MFE = # Mammospheres/25,000 Cells Plated) of T47D cells treated with PBS or 5μg/mL EPS for 4 days. Scale bar = 500μm. Data are mean values ± SD of 6 independent experiments performed as a single replicate. Statistical significance was calculated using a Student's *T*-test. * P < 0.05. **(B)** Scratch assay of T47D cells treated with PBS or 5μg/mL EPS for 24 and 48h. Experiments were repeated at least 3 times. **(C)** T47D cells treated with 5μg/mL EPS or PBS *in vitro* for 8 days, and $4x10^6$ cells injected into mammary fat pads of four female, ovariectomized, foxn1 nu/nu, athymic nude mice implanted with a capsule releasing 17β-estradiol. EPS was i.p injected with 50μg EPS or 100μl PBS 3 times/week. Tumor volume (mm^3) ± SD of 8 tumors per group (left). Tumor mass (mg) ± SD with a Student's *T*-test = * P =0.053. **(D)** T47D cells treated with 5μg/mL EPS or PBS *in vitro* for 8 days, and $4x10^6$ cells injected into the mammary fat pads of female, ovariectomized, NOD/SCID mice as in **(C)** Left graph shows tumor volume (mm^3) or tumor mass (mg) mean ± SD of 8-14 tumors per group. Student's *T*-test was used to assess statistical significance between slopes or mass.

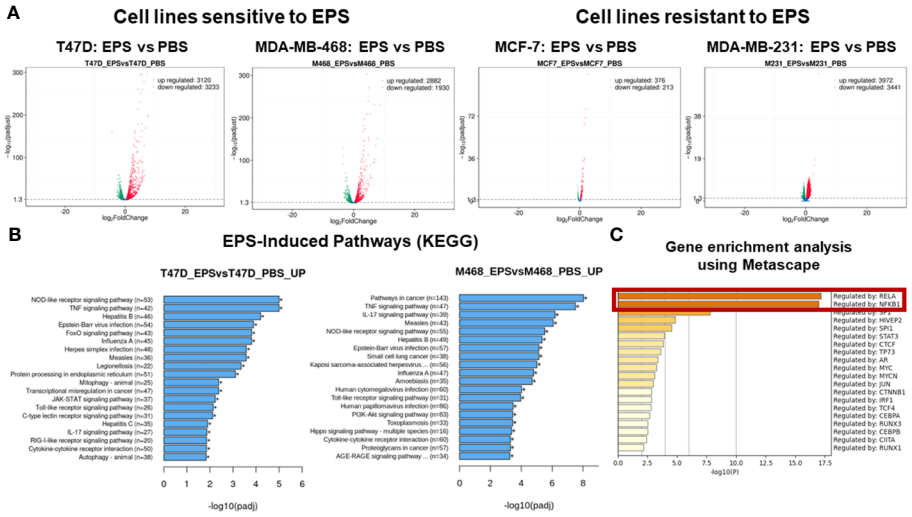

FIGURE 4
RNA-SEQ analysis of genes and pathways altered by EPS. RNA-SEQ was performed on total RNA extracted from T47D, MDA-MB-468, MCF-7, and MDA-MB-231 cells treated with 5μg/mL EPS or PBS for 24 hours. **(A)** Volcano plots were generated showing −log2 fold decrease (green) or increase (red) in expression of genes in response to EPS compared to PBS as calculated using FPKM values and −log10 padjusted values for statistical significance. **(B)** Enriched pathways for EPS compared to PBS were determined using KEGG pathway analysis. The Y-axis depicts the pathways and the X-axis shows the −log10 padjusted values. **(C)** Metascape gene enrichment analysis was performed on 290 genes-identified as being upregulated by EPS only in the sensitive but not in resistant cell lines. The data represent the -log10 p-values on the X-axis and transcriptional regulators on the Y-axis. The p-values were calculated based on 3 biological replicates.

inflammatory response in sensitive breast cancer cells, possibly through activation of TNF, interferon/JAK-STAT, and/or NF-κB signaling. We tested this possibility by treating cells with EPS and performing western blot analysis to identify phosphorylated proteins. Using three EPS sensitive cells (MDA-MB-453, MDA-MB-468, and HCC1428) and one resistant cell line (MCF-7), we found that EPS induced considerable phosphorylation of p65, IκB, p38, and STAT1 in sensitive cells, but little to none in the resistant cell line (Figure 5A). Additionally, EPS increased phosphorylation of p65, IKKα/β, IκB, and RelB within 5 min to 1.7hrs (Figure 5B), p38 within 5min, and STAT1 and STAT3 within 3.3hrs (Figure 5C) in T47D cells. The activation of canonical NF-κB, as indicated by phosphorylation of p65, occurred within 5 min of EPS treatment. Activation of STAT1 and STAT3 required at least 3hrs. These data suggest that EPS may first activate the IKK-NF-κB pathway, followed by subsequent activation of STAT1.

Requirement of IKK signaling

We tested if the IKK-NF-kB pathway is required for EPS's effect on the sensitive cell lines by using TPCA-1, a potent inhibitor of IκB kinases (IKKs). TPCA-1 has 22-fold selectivity for IKKβ over IKKα with an IC50 of 17.9 (69), and although well-known as an IKK/NF-κB inhibitor, TPCA-1 also inhibits STAT3 (70). We treated T47D cells with increasing concentrations of TPCA-1 in the presence of PBS or EPS for 2 hrs, and by western blot analysis found that TPCA-1 reduced phosphorylation of IκBα and p65 (Figure 6A,

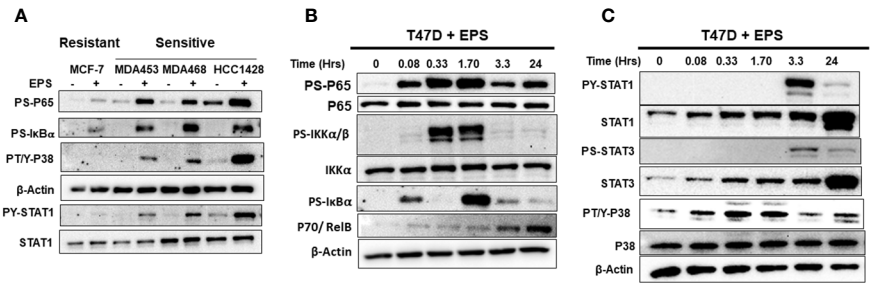

FIGURE 5
Western blot analysis of EPS activation of IKK-NF-κB, p38, and STAT1/3 pathways. **(A)** EPS-sensitive (MDA-MB-453, MDA-MB-468, and HCC1428) and resistant (MCF-7) cells were treated with PBS (–) or 5μg/mL EPS (+) for 3h. Total cell lysates were analyzed using antibodies against P-p65, P-IκBα, P-p38, β-Actin, P-STAT1, and total STAT1. **(B)** T47D cells were treated with 5μg/mL EPS for up to 24h and cell lysates were analyzed using antibodies against P-p65, total p65, P-IκBα, P-IKKα/β, total IKKα, p70, and β-Actin. **(C)** T47D cells was treated with 5μg/mL EPS for up to 24h and total cell lysates were analyzed using antibodies against indicated P-STAT1, total STAT1, P-STAT3, total STAT3, P-p38, total p38, and β-Actin. Experiments were repeated 2-3 times. Representative images for each detected protein are shown.

upper panel), but increased phosphorylation of IKKα/β, both in a concentration-dependent manner (Figure 6A). These data suggest that EPS may inhibit an upstream phosphatase in the NFκB pathway. Surprisingly, TPCA-1 decreased EPS-induced STAT1 phosphorylation in a concentration-dependent manner (Figure 6A, lower panel), while having little effect on p38 phosphorylation (not shown), indicating that the effect on STAT1 is specific (Figure 6A).

Since TPCA-1 prevented EPS-mediated activation of both NF-κB and STAT1, we tested if NF-κB and/or STAT1 are required for EPS inhibition of proliferation and for the G1/G0 cell cycle arrest of T47D cells. We found that TPCA-1 (1μM) almost completely rescued the G1/G0 cell cycle arrest induced by EPS in T47D cells (Figure 6B), as well as the inhibition of proliferation (Figure 6C). Additionally, EPS-mediated upregulation of BCSCs (Figure 6D) and increased cell migration (Figure 6E) were inhibited by TPCA-1. Although TPCA-1 was very efficient at rescuing these phenotypes induced by EPS, the mechanism of action is potentially multifaceted as TPCA-1 inhibits the activation of both IKK-NF-κB and STAT1 in response to EPS.

TPCA-1 is highly specific for IKKs, with higher selectively for IKKβ over IKKα, and we hypothesized that IKKβ maybe the direct target of TPCA-1 in EPS-treated cells. In addition, TPCA-1 potently inhibited EPS-induced STAT1 phosphorylation, suggesting that it could inhibit a kinase responsible for phosphorylating STAT1. JAK1, the upstream kinase of STAT1, is another potential target of TPCA-1 as it has been shown to inhibit JAK1 (71). To test how EPS was functioning, we performed an RNAi-mediated knockdown of IKKβ or JAK1 in T47D cells and measured cell cycle progression and proliferation in response to EPS without or with TPCA-1. IKKβ knockdown alone modestly enhanced the % of cells in S-phase and abrogated the inhibitory effects of EPS similar to TPCA-1 (Figure 7A). EPS-mediated inhibition of proliferation of T47D cells was rescued by IKKβ knockdown or treatment with TPCA-1 (Figure 7B). The effect of EPS and TPCA-1 on proliferation was due primarily to IKKβ and not to JAK1 as the knockdown of JAK1 had little effect on inhibition of proliferation by EPS nor on the rescue by TPCA-1 (Supplementary Figure 6). In addition, IKKβ knockdown alone increased BCSC survival and EPS had little effect when IKKβ was depleted (Figure 7C). Western blot analysis confirmed that IKKβ was knocked down by the siRNA (Figure 7D). These data indicate that the most likely target of TPCA-1 was IKKβ as it was required for EPS-mediated inhibition of proliferation and cell cycle arrest.

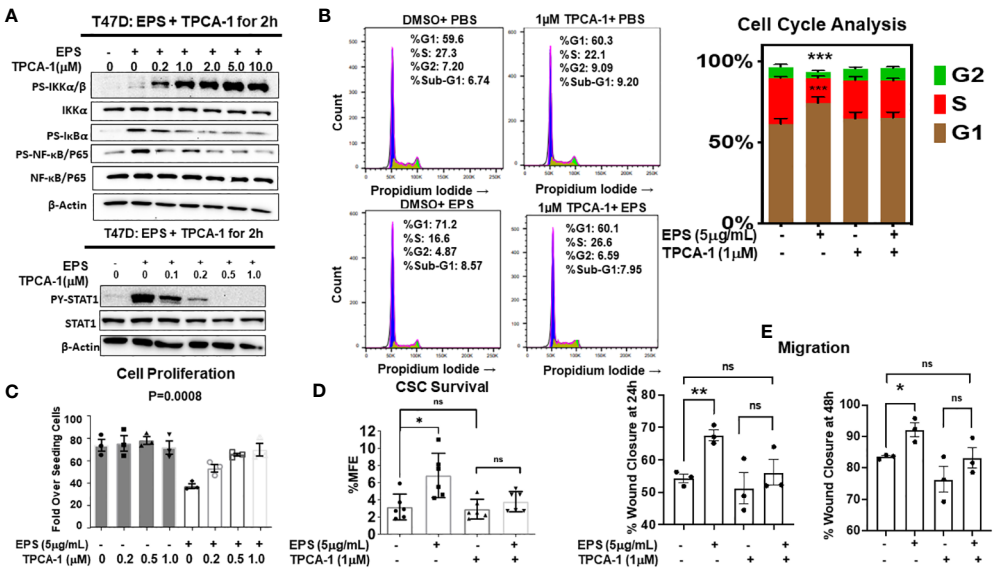

FIGURE 6
Rescue of EPS-Induced signaling and cancer associated phenotypes in T47D cells by the IKKβ inhibitor, TPCA-1. **(A)** T47D cells were pretreated with increasing amounts of TPCA-1 for 30min, then 5μg/mL EPS or PBS was added for 2h. Total cell lysates were analyzed by western blots using antibodies against: Top panel (P-IKKα/β, total IKKα, P-IκBα, P-p65, total p65, and β-actin) and Bottom panel (P-STAT1, total STAT1, P-p38, total p38 and β-actin). Experiments were repeated 3 independent times. Representative images are shown. **(B)** T47D cells were treated with PBS or 5μg/mL EPS in the presence of 1μM TPCA1 for 24h. Cells were analyzed by flow cytometry after fixing and staining with propidium iodide. Experiments were performed three independent times. Representative images are shown and the bar graph depicts data as mean ± SEM of 3 independent experiments performed in triplicate, with Student's *T*-test comparing %S of PBS vs EPS, *** $P \leq 0.001$. **(C)** Growth of T47D cells after treatment with PBS or 5μg/mL EPS, and increasing doses of TPCA-1 every 2 days for 6 days. Proliferation was calculated as in Figure 1. Data are represented as mean ± SEM of 3 independent experiments each performed in triplicate. A one-way ANOVA was performed, with P=0.0008 for EPS compared to the PBS control. **(D)** Percent mammosphere forming efficiency (%MFE = # Mammospheres/25,000 cells plated) of T47D cells pretreated with 1μM TPCA-1 for 30mins before PBS or 5μg/mL EPS treatment for 4 days. Data are represented as mean ± SD of 6 independent experiments, with statistical significance of $P < 0.05$ as calculated using a Student's *T*-test (Left). **(E)** Scratch migration assay of T47D cells pretreated with 1μM TPCA-1 for 30mins followed by PBS or 5μg/mL EPS for 24 and 48h. Data are represented as mean ± SEM of 3 independent experiments each performed in triplicate, with Student's *T*-test * $P \leq 0.05$, ** $P \leq 0.01$, ns, not statistically significant.

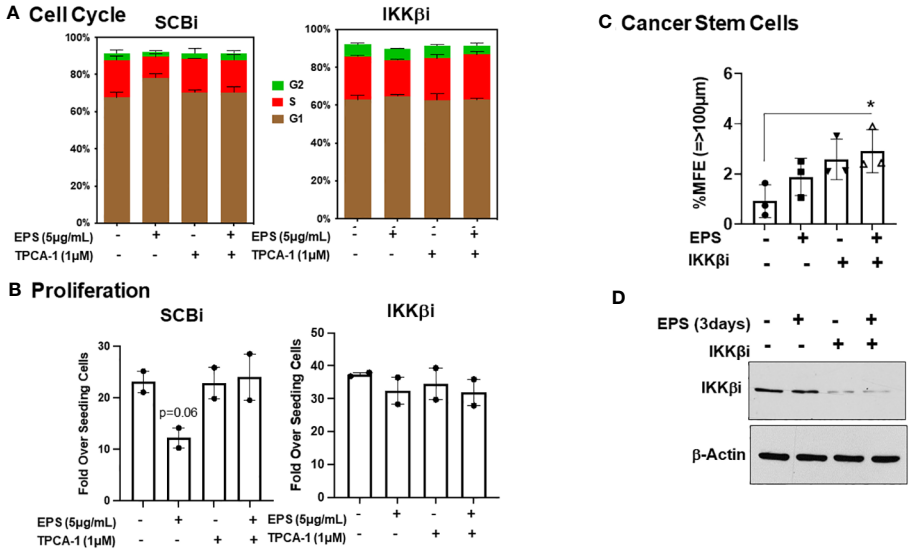

FIGURE 7
Role of IKKβ for EPS-mediated effects in T47D cells. T47D cells were transfected with IKKβ siRNA or scrambled siRNA (SCRi). **(A)** Transfected cells were plated in 12-well plates and treated with PBS or 5μg/mL EPS in the presence of DMSO or 1μM TPCA1 for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SEM of 2 independent experiments performed in duplicate. **(B)** Growth assay was performed on transfected cells in the presence of 5μg/mL EPS and TPCA1 for 6 day. Live cells were counted by trypan blue exclusion on a hemocytometer. Proliferation was calculated as Fold over seeding cells = (# Live Cells on Day 6)/(# Live Cells Plated on Day 0). Data are represented as mean ± SEM of 2 independent experiments performed triplicate. A T-test was performed for significance with p=0.06. **(C)** Percent mammosphere forming efficiency (%MFE = # Mammospheres/25,000 Cells Plated) of transfected T47D cells treated with PBS or EPS for 3 days. Data are represented as mean ± SD of 3 independent experiments, with statistical significance of $P < 0.05$ as calculated using a one-way ANOVA. **(D)** Western blot of lysates of transfected T47D cells after 3 days treatment with PBS or EPS. Blot probed with anti-IKKβ and Actin.

The role of STAT1 signaling in EPS-mediated cell cycle arrest

Neither genetic knockout nor knockdown approaches were successful at depleting STAT1 or at preventing EPS-mediated STAT1 phosphorylation (data not shown). Hence, to address the role of STAT1 in EPS-induced changes to T47D cells, a pharmacologic approach was taken to inhibit STAT1 indirectly by targeting its upstream kinase, JAK1 using Cerdulatinib. Another target of this inhibitor is IKK, which is required by EPS to induce cell cycle arrest. This inhibitor at 1μM successfully inhibited STAT1 phosphorylation in EPS-treated T47D cells (Supplementary Figure 7A), but had no effect on NF-κB activation as measured by levels of phosphorylated IκB and p65 (Supplementary Figure 7A). Cerdulatinib (1μM) also rescued the G1/G0 cell cycle arrest induced by EPS (Supplementary Figure 7B). These data suggest that STAT-1 also contributes to EPS-mediated cell cycle arrest of breast cancer cells.

Discussion

The microbiome has been recognized as being part of the tumor microenvironment. Dysbiosis induced by various factors is associated with breast cancer development (31). Microbiome studies report large-scale changes in bacterial composition, which makes it difficult to pinpoint the specific causal microbes. So far, there have been few reports regarding effects of specific commensal bacteria on breast cancer phenotypes. This study is the first to evaluate the effect of EPS produced from the commonly used probiotic strain *B. subtilis* on cancer cells, using breast cancer as a model. Although most of the work focused on T47D cells, similar results were also shown in other cell lines. We found that EPS directly modulated various phenotypes of breast cancer cells, from cell cycle arrest, inhibition of bulk cell proliferation, increased migration, increased BCSC survival, and increased tumor growth. Overall, EPS has differential activity on breast cancer cells that does not require TLR4, unlike previous studies showing that TLR4 signaling is required on myeloid cells for the anti-inflammatory effect of EPS (39–41). The receptor for EPS on breast cancer cells is yet to be identified. We performed RNA-SEQ analysis across multiple cell lines and focused on top pathways shared by sensitive and not resistant cell lines. STAT1 and IKK were activated across all four sensitive cell lines. Hence, the mechanism by which EPS exerts these effects on breast cancer cells is most likely through activation of IKKβ-NFκB signaling and possibly also STAT1 activation as shown in our current model (Figure 8). The NF-κB pathway was activated within minutes of EPS exposure. IKKβ inhibitors (TPCA-1 and Cerdulatinib) abrogated EPS-induced STAT1 phosphorylation and subsequent cancer associated phenotypes. IKKβ knockdown also seemed to rescue EPS-mediated growth inhibition. However, both genetic knockout or knockdown approaches directed at STAT1 were unsuccessful at completely depleting STAT1 (data not shown). Incomplete knockdown was also not useful because the small amount of STAT1 protein remaining was phosphorylated in response to EPS

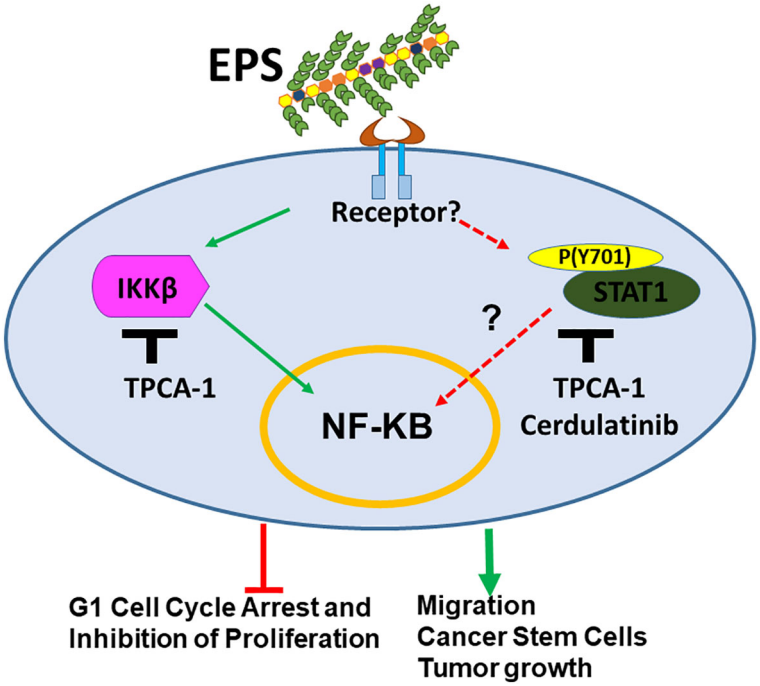

FIGURE 8
Model for mechanism by which EPS-derived from the probiotic *B. subtilis* modulates breast cancer associated phenotypes. EPS binds an unknown receptor on the cell surface activating IKKβ and STAT1 signaling. This activation leads to inhibition of cell cycle progression and proliferation of bulk cells. In addition, EPS-mediated activation of these pathways enhances cell migration, survival of cancer stem cells, and tumor growth in immunocompromised mice.

(data not shown). These data suggest that EPS may not utilize the canonical Interferon/JAK/STAT1 pathway to modulate breast cancer phenotypes as activation by interferon-γ did not induce cell cycle arrest and knocking down JAK1 did not interfere with EPS-mediated inhibition of proliferation. Instead, IKKβ may be associated with STAT1 phosphorylation at tyrosine 701. Since IKKβ is a serine/threonine kinase that phosphorylates IκBα (72), it is unlikely that IKKβ would be able to directly phosphorylate the tyrosine 701 on STAT1. Thus, an unidentified tyrosine kinase that is not JAK1 may be involved. IKKα, which is the sister kinase to IKKβ within the IKK complex, may also need to be investigated to see whether it plays a role in EPS signaling. We elected to knockdown IKKβ first because TPCA-1 has a 22-fold selectivity for IKKβ over IKKα (69). Interestingly, one study showed that silencing of IKKα significantly decreased STAT1 tyrosine phosphorylation in response to dsRNA in HeLa cells, suggesting that IKKα can mediate both type I interferon-dependent and interferon-independent STAT1 phosphorylation (73). However, no physical interaction between IKKα and STAT1 was detected (73). Future studies will focus on further delineating the interaction between IKKβ and STAT1 induced by EPS.

It is also important to understand which bacteria are beneficial or harmful for cancer phenotypes, and in which context. Probiotics, or the use of living microorganisms to promote health, have proven benefits (13). Several probiotics (mainly *Lactobacillus* and *Bifidobacterium* strains) have beneficial effects on prevention and treatment of breast cancer (13, 74–76). Probiotic supplements significantly reduced the incidence of chemotherapy-related cognitive impairment and alleviated gastrointestinal toxicity induced by chemotherapy or radiation in breast cancer patients (77, 78). Probiotic bacteria such as *Akkermansia muciniphila* improved response to anti-PD-1 immunotherapy (79, 80). However, other studies showed that there was little benefit from probiotic use in improving diarrhea associated with radiation or chemotherapy (54). Additional reports showed that long term probiotic use interferes with the gut commensal bacteria and may result in sepsis, fungemia and GI ischemia (55). Therefore, it will be important to understand which types of probiotics or molecule they secrete are beneficial or harmful in regards to cancer therapy.

Our results suggest a novel finding in which a well-established probiotic, commensal bacterium, *Bacillus subtilis* produces an EPS molecule that can directly alters breast cancer cell signaling and modulate breast cancer cell phenotypes. EPS has potent anti-inflammatory effects (39–45). While EPS appeared as a potent anti-proliferative agent across commonly used *in vitro* assays including viability assays (XTT), cell cycle progression, cell proliferation, and Annexin-V cell death analysis, EPS unexpectedly enhanced cell migration, BCSC survival, promoted tumor growth in immune compromised xenograft models. There are certainly more factors at play *in vivo* that could alter the tumor's response to a drug, from drug bioavailability to other cell extrinsic phenotypes. It is also important to note that the duration of exposure to EPS is critical for phenotypes. Longer treatment in mice led to tumor growth while shorter exposure *in vitro* predominantly inhibited proliferation. These results indicate that

EPS has multifaceted functions depending on the breast cancer cells and cellular environment and future studies are needed to fully elucidate the different mechanisms of action.

In the modern world where clean/urbanized environment and processed foods are common, exposure to *B. subtilis* is from unconventional sources such as fermented soybeans called Natto \Miso in Japan or Cheongukjang in Korea, or fermented cabbage called Kimchi in Korea (32, 33). *B. subtilis* has been isolated from the ileum and feces of healthy humans, and can persist in the gut for up to 20 days after its withdrawal from the diet according to animal studies (56, 57, 81). Although it is unknown if *B. subtilis* can be found in breast tissue, EPS produced by *B. subtilis* may exert local and systemic effects on the immune system, creating a healthy anti-inflammatory state as a commensal bacterium. EPS may also travel to breast tissue, interacting directly with breast cancer cells to modulate their growth and phenotypes. Additional experiments are needed to determine the physiological relevance of EPS on breast cancer and benefit to risk ratio of using this probiotic, EPS-derived from *B. subtilis*.

Data availability statement

The data presented in this study was deposited into the NCBI database SRA (https://www.ncbi.nlm.nih.gov/sra), accession number PRJNA1036683, and in the GEO repository (https://www.ncbi.nlm.nih.gov/geo), accession number GSE248119.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MN: Data curation, Methodology, Visualization, Writing – review & editing, Conceptualization, Formal Analysis, Investigation, Writing – original draft. EM: Data curation, Investigation, Writing – review & editing. DW: Data curation, Investigation, Methodology, Validation, Writing – review & editing. KK: Writing – review & editing, Funding acquisition, Resources, Supervision, Visualization. CO: Funding acquisition, Resources, Supervision, Visualization, Writing – review & editing, Data curation, Methodology, Project administration, Validation.

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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/fonc.2023.1292635/full#supplementary-material

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The association between serum heat shock protein 72 and intestinal permeability with intestinal microbiota and clinical severity in patients with cerebral infarction

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Objectives: We aimed to compare serum heat shock protein 72 (HSP72) and intestinal permeability in patients with cerebral infarction (CI) and healthy individuals to reveal their correlations and link to gut microbiota alterations and clinical severity of CI.

Methods and results: Stool samples of 50 patients with CI and 46 healthy volunteers were analyzed through 16S rRNA gene sequencing to characterize intestinal flora profiles. Serum HSP72 and zonulin were assayed using enzyme-linked immunoassay (ELISA). The obtained data were then subjected to comparative and correlative analysis. We found that the levels of zonulin and serum HSP72 were significantly higher in the CI group compared to the healthy group. Serum HSP72 and zonulin levels were positively correlated in the CI group and correlated positively with the clinical severity of CI. β diversity showed significant differences in intestinal microbiota composition between the two groups. In the CI patient group, the abundance of bacteria *Eubacterium_fissicatena_group*, *Eubacterium_eligens_group,* and *Romboutsia* manifested a remarkably positive correlation with serum HSP72. The abundance of bacteria *Eubacterium_fissicatena_group* and *Acetivibrio* had a significantly positive correlation with zonulin levels.

Conclusion: Our findings indicated that an increase in serum HSP72 and zonulin levels was manifested in patients with CI and was related to specific gut microbiota alterations and the clinical severity of CI.

KEYWORDS

intestinal flora, cerebral infarction, serum heat shock protein 72, intestinal permeability, zonulin, clinical severity of CI, ELISA, NIHSS

Introduction

Stroke, a devastating and lethal disease, is currently second among the leading causes of death globally and the third contributor to disability in the world (1). Besides the neurological defects caused by the infarct site, the major cause of death is the peripheral tissue damage induced by post-stroke leaky gut. Previous reports have shown that the abundance of opportunistic pathogens and the corresponding product of metabolism changes increased

significantly in patients with cerebral infarction (CI) compared to healthy controls (2). Incidentally, elevated inflammatory levels after CI efficiently facilitated the increase of intestinal mucosal permeability. Strict regulation of intestinal integrity is essentially related to host antimicrobial immune defense. Regrettably, previous studies on CI and specific alterations of intestinal flora have not effectively obtained explicit conclusions.

The level of the serum zonulin, a protein produced by the intestine epithelium, is proportional to gut permeability. *In vitro* studies demonstrated that endogenous human zonulin was responsible for increased permeability in the jejunum and ileum (3). An overgrowth of intraluminal microorganism and gluten contribute significantly to intestinal zonulin release (4). Zonulin secretion has been reported to be induced through the MyD88-dependent pathway, which is followed by dissociation of the protein ZO-1 from the tight junctional complex, thus generating leaky gut syndrome (5). Of note, zonulin is the biological door to a vast number of diseases such as autoimmune diseases, neuropsychiatric diseases, and cancer through its intestinal barrier regulation function (6, 7).

Heat shock protein 72 (HSP72), a critical component of inducible HSPs (iHSPs), is a ubiquitous molecule that exerts efficient effects on cellular survivability and tolerance to stressors. Correspondingly, animal experiments showed that the upregulation of HSP72 in ischemic cerebral tissue confirmed a crucial protective role in the outcome following stroke (8). Recent evidence indicates that bacterial components and metabolites specifically control the expression of HSPs (9, 10). HSP72 can be detected in quite a few body fluids, such as pleural fluid (11), cerebrospinal (12), synovia (12), bronchoalveolar lavage fluid (13), and serum (14). Recent evidence indicated that the concentration of the stress-inducible HSP72 homolog, HSP70, increased in patients with heart failure (15) and atherosclerosis (16). Strikingly, previous studies have documented that the serum expression level of HSP70 significantly increased following acute middle cerebral artery occlusion (MCAO) in rats (16). Nevertheless, whether there is a change in human serum HSP72 after CI has not been addressed. Thereafter, how serum HSP72 correlates with intestinal dysfunction and CI severity has not been accurately explored.

Accordingly, in this study, we deciphered gut microbiome profiles through 16S rRNA gene sequencing of stool samples, quantified serum HSP72 and zonulin via enzyme-linked immunoassay (ELISA), further compared and analyzed the levels of serum HSP72 and zonulin in patients with CI and healthy controls, and explored the link between the levels of serum HSP72 and zonulin to alterations in intestinal microbiota and clinical severity of CI.

Materials and methods

Study subjects

This cross-sectional study was conducted at Hangzhou First People's Hospital. We consecutively enrolled 81 patients with CI and 52 healthy individuals, respectively, from the Department of Neurology and Physical Examination Center. Herein, we rigorously screened out 50 patients with CI and 50 healthy controls who satisfied the inclusion criteria and ultimately participated in the study. Case groups were diagnosed within 2 weeks after sudden focal neurological deficits, with the acute infarct area in the corresponding brain region confirmed on the computed tomography (CT) and/or magnetic resonance imaging (MRI). Healthy controls reported never being diagnosed with any of the risk factors associated with CI, such as hypertension, diabetes, metabolic syndrome, and atrial fibrillation. Baseline characteristics such as demographic data including age, sex, dietary habits, medical histories (hypertension, diabetes, and atrial fibrillation), and laboratory data were collected. This study was approved by the Clinical Research Ethics Committee of Hangzhou First People's Hospital.

The inclusion criteria for the study subjects were determined as (1) aged between 40 and 80 years old; (2) grew up in southern China and maintained a healthy lifestyle, with healthy dietary and bowel habits; (3) body mass index range from 18 to 24; (4) had available blood and stool samples; and (5) signed informed consent before the experiments. Subjects were excluded if they (1) had a history of intracranial hemorrhage and other neuropsychiatric disorders; (2) were previously diagnosed with respiratory failure, heart failure, uremia, severe liver dysfunction, malignant tumors, and autoimmune disease; (3) had inflammatory bowel disease, gastrointestinal bleeding, and surgery, as well as other gastrointestinal dysfunction; and (4) had received treatment with antibiotics, probiotics, or hormone drugs within 2 months before the recruitment.

Sample collection

To avoid random error, fecal samples were taken with cotton swabs from the middle section of the stool samples provided and were kept in two 2 mL sterile frozen depository tubes with approximately 200 mg of samples in each tube. Fasting blood samples were collected in the coagulation vessels and centrifuged at 3000r for 10 min at −4°C. The serum was transferred to 1.5 mL frozen tubes. Preprocessed stool and serum samples were immediately transferred to the laboratory for storage at −80°C.

Microbial DNA extraction and sequence data analysis

The total DNA of fecal specimens was extracted using the E.Z.N.A.®Stool DNA Kit and samples were sequenced on the Illumina NovaSeq platform following the manufacturer's instructions. The complexity of the sample species diversity was characteristic of α diversity, calculated by the QIIME2. The β diversity was calculated by non-metric multidimensional scaling and plotted by the R package. Composition difference was analyzed by the Wilcoxon rank-sum test and Welch's t-test, based on which we constructed the heat map at the phyla level.

Quantitative assay of serum HSP72 and zonulin by ELISA

Serum HSP72 and zonulin were assayed through the Human HSP72 ELISA Kit and Zonulin ELISA Kit (Meimian Industrial, Jiangsu, China). A 50μL proof sample was added to the standard well.

A 40μL sample diluent and a 10μL sample being tested were added to the enzymatic coating plate so that samples were eventually diluted to 5-fold. Samples were incubated with 100μL HRP-Conjugate reagent at 37°C for 60 min. Then, each well was filled with wash fluid, and the fluid was discarded after 30 s. This process was repeated five times, and each well was dried. Each well was incubated with chromogenic agent at 37°C for 15 min and then the reaction was terminated by adding 50μL of the stop solution to each well until the blue color turned yellow. Finally, the absorbance of each well was determined within the wavelength of 450 nm.

Statistical analysis

Data were analyzed using SPSS version 26.0 software and R version 3.6.1 statistical software. The continuous variable was denoted by mean ± SD, while categorical variables were denoted by numbers and percentages. Measurement data meeting normality was indicated by independent sample Student's t-test or analysis of variance and by Wilcoxon's rank sum test if the variable violated the assumption of normality. Categorical variable differences between groups were compared by a χ^2 test. We used binary logistic regression analysis to explore the relationship between serum HSP72 and zonulin levels and the occurrence of CI, after adjustment for age and sex. The area under the receiver operating characteristic (ROC) curve (AUC) was applied to evaluate the model's diagnostic performance for investigating the ability of serum HSP72 and zonulin levels to distinguish between patients with CI and controls. Multivariable linear regression models were conducted to examine the association between serum HSP72 and zonulin levels and clinical severity. The correlation between intestinal flora and clinical data was assessed by Spearman's rank correlation analysis. The data correlation conforming to the normal distribution was analyzed with the Pearson test. p-value <0.05 was defined as statistically significant.

Results

Study population and baseline characteristics

A total of 81 patients with CI and 52 healthy individuals were enrolled in this study. However, 31 patients and two control participants failed to defecate on time or provided unqualified fecal sampling, and were thus excluded from the subsequent analysis. Four healthy samples could not meet the 16S rRNA sequencing requirements due to low fecal volume, which led to unqualified amplification. Ultimately, only 96 subjects (50 patients with CI and 46 controls) remained in the next 16S rRNA analysis, and the final included samples were tested by ELISA. The National Institute of Health Stroke Scale (NIHSS) score was less than 16 in the CI group, for which the patients with severe CI had difficult fecal discharge the early next morning. All participants in the experiment were matched for age (CI group, 66.42 ± 6.92; healthy group, 65.15 ± 5.82; $p = 0.233$) and sex (M/F: case, 26/24; control, 25/21; $p = 0.818$). The demographic and baseline clinical characteristics of patients with CI and controls are shown in Table 1.

Comparison of serum HSP72 and zonulin levels between the two groups

The quantification and comparison of serum HSP72 and zonulin between the two groups are shown in Table 1 and Figure 1. The mean serum HSP72 level was 307.46 ± 42.59 pg./g in patients with CI, while the mean value was 176.61 ± 44.75 pg./g in controls. In light of this, patients with CI had significantly higher serum HSP72 than controls ($p < 0.01$). Accordingly, the mean zonulin level was 135.70 ± 30.74 ng/mL in patients with CI, while the mean value was 105.83 ± 24.04 ng/

TABLE 1 Comparison of the baseline data between the two groups.

Clinical parameter	CI group (n = 50)	Healthy group (n = 46)	p-value
Sex, male	26 (52%)	25 (54%)	0.818
Age, y	66.42 ± 6.92	65.15 ± 5.82	0.233
NIHSS score	10.88 ± 2.67	/	/
Hypertension (n, %)	36 (72%)	0	< 0.01**
Diabetes (n, %)	25 (50%)	0	< 0.01**
Atrial fibrillation (n, %)	3 (6%)	0	< 0.01**
Smoking history (n, %)	9 (18%)	6 (13%)	0.504
TG (mmol/L)	1.53 ± 0.76	1.21 ± 0.60	0.013*
TC (mmol/L)	4.21 ± 0.97	2.64 ± 0.68	< 0.01**
LDL (mmol/L)	2.32 ± 0.84	2.43 ± 0.54	0.203
HCY (umol/L)	16.55 ± 10.90	8.81 ± 3.03	< 0.01**
UA (umol/L)	314.28 ± 89.72	350.30 ± 42.75	0.016*
Serum HSP72 (pg/g)	307.46 ± 42.59	176.61 ± 44.75	< 0.01**
Zonulin (ng/ml)	135.70 ± 30.74	105.83 ± 24.04	< 0.01**

TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HCY, homocysteine; UA, uric acid; HSP72, heat shock protein 72. Variables are expressed as mean ± SD or n (%). *$p < 0.05$. **$p < 0.01$.

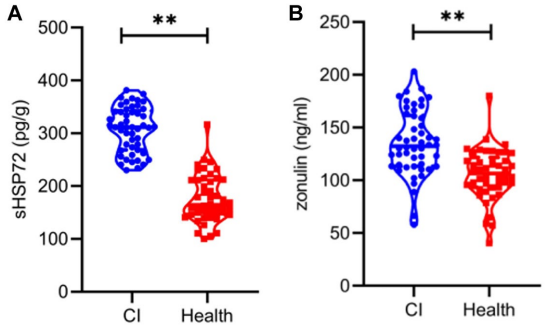

FIGURE 1
Violin plot representing data density distribution and median with interquartile range of serum HSP72 **(A)** and zonulin **(B)** between the two groups. **, $p < 0.01$.

mL in controls. Moreover, serum zonulin was significantly higher in the CI group than in healthy controls ($p < 0.01$).

We then investigated whether serum HSP72 or zonulin distinguished between patients with CI and healthy individuals. Binary logistic regression analysis indicated that higher levels of serum HSP72 (OR = 1.10, 95% CI = 1.03–1.11; $p < 0.01$) and zonulin (OR = 1.04, 95% CI = 1.02–1.07; $p < 0.01$) were more likely to associated with CI patients after adjustment for sex and age.

According to binary logistic analysis, ROC curve analysis was performed to predict CI occurrence. Strikingly, the AUC, which represented the prediction precision, raised from 0.57 in the basic model (age + sex, $p = 0.22$) to 0.98 in a way that added serum HSP72 levels, which discriminated clearly between the CI and healthy groups in the logistic regression analysis ($p < 0.01$). The AUC was 0.79 with the addition of the serum level of zonulin ($p < 0.01$), which also discriminated markedly between the CI and healthy groups in the logistic regression analysis. Furthermore, when bringing age, sex, serum HSP72, and zonulin into the full model, the AUC reached 0.98 ($p < 0.01$) (Figure 2A; Table 2).

Association between serum HSP72 and zonulin levels and the link to CI severity

Based on Pearson correlation analysis, we further observed that serum HSP72 ($\rho = 0.93$, $p < 0.01$) and zonulin ($\rho = 0.97$, $p < 0.01$) positively correlated with NIHSS scores (Figures 2B,C). Of note, we found a positive correlation between the levels of serum HSP72 and zonulin within the CI group ($\rho = 0.87$, $p < 0.01$) (Figure 2D). Similarly, after adjustment for age, sex, smoking history, previous related diseases, and routine blood test results in bias correlation analysis, serum HSP72 ($\rho = 0.93$, $p < 0.01$) and zonulin ($\rho = 0.97$, $p < 0.01$) levels continuously correlated positively with NIHSS scores in patients with CI. With the multivariate linear regression model, we found that serum HSP72 ($b = 0.06$, $t = 14.37$, $p < 0.01$) and zonulin ($b = 0.02$, $t = 7.16$, $p < 0.01$) were persistently positively correlated with NIHSS scores. These data demonstrated that higher levels of serum HSP72 and zonulin were associated with a more severe degree of CI.

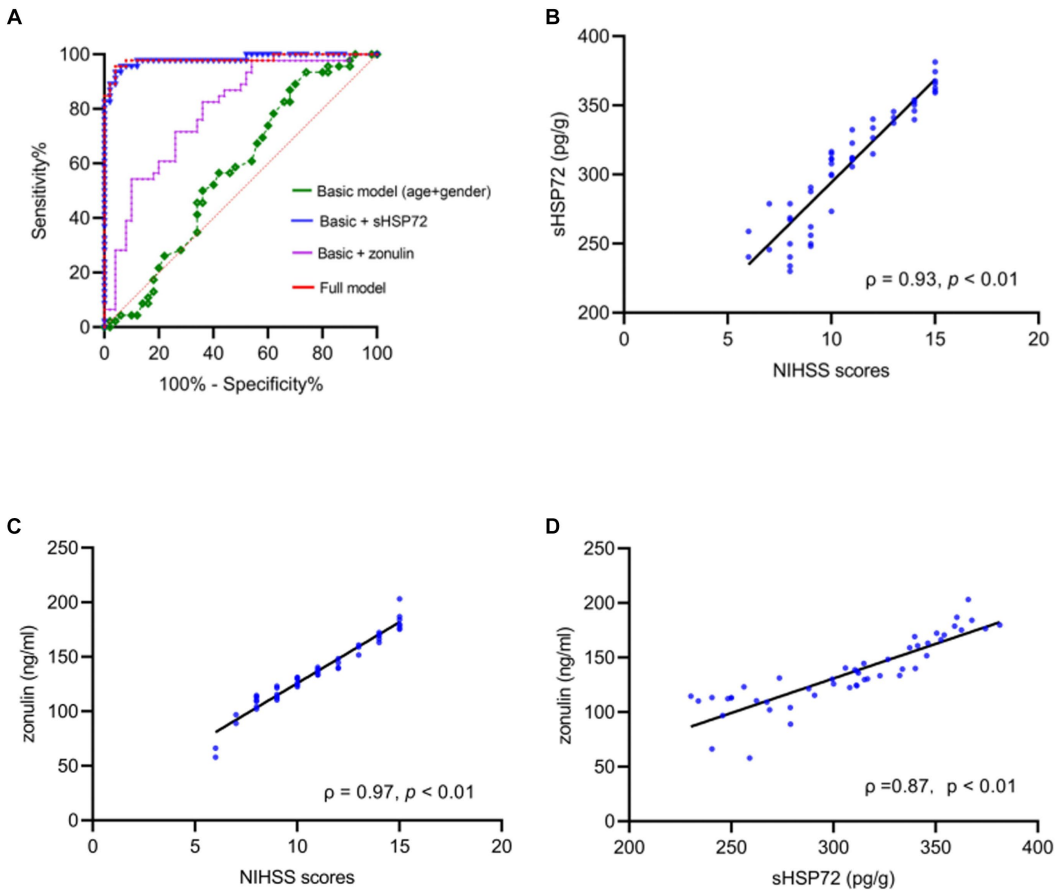

FIGURE 2
(A) ROC curves for estimating the accuracy of cerebral infarction (CI) using basic model (age + sex, AUC = 0.57, $p = 0.22$) raised with the addition of serum HSP72 and zonulin (AUC = 0.98, $p < 0.01$; AUC = 0.79, $p < 0.01$). In a full model comprising age, sex, serum HSP72, and zonulin, the accuracy of evaluating the occurrence of CI showed significant differences between CI and healthy individuals (AUC = 0.98, $p < 0.01$). **(B,C)** Scatter diagram manifested positive correlations between NIHSS scores and serum HSP72 **(B)** and zonulin **(C)**. **(D)** Scatter diagram showed correlations between serum HSP72 and zonulin. ROC = receiving operating characteristic. AUC = area under the curve.

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Intestinal flora correlated with serum HSP72 and zonulin

α diversity was used to describe the species diversity of individual samples. The Wilcoxon rank-sum test, depicted through Chao 1, Good's coverage, Simpson, and Shannon indexes, showed no statistical difference in species richness and evenness between the two groups (Figure 3A). β diversity is an index reflecting the difference in composition and distribution of bacteria between groups. NMDS plots showed significant differences in intestinal microbiota composition between the two groups (unweighted Unifrac stress = 0.17 and weighted UniFrac stress = 0.15) (Figure 3B). We then analyzed the differences in colony abundance at the 67 genus level (Figure 3C). Accordingly, the CI group was more abundant with 27 features, while the healthy group was enriched with 40 features. These data indicated that the microbial abundance in the CI group was much lower than that in the healthy group.

We further performed the Spearman correlation analysis on the differentially abundant bacteria genus with serum HSP72 and zonulin. We found significant correlations between the bacteria genus with both serum HSP72 and zonulin. Among the correlation heatmap for CI participants, the abundance of bacteria *Eubacterium_fissicatena_group*, *Eubacterium_eligens_group*, and *Romboutsia* manifested a remarkably positive correlation with serum HSP72 ($\rho = 0.28$, $p = 0.04$; $\rho = 0.30$, $p = 0.03$; $\rho = 0.28$, $p = 0.04$). The abundance of bacteria *Eubacterium_fissicatena_group* and *Acetivibrio* had a significantly

TABLE 2 Receiver operating characteristic association statistics for forecasting cerebral infarction.

	AUC (95% CI)	*p*-value
Basic (age, sex)	0.57 (0.46–0.69)	0.22
Basic + serum HSP72	0.98 (0.96–1.00)	< 0.01**
Basic + zonulin	0.79 (0.70–0.88)	< 0.01**
Full model (age, sex, serum HSP72 and zonulin)	0.98 (0.95–1.00)	< 0.01**

AUC = area under the receiver operating characteristic curve; CI = confidence interval.
**$p < 0.01$.

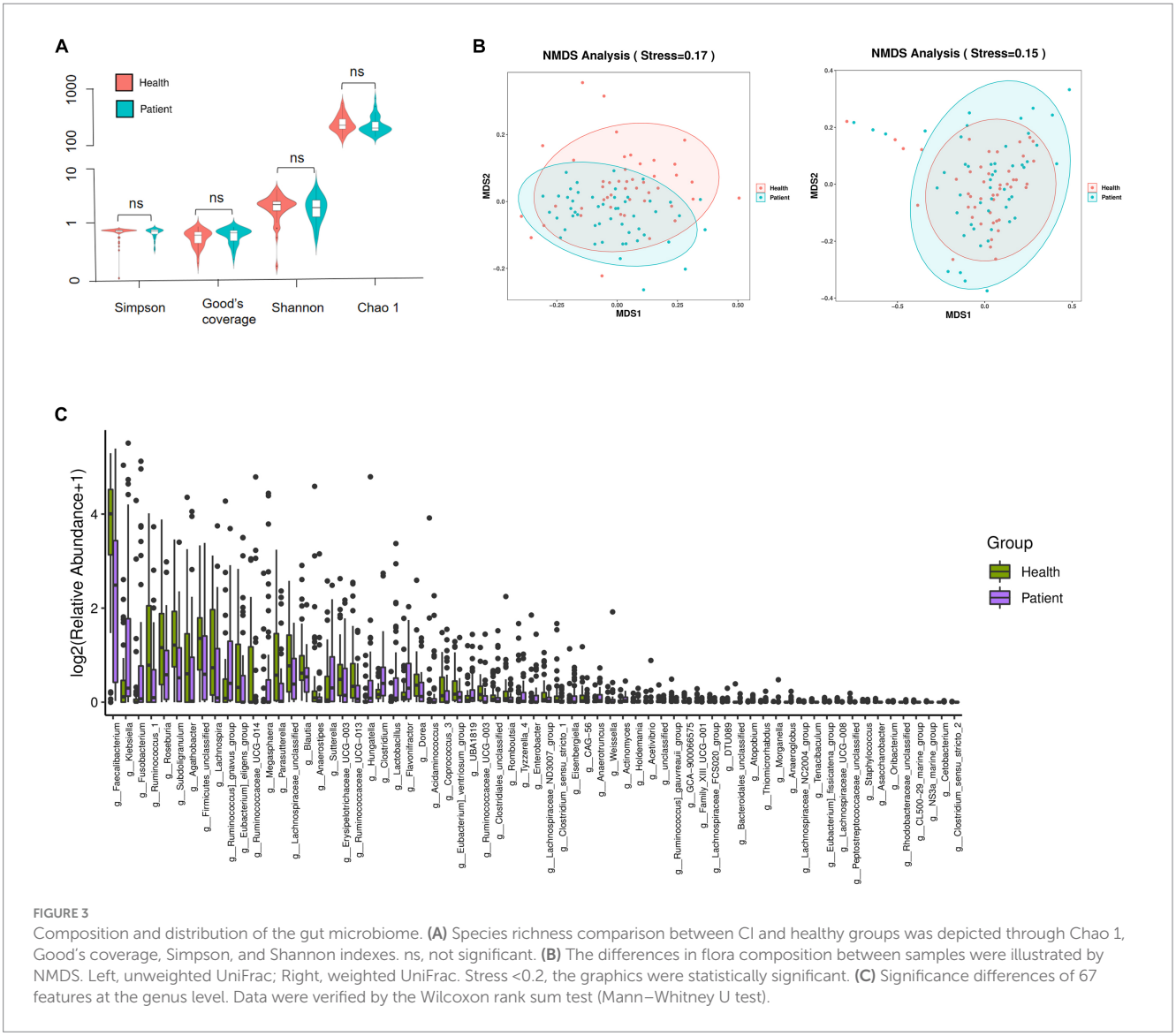

FIGURE 3
Composition and distribution of the gut microbiome. **(A)** Species richness comparison between CI and healthy groups was depicted through Chao 1, Good's coverage, Simpson, and Shannon indexes. ns, not significant. **(B)** The differences in flora composition between samples were illustrated by NMDS. Left, unweighted UniFrac; Right, weighted UniFrac. Stress <0.2, the graphics were statistically significant. **(C)** Significance differences of 67 features at the genus level. Data were verified by the Wilcoxon rank sum test (Mann–Whitney U test).

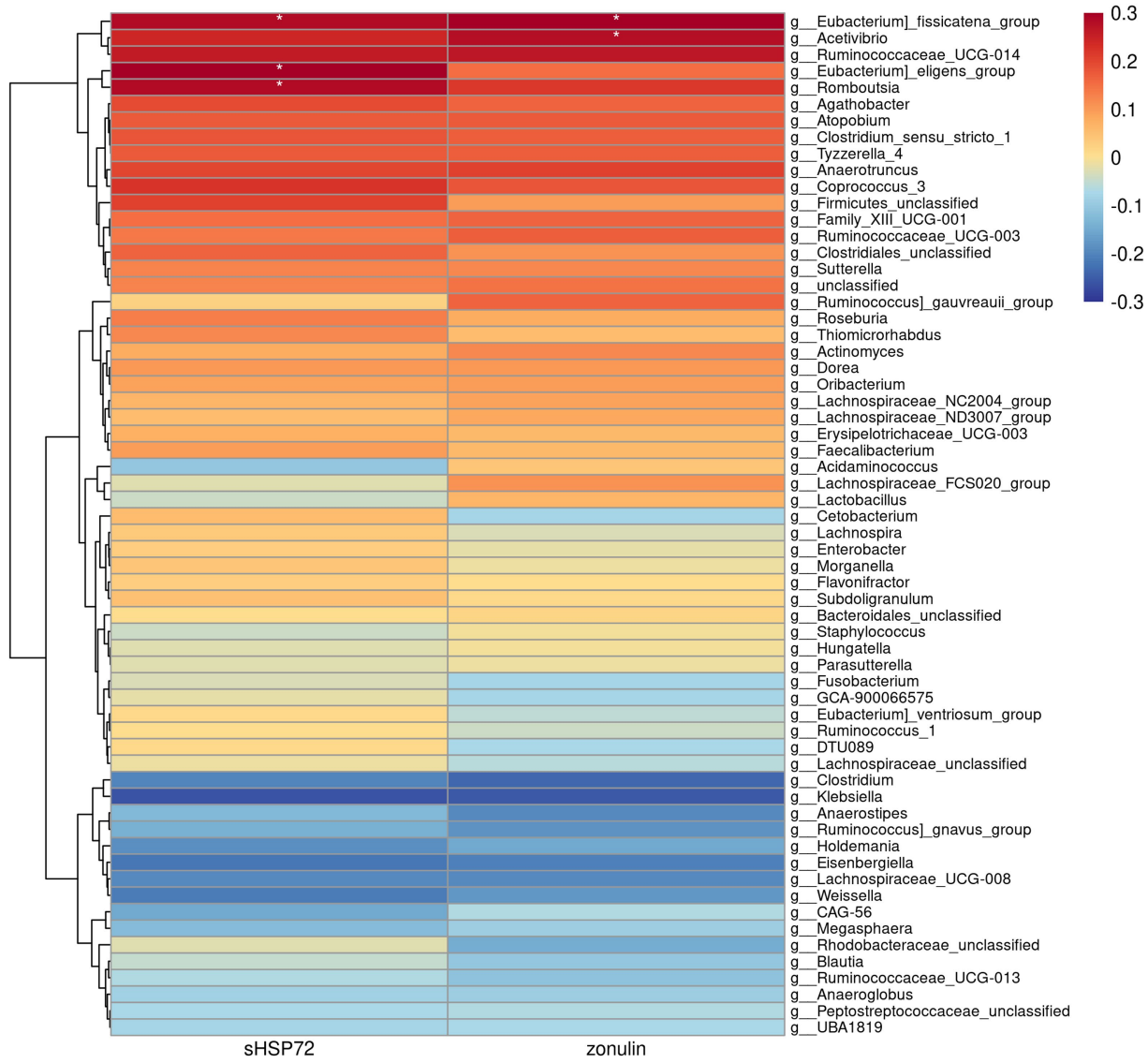

FIGURE 4
Correlation heatmap illustrating differentially abundant bacteria genus with serum HSP72 and zonulin in patients with CI. The deeper color in the figure represents a stronger correlation. The icon on the right displays the Spearman correlation coefficient gradient. Red, positive correlation. Blue, negative correlation. *, $p < 0.05$, $\rho \geq 0.28$.

positive correlation with zonulin levels ($\rho = 0.34$, $p = 0.02$; $\rho = 0.28$, $p = 0.04$) (Figure 4). Of note, the CI group was characterized by a decreased abundance of *Eubacterium_fissicatena_group*, *Eubacterium_eligens_group*, and *Romboutsia* and an increased abundance of *Acetivibrio* ($p < 0.05$).

Discussion

The present study is the first to elucidate how both serum HSP72 and zonulin levels interrelate to alterations in intestinal microbiota and CI symptom severity in patients compared with healthy controls. After adjustment for confounders, we subsequently demonstrated that an increase in serum HSP72 and zonulin was associated with the symptom severity of CI and gut microbiota alterations.

Accordingly, we demonstrated higher levels of serum HSP72 in patients with CI than in healthy controls. Notably, the levels of serum HSP72 were positively associated with CI clinical severity. Intracellular HSP72 distributes in nearly every cell of the body and provides multiple cell survival functions such as restricting protein aggregation, promoting protein refolding, and acting as protein chaperoning. Current research pays attention to confirming intra-cellular HSP72 expression levels in various diseases. In this regard, serum HSP72 may confer an immunostimulatory effect that, on the one hand, facilitates innate immune responses against acute pathogenic substances, whereas, on the other hand, serum HSP72 accelerates the

inflammatory process in people with various diseases such as hypertension and atherosclerosis (17). As a danger signal, circulation HSP72 is capable of specifically stimulating NO, TNF-α, IL-10, and IL-6 secretion from macrophages and neutrophils (18). It is noteworthy that HSP70 interacts with microglia and macrophages through toll-like receptors (TLRs) (17). The combination of HSP70 and TLRs induces the expression of genes encoding inflammation-associated molecules and cytokines through the activation of transcription factors activator protein 1 (AP-1) and nuclear factor κB (NF-κB) (19). In this connection, theoretically, HSP72 levels are related to the severity of inflammation and stress in organisms, which explains the higher levels of serum HSP72 in patients with severe CI. Previous research confirmed that overexpressing HSP72 facilitated a satisfactory prognosis of cerebral ischemia–reperfusion injury, which may be attributed to c-Jun N-terminal kinase 3 signaling pathway inhibition and Akt1 activation (20). George et al. found an association between HSP72 overexpression and reduced reactive astrocytes after stroke, which may contribute to neuroprotection (21). Moreover, Shailaja et al. suggested that HSP72 knockdown significantly upregulates apoptosis-inducing factors and ROS levels in both anoxia/reoxygenation (22). Recent work by Xu et al. (8) demonstrated that the upregulation of HSP72 in ischemic cerebral tissue has a crucial protective role in the outcome of stroke. In addition, it is noteworthy that endogenous HSP72 is released through necrotic or lytic cell death and activation of the α-adrenergic receptor pathway in response to environmental stressors in ways that can be detected in blood and excreta (23). We speculated that the elevation of the serum HSP72 level after CI mainly resulted from two leading causes. The first is that intracranial ischemic death cells rupture and release HSP72 to peripheral blood circulation. The other is that the sympathetic adrenal axis activated after CI triggers a large amount of HSP72 released from tissue cells and intracranial inflammatory cells to alleviate brain inflammation and oxidative damage. Recent evidence revealed that high levels of serum HSP72 were usually associated with enhanced oxidative profiles and ascending rates of mortality among septic patients (24). To this point, our results suggested that HSP72 may play a role in the deterioration of CI.

We observed that patients with CI had higher serum concentrations of zonulin than controls. Zonulin levels correlated with the degree of neurological deficit after CI. Our findings support previous studies that indicated that blood levels of zonulin are elevated in neuroinflammatory diseases, such as stroke, severe traumatic brain injury, neurodegenerative diseases, and psychiatric disorders (25). Produced by small intestine epithelium, the blood zonulin level becomes a reliable indicator reflecting intestinal permeability and inflammatory response level *in vivo*. Intriguingly, it is worth mentioning that intestinal epithelium plays an essential role in triggering the pathogenesis of numerous inflammatory diseases, and zonulin levels are provoked under acute stress states. Apart from the direct neural pathway connecting the intestine and the brain, it has long been appreciated that post-stroke dysbiosis gives rise to a down-modulated biosynthesis of γδT lymphocytes that directly impairs the immune system stability. It is worth mentioning that gut microbiota dysbiosis not only induces intestinal homeostasis damage but also stimulates the migration of γδT lymphocytes from the intestinal tract to the brain (26). Bacterial products and cellular components derived from the intestinal flora have an impact on the prognosis of patients with CI. Lipopolysaccharide (LPS) released by gut microbes after CI binds to TLRs and then activates the TLR4/P13K/Akt/MAPKs pathway, which sets the stage for matrix metalloproteinase 9 (MMP-9) expression in astrocytes and astrocytes migration and leads to intestinal leakage (27). Trimethylamine oxide (TMAO), a component of microbiota metabolite, is positively correlated with infarct size and severity of CI (28). Inflammatory signaling pathways mediated by TMAO involved NF-κB, pyrin dome-containing protein 3 (NLRP3) inflammasome, and the MAPK/JNK pathway in the peripheral and central nervous systems (29). Alternatively, the release of zonulin triggered by intestinal flora imbalance induces antigen influx from the intestinal lumen to the lamina propria and further exacerbates immune response, causing IFN-γ and TNF-α release (4, 30). Additionally, zonulin secretion was regarded as MyD88-dependent followed by protein ZO-1 dissociation from the tight junctional complex, which was responsible for both intestinal and extraintestinal inflammation, autoimmunity, and cancer (5, 6). Accordingly, the alterations of zonulin may be attributed to post-stroke dysbiosis and neuro-humoral mechanisms.

Moreover, we further observed the positive correlation between the levels of serum HSP72 and zonulin. A compromise of the intestinal mucosa is a result of increased severity and duration of stress and insufficient endogenous protective factors. Previous research has given insight into the endogenous protective mechanism of HSP72 within enterocytes. In 1999, an *in vitro* cell study conducted by Musch et al. (31) supported that HSP72 played a pivotal role in the integrity of the actin cytoskeleton and maintenance of epithelial barrier function under oxidant-induced stress. It remains a mystery whether serum HSP72 could be a reliable indicator reflecting intestinal permeability. We speculated that serum HSP72 may act on the brain-gut axis accelerating intestinal barrier destruction.

In the present study, the 16s rRNA sequencing results demonstrated that CI was associated with certain transformations in fecal bacteria. Consistent with the findings of previous publications, post-stroke dysbiosis was characterized by reduced diversity, decreased abundance of protective bacteria, and harmful bacterial overgrowth. Intestinal dysbiosis is effectively linked to several risk factors for stroke, such as diabetes, hypertension, and atherosclerosis, and also to stroke outcomes. However, previous studies on CI and specific alterations of intestinal flora failed to obtain unanimous and definite conclusions, which may result from different patient races, different DNA detection methods used by researchers, and different patient dietary habits.

Furthermore, we discovered that the levels of serum HSP72 and zonulin in patients with CI were correlated with the relative abundance of specific differential microbial genera. The abundance of bacteria *Eubacterium_fissicatena_group*, *Eubacterium_eligens_group,* and *Romboutsia* manifested a remarkably positive correlation with serum HSP72. The abundance of bacteria *Eubacterium_fissicatena_group* and *Acetivibrio* had a significantly positive correlation with zonulin levels. Indeed, we noted the CI group was associated with a significant decrease in the abundance of *E. fissicatena group* and *E. eligens group*. The genus *Eubacterium*, belonging to the bacterial phylum *Firmicutes*, has been identified to contribute to massive aspects of human health, for the majority of the family produce short-chain fatty acids (SCFAs), especially butyric acid. It is acknowledged and accepted that SCFAs act as a special nutrient and energy component of the intestinal epithelium, protect the intestinal mucosal barrier, and reduce inflammation levels in the body. Of note, *Eubacterium* has been shown

to detoxify toxic compounds into more benign forms in the intestine. Understandably, it has been reported recently that the reduction or absence of *Eubacterium* is associated with many diseases, such as depression, obesity, inflammatory bowel disease, type 2 diabetes, cardiovascular disease, and autism (32). However, the functional annotation of *E. fissicatena group* and *E. eligens group* remains poorly understood, partly because both of the bacterial species are rarely detected in feces in previous studies. Despite the protective nature of most members of the genus *Eubacterium*, recent evidence revealed that *E. fissicatena group* belongs to potentially disease-related bacteria that add to the risk of intestinal inflammation and metabolic disorders (33). Jing et al. reported that the increased abundance of *E. fissicatena group* had a positive correlation with serum TMAO levels, which was one of the independent risk factors of acute coronary syndrome (34). Nevertheless, *E. fissicatena group* was also regarded as butyrate-producing bacteria and beneficial bacteria suppressing intestinal inflammation (35). Another associated genus *E. eligens group* has been widely acknowledged to exhibit its probiotic effects. Using metagenomic analysis to estimate the gut microbiome profile in atherosclerosis patients, Sheng et al. revealed that the abundance of *E. eligens group* was positively correlated with propionate and butyrate production but was negatively correlated with inflammatory marker high-sensitivity C-reactive protein and visceral fat area. Similarly, *E. eligens group* played vital roles in the pathway CDP-diacylglycerol biosynthesis and was also significantly correlated with higher high-density lipoprotein-cholesterol levels, which significantly modulate the lipid metabolism (36). Afterward, *in vitro* cell-based assays found that *E. eligens group* efficiently promoted the production of the anti-inflammatory cytokine IL-10, suggesting the potential to be a therapeutic target for inflammatory diseases (37). In general, it seems reasonable in our results that *E. fissicatena* and *E. eligens group*, combined with serum HSP72 and zonulin, have the potential to be involved in the post-stroke systemic inflammatory response.

Our research also added to previous reports that the CI group has an increased abundance of genus *Acetivibrio*, which manifested a positive correlation with zonulin levels. The genus *Acetivibrio* was equipped with efficient biological machinery transferring lignocellulose into ethanol and has been known to ferment carbohydrates to produce acetic acid (38). Yuan et al. (39) provided evidence that *A. ethanolgignens group* played a pivotal role in facilitating inflammation and lipid metabolism abnormalities as well as interfering with the energy supply process of the tricarboxylic acid cycle. Normal peristalsis, digestive, and absorption functions of the gut require a series of coordinated operations of intestinal cells. Intestinal flora disorders and systemic inflammation will allow for intestinal barrier disruption and the invasion of harmful substances into circulation. We speculated that *Acetivibrio* accelerated increased intestinal permeability through the induction of metabolic disturbance and energy intake difficulty of intestinal cells.

Genus *Romboutsia* are SCFA producers and immunomodulators in the gut, which act in the maintenance of intestinal barrier integrity. Our results revealed that a significantly increased abundance of *Romboutsia* genus was observed in the healthy group as compared to the CI group, and the higher the abundance of *Romboutsia* genus, the higher the levels of serum HSP72. In earlier studies, Gerritsen et al. (40) showed that, as a dominant taxon in the small intestine of rats, *Romboutsia* displayed a restricted capacity to synthesize amino acids and vitamins, whereas it was adept at the utilization of different relatively simple carbohydrates (40). Intriguingly, *Romboutsia* has the potential to engage in obesity-related metabolic abnormalities. Previous studies conducted by Zeng et al. (41) indicated that *Romboutsia* was positively associated with body weight, serum lipids, and UA. We, therefore, speculated that the decreased abundance of *Romboutsia* could be an indicator of post-stroke dysbiosis and that the genus *Romboutsia* may have something to do with serum HSP72 levels and post-stroke immunomodulatory effects.

Taken together, our findings proposed that an increase in serum HSP72 and zonulin was observed in patients with CI. It has to be emphasized that the levels of serum HSP72 and zonulin were related to the clinical severity of CI and specific gut microbiota alterations. Our present study has some limitations. First, it was a single-center cross-sectional study with inevitable time and place biases. Second, considering timely bowel movements, the NIHSS scores of CI patients enrolled in this study did not reach more than 15, which made it impossible to assess the relationship between extremely severe CI and the levels of serum HSP72 and zonulin. Finally, a considerable part of patients in the CI group were accompanied by different coexisting diseases that may have affected the results. In general, our results provided promising research prospects that the levels of serum HSP72 and zonulin have the potential to serve as prospective markers for distinguishing patients with CI from controls and mirroring disease severity. Further investigation is required to explore the definitive mechanisms of how serum HSP72 and zonulin act on the process of post-stroke systemic inflammation and intestinal dysbiosis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the studies involving human participants were reviewed and approved by the Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from a by-product of routine care or industry. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

JZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration,

Resources, Writing – original draft. YD: Writing – original draft. BT: Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. HZ: Project administration, Resources, Software, Supervision, 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

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Glossary

CI	Cerebral infarction
ELISA	enzyme-linked immunoassay
NIHSS	National Institute of Health Stroke Scale
EGF	epidermal growth factor
TJ	tight junction
HSP72	heat shock protein 72
CT	computed tomography
MRI	magnetic resonance imaging
ROC	receiver operating characteristic
AUC	area under the curve
SCFAs	short-chain fatty acids
TMAO	trimethylamine N-oxide
TLRs	toll-like receptors
AP-1	activator protein 1
NF-κB	nuclear factor κB
LPS	lipopolysaccharide
MMP-9	metalloproteinase 9
MCAO	middle cerebral artery occlusion

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RET is a sex-biased regulator of intestinal tumorigenesis

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Ret is implicated in colorectal cancer (CRC) as both a proto-oncogene and a tumor suppressor. We asked whether RET signaling regulates tumorigenesis in an *Apc*-deficient preclinical model of CRC. We observed a sex-biased phenotype: $Apc^{Min/+}$*Ret*+/- females had significantly greater tumor burden than $Apc^{Min/+}$*Ret*+/- males, a phenomenon not seen in $Apc^{Min/+}$ mice, which had equal distributions by sex. Dysfunctional RET signaling was associated with gene expression changes in diverse tumor signaling pathways in tumors and normal-appearing colon. Sex-biased gene expression differences mirroring tumor phenotypes were seen in 26 genes, including the *Apc* tumor suppressor gene. *Ret* and *Tlr4* expression were significantly correlated in tumor samples from female but not male $Apc^{Min/+}$*Ret*+/- mice. Antibiotics resulted in reduction of tumor burden, inverting the sex-biased phenotype such that microbiota-depleted $Apc^{Min/+}$*Ret*+/- males had significantly more tumors than female littermates. Reconstitution of the microbiome rescued the sex-biased phenotype. Our findings suggest that RET represents a sexually dimorphic microbiome-mediated "switch" for regulation of tumorigenesis.

KEYWORDS

colorectal cancer, microbiome, RET, sexual dimorphism, Apc Min/+ mice

Introduction

Colorectal cancer (CRC) is one of the most common cancers globally. *Ret*, which is critical in enteric nervous system (ENS) development and maintenance, has been implicated as both a proto-oncogene (1) and tumor suppressor (2) in CRC. While RET fusions in metastatic CRC portend a poor prognosis (3), specific RET inhibitors such as selpercatinib and pralsetinib have demonstrated efficacy in targeting oncogenic RET rearrangements [reviewed at (4, 5)]. *Apc* encodes a tumor suppressor and is commonly mutated in CRC. We assessed interactions of *Apc* and *Ret* through a crossbreeding experiment using $Apc^{Min/+}$ mice and *Ret*+/- mice (6). We administered 1.5% dextran sodium sulfate (DSS) to induce colonic tumors in $Apc^{Min/+}$*Ret*+/- progeny (as well as

littermate controls with a mutation in *Apc* only, *Ret* only, or neither). We expected that $Apc^{Min/+}$ mice would develop many intestinal tumors compared to wild-type or *Ret*+/- mice, which are not predisposed to developing tumors, but it was unknown whether mice harboring mutations both in *Apc* and *Ret* would develop a greater, lesser, or equivalent number of tumors compared to $Apc^{Min/+}$ mice. We hypothesized that if tumorigenesis is regulated by RET signaling, then we should observe a change in tumor burden in $Apc^{Min/+}$*Ret*+/- mice that is either decreased compared with $Apc^{Min/+}$ mice if RET signaling promoted tumorigenesis or increased if RET suppressed tumorigenesis.

Results

Consistent with expectations, (i) $Apc^{Min/+}$ mice (n=25) had a significantly greater tumor burden than wild-type littermates (n=31) (mean ± SEM: 22.6 ± 2.2 vs 0.16 ± 0.12, $p<10^{-8}$ [females]; 31.2 ± 6.1 vs 0 ± 0, p=0.004 [males]; Figures 1A, B; Supplementary Table 1); (ii) *Ret*+/- mice (n=13) had tumor burdens not significantly different from wild-type littermates (n=31) (0.7 ± 0.7 vs 0.2 ± 0.1 [females]; 0.1 ± 0.1 vs 0 ± 0, [males]; Figure 1A; Supplementary Table 1); and (iii) compared to $Apc^{Min/+}$ mice that did not receive DSS (n=30), DSS-treated $Apc^{Min/+}$ mice developed more colonic tumors (9.3 ± 1.0 vs 1.1 ± 0.3, $p<10^{-6}$ [females]; 10 ± 1.7 vs 1.8 ± 0.3, p=0.004 [males]). In the experimental $Apc^{Min/+}$*Ret*+/- cohorts, there was no overall significant difference in tumor burden compared to $Apc^{Min/+}$ mice (two-way ANOVA; Figure 1A). However, we observed a sex-biased interaction of *Ret* and *Apc*: total (colonic plus small intestinal) tumor burden was significantly greater in female $Apc^{Min/+}$*Ret*+/- mice compared to male $Apc^{Min/+}$*Ret*+/- mice (3-way ANOVA, interaction between *Apc*, *Ret*, and sex covariates: p<0.003, $F_{1,74} = 10$; Figure 1B). Compared to males, female $Apc^{Min/+}$*Ret*+/- mice had significantly greater tumor burden in the distal colon, which we defined as the last 25% of the colon by length (2.94 ± 1.17 vs 0 ± 0, p<0.04, Student's two-tailed *t*-test; Figure 1C). In the small intestine, $Apc^{Min/+}$*Ret*+/- females had more tumors than males (20.5 ± 4.2 vs 10.6 ± 2.3, p=0.06, Student's two-tailed *t*-test). These sex-biased differences did not exist among $Apc^{Min/+}$ mice.

Male $Apc^{Min/+}$*Ret*+/- mice had longer small intestines than female $Apc^{Min/+}$*Ret*+/- mice (36.4 ± 0.37 cm vs 33.9 ± 0.85 cm; p<0.03, Student's two-tailed *t*-test). While of unclear biological significance, it excludes small intestinal length as a potential confounder for the difference in small intestinal tumor burden, which was greater in females. Otherwise, there were no sex-based differences in tumor size, intestinal lengths, or whole gut transit times (Supplementary Table 1). Within control cohorts, there were also no sex-based differences in these metrics.

To define *Ret*'s role in tumor signaling pathways, we profiled gene expression in colonic tumors (n=24) and healthy-appearing colonic tissues (n=24) from male and female $Apc^{Min/+}$ and $Apc^{Min/+}$*Ret*+/- mice (Supplementary Table 2A). Global gene expression profiles clustered predominantly by whether they represented tumors or healthy tissues (p=0.001, PERMANOVA [permutational multivariate analysis of variance using distance matrices] using Bray-Curtis dissimilarity; Figure 2A). Expression levels of a subset of genes were nonetheless affected by the interaction between sex, genotype, and tissue sample type (ANOVA: p<0.05; Figure 2B). Consistent with their established biological roles, *Apc*, *Fxr* (*Wnt* pathway antagonist; bile acid receptor), and *Vdr* (vitamin D receptor; bile acid receptor) were expressed at higher levels in healthy tissues. In tumors, *Myc* (oncogene), *Lgr5* (stem cell marker), and *Wnt5a* (*Wnt* pathway activator) were expressed at higher levels and were affected by the interaction between sex and genotype (ANOVA: p<0.05). Consistent with *Fxr*'s role as a negative regulator of bile acid synthesis, total bile acid concentrations were higher in females than males (3,604.2 ± 418.2 ng/mg stool vs 2,410.84 ± 177.2 ng/mg stool, p=0.01, Student's two-tailed *t*-test).

Ret deficiency appeared to dysregulate homeostatic gene expression networks, resulting in a dramatic increase in numbers of genes significantly correlated with *Apc* expression in tumor samples (Figure 2C). Interestingly, $Apc^{Min/+}$*Ret*+/- female mice had more positive than negative correlations, while $Apc^{Min/+}$*Ret*+/- male mice had more negative than positive correlations (Figure 2C). The genes correlated with *Apc* are implicated in diverse pathways, suggesting global effects on gene expression (Supplementary Table 2B). In healthy samples, *Apc* expression was greater in $Apc^{Min/+}$*Ret*+/- males than in females (2,452 ± 58 arbitrary units [AU] vs 2,181 ± 83 AU, p<0.03, Student's two-tailed *t*-test). In tumor samples, $Apc^{Min/+}$*Ret*+/- males had significantly higher *Apc* expression than both $Apc^{Min/+}$*Ret*+/- females (2,160 ± 80 AU vs 1,854 ± 55 AU, p<0.02, Student's two-tailed *t*-test) and $Apc^{Min/+}$ males (2,160 ± 80 AU vs 1,885 ± 29 AU, p=0.02, Student's two-tailed *t*-test). Notably, within tumor samples, *Apc* expression was higher in male $Apc^{Min/+}$*Ret*+/-

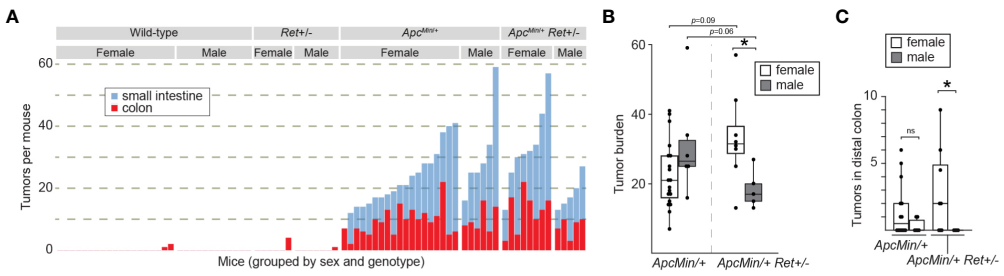

FIGURE 1
RET signaling regulates intestinal tumorigenesis in a sex-dependent manner. **(A)** Tumor burden in male and female wild-type, *Ret*+/-, $Apc^{Min/+}$, and $Apc^{Min/+}$*Ret*+/- mice treated with 1.5% DSS. Each stacked bar represents one mouse. **(B, C)** Total intestinal and distal colonic tumor burdens. *: p<0.05, ns: not significant.

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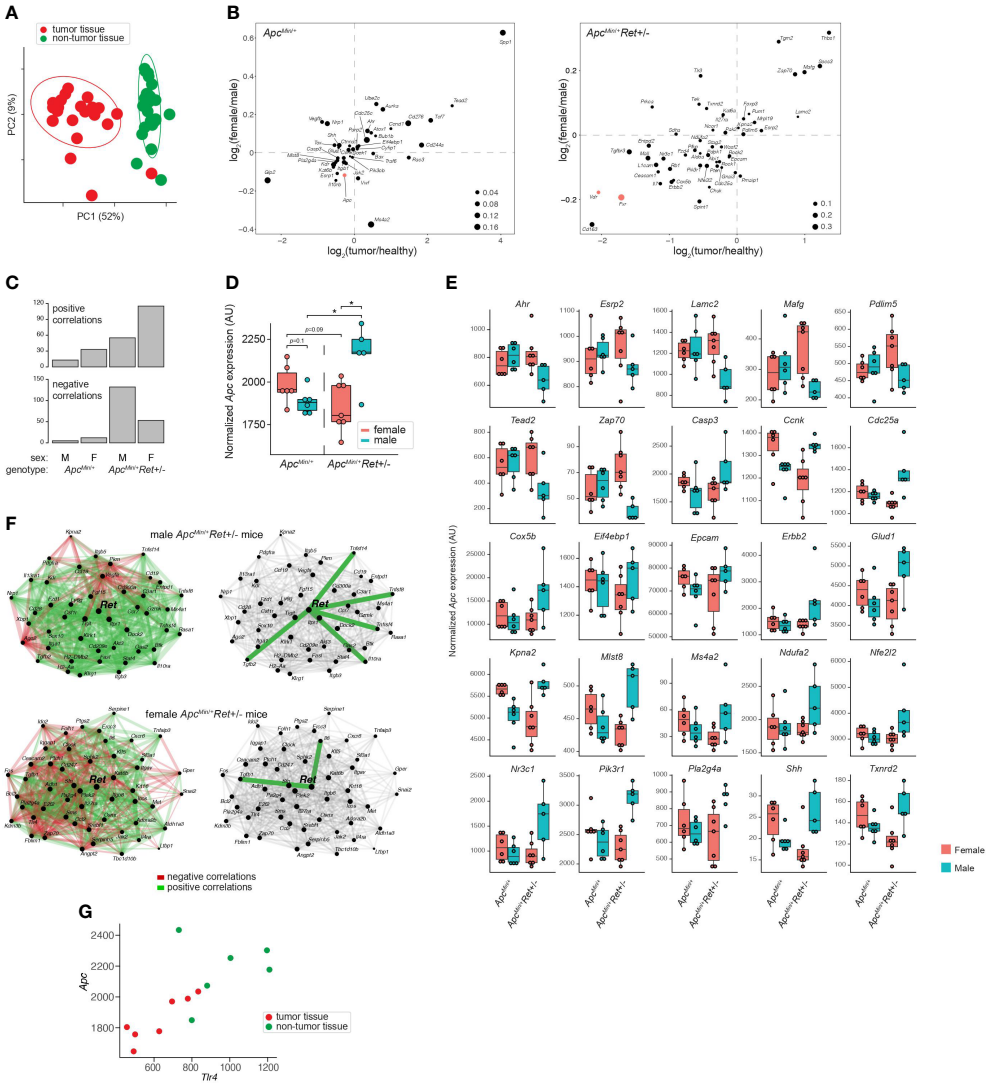

FIGURE 2
Global and gene-specific variation in colonic gene expression is regulated by RET signaling in a sex-dependent manner. **(A)** Principal coordinate analysis of global gene expression variation across samples. **(B)** Genes whose expression was significantly affected by the interaction between sex, genotype, and sample type. 2D enrichment plots of genes significantly enriched in tumors (x-axis) or in females (y-axis) in $Apc^{Min/+}$ (left) and $Apc^{Min/+}$*Ret*+/- (right) mice. The dot sizes represent log of fold change of expression in each genotype compared to the other. Three genes referenced in the text are colored red. **(C)** Numbers of genes that are significantly correlated with *Apc* expression in different sex and genotype contexts. **(D)** Tumor *Apc* expression split by cohort. **(E)** 25 genes, other than Apc, whose expression in tumors mirrored the sex-biased genotype-dependent tumor phenotype based on the mean expression for each group. Plots are organized by genes that first follow the tumor phenotype (U shaped) and then the inverse. **(F)** Sex biases in *Ret* gene networks in $Apc^{Min/+}$*Ret*+/- mice. **(G)** *Tlr4* and *Apc* correlation in $Apc^{Min/+}$*Ret*+/- females. *: p<0.05.

mice than in female $Apc^{Min/+}$*Ret*+/- mice, and this trend was opposite in $Apc^{Min/+}$ mice — the inverse of the sex-biased genotype-dependent tumor phenotype (Figure 2D). Including *Apc*, tumor-intrinsic expression levels of 26 genes mirrored tumor phenotypes (exactly or inversely) and were significantly affected by interactions between sex and genotype ($p<0.05$, two-way ANOVA) (Figure 2E). While *Apc* expression levels varied with tumor phenotypes in such a manner that it may plausibly explain the underlying biology, the biological significance of the other 25 genes is more challenging to interpret and underscores complexity of gene network interactions.

Strikingly, tumor-intrinsic *Ret*-centric gene expression networks were entirely non-overlapping between male and female $Apc^{Min/+}$*Ret*+/- mice (Figure 2F). In tumors harvested from $Apc^{Min/+}$*Ret*+/- males, we observed correlations between *Ret* and *Il10ra*, *Tnfsf14*, *Tnfsf8*, *Tnfrsf4*, and *Tgfb2* (r≥0.89, $p<0.05$, Pearson correlation), which are involved in anti-inflammatory responses. In contrast, in $Apc^{Min/+}$*Ret*+/- females, *Ret* was significantly correlated with *Tgfb1* and with *Il6* (r≥0.78, $p<0.05$, Pearson correlation), both of which are involved in upregulating the immune system. These findings suggest links between the immune system and *Ret*, whereby loss of functional RET signaling leads to a pro-inflammatory state in females and an anti-inflammatory state in males.

Intriguingly, *Ret* expression was significantly correlated with *Tlr4* expression in tumor samples from all cohorts of mice (r≥0.79, $p<0.04$, Pearson correlation) except for the male $Apc^{Min/+}$*Ret*+/- mice. TLR4 is critical in microbial pattern recognition and is overexpressed in

colorectal cancers and adenomas compared to healthy tissues in humans (7). *Tlr4* was correlated with *Apc* in $Apc^{Min/+}$*Ret*+/- female mice (tumor samples: r=0.88, $p<0.01$; all samples: r=0.73, $p<0.005$, Pearson correlation; Figure 2G) but not in other cohorts.

Thus, we next tested the role of the gut microbiome in driving sex bias in tumor phenotypes. We either administered chronic antibiotics or first depleted the native microbiota with antibiotics and then reconstituted the microbiota via oral gavage using a uniform fecal suspension. Chronic antibiotics reduced tumor burden, significantly more so in females, and resulted in complete inversion of the sex-biased tumor phenotype: microbiota-depleted $Apc^{Min/+}$*Ret*+/- females had significantly fewer tumors than males (3.7 ± 1.5 vs 12.7 ± 1.5, $p=0.005$, two-tailed Student's *t*-test; Figure 3A). The interaction between sex and microbiome status (harboring or lacking a microbiome) was significant ($p<0.02$, $F_{1,12} = 7$, two-way ANOVA).

Finally, we asked whether human CRC data support our findings. Approximately 10% of CRCs are reported as harboring *Ret* mutations (2). Intriguingly, in The Cancer Genome Atlas (8), CRCs with *Ret* mutations occurred predominantly in females (z=2.98, $p<0.003$). Further, in a reanalysis of published CRC microbiome surveys (9), we found that 68 bacterial species were differentially abundant between sexes in CRC (Figure 3B); of these, only 11 species were also differentially abundant in the healthy cohort, evidencing CRC-specific sex biases. *E. coli* was more abundant in males with CRC than in females with CRC, whereas in the healthy cohort, *E. coli* was more abundant in females than in males. In contrast, *Fusobacterium nucleatum* was more abundant in females than in males in the CRC cohort. *F. nucleatum*, which is linked to CRC progression and metastasis, has been associated with CpG methylation and a CpG island methylator phenotype, which is associated with the female sex (10).

Discussion

The answer to our initial question — is RET an oncogene (1) or tumor suppressor (2) in CRC? — appears to be complicated: it depends on sex and the microbiome. Underlying the phenotype that we discovered were significant differences in tumor burden in the distal colon. Interestingly, public datasets suggest that *Ret* mutations in CRC and CRC microbiome signatures are both sex-biased. Our study offers proof-of-principle that CRC risk-modulating gut microbial effects depend on sex and genetics, and they underscore the importance of evaluating sex as a biological variable in research and of reporting the sexes of both human and non-human study participants.

A limitation of this study is that the specific cellular players remain unknown. Our gene expression data suggest that both tumor-intrinsic and tumor-extrinsic cells are disrupted by *Ret* insufficiency, and that the resulting *Ret* gene networks starkly differ by sex. RET is a critical player in the ENS, which transmits intestinal growth signals via the GLP-2 pathway (11, 12), potentially facilitates CRC metastasis (13, 14), and regulates immunity (15) and physiology (16). Enteric neurons express estrogen receptor (17) so could form the basis for sex-biased microbiome-dependent tumorigenesis. Future studies should elucidate tumor cell-autonomous effects versus effects of a RET-deficient ENS. This research could lead to novel personalized CRC prevention tactics.

Materials and methods

Animal husbandry

Male and female $Apc^{Min/+}$, *Ret*+/-, and $Apc^{Min/+}$*Ret*+/- mice (C57BL/6 background) were studied using methods approved by the Institutional Animal Care and Use Committee of Fred Hutchinson Cancer Center (protocol 51049). Mice from the same litter were co-housed regardless of differences in genotype, thereby leading to shared microbiota due to coprophagy. Mice were fed *ad libitum* with PicoLab Mouse Diet 5058. DSS (1.5% by volume) was administered in drinking water for 7 days to mice at 6-8 weeks of age. Following DSS exposure, mice were given standard drinking water for the remainder of the experiment. Mice were euthanized

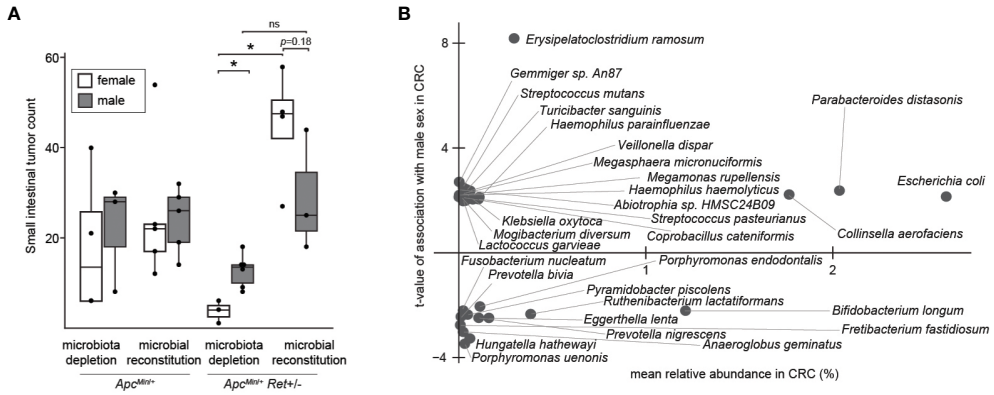

FIGURE 3
The microbiome as a potential sex-biased driver of intestinal tumorigenesis. **(A)** Effects of microbiota depletion with or without gut microbial reconstitution on tumor burden. **(B)** Bacterial species enriched in males (up) or females (down) in human CRC microbiomes, adjusting for biases in non-CRC cohorts. *: p<0.05, ns: not significant.

~6 weeks after DSS exposure (or earlier if exhibiting symptoms of high tumor burden) using aerosolized 1.5% isoflurane delivered through a precision vaporizer. At euthanasia, small intestines and colons were harvested, rinsed in ice cold phosphate buffered saline to remove fecal material, and opened longitudinally for tumor counting and characterization. Fresh fecal pellets were snap-frozen in liquid nitrogen and stored at -80°C until use. *Tumor localization analysis*. Digital images of colons were equally split into quartiles. Tumors spanning different segments of the colon had fractions of tumors counted in each segment. These fractions were estimated as 0.25, 0.5, or 0.75, and the fractions for each tumor add up to 1. *Microbiome manipulation studies*. Vancomycin (1 g/L), metronidazole (1 g/L), and neomycin (0.5 g/L) antibiotics (Millipore Sigma, St. Louis, MO) were delivered via drinking water containing 2% sucrose (20 g/L) over 10 days or, in the experiment involving chronic antibiotic administration, cyclically throughout the duration of the experiment. Recolonization was performed via oral gavage of a fecal suspension from female $Apc^{Min/+}Ret$+/- mice. Due to enhanced DSS toxicity in the setting of antibiotic use, mice receiving antibiotics were not given DSS; therefore, small intestinal tumor counts were used as readouts in these experimental cohorts.

Gut transit time measurements

Using previously published methods (18), mice were gavages with 200 µL per mouse of a sterilized 6% red carmine dye solution (Sigma C1022) and monitored for time to initial passage per rectum. Gavages were performed by the same individual (N.L.) within a consistent time frame in all mice in order to minimize variability.

RNA isolation

After flushing with PBS, gut segments were stored in RNA*later* (Thermo Fisher Scientific Inc., Waltham, MA) at 4°C for 24 hours and then transferred to -20°C for storage until use. To isolate RNA, 20 mg of each sample was placed into a tube containing 0.1 mm zirconium beads, a 4 mm steel ball, and homogenization buffer before mechanical disruption using a TissueLyser II (Qiagen, Hilden, Germany). RNA was purified using RNeasy Mini kits (Qiagen).

Colonic tumor and non-tumor gene expression profiling

Gene expression data were generated using NanoString nCounter® Tumor Signaling 360 Panels in conjunction with a 55-gene Panel Plus.

Analysis of published human microbiome datasets

We utilized *curatedMetagenomicData*, an R package linked to a database of curated data from nearly 100 microbiome surveys (9). We filtered in stool samples from individuals with CRC and healthy individuals at least 18 years of age, none of whom reported current antibiotic use. We identified 625 stool samples from individuals with CRC (392 males and 233 females) and 5,221 stool samples from otherwise healthy individuals (2,178 males and 3,043 females). To control for differences in age and study population, we included only the subset of individuals from the healthy cohort who were (i) from the same countries represented in the CRC cohort and (ii) within 2 years of age of an individual in the CRC cohort. Ultimately, this resulted in a healthy cohort comprised of 1,707 individuals (911 males and 796 females). We then used *corncob*, an R package that uses beta-binomial regression to model relative abundances and identify bacteria significantly associated with covariates of interest (19), to identify bacterial species that were significantly differentially abundant in CRC between sexes.

Data analysis

Statistical comparisons were performed in R (version 4.0.0). Figures were generated using R using native functions as well as the *ggplot2* (version 3.1.0) and *pheatmap* (version 1.0.12) packages. Figures were assembled in Adobe Illustrator.

Data availability statement

The previously published sequencing datasets re-analyzed in this study can be found in the online curated Metagenomic Data database, which lists the names of the original repositories along with corresponding accession numbers. The gene expression data we generated and present here are reported in Supplementary Table 2.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of Fred Hutchinson Cancer Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. NL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. ND: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgstr.2023.1323471/full#supplementary-material

SUPPLEMENTARY TABLE 1
Individual mouse data. Unique mouse identification numbers, cage identification numbers, sex, genotype, age at time of euthanasia, microbiota, whether DSS was used, diet, transit times, small intestinal length, cecal length, colonic length, and tumor counts by location.

SUPPLEMENTARY TABLE 2
Gene expression data. **(A)** Normalized data from the NanoString nCounter® Tumor Signaling 360 Panels in conjunction with a 55-gene Panel Plus. **(B)** Genes significantly correlated with *Apc* expression in $Apc^{Min/+}$ and $Apc^{Min/+}$*Ret*+/- mice.

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Case report: Primary CDK4/6 inhibitor and endocrine therapy in locally advanced breast cancer and its effect on gut and intratumoral microbiota

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Locally advanced breast cancer poses significant challenges to the multidisciplinary team, in particular with hormone receptor (HR) positive, HER2-negative tumors that classically yield lower pathological complete responses with chemotherapy. The increasingly significant use of CDK 4/6 inhibitors (CDK4/6i) plus endocrine therapy (ET) in different breast cancer settings has led to clinical trials focusing on this strategy as a primary treatment, with promising results. The impact of the microbiota on cancer, and vice-versa, is an emerging topic in oncology. The authors report a clinical case of a postmenopausal female patient with an invasive breast carcinoma of the right breast, Luminal B-like, staged as cT4cN3M0 (IIIB). Since the lesion was considered primarily inoperable, the patient started letrozole and ribociclib. Following 6 months of systemic therapy, the clinical response was significant, and surgery with curative intent was performed. The final staging was ypT3ypN2aM0, R1, and the patient started adjuvant letrozole and radiotherapy. This case provides important insights on primary CDK4/6i plus ET in locally advanced unresectable HR+/HER2- breast cancer and its potential implications in disease management further ahead. The patient's gut microbiota was analyzed throughout the disease course and therapeutic approach, evidencing a shift in gut microbial dominance from Firmicutes to Bacteroidetes

and a loss of microbial diversity following 6 months of systemic therapy. The analysis of the intratumoral microbiota from the surgical specimen revealed high microbial dissimilarity between the residual tumor and respective margins.

KEYWORDS

breast cancer, CDK 4/6 inhibitors, gut microbiota, gut microbiome, microbiota, microbiome, intratumoral microbiota

Introduction

Breast cancer is the second most common neoplasm worldwide and represents the leading cause of cancer-related death among women in over 100 countries (1). It is a heterogeneous disease that can be further classified into different molecular subtypes with specific prognostic and therapeutic implications. Hormone receptor-positive (HR+) and HER2-negative (HER2-) breast cancer is the most common subtype, accounting for more than 65% of all breast cancers (2).

Endocrine therapy (ET) is the mainstay of HR+/HER2- breast cancer's systemic therapy, being recommended as adjuvant treatment in early disease and as the preferred option in the metastatic setting in the absence of visceral crisis (3–5). However, in recent years, this disease's systemic approach has changed considerably with the discovery and establishment of CDK 4/6 inhibitors (CDK4/6i), initially in the metastatic setting and, more recently, in adjuvancy (6–8). The cyclin-dependent kinases (CDKs) are a large family of serine-threonine kinases that have important roles in cell cycle regulation (9). The dysregulation of mechanisms that govern the cell cycle, such as the complex interplay between cyclins and their associated CDKs, results in uncontrolled cellular proliferation and constitutes one of the hallmarks of cancer (10). Cyclin D binds CDK 4/6 and then hyperphosphorylates retinoblastoma protein (pRb), which results in cancer cell cycle progression. CDK4/6i block the hyperphosphorylation of pRb, causing G1 arrest and thereby hindering proliferation (11).

The increasing evidence that complex microbial ecosystems play a substantial role in tumorigenesis, cancer differentiation, and malignant progression has recently led to the inclusion of polymorphic microbes as an emerging hallmark of cancer (12). The most significant evidence for this integrated role comes from studying microbes within the gastrointestinal tract, also known as gut microbiota. However, there has been a growing appreciation of the role of these polymorphic microbes in other tissues and organs, including those living within tumors (intratumoral microbiota).

Gut microbiota is unique in each individual and is determined by lifestyle and genetic factors, posing a challenge in distinguishing healthy from abnormal gut microbiota. Microbial dysbiosis refers to a maladaptation or abnormal composition of the microbial community of a given organ or tissue, and increasing evidence suggests it may influence tumor biology, drug metabolism, and immune system regulation (13). To understand the differences between homeostatic and dysbiotic microbiota, it is essential to comprehend the concepts of α and β-diversity (14). α-diversity measures the diversity of microbial species within a sample and can be calculated by Operational Taxonomic Units (OTUs) count (which refers to the number of different species in the sample) or by Simpson's and Shannon's diversity indices (which measure how evenly the microbes are distributed). β-diversity is used to compare different samples, accessing the differences in microbial composition between them.

It is believed that both gut and breast microbiota may play a role in breast carcinogenesis, namely through the secretion and metabolism of hormone-like bioactive compounds (14, 15). Intratumoral microbiota is thought to contribute to cancer initiation and progression through DNA mutations, activation of carcinogenic pathways, promotion of chronic inflammation, the complement system, initiation of the metastatic process, and modulation of antitumor immunity (16). Different tumor types have distinct intratumoral microbial compositions, with breast cancer standing out for a particularly rich and diverse microbiota (17).

The prognostic value of gut and intratumoral microbiota in breast cancer is an active research area. Several studies have found correlations between specific microbiota compositions and outcomes such as tumor progression, metastases, response to therapy, and toxicities (14, 18–20). However, the clinical significance of these findings remains mainly unclear, and microbiota analysis and modulation strategies are not current practice in breast cancer management.

This paper depicts the clinical case of a postmenopausal female patient diagnosed with a locally advanced HR+/HER2- breast carcinoma that was considered primarily unresectable and was therefore proposed for systemic therapy with an aromatase inhibitor (AI) and a CDK4/6i, achieving a good clinical response. The case demonstrates the potential of this therapeutic approach in a setting in which high-level evidence is still lacking. Moreover, the patient was included in the BioBreast study, a study that aims to understand the interplay between microbiota and systemic therapy in breast cancer patients, allowing a unique analysis of the patient's gut microbiota throughout the disease course and therapeutic approach, as well as a comprehensive characterization of intratumoral microbiota.

Case description

Patient information

A female patient in her late 60s consulted a general surgeon because of a painful mass in her intermammary cleft that lasted for approximately four months. The patient's medical history revealed essential hypertension and dyslipidemia, and she was medicated accordingly with olmesartan and simvastatin. Her surgical history revealed a previous ovarian cystectomy. Her menarche was at 17 years old, menopause at 50, and she had two pregnancies, two deliveries (G2P2A0) and breastfed. The patient did not report any family history of breast, uterine, or ovarian cancer.

Clinical findings

At physical examination, she presented an enlarged right breast with lower-quadrant edema and an infiltrative ulcerated mass with a multinodular aspect in the intermammary cleft (Figure 1A). At palpation, it was possible to identify multiple right axillary adenopathies.

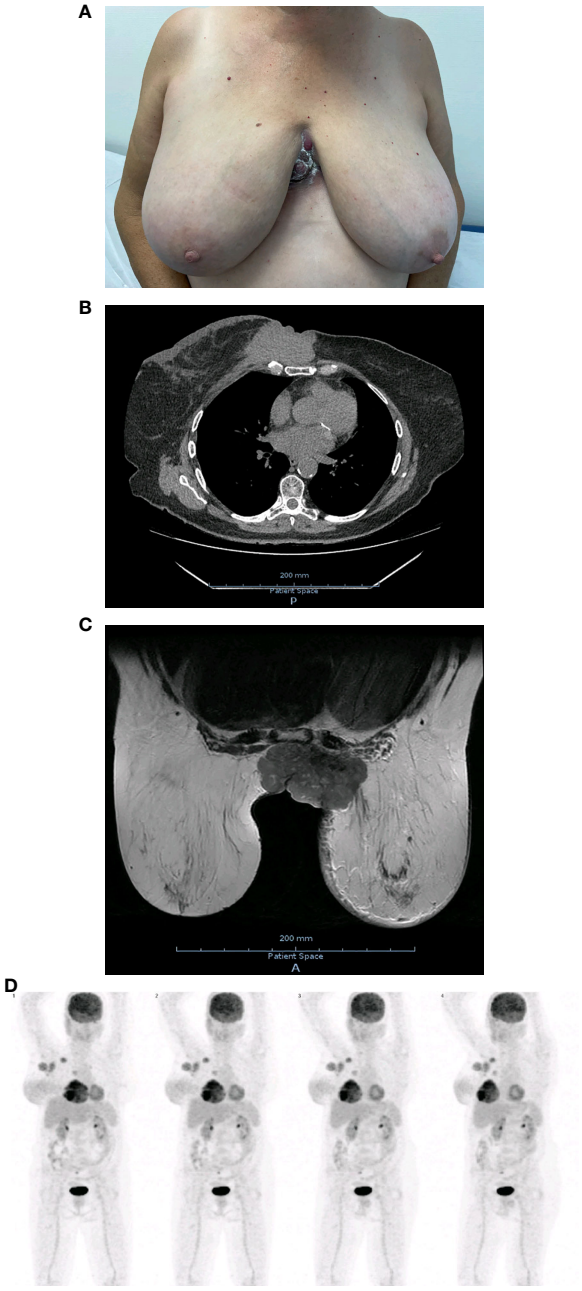
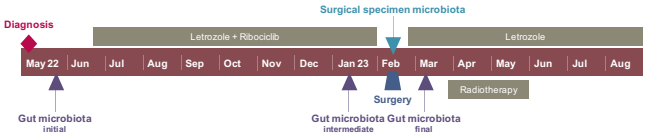


FIGURE 1
(A) Initial clinical presentation. **(B)** Initial chest CT. **(C)** Initial breast MRI. **(D)** Whole body on PET/CT with 18F-FDG at initial staging.

Timeline



Diagnostic assessment

Following this presentation, she underwent an ultrasound-guided core biopsy of the mass and a chest computerized tomography (CT). The chest CT revealed a solid mass with irregular borders in the medial area of the right breast measuring 53 x 82 x 86 mm with posterior involvement of the sternum and chondrosternal joints (Figure 1B) and multiple enlarged nodes in the right axillary region. The core biopsy confirmed an invasive breast carcinoma of no special type (NST), moderately differentiated (Grade 2), GATA3+, CK7+, with an expression of estrogen and progesterone receptors in 95% and 75% of tumor nuclei, respectively. The C-ERB-B2 score was 1+, and the tumor's proliferative index, accessed by Ki67 expression, was 30%.

In order to complete a mammary study, a breast magnetic resonance imaging (MRI), ultrasound, mammogram, and an ultrasound-guided microbiopsy of the axilla were requested. The breast MRI revealed a lesion of 90 x 55 x 76 mm occupying the totality of the lower-inner quadrant of the right breast, with a necrotic component that invaded and ulcerated the overlying skin, upper anterior abdominal wall, chest wall, and the lower-inner quadrant of the left breast (Figure 1C). The MRI and ultrasound further revealed a suspicious lymph node in the right internal mammary chain, as well as multiple enlarged lymph nodes on levels I, II and III of the right axilla. The axillary histology revealed fibroadipose tissue infiltrated with breast carcinoma of NST without any identifiable lymph node tissue.

Positron emission tomography (PET)/CT with 18F-fluorodeoxyglucose (18F-FDG) was performed to complete staging and evaluate for possible signs of distant metastases. The exam was positive for right axillary, left parasternal, and right supraclavicular lymph nodes, without evident bone involvement or other images suggesting distant metastases (Figure 1D).

Considering these studies, the patient was diagnosed with invasive breast carcinoma NST of the right breast, Luminal B-like, and was staged as cT4cN3M0, corresponding to stage IIIB according to AJCC's TNM 8th edition. The patient's case was discussed in the breast multidisciplinary meeting, and the tumor was considered locally advanced and primarily unresectable. Therefore, it was proposed to start systemic therapy with an AI with a CDK4/6i and to reassess for resectability further ahead. The patient was included in the BioBreast study, and the bacterial composition of her gut microbiota was studied by next-generation sequencing (NGS) prior to therapy initiation. Detailed information on fecal harvest and sample management is available as Supplementary Material.

Therapeutic intervention

The patient started letrozole 2.5 mg once a day and ribociclib 600 mg once daily for 21 days, followed by a 7-day break to complete a 28-day treatment cycle. She underwent 6 complete cycles of ribociclib and the first two weeks of the seventh cycle. Systemic therapy was well-tolerated, with only nausea grade 1 according to Common Terminology Criteria for Adverse Events (CTCAE version 5.0) to report.

Follow-up and outcome

The patient had a good local response from as early as the first cycle of ribociclib, and the lesion's regression was very evident following six months of systemic therapy (Figure 2A). As part of the BioBreast study, a new sample of gut microbiota was studied following 6 months of systemic treatment. At this time, she also repeated the breast MRI and PET/CT with 18F-FDG.

The breast MRI confirmed a favorable response, with a tumor size reduction from 90 x 55 x 76 mm to 72 x 39 x 73 mm, the disappearance of the vegetation in the intermammary cleft, and suggested tumor necrosis (Figure 2B). The internal mammary suspicious lymph node was no longer present, and there was a significant decrease in the number and volume of the right axillary adenopathies.

The PET/CT with 18F-FDG also evidenced a favorable response compared to the previous study. There was a marked decrease in both metabolic expression (SUV_{max} 10.95 to 2.30) and tumor's dimensions of the right breast neoplasm, as well as a complete extinction of the lymph node hypermetabolism previously documented (Figures 2C–E).

Following this significant local response, the patient was proposed for surgery with curative intent. She underwent a resection of the previously clip-marked tumor, including pre- and latersternal skin, medial portions of both pectoralis muscles, as well as part of the inner quadrants of both breasts (Figure 3A). Additionally, a right axillary lymphadenectomy was performed. The posterior margin was in contact with the sternal periosteum, precluding further margin extension. The resection was followed by an immediate reconstruction using internal mammary flaps (Figure 3B).

The pathological results evidenced a tumor bed of 77 x 55 x 26 mm of invasive breast carcinoma NST, moderately differentiated, with infiltration of surrounding muscular tissue, adipose tissue, and superficial and deep dermis (Figures 3C, D). There was a 30% size reduction compared to the tumor's original dimension. A positive posterior margin was confirmed microscopically. Following an intraoperative margin extension, the remaining margins were > 10 mm away from the tumor bed. There were 4 out of 11 axillary lymph nodes positive for metastases, none with extracapsular extension. Therefore, the tumor was restaged as ypT3ypN2aM0, R1 (posterior margin). Compared with the initial biopsy, the pathological specimen revealed estrogen receptors positivity in 50% of tumor nuclei (previously 95%); no expression of progesterone receptors (previously 75%); C-ERB-B2 score remained 1+; and the tumor's Ki67 also remained at 30%.

As part of the BioBreast study, microbiota samples of the surgical specimen (residual tumor and respective margins) were collected and analyzed, as described in the Supplementary Material section.

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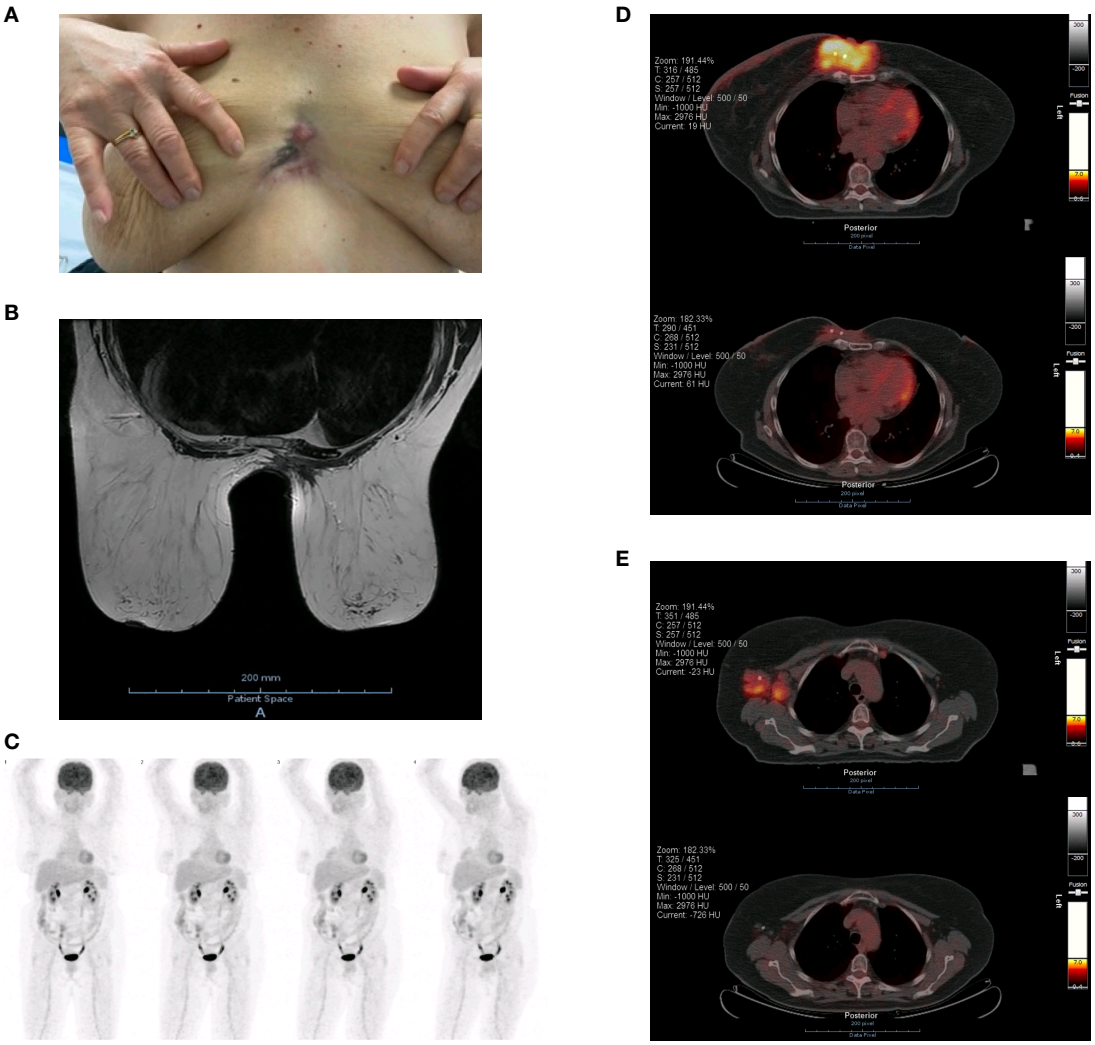

FIGURE 2
(A) Clinical response following 6 months of systemic therapy. **(B)** Breast MRI following 6 months of systemic therapy. **(C)** Whole body on PET/CT with 18F-FDG following 6 months of systemic therapy. **(D)** Right breast lesion on PET/CT with 18F-FDG prior to (upper image) and following 6 months of systemic therapy (lower image). **(E)** Lymph node involvement on PET/CT with 18F-FDG prior to (upper image) and following 6 months of systemic therapy (lower image).

One week after surgery, a new sample of gut microbiota was collected and studied. The case was then rediscussed in the breast multidisciplinary meeting, and the proposed plan was adjuvant radiotherapy and maintaining systemic therapy with letrozole and ribociclib. However, the patient did not intend to maintain CDK4/6i, so she continued letrozole alone and started radiotherapy. She completed image-guided radiotherapy (IGRT) using volumetric modulated arc therapy (VMAT) with conventional fractionation (28 + 7fx, 54.4Gy@1.8Gy) to the chest wall and right axillary, supraclavicular, and internal mammary lymph nodes.

The dynamic evolution of the gut microbiota across the three timepoints was analyzed (Figures 4A, B). There were statistically significant differences in microbial abundance between the three timepoints (p=0.015). At diagnosis, the microbial community was characterized by a notable dominance of Firmicutes phyla. Following 6 months of systemic therapy, there was a shift towards a significant prevalence of Bacteroidetes, accompanied by a marked decrease in α-diversity indices (Shannon and Simpson), suggesting a loss of microbial diversity. After surgery, Bacteroidetes remained the dominant phyla, with a partial recovery of α-diversity indices, although remaining lower than at the initial stage. The β-diversity analysis, accessed by Bray-Curtis distance, corroborates these findings, with the most significant changes observed between the initial and intermediate timepoints, and a partial shift back in the final timepoint (data not shown). At the species level, there is a clear dominance of *Prevotella copri* in the intermediate and final timepoints (Figure 4C).

The analysis from the microbiota samples of the surgical specimen revealed an interestingly high dissimilarity between the residual tumor and respective margins, with statistical significance (p<0.001), suggesting markedly different microbial compositions

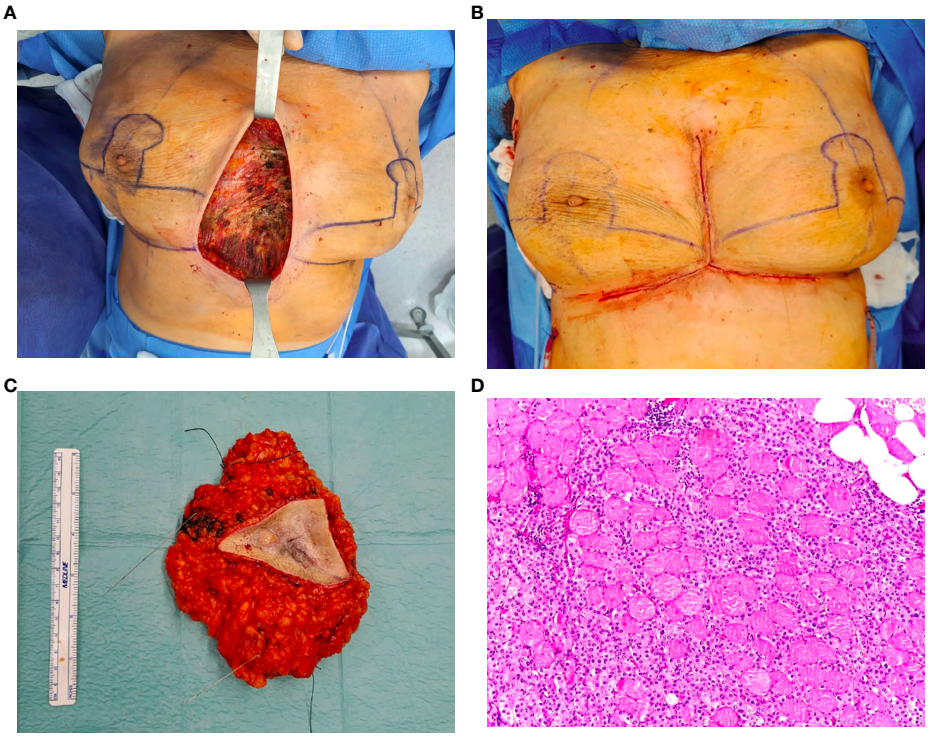

FIGURE 3
(A) Surgical resection of the tumor, pre- and latersternal skin, medial portions of both pectoralis muscles and part of the inner quadrants of both breasts. **(B)** Surgical reconstruction using internal mammary flaps. **(C)** Surgical specimen. **(D)** Pathological results of the surgical specimen showing tumoral infiltration of the muscular tissue.

(Figure 5A). While the margins revealed a more diverse distribution of microbial species, the tumor's microbial composition was dominated by fewer species, particularly *Streptococcus pneumoniae* and *Atopobium vaginae* (Figure 5B).

Discussion

This case allows for several interesting discussion points, namely the primary systemic therapy backbone selection, the available evidence supporting adjuvant therapy following a neoadjuvant approach with ET + CDK4/6i, and the microbiota analysis and its potential clinical implications.

Endocrine therapy-based vs chemotherapy-based primary systemic therapy selection

In patients whose assessment by the multidisciplinary team is an inoperable breast primary tumor, it is essential to consider primary systemic treatment. The success of this treatment can determine the possibility of surgery with curative intent later in time. Chemotherapy (ChT) has been the mainstay of neoadjuvant treatment in HR+/HER2- breast cancer due to its higher pathological complete response (pCR) rates. However, pCR rates achieved in luminal-like tumors are still much lower than those observed in triple-negative or HER2+ breast cancer (for example, 14.9% vs. 41.9% vs. 55.1%) (21). At the same time, ChT is associated with an unfavorable toxicity profile that includes myelotoxicity, gastrointestinal toxicity, and skin disorders, among others, that can have serious short- or long-term impact. ET is a valid neoadjuvant option, although less considered than ChT, being usually reserved for patients who refuse or have contraindications to cytotoxic therapy and HR+ tumors (22).

The available evidence considering ET with or without CDK4/6i versus ChT in the neoadjuvant setting has been encouraging. Still, few trials and only phase II support this strategy for a limited set of patients (23).

The CTNeoBC pooled analysis showed that pCR was associated with better Event-free survival (EFS) in high-risk (G3) HR+/HER2- breast cancer (24), highlighting the importance of developing strategies to achieve higher pCR in this patient population. Two recently presented trials, KEYNOTE-756 (NCT03725059) and CheckMate 7FL (NCT04109066), used immunotherapy in an attempt to improve pCR rates and showed significantly improved pCR rates in the experimental arm of 24.3% and 24.5% vs. 15.6% and 13.8%, respectively (25, 26). EFS data were immature for both trials. Meanwhile, ET monotherapy has been reported to achieve pCR in 0–17.5% of cases (22). Of note, no adjuvant strategy is specifically dependent upon pCR in HR+/HER2- breast cancer, in contrast with triple-negative or HER2+ tumors.

Although obtained in a pre-and perimenopausal population, the phase II RIGHT Choice trial (NCT03839823) compared ribociclib +

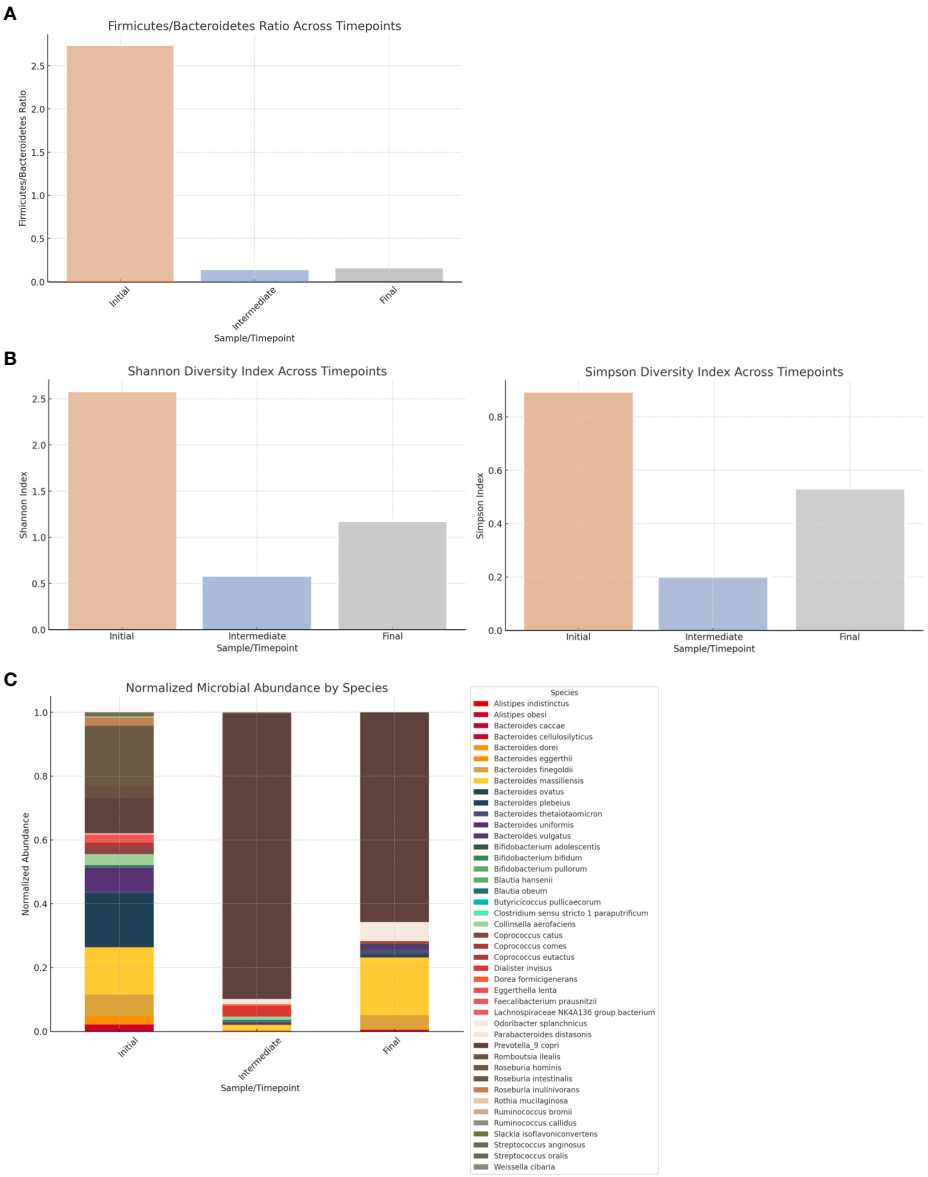

FIGURE 4
(A) Firmicutes/Bacteroidetes (F/B) ratio in gut microbiota across timepoints. The F/B ratio decreases from 2.73 at the initial timepoint (diagnosis) to 0.13 at the intermediate timepoint (following 6 months of systemic therapy) and 0.15 at the final timepoint (following surgery), reflecting a major shift from a Firmicutes-dominant profile at the initial timepoint to a Bacteroidetes-dominant profile in the subsequent timepoints. **(B)** α-diversity indices in gut microbiota across timepoints. At diagnosis, Shannon's and Simpson's diversity indices were 2.58 and 0.89, respectively, indicating a high diversity at this stage. There is a significant decrease in both indices at the intermediate timepoint (0.58 and 0.20), followed by a partial recovery by the final timepoint (1.17 and 0.53). **(C)** Relative abundance of microbial species in gut microbiota across timepoints.

ET versus physician's choice combination ChT in patients with aggressive HR+/HER2− advanced breast cancer, including inoperable locally advanced tumors like the case described (27). Patients treated with ribociclib + ET had a statistically significant and clinically meaningful PFS benefit of approximately 1 year, with similar overall response rates for both strategies. Furthermore, lower rates of treatment-related serious adverse events and discontinuation were seen in the ribociclib + ET group.

This patient's tumor was considered primarily inoperable, and she was, therefore, proposed for primary systemic treatment. Following 6 months of ET + CDK4/6i, there was an almost 19% reduction in tumor size from 90 mm at baseline to 73 mm (using RECIST per MRIs measurements), a complete disappearance of the vegetation in the intermammary cleft, and a marked decrease in the metabolic profile on PET-FDG, which motivated a R0-intended surgery. However, the surgery was R1 due to the tumor's posterior margin contact with the sternal periosteum, which precluded further margin extension. In fact, when analyzing the clinical-to-pathological downstaging of the tumor (cT4cN3M0 to ypT3ypN2aM0), it seems that the response achieved was far more modest than clinically assumed. Furthermore, the tumor's Ki67 index remained untouched at 30% from the diagnostic biopsy to the surgical specimen. A high Ki67 index after neoadjuvant endocrine therapy is known to be a strong prognostic biomarker, being

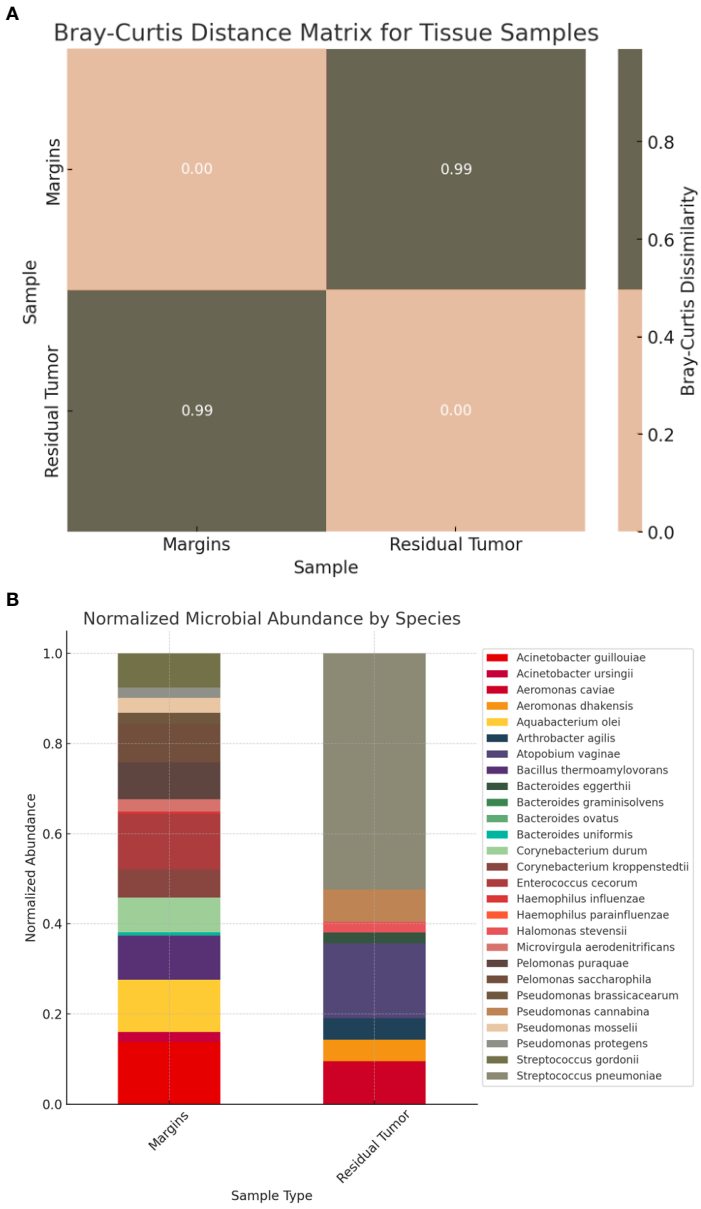

FIGURE 5
(A) Bray-Curtis distance matrix for tissue samples. The Bray-Curtis distance matrix shows a very high dissimilarity (approximately 0.995) between the two samples, suggesting that the microbial compositions of the residual tumor and respective margins are markedly different. **(B)** Relative abundance of microbial species in tissue samples (residual tumor and respective margins).

associated with worse recurrence-free survival and overall survival (28). Another interesting aspect in this case is the progesterone receptor downregulation following ET exposure, suggesting an *in vivo* selection of potentially ET-resistant clones.

Adjuvant individualized decisions

After surgery, the patient's case was rediscussed to define the adjuvant therapeutic plan. Adjuvant ET is an essential component of the treatment of HR+ breast cancer. AIs are the preferred adjuvant treatment for postmenopausal women, when compared to tamoxifen, with a favorable impact on recurrence and survival and a generally acceptable toxicity profile.

In the PENELOPE-B trial (NCT01864746), adjuvant palbociclib for 1 year in addition to ET did not improve invasive disease-free survival (iDFS) in women with residual invasive disease and at a high risk of relapse after taxane-containing neoadjuvant ChT (29). This strategy was not applicable in this patient's particular case since no ChT was used pre-surgically. The only CDK4/6i currently approved in adjuvancy is abemaciclib (150 mg orally twice a day for 2 years, in addition to ET) according to the monarchE trial that showed a sustained recurrence risk reduction (about 32%) in high-risk patients (7,

30). However, the monarchE trial excluded patients who had previously received treatment with CDK4/6i; therefore, no recommendation could be made for this patient based on this trial. Recently, the first data regarding the NATALEE trial (NCT03701334) were made available, supporting ribociclib plus ET in patients with stage II or III breast cancer (8). Prior (neo) adjuvant ET was allowed if initiated ≤ 12 months before randomization, but previous CDK4/6i use was an exclusion criterion, so, again, no recommendation can be made based on this trial. Therefore, despite this patient's proposed adjuvant therapeutic plan, there is no current evidence supporting the use of adjuvant CDK4/6i in patients previously treated with CDK4/6i. Another important consideration is that, in this case, no surgical complications were potentially attributable to the previous use of ET plus CDK4/6i.

Olaparib, a poly (ADP-ribose) polymerase inhibitor (PARPi), has been approved in the adjuvant setting in high-risk BRCA1/2 mutated patients following the results of the OlympiA trial (NCT02032823) (31). Although the patient has not reported a family history consistent with breast-ovarian cancer syndrome and appears to have no personal criteria for genetic counseling, a BRCA1/2 germline mutation test could be useful to determine the benefit of adjuvant PARPi treatment. In this particular case, since the OlympiA trial only included patients treated with neo/adjuvant ChT, this evaluation was not considered useful at this stage, and with a low probability of mutation since there was no family history. Nevertheless, genomic testing is advised considering the patient's high risk of recurrence since PARPi may constitute a future therapeutic option, according to EMBRACA (NCT01945775) and OlympiAD (NCT02000622) trials.

Regarding radiotherapy, being a locally advanced surgically removed R1 breast cancer, evidence strongly supports adjuvant radiotherapy.

Microbiota insights and correlation with potential outcomes

Gut microbiota

Firmicutes and Bacteroidetes are the dominant phyla inhabiting the gut, accounting for approximately 90% of the entire gut microbiota (32). The shift from a Firmicutes-dominant to a Bacteroidetes-dominant profile in this patient's gut microbiota across the therapeutic approach may have multifaceted implications.

The F/B ratio is known to have an important effect on maintaining gut homeostasis and is imbalanced in various health conditions (33). As an example, high F/B ratios have been seen in obesity, a recognized risk factor for breast cancer, though this association is still controversial (33, 34). A study conducted on 95 breast cancer patients showed that the F/B ratio was three times lower in patients with breast cancer in comparison to healthy controls (35). Luminal subtypes had higher F/B ratios than HER2+ or triple-negative breast cancers, and the ratio tended to decrease as cancer stage increased. The same study defined an optimal cutoff value for F/B ratio at 3.37, meaning that there is a break of gut microbial symbiosis and an increase in breast cancer risk below this value. Our patient had an F/B ratio at diagnosis of 2.73, thereby suggesting dysbiosis and increased risk for breast cancer.

Cancer therapy can influence microbiota composition as described in the case. Letrozole, for instance, has been shown to cause a time-dependent shift in gut microbiota in a mouse model (36). In that study, letrozole-treated mice evidenced different relative abundance of specific bacterial OTUs, most of them Bacteroidetes and Firmicutes phyla members, accompanied by a substantial reduction in overall species and phylogenetic richness. However, this relationship between microbiota and cancer therapy is not unidirectional. Changes in microbiota composition can also influence drug metabolism, thereby impacting cancer treatments' efficacy and toxicity, a field known as pharmacomicrobiomics (14, 37).

Despite existing evidence on gut microbiota's predictive utility in other tumor types, including HER2+ breast tumors, evidence in HR+/HER2- breast cancer is still scarce and preliminary (38, 39). A study conducted on 14 HR+/HER2- metastatic breast cancer patients recently addressed the potential relationship between gut microbiota and response to CDK4/6i (40). Although no significant differences were observed between responders and non-responders in terms of α-/β-diversity at the phylum or species level, four bacterial species were collectively able to predict response to CDK4/6i. The phyla analysis from that study shows a dominance of Firmicutes in both responder and non-responder cohorts, with F/B ratios of 2.7 and 2.1, respectively.

The Firmicutes-to-Bacteroidetes switch observed in our patient was mostly due to a noteworthy increase in the relative abundance of *Prevotella copri* following 6 months of letrozole and ribociclib. *Prevotella copri* is an abundant member of the human gut microbiota, whose relative abundance has curiously been associated with positive and negative impacts on several diseases, alongside some pharmacomicrobiomic implications (41). The link between *Prevotella copri* and different types of cancer remains inexplicit, although some hypothesize that Prevotella genera may be involved in breast disease due to its estrogen-deconjugating enzymatic activity (41, 42). The role of *Prevotella copri* and other bacterial species capable of metabolizing estrogens in breast cancer is a field of particular interest for future research.

Finally, a third dimension of the interaction between gut microbiota and cancer therapy comes from the observation that gut microbial shifts can influence gut health, which may greatly impact the patient's quality of life through the gastrointestinal side effects often associated with cancer treatments (43).

This case report is unique because of the longitudinal analysis of the patient's gut microbiota throughout the therapeutic approach with ET + CDK4/6i. Additional analyses would be of value in order to confirm the persistence of this microbial shift in the long term, namely after completing therapy with adjuvant letrozole or in case of recurrence. Unfortunately, such analyses are not possible due to BioBreast's study protocol, thereby constituting a limitation to this case report.

Intratumoral microbiota

The analysis of the microbial composition of the residual tumor and respective margins revealed a high dissimilarity and differing

dominant species between both samples, suggesting that they may come from different tissue conditions. These findings are aligned with a previous study that reported different microbial distributions between breast tumors and tumor-adjacent normal breast tissues (17). However, in contrast with that study, our analysis revealed a less diverse microbial population in the residual tumor in comparison with the respective margins, a finding that may be potentially related to the systemic therapy. The dominance of certain species in the residual tumor, like *Streptococcus pneumoniae* and *Atopobium vaginae*, might be of particular interest for further investigation in this field. A preclinical study showed that Streptococcus in breast cancer cells can inhibit the RhoA-ROCK signaling pathway to reshape the cytoskeleton and help tumor cells resist mechanical stress in blood vessels, thus promoting hematogenous metastasis (20). Although both samples were collected and conserved in similar conditions, the absence of direct controls may constitute a limitation to this preliminary analysis.

Conclusions

This case provides an example of primary CDK4/6i + ET in locally advanced breast cancer considered primarily unresectable. This strategy allowed us to consider and perform a curative-intended surgery later in time. High-level evidence on the use of neoadjuvant CDK4/6i is highly awaited, but the increasing use of this approach will also raise more questions, namely on the potential implications on adjuvancy. Most adjuvant options currently available in HR+/HER2- breast cancer were approved based on trials that excluded patients previously treated with CDK4/6i, making decisions on adjuvancy potentially less evidence-based.

This case is also unique in that, as part of an investigational study, it reports an analysis of the patient's gut microbiota throughout the disease course, something not currently performed in clinical practice. This analysis revealed a modulatory effect at this level following 6 months of ET + CDK4/6i. Future research might delve into how specific microbial alterations correlate with clinical outcomes and whether targeted microbiota modulation, such as probiotics or dietary interventions, might be employed as an adjuvant strategy in cancer management.

Patient perspective

After multiple biopsies and exams, I was diagnosed with breast cancer in a slightly advanced stage. This diagnosis was the beginning of a 14-month journey. My first battle was taking ribociclib for 6 months. After that, I underwent surgery. I vividly remember waking up after the procedure with someone whispering in my ear that I had kept both breasts. I cannot express the immense happiness I felt upon hearing those words. Everything went down perfectly, and I am deeply grateful to my exceptional medical team for this success. Lastly, I had to endure more than 30 painful radiotherapy sessions, along with some physical therapy.

Despite the difficulties, everything ultimately turned out well. After these 14 months, I must extend my heartfelt thanks to my oncology team, who went above and beyond to ensure a positive outcome. I fought for my will to live and held onto my faith that everything would turn out well.

Data availability statement

All relevant data is contained within the article: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by The ethics committee of the CUF Descobertas Hospital. 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

GV: Writing – original draft, Writing – review & editing. DC: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. MF-S: Supervision, Writing – review & editing. PCR: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – review & editing, Validation, Visualization. FM: Resources, Supervision, Writing – review & editing. CBS: Supervision, Writing – review & editing. CRS: Supervision, Writing – review & editing. IN: Writing – review & editing, Supervision. AC: Methodology, Validation, Writing – review & editing. PB: Methodology, Supervision, Validation, Writing – review & editing. AGP: Methodology, Supervision, Validation, Writing – review & editing. CM: Supervision, Validation, Writing – review & editing, Methodology. JGS: Supervision, Writing – review & editing, Resources. RC: Investigation, Supervision, Writing – review & editing. JCR: Investigation, Supervision, Validation, Writing – review & editing. CC: Investigation, Project administration, Supervision, Validation, Visualization, Writing – review & editing. AF: Formal analysis, Investigation, Methodology, Project administration, Supervision, 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.

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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.2024.1360737/full#supplementary-material

GRAPHIC 1
(A) Firmicutes/Bacteroidetes (F/B) ratio in gut microbiota across timepoints. The F/B ratio decreases from 2.73 at the initial timepoint (diagnosis) to 0.13 at the intermediate timepoint (following 6 months of systemic therapy) and 0.15 at the final timepoint (following surgery), reflecting a major shift from a Firmicutes-dominant profile at the initial timepoint to a Bacteroidetes-dominant profile in the subsequent timepoints. **(B)** α-diversity indices in gut microbiota across timepoints. At diagnosis, Shannon's and Simpson's diversity indices were 2.58 and 0.89, respectively, indicating a high diversity at this stage. There is a significant decrease in both indices at the intermediate timepoint (0.58 and 0.20), followed by a partial recovery by the final timepoint (1.17 and 0.53). **(C)** Relative abundance of microbial species in gut microbiota across timepoints.

GRAPHIC 2
(A) Bray-Curtis distance matrix for tissue samples. The Bray-Curtis distance matrix shows a very high dissimilarity (approximately 0.995) between the two samples, suggesting that the microbial compositions of the residual tumor and respective margins are markedly different. **(B)** Relative abundance of microbial species in tissue samples (residual tumor and respective margins).

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Causal relationship between gut microbiota and differentiated thyroid cancer: a two-sample Mendelian randomization study

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Background: The gut microbiota has been significantly associated with differentiated thyroid cancer (DTC). However, the causal relationship between the gut microbiota and DTC remains unexplored.

Methods: Genome-wide association study (GWAS) summary databases were utilized to select exposures and outcomes. The Mendelian randomization (MR) method was employed to investigate the causal relationship between the gut microbiota and DTC. A sensitivity analysis was performed to assess the reliability of the findings.

Results: Four bacterial traits were associated with the risk of DTC: Class Mollicutes [odds ratio (OR) = 10.953, 95% confidence interval (95% CI): 2.333–51.428, p = 0.002], Phylum Tenericutes (OR = 10.953, 95% CI: 2.333–51.428, p = 0.002), Genus Eggerthella (OR = 3.219, 95% CI: 1.033–10.024, p = 0.044), and Order Rhodospirillales (OR = 2.829, 95% CI: 1.096–7.299, p = 0.032). The large 95% CI range for the Class Mollicutes and the Phylum Tenericutes may be attributed to the small sample size. Additionally, four other bacterial traits were negatively associated with DTC: Genus Eubacterium fissicatena group (OR = 0.381, 95% CI: 0.148–0.979, p = 0.045), Genus Lachnospiraceae UCG008 (OR = 0.317, 95% CI: 0.125–0.801, p = 0.015), Genus Christensenellaceae R-7 group (OR = 0.134, 95% CI: 0.020–0.886, p = 0.037), and Genus Escherichia Shigella (OR = 0.170, 95% CI: 0.037–0.769, p = 0.021).

Conclusion: These findings contribute to our understanding of the pathological mechanisms underlying DTC and provide novel insights for the clinical treatment of DTC.

KEYWORDS

causality, gut microbiota, differentiated thyroid cancer, Mendelian randomization, genome-wide association study

Introduction

Thyroid cancer is the most prevalent malignancy of the endocrine system (1). According to the GLOBOCAN (2020) database, there were 586,202 new cases of thyroid cancer in 2020 worldwide, constituting 3.0% of all cancer incidences (2, 3). Thyroid cancer encompasses four primary pathological classifications: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), and anaplastic thyroid carcinoma (ATC) (4, 5). Differentiated thyroid cancer (DTC), comprising PTC and FTC, accounts for most thyroid cancer cases and typically presents a favorable prognosis (6). However, DTC is susceptible to local lymph node metastasis, contributing to a recurrence rate of up to 20% within 10 years (7). Certain subtypes, such as the diffuse sclerosing variant (DSV), exhibit relatively high invasiveness and are associated with a dismal prognosis (8). Therefore, a profound comprehension of the mechanisms underlying the onset and development of DTC is imperative.

Accumulating evidence suggests that the gut microbiota plays a crucial role in malignant tumors, including colorectal cancer, lung cancer, and breast cancer (9–11). Yu et al. (12) observed a significant decline in the richness and diversity of the gut microbiota in patients with DTC compared to healthy individuals. They developed a 10-genus microbial signature capable of effectively distinguishing patients with DTC from healthy individuals. Moreover, the gut microbiota is closely associated with the therapeutic response to radioactive iodine (RAI) following thyroidectomy (13). Before RAI treatment, thyroid hormone withdrawal (THW) is typically necessary to stimulate the secretion of thyroid stimulating hormone; however, THW-related complications significantly decrease quality of life. A recent randomized clinical trial showed that probiotics improve multiple symptoms induced by THW, including constipation and excessive weight gain (14). These findings indicate the involvement of the gut microbiota in the progression of DTC. Nevertheless, the causal relationship between the gut microbiota and DTC remains unexplored.

In the present study, the Mendelian randomization (MR) method was employed to ascertain whether there exists a causal relationship between the gut microbiota and DTC. Our findings may provide crucial insights into the pathological mechanisms underlying DTC.

Patients and methods

Patient data

A genome-wide association study (GWAS) dataset of the gut microbiota was downloaded from MiBioGen (https://mibiogen.gcc.rug.nl/menu/main/home/) and utilized as an exposure variable. To investigate the interactive effects of human genetics and gut microbiota, 16S rRNA gene sequencing was performed on 18,340 individuals from 24 different cohorts. Following the exclusion of entries with unknown taxonomic information, a total of 196 bacterial traits were selected, comprising 9 phyla, 16 classes, 20 orders, 32 families, and 119 genera. A GWAS dataset of DTC (GWAS ID: ieu-a-1082) was obtained from the IEU OpenGWAS project (https://gwas.mrcieu.ac.uk/) and employed as the outcome variable (15). Initially, a total of 701 Italian individuals with DTC (median age, 46 years) were included, of whom 649 remained after rigorous quality control measures. All cases were histologically validated as DTC, without further differentiation between PTC and FTC subtypes.

To establish a definitive causality between the gut microbiota and DTC, we employed the following screening criteria for instrumental variables (IVs): 1) single-nucleotide polymorphisms (SNPs) had a strong correlation with exposure (gut microbiota). The level of significance (p-value) was set to $p < 1 \times 10^{-5}$, consistent with the established protocol in prior studies (16, 17). 2) An F-statistic threshold >10 was utilized to mitigate weak IV bias. 3) SNPs exhibiting linkage disequilibrium effects were excluded, employing an R^2 cutoff of 0.001 and a clumping window size of 10,000 kb. 4) In cases where SNPs were absent in the outcome, proxy SNPs ($R^2 > 0.8$) were obtained from the 1000 Genomes Project (http://www.internationalgenome.org/). 5) The threshold for allele frequencies was set to 0.01. The study was approved by the Ethics Committee of Suzhou Ninth Hospital Affiliated to Soochow University.

Statistical analysis

Before conducting MR analysis, palindromic SNPs were excluded to harmonize the effects of the SNPs, and Steiger filtering was performed to ensure the correct directionality of each SNP. Five common MR methods were utilized in this study: inverse variance weighted (IVW), weighted median, weighted mode, simple mode, and MR-Egger methods. The IVW method estimates causal relationships by integrating the effect sizes of multiple genetic variations and applying inverse variance weighting. This approach enhances statistical power, mitigates estimation bias, and improves the accuracy of causal relationship assessment, making it a preferred choice in MR-related studies. The odds ratio (OR) and 95% confidence interval (95% CI) were determined. The MR-Egger intercept test was employed to assess directional horizontal pleiotropy (18). Cochran's Q statistic was used to assess the heterogeneity in the data (19). MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) analysis was performed to assess the presence of outliers (20). All statistical analyses were conducted using R software (version 4.3.1), primarily utilizing the R packages "TwoSampleMR" (version 0.5.7), "MRPRESSO" (version 1.0), "ieugwasr" (version 0.1.5), and "plinkbinr" (version 0.0.0.9000). A p-value <0.05 indicated statistical significance.

Results

MR analysis

The outcome comprised a total of 1,080 individuals, including 649 DTC cases and 431 controls (Figure 1). The IVW analysis showed that the Class Mollicutes and the Phylum Tenericutes were positively correlated with DTC, suggesting that Mollicutes and

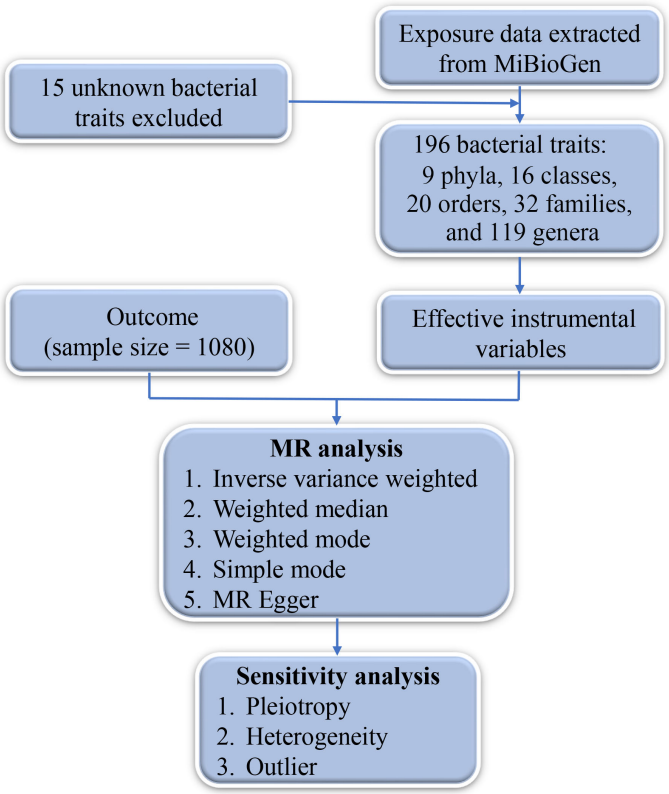

FIGURE 1
Flowchart depicting the MR analysis. MR, Mendelian randomization.

Tenericutes are associated with an increased risk of DTC (Figures 2, 3A, B, Table 1; Class Mollicutes, OR = 10.953, 95% CI: 2.333–51.428, $p = 0.002$; Phylum Tenericutes, OR = 10.953, 95% CI: 2.333–51.428, $p = 0.002$). The risk effects associated with both entities on DTC were nearly identical, as the Class Mollicutes falls within the Phylum Tenericutes. The weighted median method further confirmed the results (Table 1; Class Mollicutes, OR = 13.375, 95% CI: 1.731–103.376, $p = 0.013$; Phylum Tenericutes, OR = 13.375, 95% CI: 1.730–103.393, $p = 0.013$). In addition, no directional horizontal pleiotropy was observed (Supplementary Table 1; Class Mollicutes, Egger intercept = −0.482, $p = 0.482$; Phylum Tenericutes, Egger intercept = −0.482, $p = 0.482$). Furthermore, Cochran's Q test indicated no heterogeneity in individual causal effects (Supplementary Table 2; Class Mollicutes, Cochran's Q = 0.290, $p = 0.590$; Phylum Tenericutes, Cochran's Q = 0.290, $p = 0.590$). Additionally, the MR-PRESSO analysis did not identify any potential outliers (Supplementary Table 3).

IVW analysis identified the Genus Eggerthella and the Order Rhodospirillales as risk factors for DTC (Figures 2, 3C, D, Table 1; Genus Eggerthella, OR = 3.219, 95% CI: 1.033–10.024, $p = 0.044$; Order Rhodospirillales, OR = 2.829, 95% CI: 1.096–7.299, $p = 0.032$). However, these results lacked support from the weighted median method (Table 1; Genus Eggerthella, OR = 3.716, 95% CI: 0.891–15.503, $p = 0.072$; Order Rhodospirillales, OR = 2.861, 95% CI: 0.845–9.688, $p = 0.091$). MR-Egger analysis indicated no directional horizontal pleiotropy (Genus Eggerthella, Egger intercept = 0.523, $p = 0.490$; Order Rhodospirillales, Egger intercept = −0.455, $p = 0.246$). Cochran's Q test revealed no heterogeneity in individual causal effects (Supplementary Table 2; Genus Eggerthella, Cochran's Q = 1.139, $p = 0.286$; Order Rhodospirillales, Cochran's Q = 1.288, $p = 0.863$).

In addition to the gut microbiota associated with an increased risk of DTC, the microbiota protecting against DTC was identified. IVW analysis revealed that the Genus Eubacterium fissicatena group and the Genus Lachnospiraceae UCG008 were negatively correlated with DTC, indicating their potential protective roles against DTC (Figures 2, 4A, B, Table 1; Genus Eubacterium fissicatena group, OR = 0.381, 95% CI: 0.148–0.979, $p = 0.045$; Genus Lachnospiraceae UCG008, OR = 0.317, 95% CI: 0.125–0.801, $p = 0.015$). These results were further supported by the weighted median analysis (Table 1; Genus Eubacterium fissicatena group, OR = 0.322, 95% CI: 0.114–0.910, $p = 0.033$; Genus Lachnospiraceae UCG008, OR = 0.265, 95% CI: 0.077–0.907, $p = 0.034$). In addition, no directional horizontal pleiotropy was observed (Supplementary Table 1; Genus Eubacterium fissicatena group, Egger intercept = −0.169, $p = 0.790$; Genus Lachnospiraceae UCG008, Egger intercept = −0.280, $p = 0.593$). Cochran's Q test indicated no heterogeneity in the individual causal effects (Supplementary Table 2; Genus Eubacterium fissicatena group, Cochran's Q = 4.218, $p = 0.121$; Genus Lachnospiraceae UCG008, Cochran's Q = 0.860, $p = 0.835$).

The Genus Christensenellaceae R-7 group and the Genus Escherichia Shigella emerged as protective factors against DTC, as

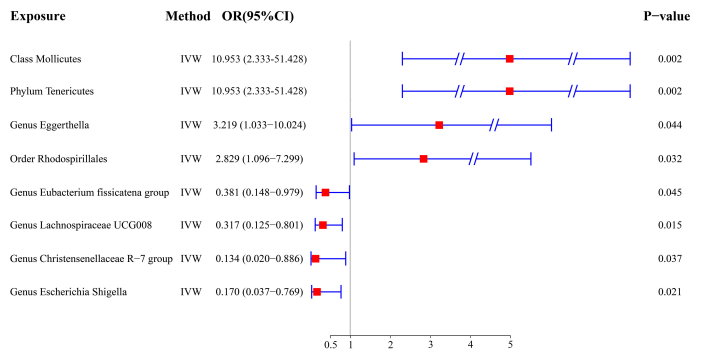

FIGURE 2
A forest plot illustrating the MR results. MR, Mendelian randomization.

revealed by the IVW analysis (Figures 2, 4C, D, Table 1; Genus Christensenellaceae R-7 group, OR = 0.134, 95% CI: 0.020–0.886, $p = 0.037$; Genus Escherichia Shigella, OR = 0.170, 95% CI: 0.037–0.769, $p = 0.021$). However, these results were not supported by the weighted median analysis (Table 1; Genus Christensenellaceae R-7 group, OR = 0.174, 95% CI: 0.016–1.950, $p = 0.156$; Genus Escherichia Shigella, OR = 0.154, 95% CI: 0.023–1.030, $p = 0.054$). Moreover, the MR-Egger analysis indicated no directional horizontal pleiotropy (Supplementary Table 1; Genus Christensenellaceae R-7 group, Egger intercept = 0.069, $p = 0.796$; Genus Escherichia Shigella, Egger intercept = 0.030, $p = 0.921$). Additionally, Cochran's Q test revealed no heterogeneity in

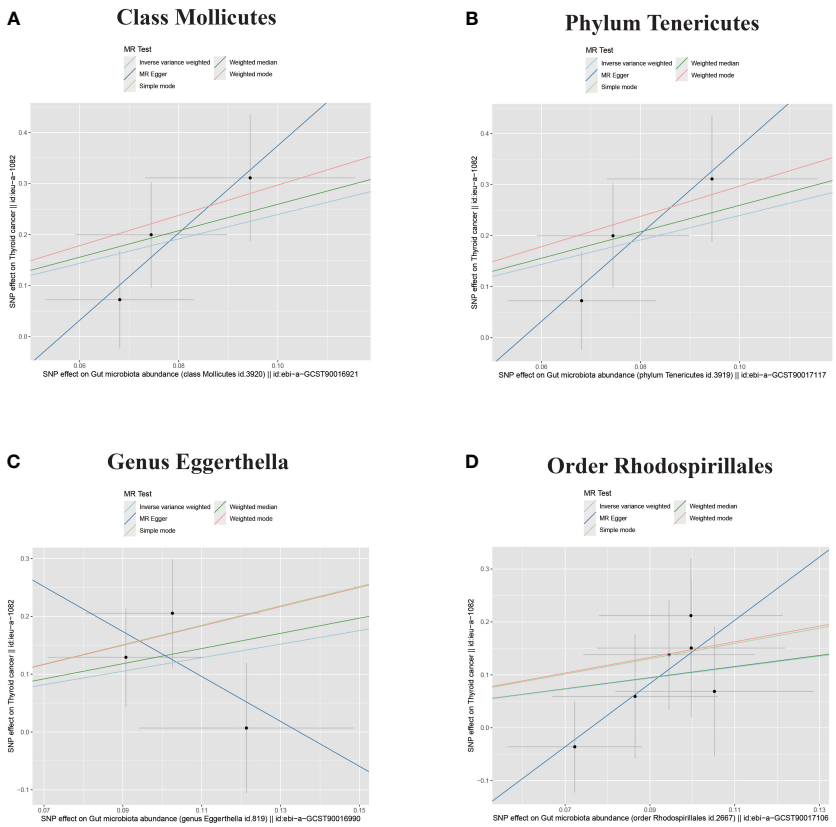

FIGURE 3
Scatter plots of four bacterial traits positively associated with DTC. **(A)** Class Mollicutes. **(B)** Phylum Tenericutes. **(C)** Genus Eggerthella. **(D)** Order Rhodospirillales. DTC, differentiated thyroid cancer; MR, Mendelian randomization; SNP, single-nucleotide polymorphism.

TABLE 1 Causal effects of gut microbiota on DTC.

Exposure	No. of SNPs	Methods	Beta	SE	OR (95% CI)	*p*-Value
Class Mollicutes	3	Inverse variance weighted	2.394	0.789	10.953 (2.333–51.428)	0.002
	3	Weighted median	2.593	1.043	13.375 (1.731–103.376)	0.013
	3	Weighted mode	2.970	1.310	19.491 (1.494–254.263)	0.152
	3	Simple mode	2.970	1.345	19.491 (1.395–272.254)	0.158
	3	MR-Egger	8.559	5.872	5211.449 (0.052–5.19e+8)	0.383
Phylum Tenericutes	3	Inverse variance weighted	2.394	0.789	10.953 (2.333–51.428)	0.002
	3	Weighted median	2.593	1.043	13.375 (1.730–103.393)	0.013
	3	Weighted mode	2.970	1.286	19.491 (1.568–242.297)	0.147
	3	Simple mode	2.970	1.324	19.491 (1.454–261.229)	0.154
	3	MR-Egger	8.559	5.872	5211.45 (0.052–5.19e+8)	0.383
Genus Eggerthella	3	Inverse variance weighted	1.169	0.580	3.219 (1.033–10.024)	0.044
	3	Weighted median	1.313	0.729	3.716 (0.891–15.503)	0.072
	3	Simple mode	1.678	0.938	5.354 (0.852–33.653)	0.216
	3	Weighted mode	1.667	0.935	5.295 (0.847–33.084)	0.217
	3	MR-Egger	−3.883	4.933	0.021 (0–525.661)	0.575
Order Rhodospirillales	6	Inverse variance weighted	1.040	0.484	2.829 (1.096–7.299)	0.032
	6	Weighted median	1.051	0.622	2.861 (0.845–9.688)	0.091
	6	MR-Egger	5.979	3.674	395.189 (0.295–529391.600)	0.179
	6	Simple mode	1.448	0.931	4.253 (0.686–26.364)	0.181
	6	Weighted mode	1.477	0.982	4.381 (0.640–29.996)	0.193
Genus Eubacterium fissicatena group	4	Inverse variance weighted	−0.966	0.482	0.381 (0.148–0.979)	0.045
	4	Weighted median	−1.133	0.530	0.322 (0.114–0.910)	0.033
	4	Weighted mode	−1.320	0.651	0.267 (0.075–0.956)	0.136
	4	Simple mode	−1.320	0.754	0.267 (0.061–1.171)	0.178
	4	MR-Egger	0.332	4.317	1.394 (0–6586.220)	0.946
Genus Lachnospiraceae UCG008	5	Inverse variance weighted	−1.150	0.473	0.317 (0.125–0.801)	0.015
	5	Weighted median	−1.328	0.628	0.265 (0.077–0.907)	0.034
	5	Simple mode	−1.467	0.743	0.231 (0.054–0.990)	0.120
	5	Weighted mode	−1.479	0.810	0.228 (0.047–1.114)	0.142
	5	MR-Egger	1.517	4.498		0.758

(Continued)

TABLE 1 Continued

Exposure	No. of SNPs	Methods	Beta	SE	OR (95% CI)	*p*-Value
					4.560 (0.001–30754.110)	
Genus Christensenellaceae R-7 group	3	Inverse variance weighted	−2.010	0.964	0.134 (0.020–0.886)	0.037
	3	Weighted median	−1.746	1.232	0.174 (0.016–1.950)	0.156
	3	Weighted mode	−1.598	1.271	0.202 (0.017–2.444)	0.336
	3	Simple mode	−1.598	1.401	0.202 (0.013–3.149)	0.372
	3	MR-Egger	−2.934	2.952	0.053 (0–17.299)	0.502
Genus Escherichia Shigella	3	Inverse variance weighted	−1.774	0.772	0.170 (0.037–0.769)	0.021
	3	Weighted median	−1.874	0.971	0.154 (0.023–1.030)	0.054
	3	Simple mode	−1.968	1.074	0.140 (0.017–1.147)	0.208
	3	Weighted mode	−1.960	1.121	0.141 (0.016–1.267)	0.222
	3	MR-Egger	−2.193	3.434	0.112 (0–93.381)	0.638

SNP, single-nucleotide polymorphism; SE, standard error; OR, odds ratio; 95% CI, 95% confidence interval; DTC, differentiated thyroid cancer.

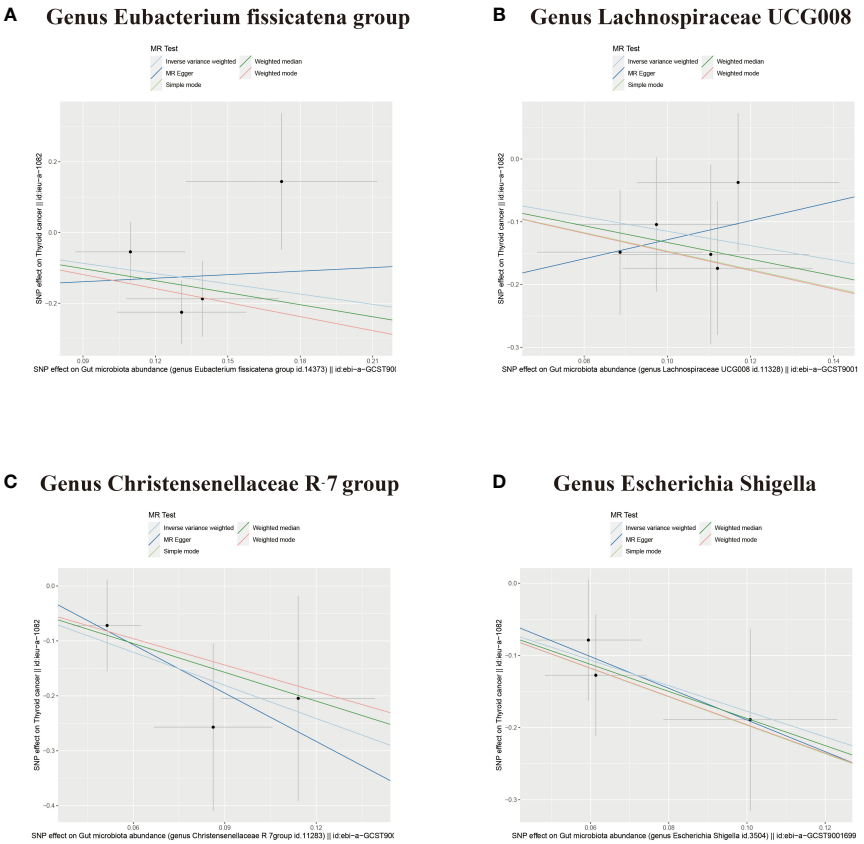

FIGURE 4
Scatter plots depicting four bacterial traits negatively associated with DTC. **(A)** Genus Eubacterium fissicatena group. **(B)** Genus Lachnospiraceae UCG008. **(C)** Genus Christensenellaceae R-7 group. **(D)** Genus Escherichia Shigella. DTC, differentiated thyroid cancer; MR, Mendelian randomization; SNP, single-nucleotide polymorphism.

individual causal effects (Supplementary Table 2; Genus Christensenellaceae R-7 group, Cochran's Q = 0.353, p = 0.552; Genus Escherichia Shigella, Cochran's Q = 0.142, p = 0.707).

Discussion

Most studies on the gut microbiota have predominantly focused on malignant tumors of the digestive tract (21). Gradually, it has been acknowledged that the gut microbiota also impacts non-gastrointestinal tumors through various mechanisms, including inflammation and immunoregulation, metabolic pathways, and bacterial translocation. For example, *Bacteroides fragilis* induces the differentiation of Treg cells, thereby promoting the formation of an immunosuppressive microenvironment through the production of immunosuppressive factors such as IL-10 and TGF-β, ultimately contributing to the development of gliomas (22, 23). Additionally, *Ruminococcus* sp. DSM_100440 has been found to convert androgen precursors into androgens, expediting the progression of castration-resistant prostate cancer (CRPC) (24). In this study, we identified eight bacterial traits significantly associated with DTC. Among these, four bacterial traits (Class Mollicutes, Phylum Tenericutes, Genus Eggerthella, and Order Rhodospirillales) were associated with the risk of DTC, while the remaining four traits (Genus Eubacterium fissicatena group, Genus Lachnospiraceae UCG008, Genus Christensenellaceae R-7 group, and Genus Escherichia Shigella) exhibited a protective effect against DTC. These findings contribute to our comprehension of the role of the gut microbiota in non-gastrointestinal tumors and offer a novel avenue for DTC treatment.

Christensenellaceae is widely distributed throughout the digestive tract and is intricately linked to human health (25). We found that the Genus Christensenellaceae R-7 group had a significant negative causality with DTC. Consistent with our findings, Lu et al. showed that the Genus Christensenellaceae R-7 group is significantly associated with DTC (26). They suggested that the Genus Christensenellaceae R-7 group may promote the occurrence and development of DTC by regulating lipid metabolism, as indicated by the marked inhibition of lipid digestion and steroid biosynthesis pathways. Similarly, a reduced abundance of the Genus Christensenellaceae R-7 group has been observed in patients with colorectal cancer (27), further underscoring the potential of this genus as a probiotic.

The Family Lachnospiraceae has been reported to play a protective role against colorectal carcinogenesis by bolstering the tumor immunosurveillance function of $CD8^+$ T cells (28). Additionally, it has been demonstrated to serve a protective role in the regulation of radiation-induced intestinal damage (29). However, Zheng et al. (13) elucidated a greater abundance of the Family Lachnospiraceae in patients with DTC exhibiting a non-excellent response to RAI compared to those exhibiting an excellent response. We speculate that the absence of further taxonomic classification within the Lachnospiraceae family may have contributed to these discrepant findings. A genus-level analysis showed that the abundance of the Genus Lachnospiraceae UCG010 is significantly higher in patients with DTC exhibiting an excellent response to RAI than in those exhibiting a non-excellent response, suggesting that the Genus Lachnospiraceae UCG010 is a protective factor. Similarly, our analysis indicated that Lachnospiraceae UCG008 exerts protective effects against DTC.

The abundance of Mollicutes/Tenericutes was significantly increased in various tumor tissues, including gastric and lung cancers (30, 31). Employing animal models, Lee et al. (32) elucidated a positive correlation between gut Mollicutes/Tenericutes and tumor burden in colitis-associated cancer. To the best of our knowledge, our study is the first to demonstrate the causal relationship between gut Mollicutes/Tenericutes and DTC. The large 95% CI range observed for the Class Mollicutes and Phylum Tenericutes may be attributed to the small sample size. Further studies are warranted to elucidate the pathogenic mechanisms of Mollicutes/Tenericutes.

This study has a few limitations. First, it included only one cohort comprising 1,080 participants, potentially resulting in a limited number of valid SNPs. Studies involving larger cohorts are necessary to establish more robust causal links between the gut microbiota and DTC. Second, although taxonomic classification has identified approximately 1,000 species of gut microbiota (33), our analysis was limited to 119 genera, precluding consideration at the species level. Third, due to the small sample size, DTC was treated as a single entity in this study. In fact, DTC can be classified into various subtypes, each exhibiting distinct biological behaviors. In future studies, histopathological subclassifications will be pivotal for refining precision treatments for DTC. Fourth, potential unaddressed confounding factors could impact the accuracy and generalizability of our findings. Finally, discrepancies observed across MR methods raise concerns regarding the robustness of certain associations, while the lack of functional insights leaves unanswered questions regarding biological mechanisms.

In summary, our investigation revealed that eight bacterial traits exert a significant causal effect on DTC. These findings enhance our comprehension of the pathological mechanisms underlying DTC and provide a novel avenue for its treatment.

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.

Ethical statement

The experiments were ethically approved by the Suzhou Ninth People's Hospital.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Suzhou Ninth Hospital Affiliated to Soochow University. The studies were conducted in accordance with the

local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

SH: Data curation, Formal analysis, Methodology, Validation, Software, Writing – original draft. FF: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. CT: Data curation, Investigation, Methodology, Resources, Validation, Writing – original draft. LW: Conceptualization, Data curation, Formal analysis, Supervision, Writing – original draft. XL: Conceptualization, Formal analysis, Supervision, Visualization, Writing – review & editing. MS: Conceptualization, Data curation, Methodology, Software, Supervision, Writing – review & editing. LY: Conceptualization, Investigation, Project administration, Resources, Supervision, 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/fonc.2024.1375525/full#supplementary-material

SUPPLEMENTARY TABLE 1
Results of the pleiotropy test.

SUPPLEMENTARY TABLE 2
Results of the heterogeneity test.

SUPPLEMENTARY TABLE 3
Results of the MRPRESSO test.

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