frontiers | Research Topics

Targeting the microbiota to attenuate chronic inflammation

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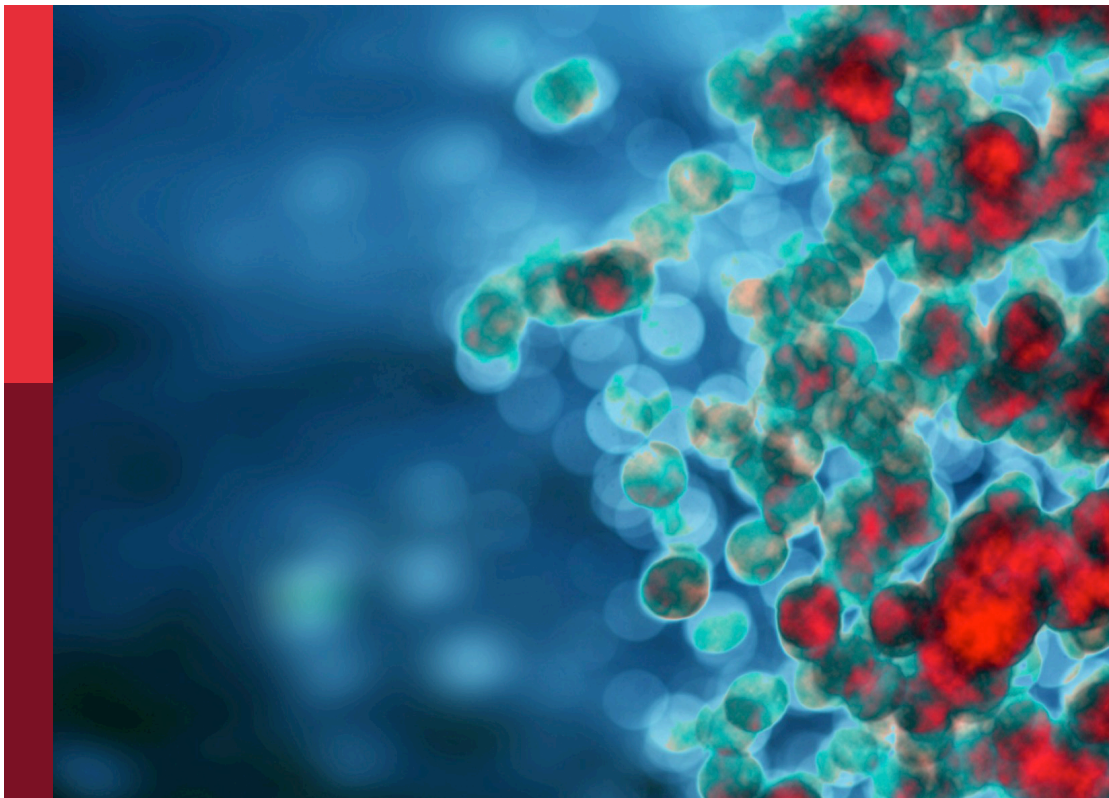
Published in
Frontiers in Immunology
Frontiers in Microbiology



ISSN 1664-8714
ISBN 978-2-8325-2336-0
DOI 10.3389/978-2-8325-2336-0

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Targeting the microbiota to attenuate chronic inflammation

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Citation

Serrano-Villar, S., Trøseid, M., Vujkovic-Cvijin, I., Marchetti, G. C., eds. (2023). *Targeting the microbiota to attenuate chronic inflammation*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2336-0

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 18 April 2023
DOI 10.3389/fimmu.2023.1202222

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 07 April 2023
ACCEPTED 12 April 2023
PUBLISHED 18 April 2023

CITATION
Moreno E, Trøseid M, Vujkovic-Cvijin I, Marchetti G, Martín-Pedraza L and Serrano-Villar S (2023) Editorial: Targeting the microbiota to attenuate chronic inflammation.
Front. Immunol. 14:1202222.
doi: 10.3389/fimmu.2023.1202222

Editorial: Targeting the microbiota to attenuate chronic inflammation

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KEYWORDS

microbiota, inflammation, biomarkers, omics, personalized medicine (PM)

Editorial on the Research Topic
Targeting the microbiota to attenuate chronic inflammation

Introduction

Although the whole human body is a complex ecosystem inhabited by very different microorganisms, including bacteria, fungi, archaea, and viruses, studies about gut bacteria have received the most attention. The relationship between the gut microbiota and the immune system has been the focus of intense research [recently reviewed at (1)]. Chronic inflammation correlates with an altered microbiota composition in the context of inflammatory bowel disease, colorectal cancer, frailty, metabolic endotoxemia, and non-communicable diseases (2–6). Specifically, many studies have addressed the role of different microbiome components on chronic inflammation (7, 8). For example, some microbes adhered to the intestinal epithelium can locally induce Th17 responses (9), while others can exert distant effects on different organ systems through metabolite production (10, 11). This has fueled research aimed at shaping these interactions by either redesigning the entire bacterial community or administering specific relevant bacterial strains that are presumably beneficial (12). However, most studies targeting the microbiota to elicit protective immune responses are only exploratory, with limited sample sizes and assessing multiple outcomes. Therefore, it is unclear how we can induce stable, beneficial changes in the gut microbiota.

This Research Topic highlights translational research and clinical trials evaluating the immunological effects of interventions on the gut microbiota, such as dietary or pharmacological interventions, probiotics, fungus, and other compounds. In addition, it focuses on specific mechanisms by which the microbiota can affect chronic inflammation in different diseases.

Interventions on the gut microbiota in chronic inflammation diseases

This Research Topic addresses interventions on the gut microbiota from different perspectives.

According to new probiotic characterizations, Blázquez-Bondia et al. show the effect of a novel probiotic in a double-blind placebo-controlled clinical trial (RECOVER study). The i3.1 probiotic (a mixture of *L. plantarum* and *P. acidilactici* and a fiber-based prebiotic) improved immune reconstitution in people with HIV with impaired immunological recovery under stable antiretroviral therapy. There were no major adverse effects related to the intervention, and a slight increase in CD4/CD8 ratio as well as a decrease in pathways abundances were found in the active arm. Saghari et al. report the effects of three monoclonal microbial formulations of *L. lactis* spp. *cremoris* (EDP1066) on the immune response to a marine mollusk protein used to "mimic" an immune response in healthy volunteers. They assessed three different probiotic formulations to evaluate various exposure sites within the gastrointestinal tract. The immunomodulatory effect was assessed by quantifying circulating regulatory T cells and by stimulation of monocyte and lymphocyte with the Toll-like receptor 4 ligand lipopolysaccharide (LPS) and phytohemagglutinin (PHA), respectively. However, the results did not show a significant immune modulation measured as an antibody response to the challenge with the mollusk protein.

Plant compounds have mainly been used in the history of medicine. Liu et al. assessed the role of caesaldekarine, a cassane diterpenoid isolated from the plant *Caesalpinia bonduc*, to ameliorate colitis in mice. The mechanisms involved suppression of tissular inflammation, intestinal barrier integrity maintenance, and increased Lactobacillus abundance.

Fungi have been rarely been assessed as probiotics, with the exception of Saccharomyces Boulardii. Wang et al. evaluated the effects of dietary supplementation with *Tolypocladium sinense*, a mycelium isolated from a Chinese caterpillar, against obesity. This intervention affected the inflammatory response and oxidative stress levels by regulating lipid metabolism, such as decreasing short-chain fatty acid content. These results were further confirmed after fecal transplantation in mice.

Lastly, some traditional Chinese medicine products, as presented by Zhu et al., have been potentially related to improving different liver-related diseases through microbiota regulation mechanisms. Their review describes targeting microbiota studies to treat liver conditions, such as alcoholic disease, nonalcoholic disease, autoimmune disease, liver injury, and cancer.

Advancing translational research in the microbiome field

Direct intervention studies are essential for determining the causal effects of the microbiome on disease pathogenesis. But it is also important to understand the mechanisms underlying specific microbiota actions in the host to design efficient and effective treatments. However, as described in Moreno et al., for the particular cases of HIV and HPV infection, most studies are based on highly dimensional datasets and address multiple outcomes, which hampers transferring the results to the clinic. The review also highlights the need for standardization of methods and encourages more hypothesis-driven studies.

Other studies in this Research Topic report novel mechanisms by which the microbiota can contribute to inflammation.

Ling et al. evaluated 140 school-aged children (6-12 years) from China (92 with depression and 48 healthy controls). They analyzed the correlations between gut microbiota profiles and host immune response measured as the expression of 27 cytokines. Patients with depression exhibited enrichment for proinflammatory genera (*Streptococcus*) and some inferred immunomodulatory metabolites (e.g., increase in membrane transport, signal transduction, and metabolism of other amino acids in children with depression), which correlated with increased levels of proinflammatory cytokines such as IL-17.

Fonseca et al. describe the anti-inflammatory and immunomodulatory properties of extracellular vesicles produced by the prominent human gut commensal bacterium *Bacteroides thetaiotaomicron*. By administering these vesicles to mice with colitis, they report important factors in anti-inflammatory and immunomodulatory responses, showing a reduction in intestinal inflammation, upregulation of the anti-inflammatory cytokine IL-10, and even epigenetic reprogramming.

As mentioned above, inflammation and HIV are two deeply interconnected factors. Littlefield et al. described the etiology of gastrointestinal inflammation among men who have sex with men and their link with gut microbiome composition. They found specific fecal soluble immune factors, such as calprotectin, a clinically relevant marker of gastrointestinal inflammation in men who have sex with men independently of their HIV status. They also observed differences in markers of bacterial translocation (elevated levels of plasma, sCD14, and sCD163) and in an *in vitro* system. These data indicate a connection between fecal soluble immune factors composition, decreased intestinal barrier function, and bacterial-induced systemic inflammation.

Additional mechanisms by which some therapeutic anti-inflammatory interventions could affect, and be affected by, the microbiota are addressed in this Research Topic.

Johnson et al. assess the effect of treatment with the humanized monoclonal antibody anti-α4β7 on microbiota composition. Vedolizumab administration to SIV-infected macaques led to different results than previous studies. The intervention elicited the maturation of macrophages associated with dysbiosis markers previously identified as predictors of HIV replication, immune activation, and changes in viral loads in tissues. This point towards a possible future modulation of gut immune functions to improve treatments for HIV infection.

Zhou et al. evaluate the involvment of the gut microbiota in the efficacy of the anti-rheumatic drug methotrexate. Patients with rheumatoid arthritis showed a decreased abundance of intestinal *Bacteroides fragilis* after methotrexate treatment. Transplantation of *Bacteroides fragilis* or supplementation with butyrate restored the

methotrexate efficacy in collagen-induced arthritis mice pretreated with antibiotics.

Finally, four articles suggest a potential role of the microbiota in the activation of host pathways that have been linked to thepathogenesis of different conditions. First, Ancona et al. review the implications of gut dysbiosis in COVID-19 and long-COVID syndrome. They focus on the confounding factors in the previous literature and, more specifically, on studies of airway microbiota and long-COVID with neurological symptoms. Second, Omaru et al. reviewed the activation of NOD1/NOD2 receptors in chronic liver disease. This occurs through the regulation of proinflammatory cytokine responses leading to the development of chronic liver diseases, including hepatocellular carcinoma. Third, Fan et al. propose using the aryl hydrocarbon receptor as a therapeutic target for ischemic stroke by describing its role in the "microbiota-gut-brain axis" as a receptor of tryptophan metabolites that is impacted by gut microbiota. Finally, Li et al. review the impact of the oral microbiota on cardiometabolic health. Microbiota metabolites in the oral cavity, affected by oral dysbiosis, periodontal disease, and dental plaque, have been associated with cardiovascular disease occurrence. According to this, they discuss the potential of oral microbiota transplantation as a therapeutic intervention.

Perspectives

This Research Topic includes studies that evaluate the potential of microbiota to attenuate chronic inflammation. Some of these studies assessed direct interventions on gut microbiota in different diseases and aimed to characterize the immunological effects. Other studies describe specific mechanisms underpinning these relationships, such as changes in metabolic routes or regulation of particular host factors related to immune responses. Finally, some of these studies review host-microbiota interactions in different conditions and suggest novel approaches to improve health

However, this field is still in its infancy, and more studies are required. We must unravel the specific mechanisms by which microbiota modulates the immune system. Such approaches could become helpful in improving outcomes in diseases characterized by chronic inflammation.

Author contributions

EM drafted the manuscript; all authors contributed to the Research Topic, reviewed and accepted the last 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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 12 August 2022
DOI 10.3389/fimmu.2022.967300

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

RECEIVED 12 June 2022
ACCEPTED 26 July 2022
PUBLISHED 12 August 2022

CITATION
Fan X, Wang S, Hu S, Yang B and Zhang H (2022) Host-microbiota interactions: The aryl hydrocarbon receptor in the acute and chronic phases of cerebral ischemia. *Front. Immunol.* 13:967300. doi: 10.3389/fimmu.2022.967300

Host-microbiota interactions: The aryl hydrocarbon receptor in the acute and chronic phases of cerebral ischemia

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The relationship between gut microbiota and brain function has been studied intensively in recent years, and gut microbiota has been linked to a couple of neurological disorders including stroke. There are multiple studies linking gut microbiota to stroke in the "microbiota-gut-brain" axis. The aryl hydrocarbon receptor (AHR) is an important mediator of acute ischemic damage and can result in subsequent neuroinflammation. AHR can affect these responses by sensing microbiota metabolites especially tryptophan metabolites and is engaged in the regulation of acute ischemic brain injury and chronic neuroinflammation after stroke. As an important regulator in the "microbiota-gut-brain" axis, AHR has the potential to be used as a new therapeutic target for ischemic stroke treatment. In this review, we discuss the research progress on AHR regarding its role in ischemic stroke and prospects to be used as a therapeutic target for ischemic stroke treatment, aiming to provide a potential direction for the development of new treatments for ischemic stroke.

KEYWORDS

aryl hydrocarbon receptor, microbiota-gut-brain axis, cerebral ischemia, tryptophan metabolism, gut microbiota

Introduction

Cerebrovascular accident, commonly known as stroke and being a global health concern, is characterized by high mortality and disability rates, and is one of the leading cause of dementia and depression (1). According to neuropathology, stroke can be classified into two major subtyes: ischemic and hemorrhagic, with the former and latter accounting for 85% and 15% of all cases, respectively (2). The relationship between gut

microbiota and brain function has been studied intensively in recent years, and gut microbiota has been linked to a couple of neurological disorders, including Alzheimer's disease (AD) (3), Parkinson's disease (PD) (4), multiple scleroses (MS) (5), neurodevelopmental (6) and psychiatric disorders (7, 8), and stroke (9–15). Communication between the brain and gut microbiota is mainly mediated by neurogenic signaling molecules and microbial metabolites; specifically, four pathways related to neuro, metabolism, endocrine, and immune signaling, are involved in this process (16). In turn, the central nervous system (CNS) can regulate neurotransmitters to achieve bidirectional communications by shaping microbial community composition and function. These processes that link microbiota and the brain are termed the "microbiota-gut-brain" axis. Study have proven that the gut microbiota can influence stroke prognosis by modulating the immune response and neuroinflammation (13). In turn, stroke can induce a shift in the gut microbiota, affecting intestinal motility and permeability, stress response, and systemic infection after stroke (10, 14, 15, 17). These findings highlight the close connection between gut microbiota and stroke in the "microbiota-gut-brain" axis.

Gut microbiota interacts with the host mainly through its metabolites. Tryptophan is an essential amino-acid that must be obtained from the diet. It can be metabolized by gut microbiota directly or indirectly and participates in a variety of physiological processes. Abnormal tryptophan metabolism has been associated with many diseases. The AHR is an important mediator of acute ischemic damage and can result in subsequent neuroinflammation (18, 19). AHR can affect these responses by sensing microbiota metabolites. For instance, it can be activated predominantly by ligands produced from gut microbes metabolizing diet-derived tryptophan (20, 21). Indeed, aberrant tryptophan metabolism and dysbiosis of gut microbiota have been observed in both acute and chronic stages of cerebral ischemia (22, 23). Actually, ischemic injuries and subsequent neuroinflammation have been recognized as key elements in stroke development. Neuroinflammation exists in both acute and chronic phases of cerebral ischemia, affecting the prognosis and survival of stroke patients to some extent. Persistent neuroinflammation could induce neurodegeneration, leading to post-stroke dementia and depression (24, 25). Activated microglia and astrocyte play an important role in neuroinflammation after stroke, which may be achieved through the binding of the ligand to AHR (5, 26–30).

AHR is engaged in the regulation of acute ischemic brain injury and may be involved in chronic neuroinflammation after stroke. As an important regulator in the "microbiota-gut-brain" axis, AHR has the potential to be used as a new therapeutic target for ischemia stroke treatment. In this review, we discuss the research progress on AHR regarding its role in ischemia stroke and prospects to be used as a therapeutic target for ischemia stroke treatment, aiming to provide a potential direction for the development of new treatments for ischemia stroke.

Role of the "microbiota-gut-brain" axis in the development of ischemia stroke

The communication between the gut microbiota and CNS is mediated through at least 4 interacting components, including the immune system, metabolites, neurotransmitters, and activated vagal nerve (19). In the top-down signaling pathway, ischemia stroke can disrupt the structure and function of the gut microbiota through the autonomic nervous system, increasing the gut permeability and reducing its motility, which further induces an intestinal immune response and bacterial translocation. In the bottom-up signaling pathway, post-stroke gut microbiota dysbiosis can result in changes in bacterial metabolites, leading to systematic infection due to bacterial translocation, abnormal immune cell migration, and release of immunomodulatory cytokines, which further mediates neuroinflammation that causes severe ischemia stroke and worse prognosis (31) (Figure 1).

Preclinical and clinical studies demonstrated that gut microbiota plays an important role in the pathogenesis and prognosis of ischemia stroke (10, 12–16, 32–47) (Table 1). For example, many studies indicate that gut microbiota can affect risk factors related to ischemia stroke directly or indirectly, including hypertension, diabetes, hypercholesterolemia, obesity and atherosclerosis, as well as aging (35, 48–52). However, so far, there is no large prospective study exploring how gut microbiota relates to the long-term risk of ischemia stroke. In addition, ischemia stroke could change the gut microbiota composition. For instance, *Enterobacteriaceae*, *Ruminococcaceae*, *Veillonellaceae* and *Lachnospiraceae* were significantly enriched after stroke, while *Bacteroidaceae* and *Prevotellaceae* were significantly reduced. *Enterobacteriaceae* showed notably increased in patients with poor prognosis of cerebral infarction (47). Another study demonstrated that dysbiosis of the gut microbiota relates to ischemia stroke severity in mice; specifically, germ-free (GF) mice can develop more severe brain injury after receiving fecal transplants from high-stroke disequilibrium index (SDI) mice (36). Pre-existing microbiota ensures intestinal protection, and transplantation of the gut microbiota from post-stroke mice to GF mice exacerbates the brain damage and functional deficits compared to those in the controls. GF mice present enlarged brain lesions compared to recolonized (Ex-GF) and specific pathogen-free (SPF) mice after stroke (11). Changes in gut microbiota induced by antibiotics such as ampicillin can reduce ischemic brain injury and gut inflammation, leading to improved long-term prognosis (16, 38). However, another study showed the opposite result; mortality in

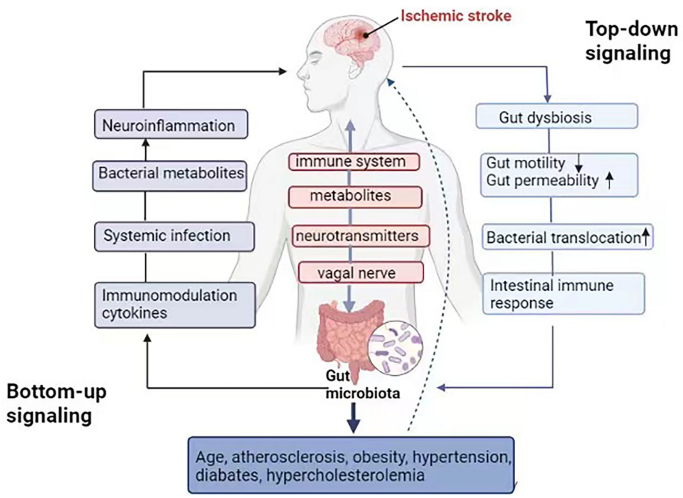

FIGURE 1
"Microbiota-gut-brain" axis in the ischemic stroke. Gut microbiota communicates to the CNS through the immune system, metabolites and neurotransmitters, as well as activation of the vagal nerve. In the top-down signaling pathway, ischemic stroke can affect the community structure and function of the gut microbiota through the autonomic nervous system, increase the gut permeability and reduce `gut motility, meanwhile, inducing an intestinal immune response and bacterial translocation. In the bottom-up signaling pathway, gut microbiota dysbiosis after stroke leads to changes in bacterial metabolites, systematic infection due to bacterial translocation, immune cell migration and the release of immunomodulation cytokines, which further mediate neuroinflammation, related to the severity of ischemic stroke and worse prognosis. Gut microbiota can affect risk factors related to ischemia stroke directly or indirectly, including hypertension, diabetes, hypercholesterolemia, obesity and atherosclerosis, as well as aging.

mice with disturbed gut microbiota was significantly higher following inhibition of gut microbiota by broad-spectrum antibiotics (12). Therapeutic fecal microbiota transplantation (FMT) can normalize the microbiota imbalance induced by brain injury and improve stroke prognosis (10). This effect may be particularly pronounced when aged stroke mice received FMT from young mice. The aged mice showed fewer behavioral abnormalities and neuroinflammation, which may be due to the fact that gut microbiota could produce high levels of short-chainfatty acids (SCFAs). Mechanistically, SCFAs can improve neuronal connectivity and synaptic plasticity after stroke by modulating microglia activation through recruitment of T-lymphocytes, thereby improving behavioral recovery. Studies have shown that supplementation of *Lactobacilli* after stroke can reduce neuroinflammation and improve cognitive function and depression (35, 40, 45). In addition, new evidence indicates that lactulose and atorvastatin may regulate the structure of gut microbiota by regulating intestinal immune function and reducing neuroinflammation after stroke (43, 46).

Acute ischemia stroke is characterized by loss of species diversity and overgrowth of opportunistic pathogens. A previous study has shown that acute ischemia stroke patients can develop significant gut microbiota disturbances at 3 days post-stroke, which returned to similar levels pre-stroke by day 5 (39). However, cerebral ischemia can induce persistent gut microbiota dysbiosis, disrupt the gut barrier, and lead to chronic systemic inflammation of the host, which is associated with worsening stroke and neurodegenerations. One study demonstrated that gut dysbiosis could last for more than 3 weeks after stroke, and then the disturbed gut microbiota could gradually recover but microbiota diversity was still decreased significantly after 4 weeks (36). Gut microbiota dysbiosis in Cynomolgus monkeys is still observed 6 and 12 months after cerebral ischemia, with notably increased *Bacteroidetes phylum* and *Prevotella* genus and significantly reduced *Firmicutes phylum*, *Faecalibacterium*, *Oscillospira*, and *Lactobacillus* genera, accompanied by a significant increase in levels of plasma D-lactate, zonulin, LPS, TNF-α, IFN-γ, IL-6 and a significant decrease in levels of SCFAs (23).

Alterations of levels of tryptophan metabolites and AHR after ischemia stroke

Tryptophan metabolism in the gastrointestinal tract can be regulated by three main pathways, i.e., the kynurenine pathway, serotonin pathway, and indol pathway. Approximately 90% of ingested tryptophan is degraded through the kynurenine pathway in immune and epithelial cells (53, 54). More specifically, tryptophan is transferred into the brain crossing the blood-brain barrier (BBB); then, two key enzymes in the kynurenine pathway, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), metabolize L-tryptophan

TABLE 1 Summarizes the pre-clinical and clinical evidences regarding the relationship between gut microbiota and ischemic stroke.

Author	Year of publication	Type of study	Subjects	Key findings
Caso et al. (32)	2009	Pre-clinical study	CCAO and MCAO rat	Bacterial translocation to mesenteric lymph nodes, spleen, liver, and lung after stroke, and it was associated with worsening stroke.
Benakis et al. (16)	2016	Pre-clinical study	MCAO mice	Antibiotic-induced alterations in the gut microbiota can reduce ischemic brain injury, the effect can be transmitted by FMT.
Singh et al. (10)	2016	Pre-clinical study	MCAO mice	Reduced species diversity and bacterial overgrowth of bacteroidetes were associated with intestinal barrier dysfunction and reduced intestinal motility; gut dysbiosis intensifies the ingress of Th17- and IL17-secreting γ δ T-cells (γ δ T-cells) into the CNS from the intestine, leading to chronic systemic and neuroinflammation. Higher numbers of proinflammatory lymphocyte populations correlate negatively with stroke outcome, which is reflected as larger infarct size, brain edema, and neurological deficits; FMT improves stroke outcome.
Houlden et al. (15)	2016	Pre-clinical study	MCAO mice	Specific changes in Peptococcaceae and Prevotellaceae were related with the severity of the stroke; changes in gut microbiota after stroke may affect recovery and treatment.Gut dysbiosis affects the local immune cells in the intestine and brain. In the early stage of stroke, engages both innate and adaptive immunity, microglial activation is followed by infiltration of peripheral immune cells, including monocytes, T- and B-lymphocytes.
Winek et al. (12)	2016	Pre-clinical study	MCAO mice	Conventional microbiota ensures intestinal protection; microbial colonization or specific microbiota are crucial for stroke outcome.
Stanley et al. (14)	2016	Pre-clinical study	MCAO mice	Stroke promotes the translocation and dissemination of selective strains of bacteria that originated from the host gut microbiota.
Crapser et al. (33)	2016	Pre-clinical study	MCAO mice	Ischemic stroke induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice.
Yamashiro et al. (34)	2017	Clinical study	41 patients: 40 controls	Ischemic stroke was independently associated with increased bacterial counts of Atopobium cluster and Lactobacillus ruminis, and decreased numbers of Lactobacillus sakei subgroup, changes in the prevalence of Lactobacillus ruminis were positively correlated with serum IL-6 levels.
Spychala et al. (35)	2018	Pre-clinical study	MCAO mice	The Firmicutes to Bacteroidetes ratio in aged mice increased 9-fold compared to young; gut microbiota can be modified to positively impact outcomes from age-related diseases.
Singh et al. (11)	2018	Pre-clinical study	MCAO mice	Bacterial colonization reduces stroke volumes by increasing cerebral expression of cytokines and microglia/macrophage cell counts; lymphocyte-driven protective neuroinflammation after stroke under control of the microbiome.
Xia et al. (36)	2019	Clinical study	83 patients: 70 controls	Dysbiosis of the gut microbiota correlated with ischaemic stroke severity, mice receiving FMT from patients with a high stroke disequilibrium index (SDI) developed more severe brain damage
Chen et al. (23)	2019	Pre-clinical study	MCAO cynomolgus monkeys.	The levels of Bacteroidetes phylum and Prevotella genus were significantly increased, the Firmicutes phylum, the Faecalibacterium, Oscillospira, and Lactobacillus genera were decreased after cerebral infarction in monkeys; Cerebral infarction induces persistent host gut microbiota dysbiosis, intestinal mucosal damage, and chronic systemic inflammation in cynomolgus monkeys.
Zeng et al. (37)	2019	Clinical study	141 patients	Compared with the low-risk group, opportunistic pathogens (Enterobacteriaceae and Veillonellaceae) and lactate-producing bacteria (Bifidobacterium and Lactobacillus) were increased, butyrate-producing bacteria (Lachnospiraceae and Ruminococcaceae) were decreased in the high-risk group.
Benakis et al. (38)	2020	Pre-clinical study	MCAO mice	Bacteroidetes S24.7 and the enzymatic pathway for aromatic metabolism were correlated with infarct volume; The gut microbiota composition in the ampicillin-treated mice was associated with reduced gut inflammation, a long-term favorable outcome, and a reduction of brain tissue loss.
JeoJeonn et al. (39)	2020	Pre-clinical study	MCAO pig	Abundance of the Proteobacteria was significantly increased, while Firmicutes decreased at 3 days poststroke, compared to prestroke populations, abundance of the lactic acid bacteria Lactobacillus was reduced. By day 5, the microbial pattern returned to similar values as prestroke,
Lee et al. (40)	2020	Pre-clinical study	MCAO mice	Aged stroke mice receiving young fecal transplant had less behavioral impairment and inflammation, which is related to Bifidobacterium longum, Clostridium symbiosum, Faecalibacterium prausnitzii and Lactobacillus fermentum, for they can produce more SCFAs
Ling et al. (41)	2020	Clinical study	93 patients	The abundance of Firmicutes and its members, including Clostridia, Clostridiales, Lachnospiraceae, and Lachnospiraceae_other, was significantly decreased in the age-matched PSCI group; PSCI was significantly correlated with the abundance of Enterobacteriaceae after adjustments

(Continued)

TABLE 1 Continued

Author	Year of publication	Type of study	Subjects	Key findings
Xiang et al. (42)	2020	Clinical study	20 patients: 16 controls	Stroke patients had fewer Firmicutes than controls. Lachnospiraceae (OTU_45) and Bacteroides served as markers of lacunar infarction. Bilophila and Lachnospiraceae (OTU_338), served as markers of non-lacunar acute ischemic infarction. Three optimal bacterial species, Pseudomonas.
Yuan et al. (43)	2021	Pre-clinical study	MCAO mice	Lactulose promotes functional outcomes after stroke in mice, which may be attributable to repressing harmful bacteria, and metabolic disorder, repairing gut barrier disruption, and reducing inflammatory reactions after stroke.
Wu et al. (44)	2021	Pre-clinical study	MCAO rat	The abundance of the Firmicutes phylum was decreased, whereas Proteobacteria and Deferribacteres were increased after stroke; Ruminococcus_sp_15975 might serve as a biomarker for the stroke; Many metabolites, such as L-leucine, L-valine, and L-phenylalanine, differed between the stroke and sham groups
Huang et al. (45)	2021	Pre-clinical study	MCAO mice	Bifidobacterium was enriched in calorie-restriction mice; Bifidobacterium administration improved the long-term rehabilitation of stroke mice
Zhang et al. (46)	2021	Pre-clinical study	MCAO mice	Atorvastatin increased the abundance of Firmicutes and Lactobacillus, decreased Bacteroidetes abundance, increased fecal butyrate level, promoted intestinal barrier function, regulated intestinal immune function, and reduced microglia-mediated neuroinflammation after stroke; FMT of atorvastatin-treated mice alleviated neuroinflammation in MCAO mice.
Xu et al. (47)	2021	Clinical study/ Pre-clinical study	28patients: 28controls	Enterobacteriaceae, Ruminococcaceae, Veillonellaceae and Lachnospiraceae were significantly enriched after stroke, while Bacteroidaceae and Prevotellaceae were significantly reduced. Enterobacteriaceae showed significant enrichment in patients with poor prognosis of cerebral infarction. Enterobacteriaceae exacerbates cerebral infarction by accelerating systemic inflammation and alleviates cerebral infarction by inhibiting its excessive growth.

CCAO, common carotid artery occlusion.

into L-Kyn (55, 56), which plays a key role in this pathway. L-Kyn can be further catabolized in two types of cells, astrocytes and microglia, in the brain. In astrocytes, L-Kyn was transformed into kynurenic acid (KYNA) under the catalyzation of kynurenine aminotransferase (KAT) family enzymes. KYNA is a well-recognized N-methyl-D-aspartate receptor (NMDAR) antagonist and is thought to be neuroprotective (57). In microglia, L-Kyn can be hydroxylated by kynurenine 3-monooxygenase (KMO) to generate 3-HK and its major metabolites, such as quinolinic acid (QUIN), which is considered to be an NMDAR agonist with neurotoxic properties (58). Both QUIN and KYNA act on NMDAR in the postsynaptic membrane of neurons. L-Kyn is shown to be a key AHR ligand and is associated with ischemia stroke severity and prognosis (18).

Preclinical and clinical studies have shown altered kynurenine pathway and tryptophan catabolism after cerebral ischemia. An increased level of brain QUIN was observed in gerbils, which was mediated by the activation of IDO, KYN, and 3-HK after transient ischemic attack (TIA), ultimately leading to an abnormal increase in the QUIN/KYNA ratio, which might contribute to the progression of post-stroke injury (59–63). QUIN is primarily detected in microglia and infiltrating macrophages 2-7 days after cerebral ischemia, which is consistent with a peak in immune infiltration, glial activation and inflammation during this period (62). An altered kynurenine pathway metabolism was observed in a permanent middle cerebral artery occlusion (MCAO) mouse model after ischemia stroke (22). The level of L-Kyn was increased in the brain as early as 3 hours after MCAO and remained at an increased level for 24 hours, in contrast to a decrease in L-tryptophan level between 3 and 24 hours and slight changes in plasma L-Kyn or L-Trp. An increase in AHR protein level, nuclear translocation and transcriptional activity of cortical neurons in this mouse model was also observed. In addition, the L-Kyn/L-Trp ratio is much higher in stroke patients than that in healthy controls and is positively correlated with infarct volume (63). The most common long-term complications after ischemia stroke are dementia and depression. A study found that abnormal alterations in kynurenine pathway catabolism persisted for at least 1 year after stroke, suggesting that it might be the cause of persistent brain dysfunction in these patients (64). The association between cognitive impairment and the kynurenine pathway after ischemia stroke has been described in only one study (65). Decreased levels of 5-HT and increased levels of kynurenine pathway catabolites have been reported in post-stroke patients with depression, and activation of key enzymes in the kynurenine pathway can lead to increased production of 3-HK, QUIN, L-Kyn, and KYNA (66), which induce the production of neurotoxic agents (67, 68). Finally, these alterations can cause damages to multiple brain regions such as the hippocampus, inhibiting neurogenesis and activating apoptotic signaling pathways, and thus leading to depression (69), which has been referred to as the kynurenine hypothesis of depression (70).

However, no correlations between depressive symptoms in post-stroke populations and blood L-Kyn/L-Trp ratios have been found (71, 72).

Gut microbiota affects levels of tryptophan metabolites and AHR

Gut microbes can metabolize tryptophan through several metabolic pathways and produce various tryptophan metabolites (73). For instance, some bacterial species, such as *Escherichia coli*, *Clostridium* spp. *Bacteroides* spp. *Clostridium sporogenes*, *Peptostreptococcus* spp. *Peptostreptococcus russellii*, *Peptostreptococcus anaerobius* and *Peptostreptococcus stomatis*, *Clostridium botulinum*, and *Peptostreptococcus anaerobius*, can produce indole propionic acid (IPA), indoleacetic acid (ILA), and indoleacetic acid (IA). While other species, such as *Lactobacilli*, *Ruminococcus gnavus*, *Clostridium bartlettii*, and *Bifidobacterium* spp., can produce indole aldehydes (IAld), indoleacetic acid (IAA), and ILA. Some others, such as *Bacteroides* spp. and *Clostridium* spp., can produce 3-methylindole (skatole) by decarboxylation of IAA.

Gut microbiota can directly or indirectly metabolize tryptophan, generating various metabolites, including indole, tryptamine, indole ethanol (IE), IPA, ILA, IAA, skatole, IAld and IA. Some of them, including Indole, IPA, and IA, can reduce intestinal permeability by disrupting mucosal homeostasis. Several other tryptophan catabolic products can regulate innate and adaptive immune responses by binding to AHR in intestinal immune cells. For example, IAld can increase IL-22 production by activating the AHR signaling pathway. Some other tryptophan metabolites, such as IPA, IE and IA, can be absorbed into the blood through the intestinal epithelium and exert antioxidant and anti-inflammatory effects (73). Tryptophan catabolic products, including IAA, IA, IAld, ILA, tryptamine, and skatole, are all ligands for AHR (74–77). Some agonists can facilitate AHR in crossing the BBB. In astrocytes and microglia, AHR can inhibit pro-inflammatory nuclear factor-κB (NF-κB) signaling, thus interfering chemokine production and transcriptional programs associated with inflammatory monocyte recruitment, and activating CNS-resident myeloid cells and producing direct neurotoxicity to regulate CNS inflammation (5).

The role of AHR in ischemia stroke

The basic characteristics of AHR

AHR is a ligand-controlled transcription factor (5), which is implicated in multiple physiological and pathological processes of many diseases, including inflammatory bowel disease (78), metabolic syndrome, and CNS diseases (79, 80). Expression of AHR is widely detected in the CNS, such as in neurons, oligodendrocytes, monocytes/macrophages, astrocytes, microglia, and cerebral endothelial cells (81). AHR can regulate the expressions of target genes which relate to cell proliferation, metabolism and immune response (82). Significant upregulation of AHR expression after stroke has been reported, which is shown to play a role in the cerebral ischemic injury (22, 83–86) (Table 2). In addition, the integrity of the BBB is also compromised upon activation of the AHR signaling (87–89). The BBB is essential for maintaining CNS homeostasis, and impairment of BBB is thought to contribute to neurodegeneration, leading cognitive impairment in humans (90).

The role of AHR in the neurological and immune systems has received increasing attention (91). The role of neuroinflammation in acute and chronic ischemia stroke has also been recognized (92). In fact, one of the pathological features of neurodegenerative diseases is neuroinflammation, mainly manifested by chronic activation of microglia (93). AHR can mediates inflammatory effects of microglia through dietary and microbial metabolites, particularly tryptophan metabolites (5, 93). Given the links between tryptophan metabolism, AHR and immune cell activation (94), we will highlight the role of the AHR signaling pathway (i.e., tryptophan metabolites as AHR ligand can bind to AHR) in ischemia stroke and potential targets for pharmacological modulation of ischemia stroke, in the following discussions.

AHR in acute phases of ischemia stroke

Cuartero et al. used mouse models to verify the hypothesis that activation of the L-Kyn-AHR signaling pathway can exacerbate acute ischemic brain injury (22). They identified increased AHR protein level, nuclear translocation and transcriptional activity of cortical neurons in a permanent MCAO mouse model. In the core of the infarct, the AHR protein level rose to a peak at around 5 hours after stroke and returned to baseline levels by day 7 after stroke; in the peri-infarct area, the AHR protein level started to increase at 18 hours after stroke and reached the peak at day 3 after stroke and then started to decrease. Treating with an AHR antagonist or using AHR-deficient mice resulted in a smaller infarct size and lower National Institutes of Health Stroke Scale (NIHSS) in mice model (22). However, another group showed an opposite result when treating ischemia stroke using the AHR agonists. Mechanistically, activation of the AHR signaling during cerebral ischemia may mediate specific pathological effects by inhibiting the cAMP response element-binding protein (CREB) signaling pathway. Further experiments demonstrated that L-Kyn could accumulate in the brain during acute ischemia stroke and act as

TABLE 2 A summary of the role of AHR in cerebral ischemia.

Reference	Subjects	Key findings	Moechanism
Cuartero et al., 2014 (22)	MCAO mice	Ischemic insult increases total and nuclear AHR levels as well as AHR transcriptional activity in neurons *in vivo* and *in vitro*, increasing infarct size and neurological deficits. L-kynurenine-AHR pathway mediates acute brain damage after stroke.	L-Kyn increased the expression of the AHR target genes Cyp1a1 and Cyp1b1 mRNA in cortical neurons; L-Kyn decreased CRE-mediated transcription in neurons, demonstrated by a reduction in both BDNF and NPAS4 mRNA expression to increase apoptosis.
Chen et al., 2019 (83)	MCAO mice	The kynurenine/AHR activation mediated acute ischemic injury. Compared to normal WT controls and AHRcKO mice. AHR immunoreactivities were increased predominantly in activated microglia and astrocytes, leading to a significantly aggrandized ischemic brain infarction, sensorimotor deficits, and nonspatial working memory after MCAO.	AHR affected pro-inflammatory cytokines IL-1β, IL-6, IFN-γ, CXCL1, as well as S100β, NGN2, and NGN1 gene and protein expression after MCAO. TMF treatment modulated gene and protein expression related to neurogenesis after stroke, leading an increased proliferation of neural progenitor cells at the ipsilesional neurogenic zones.
Kwon et al., 2020 (84)	TMCAO rat	The inhibition of AHR activation before reperfusion alleviates brain damage due to apoptosis. AHR antagonism at a delayed time point after ischaemia is also effective in suppressing cerebral I/R injury and this effect was most pronounced in the 10 min and 50 min post-stroke administration groups.	AHR antagonists after ischaemia affected the inhibition of the formation of cellular and vasogenic oedemas due to cerebral I/R.
Tanaka et al., 2021 (85)	MCAO mice	MCAO upregulated AHR expression in microglia during ischemia. MCAO increased the expression of TNFα and then induced edema progression, and worsened the modified neurological severity scores, with these being suppressed by administration of an AHR antagonist, CH223191.	In MCAO model mice, the NOX subunit p47phox expression was upregulated in microglia by ischemia, aggrandized the expression of Tnfa and edema progression. AHR antagonist can relieve hypoxia/ischemia and edema progression and improve the neurological severity scores in mice *via* inhibition of the AHR signaling pathway.
Rzemieniec et al., 2019 (86)	mice	A selective AHR modulator, DIM protects neurons against ischemia-induced damage at earlier and later stages of neuronal development,	Ischemia-induced apoptosis and autophagy and possibly corresponds to ischemia-evoked disruption of HDAC activity and AhR/CYP1A1 signaling pathway. DIM partially reversed OGD-induced apoptosis, autophagy and AHR/CYP1A1 signaling as well as OGD-inhibited HDAC activity.

OGD, Oxygen and glucose deprivation; DIM, 3,3′-diindolylmethane; TNFα, tumor necrosis factor α; NOX, NADPH oxidase; TMCAO, transient middle cerebral artery occlusion.

an endogenous activator of AHR. Exogenous supplementation of L-Kyn aggravates strokes in an AHR-dependent manner and increases infarct volume. Most interestingly, the authors also demonstrated that inhibition of L-Kyn production by pharmacological blockade of TDO could decrease the activation of AHR signaling and reduce infarct volume in the MCAO stroke model. Taken together, this study identified the L-Kyn-AHR pathway as a novel mediator of brain injury during stroke, and validated TDO and AHR as new "druggable" targets for acute ischemia stroke.

Another study suggested that AHR inhibition in acute ischemia stroke might be benefits regarding functional outcomes through reducing pro-inflammatory glial cell proliferation and promoting neurogenesis. Compared to respective controls, wild-type (WT) and AHRcKO mice that were treated with the AHR antagonist, 6,2',4'-trimethoxyflavone (TMF), showed significantly smaller infarct volumes and improved sensorimotor and non-spatial working memory functions. AHR Immunoreactivity was increased mainly in activated microglia and astrocytes after AHRcKO. TMF-treated WT and AHRcKO mice showed remarkably increased astrocyte and microglia proliferation (28). In a cerebral ischemia-reperfusion injury (CIRI) rat model, TMF-treated rats displayed lower cell apoptosis levels and smaller infarct volumes than those not treated with TMF at 24 h after cerebral ischemia, which were most pronounced in the 10 min and 50 min after stroke. This study indicated that the AHR antagonists might reduce CIRI-related cellular injury.

AHR in chronic ischemia stroke

Ischemia stroke can induce long-term host gut microbiota dysbiosis, impairing the intestinal barrier and leading to chronic neuroinflammation. This inflammatory response is associated with cognitive impairment, depression, and anxiety in post-stroke patients (28). One year after FMT, elevated plasma pro-inflammatory cytokines, such as IFN-g, IL-6 and TNF-α, were decreased in focal cerebral ischemia of monkey models, suggesting the persistence of systemic inflammation post ischemia stroke (23). Numerous studies have shown that resident inflammatory cells and microglia can first respond to CIRI and amplify neuroinflammation by interacting with astrocytes (95–98). The inflammatory response in the brain of rats surviving 2 years after ischemic brain injury was evident but varied in the extent regarding microglia and astrocyte responses in different brain tissues (25). In another rat model of dementia in which the rats survived 2 years after cerebral ischemia, it was shown that this neuroinflammatory process was mainly regulated by microglia and astrocyte activity. In conclusion, microglia and astrocytes play an important role in post-stroke neurodegeneration (99).

Stroke injuries can induce the activation of microglia, which are generally classified into detrimental M1 and protective M2 subtypes (Figure 2). M1 microglia can secrete pro-inflammatory cytokines while M2 microglia can secrete anti-inflammatory cytokines. M2 microglia can stimulate neural stem/progenitor cell proliferation and neuronal differentiation in the ipsilateral subventricular zone through upregulation of TGFα expression levels. Studies have shown that in acute ischemia stroke, activated microglia predominantly express M2 phenotypic markers. However, there is a gradual shift to the M1 phenotype at around 1 week since the acute initiation of ischemia stroke, which persists for several weeks or even months. The sustained activation microglia is also thought to be associated with the onset and progression of neurodegenerative diseases (100). NF-κB, which is a key molecule in the microglia inflammatory pathway, induces activation and polarization of M1 microglia (101). Astrocytes can proliferate reactively after ischemic stroke. Liddelow et al. classified these astrocytes into the A1 and A2 subtypes, which are neurodamaging and neuroprotective, respectively (102). AHR plays an important role in activating microglia and activating astrocytes, which participate in the pro-inflammatory and anti-inflammatory processes, respectively. AHR inhibits the pro-inflammatory NF-κB signaling pathway while deletion of AHR or AHR ligands in microglia results in a dysregulated inflammatory response. Microglia and astrocytes intercommunicate with each other in many ways and may also be involved in the "gut-microbiota-brain" axis (103). Based on the fact that gut microbial metabolites can affect the CNS *via* the AHR-dependent signaling pathway, role of the commensal microbiota-mediated AHR signaling in the regulation of inflammation-promoting activity mediated by microglia and astrocytes has been investigated in recent years. Agonists derived from diet, gut microbiota and host metabolism can activate the AHR through the BBB. The AHR promotes TGFα expression in microglia, which acts on astrocytes and inhibits their pro-inflammatory activity. Further, AHR on microglia inhibits NF-κB-driven vascular endothelial growth factor B (VEGFB) expression, thereby promoting astrocytes to exert anti-inflammatory activity (104). Gut microbiota dysbiosis after stroke leads to abnormal tryptophan metabolism, and the decreased levels of AHR agonists may lead to enhanced neuroinflammation.

AHR as a potential therapeutic target for treatment of ischemia stroke

As previously described, modulation of the AHR signaling may provide new therapeutic strategy to attenuate neuronal damage after acute ischemia stroke and prevent the development of post-stroke neurodegeneration, thereby improving the short- and long-term ischemia stroke prognosis. In the permanent MCAO mouse model, L-Kyn mediates ischemic neuronal injury as an endogenous activator of AHR (22). Therefore, pharmacological inhibition of the kynurenine pathway or

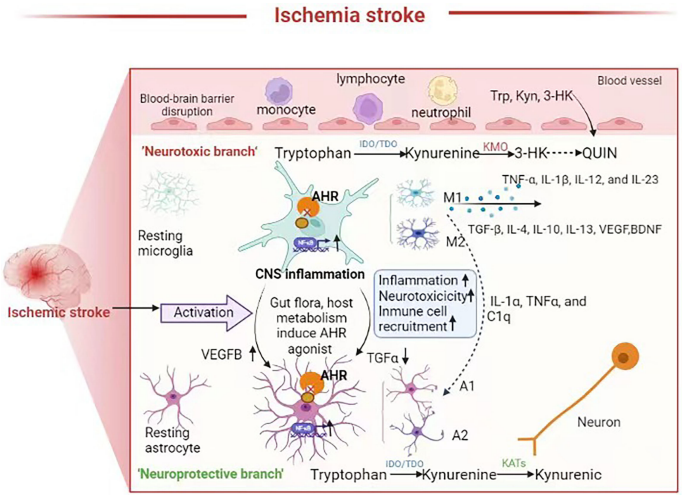

FIGURE 2
Neuroinflammation in the brain after stroke, the role of AHR and tryptophan metabolites in neuroinflammation. Microglia and astrocytes are activated and interact with each other to mediate neuroinflammation following ischemic stroke Some metabolites such as 3-HK, Kyn, QUIN produced by tryptophan metabolism can cross the BBB plays a neuroprotective or neurotoxic role. Gut flora and the host tryptophan metabolism produce AHR agonist. In astrocytes and microglia, AHR can inhibit pro-inflammatory nuclear factor-κB (NF-κB) signaling, and reduction of AHR agonists after gut microbiota dysbiosis result in an upregulated neuroinflammation and neurotoxic responses and immune cell recruitment, which are amplified through microglia-astrocyte interactions.

activation of the AHR pathway in acute ischemia stroke might prevent neurological injury. On the one hand, early administration of TMF, an AHR antagonist, can be a simple approach for the treatment of acute ischemia stroke. On the other hand, synthesis of L-Kyn under the action of TDO as the primary pathway in ischemic brain tissue and inhibition of L-Kyn production by the TDO inhibitor compromises the activation of the AHR signaling, leading to reduced infarct volume. Interestingly, pharmacological blockade of IDO, another key enzyme in L-Kyn production, by the IDO inhibitor 1-MT, does not show a beneficial effect in reducing infarct size and improving neurological prognosis, despite increased IDO expression and activity in transient MCAO mouse model (105).

AHR can mediate the inflammatory response in glial cells of the CNS (106). Dietary and microbial metabolites, particularly tryptophan metabolites, have recently been shown to act as AHR activators and thus regulate microglia and astrocyte activity and neuroinflammation in the CNS (5, 94). These studies linked the gut microbiota to neurological inflammation in the brain *via* the AHR signaling pathway. In mice with autoimmune encephalomyelitis, the AHR signaling was activated in astrocytes, which was proven to limit the inflammatory response in astrocytes. Moreover, this anti-inflammatory response could become increasingly evident when dietary tryptophan was ingested by mice. To demonstrate that this effect is regulated by microbiota-mediated tryptophan metabolites, a broad-spectrum antibiotic-ampicillin was applied to the mice to clear their gut microbiota, followed by treatment of the mice with indirubin-3'-oxime, a microbial metabolite of tryptophan. As a result, AHR-mediated anti-inflammatory effects were observed (5), indicating the effect is indeed mediated by tryptophan metabolites. Indirubin-3'-oxime has also been shown to inhibit the inflammatory activation in microglia in the rat brain (107). *Lactobacillus* was found to be an important host probiotic, and its levels were reduced after cerebral ischemia in monkeys (23). There are also studies showing that *Lactobacillus* supplementation can improve cognitive function and mood and reduce aging-related inflammation in mice and rats (28, 108). *Lactobacillus casei subsp. casei 327* (327 strain) can indirectly promote colonic 5-HT synthesis (109). *Lactobacillus reuteri* can degrade tryptophan into indolic compounds, such as IAld, ILA, and IAA (74, 110). A decreasing trend in serum kynurenine: tryptophan ratios was observed in humans after 8 weeks of oral administration of *Lactobacillus johnsonii* (111). As an important source of essential amino acids, diet is considered to be an important factor in shaping microbial tryptophan metabolism. A recent study indicated that the microbial tryptophan degradation pathway could be weakened under a high-fat diet (112). In addition, increasing carbohydrate availability promotes intestinal serotonin synthesis (113). Thus, probiotic supplementation and a reasonable diet can theoretically improve ischemia stroke prognosis; however, whether it is indeed beneficial in post-stroke patients needs to be tested in future clinical trials. Ramos et al. showed that the function of AHR and its downstream signaling pathways are impaired in the elderly and AD patients (114). The role of AHR ligands in improving learning memory deficits was also confirmed in a mouse model (104). Activation of the AHR signaling pathway by endogenous ligands such as L-Kyn and 6-Formylindolo[3,2-b]carbazole (FICZ), or exogenous ligands such as diosmin and indole-3-carbinol, could increase the expression and enzymatic activity of neprilysin in amyloid precursor protein/presenilin 1 (APP/PS1) mice, and improve cognitive impairment effectively in these mice. Tryptophan metabolites, such as 5-hydroxy indole-acetic acid and kynurenic acid, could reduce cognitive impairment in mice and Aβ load in patients with mild cognitive impairment by activating AHR (115–118).

Present shortcomings and future perspectives

New therapies, such as the application of recombinant thrombolytic tissue plasminogen activator (r-tPA) and intra-arterial thrombectomy, have been developed for the treatment of acute ischemia stroke (119). However, due to the narrow time windows and the limitations of endovascular treatment techniques, only a small number of patients with acute ischemia stroke can benefit from these new therapies. There are limited treatment options for patients with subacute and chronic ischemia stroke. In light of this, AHR can be used as a potential therapeutic target for the treatment of these patients (85). Inhibition of AHR signaling in acute ischemia stroke has the potential to benefit the patients by reducing pro-inflammatory gliosis and enhancing neurogenesis. In contrast, tryptophan metabolites, as the AHR ligands, can interact with microglia and astrocytes and prevent neurodegeneration. Supplementation of tryptophan metabolites, probiotics producing AHR agonists, and FMT from normal donors may be potential therapeutic strategies that can improve the prognosis of certain types of ischemia stroke. Delivering drugs to the brain directly has long been a major challenge in treating neurodegeneration, and thus these proposed strategies might overcome this barrier.

However, there is still a long way to go for researchers despite the substantial progress. Firstly, the composition and immunological characteristics of human gut microbiota are not completely the same as those of animals such as mice. Secondly, the effects of intestinal fungi and protozoa on tryptophan metabolism and severity of ischemia stroke are unclear. Whether there are other endogenous or exogenous AHR ligands besides tryptophan that have not been identified and

whether there are any other endogenous inhibitors of the AHR pathway are unknown as well. Tryptophan can also directly be absorbed by the host gut and the complex interactions between intestinal flora, intestinal lumen tryptophan availability, and host tryptophan metabolism need further study. Thirdly, it requires validation that whether the results from animal studies could be used for the effective treatment of human diseases such as ischemia stroke. Developing a humanized mouse model might help explain the well-known differences regarding AHR between humans and mice. Finally, large-scale, highly controlled clinical studies are urgently needed to further validate the role of AHR in ischemia stroke development.

Conclusion

The role of AHR and tryptophan metabolism in the communication between the gut microbiota and CNS has been increasingly well known. Tryptophan metabolism is directly or indirectly regulated by the gut microbiota and many tryptophan metabolites can act as endogenous AHR activators, activating AHR, which can further regulate neuroinflammation by interacting with microglia and astrocytes. Since many factors can affect the gut microbiota composition and metabolism, including diet, antibiotics, and probiotics, as well as FMT, the manipulation of the gut microbiota modulating tryptophan availability may be a therapeutic method for neuroinflammation after ischemia stroke. In conclusion, we argue that the AHR and tryptophan metabolism play an important role in ischemia stroke.

Author contributions

Conception and design: SW and XF; writing of the manuscript: XF; final approval of the manuscript: HZ. All authors read and approved the final manuscript.

Funding

This work was supported by the Construction Fund of Medical Key Disciplines of Hangzhou (discipline number OO20200485).

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 19 August 2022
DOI 10.3389/fimmu.2022.964910

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 09 June 2022
ACCEPTED 18 July 2022
PUBLISHED 19 August 2022

CITATION
Ling Z, Cheng Y, Chen F, Yan X, Liu X, Shao L, Jin G, Zhou D, Jiang G, Li H, Zhao L and Song Q (2022) Changes in fecal microbiota composition and the cytokine expression profile in school-aged children with depression: A case-control study.
Front. Immunol. 13:964910.
doi: 10.3389/fimmu.2022.964910

Changes in fecal microbiota composition and the cytokine expression profile in school-aged children with depression: A case-control study

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Depression in childhood negatively affects the growth and development, school performance, and peer or family relationships of affected children, and may even lead to suicide. Despite this, its etiology and pathophysiology remain largely unknown. Increasing evidence supports that gut microbiota plays a vital role in the development of childhood depression. However, little is known about the underlying mechanisms, as most clinical studies investigating the link between gut microbiota and depression have been undertaken in adult cohorts. In present study, a total of 140 school-aged children (6–12 years) were enrolled, including 92 with depression (male/female: 42/50) and 48 healthy controls (male/female: 22/26) from Lishui, Zhejiang, China. Illumina sequencing of the V3–V4 region of the 16S rRNA gene was used to investigate gut microbiota profiles while Bio-Plex Pro Human Cytokine 27-plex Panel was employed to explore host immune response. We found that, compared with healthy controls, children with depression had greater bacterial richness and altered β-diversity. Pro-inflammatory genera such as *Streptococcus* were enriched in the depression group, whereas anti-inflammatory genera such as *Faecalibacterium* were reduced, as determined by linear discriminant analysis effect size. These changes corresponded to altered bacterial functions, especially the production of immunomodulatory metabolites. We also identified the presence of a complex inflammatory condition in children with depression, characterized by increased levels of pro-inflammatory cytokines such as IL-17 and decreased levels of anti-inflammatory cytokines such as IFN-γ. Correlation analysis demonstrated that the differential cytokine abundance was closely linked to changes in gut

microbiota of children with depression. In summary, key functional genera, such as *Streptococcus* and *Faecalibacterium*, alone or in combination, could serve as novel and powerful non-invasive biomarkers to distinguish between children with depression from healthy ones. This study was the first to demonstrate that, in Chinese children with depression, gut microbiota homeostasis is disrupted, concomitant with the activation of a complex pro-inflammatory response. These findings suggest that gut microbiota might play an important role in the pathogenesis of depression in school-aged children, while key functional bacteria in gut may serve as novel targets for non-invasive diagnosis and patient-tailored early precise intervention in children with depression.

KEYWORDS

children, depression, dysbiosis, microbiota-targeted diagnosis, inflammation

Introduction

Depression in school-aged children, a long-overlooked psychiatric disorder, affects approximately 2.8% of children under the age of 13 and 5.6% of 13–18-year-olds (1). Childhood depression is characterized by sad or irritable mood, decreased self-esteem, sleep disturbance, anhedonia, decreased capacity for fun, social withdrawal or impaired social relationships, and impaired school performance (2, 3). This disorder tends to recur throughout life and is associated with serious complications, including self-harm and suicide. Notably, the prevalence of depression increases markedly after the transition from childhood to adolescence (4). The high prevalence and adverse outcomes have rendered childhood depression a significant public health problem. Additionally, major depressive disorder (MDD) has become a leading risk factor for suicide amongst children. Unlike in adults, depression is difficult to diagnose in children because the symptoms are non-specific and the manifestations may overlap with those normally witnessed at this stage of life. Accordingly, most children with depression go undiagnosed and untreated (2, 5).

Over recent years, childhood depression has received increasing attention from mental health professionals as well as parents. An awareness of the possibility of depression in children can expedite its diagnosis and treatment, thereby avoiding more severe complications later in life. Depression in children can be caused by any combination of factors, including physical health, family history, environment, genetic vulnerability, and biochemical disturbance. Evidence for the heritability of depression in children includes familial transmission; heightened risk for depression in the adult relatives of depressed youths; and estimates of heritability of 40%–80% in studies of twins (2, 6, 7). Environmental factors, together with genetic predisposition, also confer an increased risk for depression (8). A comprehensive meta-analysis of twins indicated that environmental effects account for a 55%-66% risk for major depression (9). Recent preclinical and clinical findings strongly support the existence of a link between gut dysbiosis and depression *via* the microbiota–gut–brain axis. We have previously reported that gut microbiota homeostasis is disrupted in adult patients with MDD, characterized by increased levels of Enterobacteriaceae and *Alistipes* and reduced numbers of *Faecalibacterium*. Notably, we found that *Faecalibacterium* abundance was negatively correlated with the severity of depressive symptoms (10). Interestingly, the transplantation of fecal microbiota derived from patients with depression ("depression microbiota") into germ-free mice can induce depression-like behavior by altering host metabolism, demonstrating a causal correlation between gut dysbiosis and the development of depression (11). A recent multi-omics-based analysis also revealed that neuroactive metabolites (multiple B vitamins, kynurenic acid, gamma-aminobutyric acid, and short-chain fatty acids) derived from specific depression-associated microbes are involved in the interactions between the gut and the brain and contribute to the pathophysiology of depression (12). Meanwhile, "depression microbiota"-derived molecules and metabolites can promote inflammation in the central nervous system, thereby greatly contributing to the onset of depression (13). Additionally, patients with depression reportedly have higher levels of pro-inflammatory cytokines, acute-phase proteins, chemokines, and cell-adhesion molecules (14, 15). Besides social, psychological, and environmental factors, recent evidence has indicated that the gut microflora, acting through the microbiota–gut–brain axis, may be a key environmental determinant for depression in adults.

Emerging evidence has indicated that the gut microbiota undergoes age-related changes; however, relatively few studies have considered the effects of age on the gut microbiota when exploring the pathogenesis of depression. Moreover, most clinical studies investigating the association between the gut microbiota and this condition have involved adult cohorts. Given the differences in environmental exposures and life trajectories between children and adults, further studies investigating the connection between gut dysbiosis and depression in childhood are urgently needed. Accordingly, the aim of this study was to explore in detail the structure and composition of the fecal microbiota in pediatric patients with MDD from Lishui using high-throughput 16S rRNA gene sequencing on the MiSeq platform, as well as identify putative associations between altered microbial profiles and host cytokine expression levels. Our findings provide novel insights into the etiology of depression in children as well as contribute to non-invasive diagnosis and personalized microbiota-targeted therapy for depression in childhood.

Methods

Participants' enrollment

A total of 92 pediatric patients with newly diagnosed MDD (age 6-12 years) according to the criteria of the Hamilton Depression Scale (HAMD), and/or the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V), and/or the third version of Chinese Classification of Mental Disorder (CCMD-3), were recruited from Lishui, Zhejiang (China) from November 2019 to April 2021, while 48 healthy children with similar age and sex distribution were enrolled as controls. The pediatric MDD patients were enrolled in the outpatient clinics of Lishui Second People's Hospital (Zhejiang, China) and diagnosed by two experienced pediatric psychiatrists. Meanwhile, their schoolmates were recruited randomly, evaluated systematically, and selected as healthy control. Those schoolmates with HAMD more than 7 would be exclude from healthy controls. Dietary and other socio-demographic information was obtained *via* questionnaires. All these participants lived in the Liandu district of Lishui, with similar birth modes, dietary habits, lifestyles, and environment. These protocols for the study were approved by the Ethics Committee of Lishui Second People's Hospital and written informed consent was obtained from their guardian before enrollment. The detailed demographic data and medical history were collected using a set of questionnaires (Table S1). The exclusion criteria included: age < 6 or > 13 years; body mass index (BMI) > 28 kg/m^2; active respiratory or intestinal infections; autism spectrum disorder, anorexia nervosa, bipolar disorder, attention-deficit/hyperactivity disorder, mania; antibiotic, prebiotic, probiotic, or synbiotic administration in the previous month; antidepressant, mood stabilizers or other psychiatric drugs in last 1 months; autoimmune diseases.

Sample collection and bacterial DNA extraction

The sample collection, processing and banking are according to our previous standardized protocols. Briefly, approximately 2g of a fresh fecal sample was collected in a sterile plastic cup, and stored at -80°C after preparation within 15 min until use. Serum samples from these participants were obtained using their fasting blood in the early morning. Bacterial genomic DNA was extracted from 300 mg of homogenized feces using a DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (16, 17). The amount of DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation, Boston, MA, USA) and the quality of DNA was checked by agarose gel electrophoresis. All DNA was stored at -20°C before further analysis.

Amplicon library construction and sequencing

The protocols of amplicon library construction and sequencing were conducted as our previous studies (16–18). The details were shown as follows: amplicon libraries were constructed with Illumina sequencing-compatible and barcode-indexed bacterial PCR primers 341F (5'-CCTACGGG NGGCWGCAG-3')/785R (5'-ACTACHVGGGTATCTAATCC-3'), which target the V3-V4 regions of the 16S rRNA gene (19). All PCR reactions were performed with KAPA HiFi HotStart ReadyMix using the manufacturer's protocol (KAPA Biosystems) and approximately 50 ng of extracted DNA per reaction. Thermocycling conditions were set at 95°C for 1 min, 55°C for 1 min, then 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 5 min. All PCR reactions were performed in 50 µl triplicates and combined after PCR. The amplicon library was prepared using a TruSeq™ DNA sample preparation kit (Illumina Inc, San Diego, CA, USA). Prior to sequencing, the PCR products were extracted with the MiniElute® Gel Extraction Kit (QIAGEN) and quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Electron Corporation) and Qubit 2.0 Fluorometer (Invitrogen). The purified amplicons were then pooled in equimolar concentrations and the final concentration of the library was determined by Qubit (Invitrogen). Negative DNA extraction controls (lysis buffer and kit reagents only) were amplified and sequenced as contamination controls. Sequencing was performed on a MiSeq instrument (Illumina) using a 300 × 2 V3 kit together with PhiX Control V3. MiSeq sequencing and

library construction were performed by technical staff at Hangzhou KaiTai Bio-lab.

Bioinformatic analysis

Based on our previous studies, the 16S rRNA gene sequence data set generated from the Illumina MiSeq platform was inputted to QIIME2 (version 2020.11), and all steps of sequence processing and quality control were performed in QIIME2 with default parameters (16, 17, 20, 21). Before the following data analysis, these reads of each sample were normalized to even sampling depths and annotated using the Greengenes reference database (version 13.8) with both the RDP Classifier and UCLUST version 1.2.22 methods implemented in QIIME2. α-diversity indices, including the observed species, abundance-based coverage estimator (ACE), Chao1 estimator, Shannon, Simpson, Evenness and PD whole tree indices, were calculated at a 97% similarity level. β-diversity was measured by the unweighted UniFrac, weighted UniFrac, jaccard and Bray-Curtis distances calculated by QIIME2, which were visualized by principal coordinate analysis (PCoA). The differences in the composition of the fecal microbiota at different taxonomic levels were analyzed with Statistical Analysis of Metagenomic Profiles (STAMP) software package v2.1.3 and the linear discriminant analysis (LDA) effect size (LEfSe) method. Only bacterial phylotypes with an average relative abundance of more than 0.01% were selected for the LEfSe analysis. Krona chart was plotted using taxonomy summary data obtained from QIIME Krona chart displays abundance and hierarchy simultaneously using a radial space-filling display and features a red-green color gradient, signifying the average BLAST hits e-values within each taxon (22). PiCRUSt v1.0.0 was used to identify predicted gene families and associated pathways from inferred metagenomes of taxa of interest identified from the compositional analyses.

Multiplex cytokine analysis

Serum cytokines, chemokines and growth factors were probed using Bio-Plex Pro Human Cytokine 27-plex Panel (M50-0KCAF0Y, Bio-Rad, Hercules, CA, USA) multiplex magnetic bead-based antibody detection kits following manufacturer's instructions. Based on the Luminex® xMAP® technology, the assays are capable of simultaneously quantifying 27 targets including interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, Fibroblast growth factor-basic (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophages colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon gamma-inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophages inflammatory protein-1α (MIP-1α), platelet-derived growth factor (PDGF-bb), MIP-1β, regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). The assays were run on the Luminex® 200™ system (Bio-Rad) and fluorescence values were collected. A standard curve was derived using the different concentrations of the assay standards. Data was acquired using the Bio-Plex Array Reader system 2200. The results expressed as picogram per milliliter (pg/mL) using the standard curves integrated into the assay and Bio-Plex Manager v5.0 software with reproducible intra- and inter-assay CV values of 5-8% (16, 17, 23).

Statistical analysis

White's nonparametric *t*-test, independent *t*-test, or Mann-Whitney *U*-test were applied for continuous variables. Pearson chi-square or Fisher's exact test were used for categorical variables between groups, Spearman's rank correlation test was utilized for correlation analyses. Statistical analysis was performed using the SPSS v19.0 (SPSS Inc., Chicago, IL) and STAMP v2.1.3 (24). False-discovery rate (FDR) was calculated according to Benjamini-Hochberg, FDR-corrected p values were denoted as Q_{FDR} and was used when performing all untargeted screening analyses of different taxa. The predictive power was evaluated by receiver operating characteristics (ROC) and area under the curve (AUC) analysis to determine the ability of the differential bacteria to accurately predict childhood depression. R software ggplot2 and pheatmap packages and GraphPad Prism v6.0 were used for preparation of graphs. All tests of significance were two sided, and $p<0.05$ or corrected $p<0.05$ was considered statistically significant.

Accession number

The sequence data from this study are deposited in the GenBank Sequence Read Archive with the accession number PRJNA846994.

Results

Characteristics of patients

A total of 140 school-aged children were included in this study, including 92 recently diagnosed with MDD (male/female: 42/50; age: 8.84 ± 1.89 years) and 48 healthy controls (male/female: 22/26; age: 9.27 ± 2.11 years). No differences in clinical characteristics such as BMI (21.86 ± 2.33 in MDD patients *vs.* 21.33 ± 2.27 in the controls), birth mode (vaginal delivery: 68 for MDD patients *vs.* 37 for the controls; cesarean section: 24 among

MDD patients vs. 11 among the controls), and feeding mode (all mixed feeding) were detected between the two groups ($p > 0.05$). All the children newly diagnosed with MDD were treatment-naive. The average HAMD score for children with MDD was 24.0 ± 4.52, significantly higher than that for the healthy controls (4.2 ± 2.48; $p < 0.05$). Among the children with MDD, 12 had a family history of a psychiatric disorder such as MDD or schizophrenia.

Fecal microbiota structure was altered in children with MDD

For microbiota analyses, we obtained 3,883,979 high-quality reads (1,222,781 for healthy controls and 2,661,198 for children with MDD), with an average of 27,742 reads per sample. In total, we identified 3,673 OTUs (unique bacterial phylotypes) among the fecal microbiota, attaining a Good's coverage of 98.87%, indicating that most of the fecal bacteria had been detected. The α-diversity of the fecal microbiota defines its richness (number of OTUs) and evenness (relative abundance of the OTUs) either qualitatively (richness and Chao1 indices) or quantitatively (Shannon and Simpson indices). Interestingly, the Shannon and Simpson index values were not significantly different between the healthy controls and the children with MDD (Figures 1A, B), whereas the richness index values—ACE, Chao1, and observed OTUs—were significantly higher in children with MDD than in the healthy controls (Figures 1C–E). To characterize the global differences between the fecal microbial communities of the two groups, PCoA plots (bacterial β-diversity) were generated based on the Bray–Curtis (R^2 = 0.079), Jaccard (R^2 = 0.052), unweighted UniFrac (R^2 = 0.054), and weighted UniFrac (R^2 = 0.181) distances. The results showed significant separation between the fecal samples of the pediatric patients with MDD and those of healthy children despite the significant interindividual variation observed (ADONIS test: $p < 0.01$; Figures 1F–I). Additionally, a Venn diagram of the shared OTUs between the children with MDD and healthy children

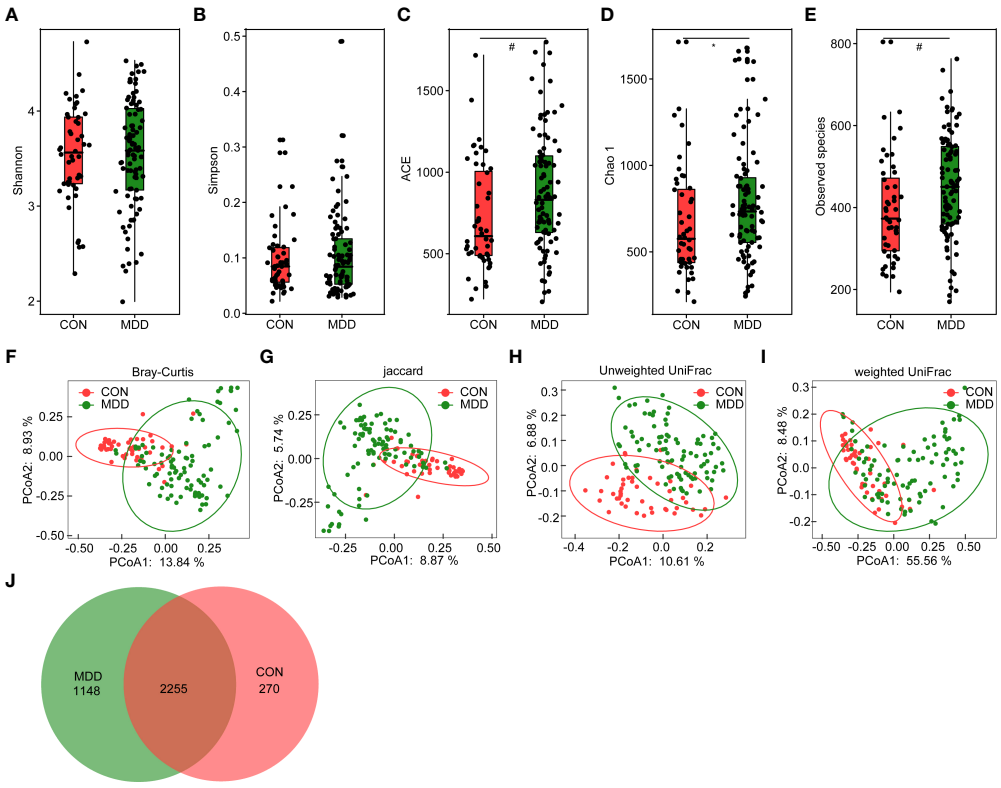

FIGURE 1
Altered overall structure of the fecal microbiota in patients with childhood major depressive disorders (MDD). The diversity indices of Shannon **(A)** and Simpson **(B)**, and the richness indices of ACE **(C)**, Chao1 **(D)**, and the observed OTUs **(E)** were used to evaluate the overall structure of the fecal microbiota in childhood MDD patients and the healthy controls. The data are presented as mean ± standard deviation. Unpaired *t* tests (two tailed) were used to analyze the variation between the groups. *$p < 0.05$ and $^{\#}p < 0.01$ compared with the control group. Principal coordinate analysis (PCoA) plots of individual fecal microbiota based on Bray–Curtis **(F)**, jaccard **(G)**, and unweighted **(H)** and weighted **(I)** UniFrac distances in childhood MDD patients and the healthy controls. Each symbol represents a sample. The Venn diagram illustrates the overlap of OTUs in childhood MDD-associated microbiota and healthy controls **(J)**.

showed that a total of 2,255 OTUs were shared between the two groups, while 1,148 and 270 OTUs were unique to children with MDD and healthy children, respectively (Figure 1J).

Differences in fecal microbiota composition between children with MDD and healthy controls

Bacterial community composition at different taxonomic levels was compared between patients with MDD and healthy children to identify the drivers of community separation. In total, the sequence reads were classified into 11 phyla, 89 families, and 246 genera in the fecal microbiota of the children using the RDP classifier. Krona radial space-filling charts showed the mean relative abundances of bacterial taxa in children with MDD and healthy children from phylum to genus levels (starting at the inner circle; Figure 2). The charts clearly demonstrated that the fecal microbiota of the children was dominated by the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Figure 3 illustrates the differentially abundant bacteria at different taxonomic levels between the pediatric patients with MDD and healthy children. Specifically, regarding the relative abundance of microbiota at the phylum level, the proportion of Firmicutes, Actinobacteria, Proteobacteria, and Candidatus_Saccharibacteria was significantly higher in child patients with MDD while that of Bacteroidetes was lower ($p < 0.05$, Figure 3A). Interestingly, the ratio of Firmicutes to Bacteroidetes (changes in which can be an indicator of gut dysbiosis) was significantly greater in children with MDD than in healthy children ($p < 0.05$, Supplementary Figure 1). At the family level, the proportions of Lachnospiraceae, Prevotellaceae, Bifidobacteriaceae, Enterobacteriaceae, Streptococcaceae, and Coriobacteriaceae were significantly greater, while those of other families, such as Bacteroidaceae and Porphyromonadaceae, were smaller, in patients with MDD than in the healthy controls ($p < 0.05$, Figure 3B). At the genus level, the proportions of 26 genera, such as *Prevotella*, *Bifidobacterium*, *Escherichia/Shigella*, *Agathobacter*, *Gemmiger*, *Streptococcus*, *Megasphaera*, *Clostridium*_XlVa, and *Collinsella*, were significantly greater in children with MDD than in the controls, while those of 6 other functional genera—*Bacteroides*, *Phocaeicola*, *Faecalibacterium*, *Parabacteroides*, *Flavonifractor*, and *Dysosmobacter*—were markedly lower ($p < 0.05$, Figure 3C). LEfSe analysis showed that many key functional taxa (biomarkers) were different between the children with MDD and the healthy controls at all taxonomic levels (LDA score > 3, $p < 0.05$) (Figure 4). A representative cladogram of the most differentially abundant taxa between the two cohorts, demonstrating the changes in fecal microbiota composition in child patients with MDD, is shown in Figure 4A. At the genus level, *Prevotella* (LDA=4.7, p<0.01), *Escherichia/Shigella* (LDA=4.5, p<0.01), *Bifidobacterium* (LDA=4.5, p<0.01), *Streptococcus* (LDA=4.1, p<0.01), *Gemmiger* (LDA=4.1, p<0.01), *Agathobacter* (LDA=4.1, p<0.01), *Klebsiella* (LDA=3.8, p<0.01), and *Collinsella* (LDA=3.7, p<0.01), among others, were biomarkers for the childhood MDD group, while *Bacteroides* (LDA=4.9, p<0.01), *Faecalibacterium* (LDA=4.4, p<0.01), *Parabacteroides* (LDA=4.2, p<0.01) and *Akkermansia* (LDA=3.4, p<0.01) were biomarkers for the healthy control group (Figure 4B).

A fecal microbiota-based signature could discriminate between children with MDD and healthy controls

To identify biomarkers that made a significant contribution to the prediction performance, we performed a receiver operating

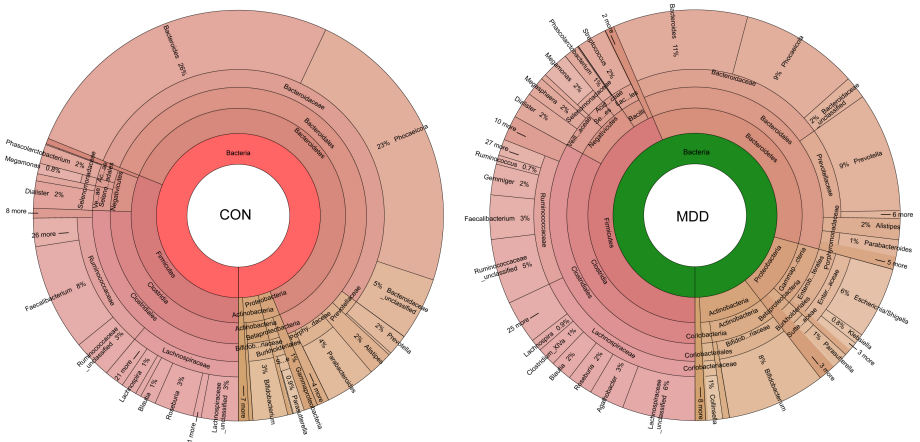

FIGURE 2
Krona charts showing the taxonomic identification and relative abundance of the most abundant bacterial OTUs recorded in childhood MDD patients and healthy controls. These taxa represent the internal core microbiota at the individual level.

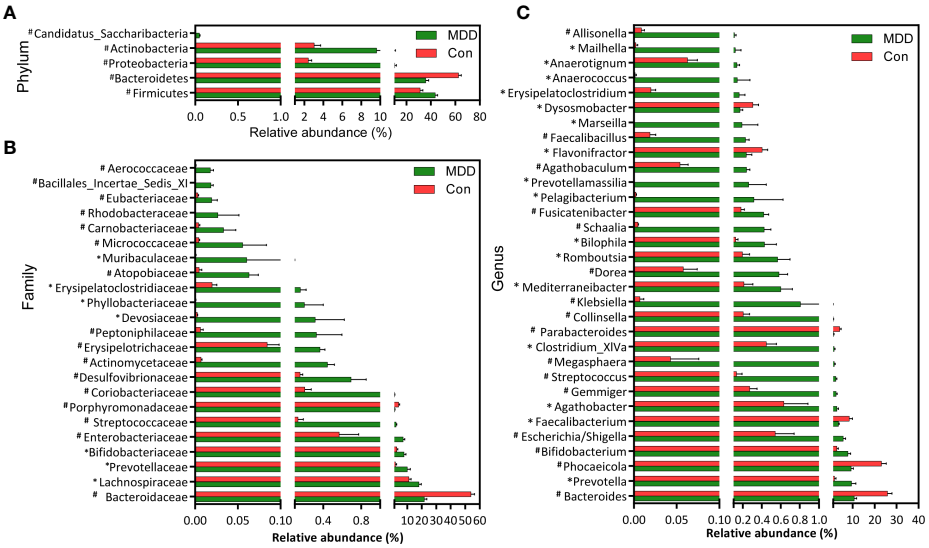

FIGURE 3
Differential bacterial taxa between childhood MDD patients and the healthy controls. Comparisons of the relative abundance of the abundant bacterial taxa at the level of bacterial phylum **(A)**, family **(B)**, and genus **(C)**. The data are presented as the mean ± standard deviation. Mann–Whitney *U*-tests were used to analyze variation between childhood MDD patients and the healthy controls. *$p < 0.05$ and #$p < 0.01$ compared with the control group.

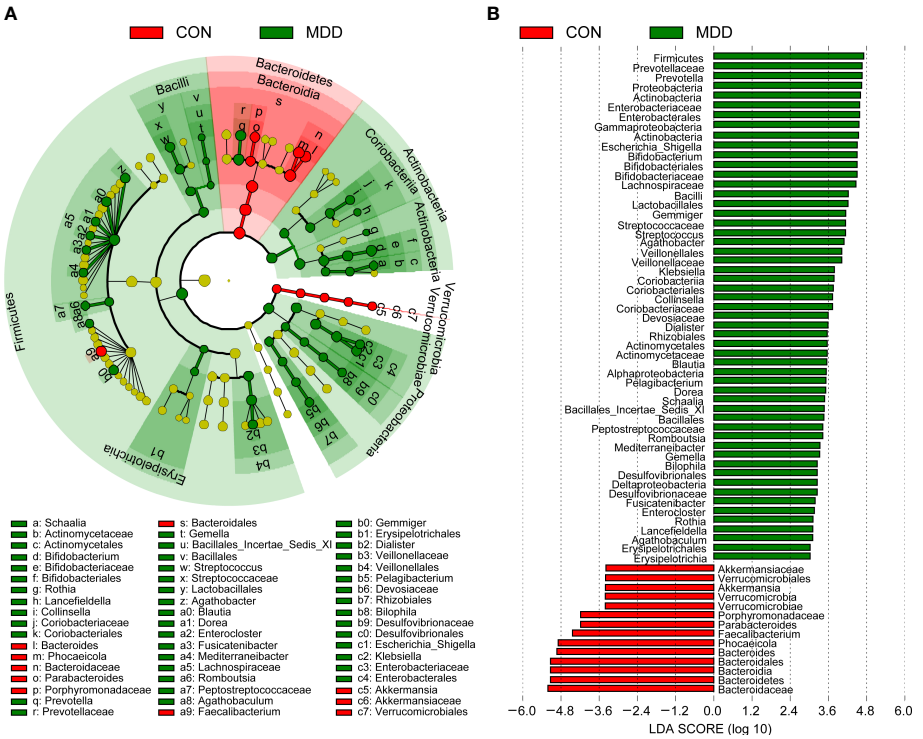

FIGURE 4
Taxonomic differences of the fecal microbiota between childhood MDD patients and the healthy controls. LEfSe identified the features of the fecal microbiota that are discriminative with respect to childhood depression using the LDA model results for the bacterial hierarchy **(A)**, while LDA coupled with effect size measurements identified the most differentially abundant taxa between the two groups **(B)**. Only the taxa meeting a significant LDA threshold value of > 3 are shown.

characteristic (ROC) curve analysis. The results of the LEfSe analysis indicated that several genera could be used as potential biomarkers to discriminate between child patients with MDD and healthy children. Important biomarkers, such as *Bacteroides*, *Phocaeicola*, *Faecalibacterium*, *Escherichia/Shigella*, *Parabacteroides*, *Gemmiger*, *Streptococcus*, *Klebsiella*, *Romboutsia*, and *Dorea*, were assessed for their potential discriminating value. Using only one of the differentially abundant genera as a predictor, we obtained an area under the ROC curve (AUC) ranging from 0.174 to 0.891 (Figure 5A). The results indicated that an increased abundance of *Streptococcus* was the best predictor for MDD in children (AUC: 0.891). We further utilized multiple logistic regression analysis to identify the best combinations of the key functional genera that could distinguish child patients with MDD from healthy controls. We found that combinations of six of the above-mentioned genera—*Bacteroides*, *Streptococcus*, *Faecalibacterium*, *Dorea*, *Romboutsia*, and *Parabacteroides*—yielded improved diagnostic performance, relative to each bacterium alone (AUC: 0.987) (Figure 5B). Meanwhile, *Phocaeicola*, *Escherichia/Shigella*, *Gemmiger*, and *Klebsiella* were excluded from further analysis because combinations with other key functional genera yielded lower AUC values compared with each bacterium alone.

General functional profile of the microbiota associated with MDD in childhood

The function of the microbiota associated with MDD in children was explored using the PiCRUSt algorithm. This algorithm can predict the abundances of functional categories within the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) database based on closed-reference OTU picking, thereby identifying metabolic and functional changes in fecal microbiota. The general functional profile of the childhood MDD-associated fecal microbiota is shown in Figure 6. Among the 64 level-2 KEGG pathways, we identified 11 categories displaying marked differential abundance between children with MDD and healthy children ($p < 0.05$), 3 of which were enriched (membrane transport, signal transduction, and metabolism of other amino acids) and 8 decreased (folding, sorting and degradation, biosynthesis of other secondary metabolites, amino acid metabolism, lipid metabolism, metabolism of cofactors and vitamins, energy metabolism, carbohydrate metabolism, and glycan biosynthesis and metabolism) in the MDD group. At level 3, a total of 42 KEGG pathways were identified as displaying significantly differential activity between the fecal microbiota of the two groups ($p < 0.05$). Specifically, 25 pathways, including fatty acid metabolism, biosynthesis of unsaturated fatty acids, bacterial secretion system, and lysine biosynthesis, showed higher activity in the childhood MDD-associated fecal microbiota, while 17 pathways, such as lipopolysaccharide biosynthesis, secondary bile acid biosynthesis, glycosaminoglycan degradation, and primary bile acid biosynthesis, showed a prominent reduction in activity. Collectively, our findings suggested that the altered functional potential of the bacterial assemblages in the fecal microbiota associated with MDD in childhood, such as increased fatty acid metabolism and decreased bile acid biosynthesis, may play a role in the pathogenesis and progression of the condition.

Correlations between differentially abundant genera and host cytokines levels

As shown in Figure 7, children with MDD exhibited complex changes in cytokine expression levels. Of the 27 cytokines examined, 8 (IL-1β, IL-4, IL-8, IL-17, IP-10, MCP-1,

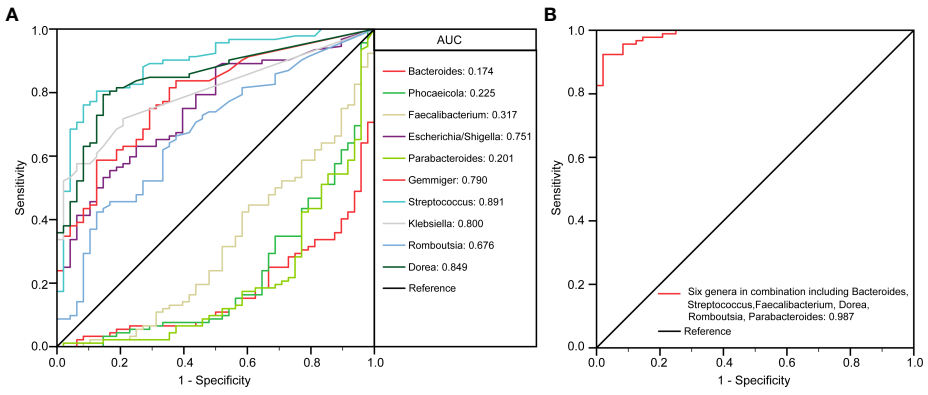

FIGURE 5
The differential genera as childhood MDD diagnostic markers. Receiver operating characteristic (ROC) curves for the differential genera such as *Bacteroides*, *Phocaeicola*, *Faecalibacterium*, *Escherichia/Shigella*, *Parabacteroides*, *Gemmiger*, *Streptococcus*, *Klebsiella*, *Romboutsia*, *Dorea* alone **(A)** or in combination including *Bacteroides*, *Streptococcus*, *Faecalibacterium*, *Dorea*, *Romboutsia* and *Parabacteroides* **(B)** used to discriminate childhood MDD patients from healthy controls. AUC, the area under the receiver operating characteristic curve.

FIGURE 6
PiCRUSt-based examination of the fecal microbiota of childhood MDD patients and the healthy controls. The different bacterial functions were evaluated between them based on two-sided Welch's *t*-test. Comparisons between the groups for each KEGG functional category (levels 2 and 3) are shown by percentage. The Benjamini–Hochberg method was used for multiple testing correction based on the false discovery rate (FDR) through STAMP.

MIP-1α, and TNF-α) were noticeably upregulated in child patients with MDD relative to healthy children while 3 (IFN-γ, MIP-1, and RANTES) were markedly downregulated (all $p < 0.05$). Next, to assess whether there was a reciprocal relationship between altered host immunity and the key functional bacteria in the child patients with MDD, we performed a Pearson's correlation analysis. Heatmaps were created based on Pearson's correlation coefficient (r, Figure 8). Interestingly, in children with MDD, the key functional genus, *Bacteroides*, was negatively associated with the above-mentioned upregulated cytokines and positively correlated with the downregulated cytokines. The genera displaying increased abundance, such as *Prevotella*, *Bifidobacterium*, *Escherichia/Shigella*, *Agathobacter*, *Gemmiger*, *Streptococcus*, were negatively correlated with IFN-γ expression levels, whereas the opposite was observed for the genera with reduced abundance, such as *Phocaeicola* and *Parabacteroides*. In children with MDD, IL-17, a pro-inflammatory cytokine, was positively correlated with the genera displaying increased abundance and negatively correlated with those showing decreased abundance. Importantly, the beneficial butyrate-producing genus, *Faecalibacterium*, was negatively correlated with IL-17 expression. Our results suggested that changes in the cytokine profile of children with MDD were closely correlated with alterations in fecal microbiota abundance, and may be involved in the pathophysiology of MDD in childhood.

Discussion

In our present study, we characterized the structure and composition of the fecal microbiota and host cytokine expression profile in drug-naïve Chinese school-aged children

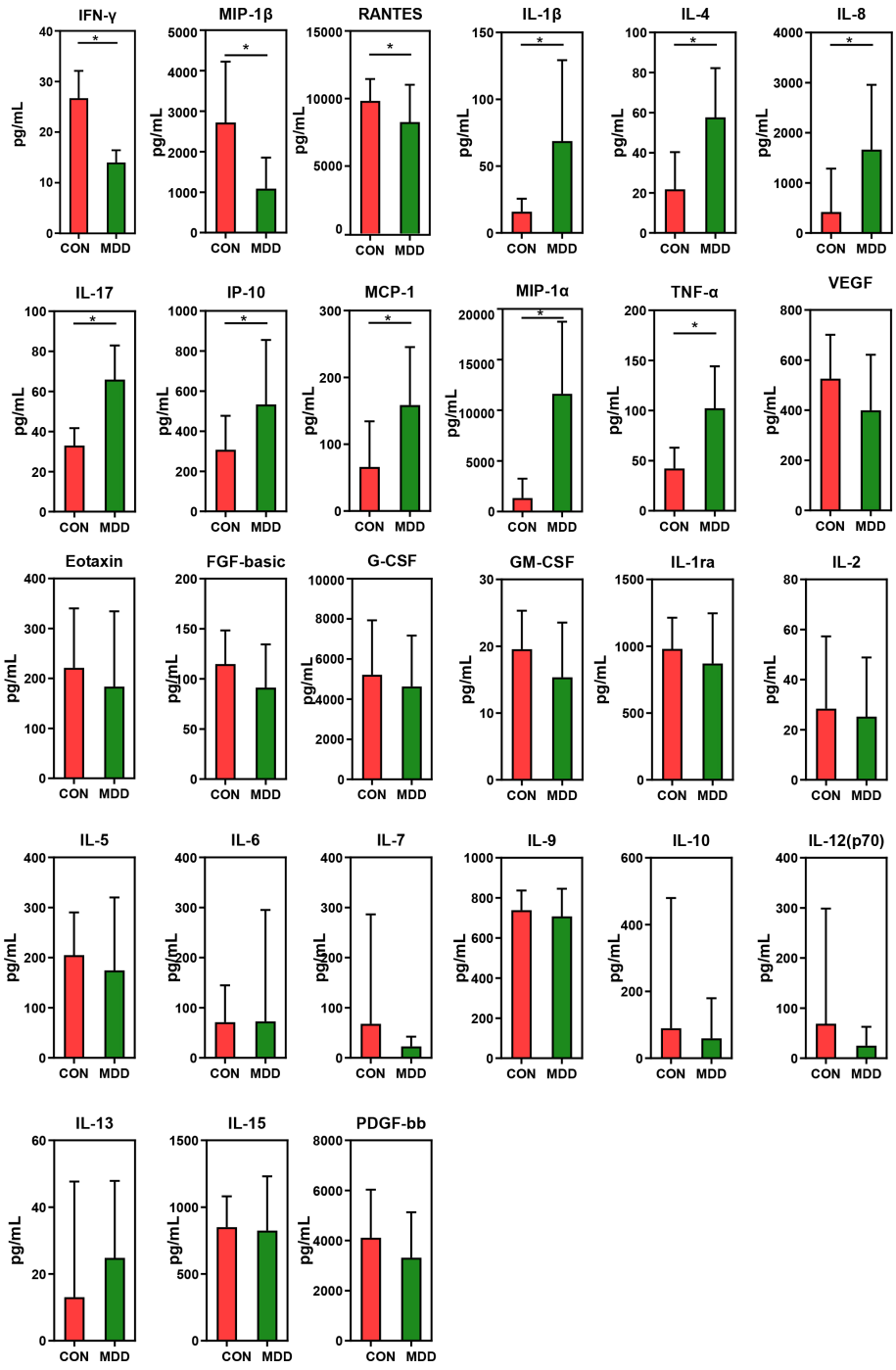

FIGURE 7
Mean (SEM) concentrations (pg/ml) of 27 pro- and anti-inflammatory cytokines and chemokines in childhood MDD patients and in healthy controls determined using Bio-Plex immunoassays. The concentrations of IL-1β, IL-4, IL-8, IL-17, IP-10, MCP-1, MIP-1α and TNF-α increased significantly in childhood MDD patients, while those of IFN-γ, MIP-1β and RANTES decreased significantly. *$p < 0.05$.

with MDD for the first time. In our cohort, we found changed overall structure of the fecal microbiota in children with MDD when compared with the healthy controls, specifically, unaltered bacterial α-diversity, increased richness indices such as ACE, Chao1, and observed OTUs and altered β-diversity. LEfSe identified several pro-inflammatory genera such as *Streptococcus* increased and anti-inflammatory genera such as *Faecalibacterium* decreased in pediatric MDD patients. These key differential

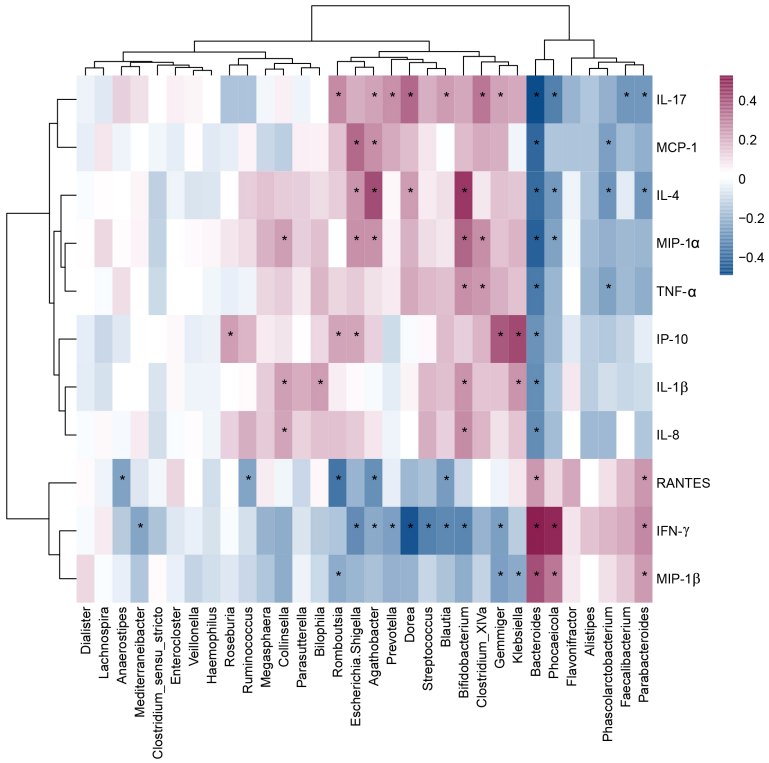

FIGURE 8
Correlation between fecal microbiota, and pro- and anti-inflammatory cytokines and chemokines in childhood depression. The heatmap shown Pearson's correlation coefficients between differential genera and host immunity in childhood MDD patients. Pearson correlation (r) and probability (p) were used to evaluate statistical importance. *p < 0.05.

functional genera, alone or in combination, could serve as novel and powerful non-invasive biomarkers to distinguish between pediatric MDD patients from healthy ones. Meanwhile, altered host cytokine expression profiles, increased levels of pro-inflammatory cytokines such as IL-17 and decreased levels of anti-inflammatory cytokines such as IFN-γ, were observed in pediatric MDD patients. Additionally, the pronounced correlation was detected between the differential cytokine abundance and changes in gut microbiota of children with MDD. These findings demonstrated gut dysbiosis concomitant with the activation of a complex pro-inflammatory response in pediatric MDD patients, which might be play vital roles in the pathogenesis of children depression.

As far as the bacterial diversity was concerned, our finding in children with MDD was inconsistent with previous case-control studies involving adolescent and adult patients with depression. Bacterial α-diversity is often used as a proxy for community stability and function, with increasing α-diversity values indicating a greater number of species, with more even representation, and/or greater biodiversity. In fact, there were no consensus on the changing patterns of the bacterial α-diversity among MDD-related clinical studies (25). In adult MDD patients, higher bacterial α-diversity as indicated by Shannon was observed in our previous study (10), while another two Chinese studies demonstrated lower Shannon index (26, 27). Similar to our present study on pediatric patients with MDD, five Chinese studies involving drug-naive adult patients with MDD from different regions showed that α-diversity indices, such as the Shannon and Simpson indices, did not differ significantly between patients and controls (11, 28–31). A comparative meta-analysis of α-diversity between adult patients with MDD and controls conducted by Sanada et al. also observed unaltered α-diversity in MDD patients (32). This might be associated with differences in the number of OTUs as well as in relative abundances (33). In addition, no significant differences were observed in bacterial α-diversity in studies investigating depression in adolescents (34) or attention-deficit/hyperactivity disorder in children (35). Regarding β-diversity in depression, studies have been relatively consistent in reporting differences in the overall community composition of the gut microbiota between patients and controls. In a systematic review of gut microbiota composition in MDD-related observational studies, differences in gut microbiota composition were reported in 87% of MDD β-diversity analyses (36). Similar patterns were found in several studies on depression in adults. In these latter studies, PCoA could separate patients with MDD and healthy controls into different clusters despite significant

interpersonal variability (11, 26, 30, 37, 38). Jackson et al. reported that β-diversity distances were associated with symptoms of depression (39), while another study found no such association (40). Regarding richness, in our study, the greater number of OTUs was observed in child patients with MDD than in healthy children. These findings were consistent with those of our previous study on adult patients with MDD (10), but contrasted with the observed decrease in the number of species reported for adult patients with MDD in Irish and Chinese studies (26, 37). In Chinese children with autism spectrum disorder, higher microbial richness was also observed when compared with age- and BMI-matched normally developing children (41). Despite the reported inconsistencies, these bacterial diversity indices have been explored as non-invasive diagnostic biomarkers and treatment outcome predictors for depression. The discrepancy in MDD-associated gut microbiota diversity between children and adults might be due to the differences in baseline profiles of the gut microbiota (42–45). The gut microbiota of healthy children displays both functional and taxonomic differences with respect to those of adults and may also be more susceptible to environmental factors (46). Generally, the age-related baseline gut microbiota profile can be strongly influenced by intrinsic factors, the accumulation of environmental and dietary exposures, life-style, and intestinal maturation, among other factors (47). The existence of a strong correlation between gut microbiota diversity and health suggests that gut dysbiosis in childhood may contribute to the occurrence and development of MDD in children.

Although diversity-related findings have been inconsistent among age groups, specific bacterial taxa have been associated with MDD in studies that compared the gut microbiota of patients with that of controls. Overall, the fecal microbiota of children with MDD is characterized by a greater abundance of pro-inflammatory bacteria, such as *Escherichia*/*Shigella*, and a lower abundance of anti-inflammatory bacteria, such as *Bacteroides* and *Faecalibacterium*. *Bacteroides* (the core genus of the phylum Bacteroidetes) is one of the most abundant bacterial genera in the human colon, and members of this genus in the gut microbiota have been associated with health benefits, resistance to pathogens, as well as other host physiologic, metabolic, and immunologic phenotypes (48, 49). In agreement with our previous study on depression in adults, *Bacteroides* abundance was reported to be decreased in children with depression (10, 50); nevertheless, the specific contributions of *Bacteroides* to behavioral changes and the underlying mechanisms remain largely elusive. Interestingly, our data also demonstrated that *Bacteroides* genus was associated with systemic inflammation, suggesting that this genus may participate in modulating the host immunity in childhood depression (51). In fact, reports on the changing pattern of *Bacteroides* abundance and its association with depression have been inconsistent among studies. Overall, increasing evidence supported the beneficial roles of *Bacteroides* in regulating the development of childhood depression. Valles-Colomer et al. found that a lower relative abundance of *Bacteroides* in adults with depression was correlated with lower quality of life and higher prevalence of depression (52). Rhee et al. observed that the *Bacteroides* genus was negatively associated with the total HAMD score in adult patients with MDD (53). Strandwitz et al. demonstrated that the relative abundance of *Bacteroides* in feces was negatively correlated with brain signatures associated with depression (54). In fact, the functions of *Bacteroides* were significantly influenced by which *Bacteroides* species or strain is dominant in the gut (55). One study found that different *Bacteroides* species differentially modulate depression-like behavior *via* metabolism-mediated gut–brain signaling, especially through the tryptophan pathway (56). Specifically, recent studies found that colonization by *B. fragilis*, *B. uniformis*, and *B. caccae*, but not *B. ovatus*, recapitulated the negative effects of "depression microbiota" on behavior and neurogenesis, suggesting differential behavioral impacts of bacteria from the same genus. *B. ovatus*, a human gut commensal bacterium with anti-inflammatory properties and potential as a next-generation probiotic (57, 58), can reportedly influence the abundance of intestinal short-chain fatty acids (SCFAs) and neurotransmitters such as γ-aminobutyric acid (GABA) (59). *Bacteroides* spp. are the major bacterial producers of inhibitory neurotransmitter GABA in the human gut, and the reduced GABA levels have been associated with depressive-like behavior (60). The abundance of *Parabacteroides*, a genus in the phylum Bacteroidetes, was also found to be decreased in the fecal microbiota of children with MDD. *Parabacteroides* has been associated with positive health states, while its reduced abundance has been linked to negative effects on health. Preclinical studies have also found that *Parabacteroides* is negatively correlated with depressive behaviors but positively correlated with neurotransmitter metabolism (61, 62). Its regulatory roles in host metabolism, especially the production of succinate and secondary bile acids, might play a role in MDD in childhood (63).

One beneficial bacterium, *Faecalibacterium* (typical strain *F. prausnitzii*), has been proposed as a major factor in human intestinal health as well as a health biosensor (64). Interestingly, several studies have indicated that *Faecalibacterium* abundance is reduced in both children and adults with depression (32), as well as in several neuropsychiatric disorders such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (16, 17, 65). These observations suggest that *Faecalibacterium* plays a positive role in the modulation of the gut–brain axis. In adults with depression, *Faecalibacterium* abundance has been negatively correlated with the severity of depressive symptoms, as evidenced by HAMD and Montgomery–Asberg Depression Rating Scale (MADRS) scores (10). In the present study, we also identified a negative correlation between *Faecalibacterium* abundance and the levels of the pro-inflammatory cytokine IL-17 in children with MDD. *Faecalibacterium* is an acetate consumer and can produce butyrate and various bioactive anti-inflammatory molecules such

as shikimic and salicylic acids (66). These bioactive metabolites can promote vagus nerve stimulation in the colon, microglia maturation and activation, and the production of brain-derived neurotrophic factor (BDNF), which might be directly involved in the pathogenesis of childhood depression *via* its effect on the gut–brain axis. Romano et al. observed that the butyrate-producing *Faecalibacterium* was consistently associated with improved quality-of-life indicators (65). Given its lower abundance in patients with depression, *Faecalibacterium* may serve as a biomarker for discriminating between individuals with MDD and healthy controls.

Additionally, the abundance of the genera *Prevotella* and *Klebsiella* was also found to be increased in Chinese pediatric patients with autism spectrum disorder and Chinese adult patients with MDD (67–69). Interestingly, Lin et al. reported that increased numbers of *Prevotella* and *Klebsiella* were significantly and positively correlated with the HAMD score (69), while the abundances of both genera were found to be reduced after successful MDD treatment (33, 69, 70). The authors proposed that these findings should be considered in the diagnosis and therapeutic monitoring of MDD in the future. Although members of the genus *Prevotella* are not normally considered to be pathogenic, the role of *Prevotella* in mucosal inflammation and the subsequent dissemination of pro-inflammatory mediators might be involved in the pathogenesis of MDD in childhood. *Klebsiella*, a genus of Gram-negative bacteria, can translocate lipopolysaccharides (LPS), thereby activating pro-inflammatory responses and inducing depressive-like behaviors. This effect may play a role in MDD pathophysiology in children. Interestingly, the abundance of the normally beneficial genus, *Bifidobacterium*, was found to be increased in child patients with MDD, which was in accordance with that reported for adults with this disorder (30, 33, 38, 71). These results may challenge the notion that *Bifidobacterium* always exerts beneficial effects on the host given that the functions of *Bifidobacterium* seem to be species- or strains-specific. Some species or strains of *Bifidobacterium* have been associated with lower scores on depression scales after regular consumption as probiotics (72). However, metagenomic-based studies have found that the relative abundance of two *Bifidobacterium* species—*B. longum* and *B. dentium*—is increased in adult patients with MDD (33, 38). Therefore, it seems that the predominant *Bifidobacterium* species or strain determines the effects (positive or negative) of this genus on depressive behavior. *Escherichia/Shigella* (family Enterobacteriaceae) are thought to be harmless; however, this group has been reported to be overrepresented in both children and adults with depression. In this study, we found that *Escherichia/Shigella* abundance was significantly and positively correlated with systemic inflammation, which may induce depressive symptoms (73). A greater abundance of *Escherichia/Shigella* could lead to the release of greater amounts of LPS into the plasma, resulting in increased blood–brain barrier permeability and chronic and persistent neuroinflammation, finally leading to depression (74). In addition, Chen et al. found that *Escherichia/Shigella* was positively associated with the severity of anxiety (75), while Cattaneo et al. proposed *Escherichia/Shigella* as a candidate pro-inflammatory taxon given that its abundance showed a positive correlation with the blood levels of IL-1β, CXCL2, and NLRP3 (76). As previously mentioned, gut dysbiosis, especially an increase in the numbers of pro-inflammatory bacteria and a decrease in those of anti-inflammatory bacteria, might contribute to the disruption of the intestinal mucosal barrier and the blood–brain barrier, leading to intestinal inflammation and neuroinflammation, a decrease in the concentrations of neurotransmitters and bacterial metabolites (e.g., SCFAs), and, consequently, childhood depression.

The criterium used for the diagnosis of depression in children is the same as that employed for the diagnosis of depression in adults, and is highly dependent on a wide variety of behavioral changes in the patients. In this study, the Firmicutes/Bacteroidetes ratio was significantly increased in pediatric patients with MDD relative to that in the controls, demonstrating that gut microbiota homeostasis was disrupted in the affected children. However, the Firmicutes/Bacteroidetes ratio in healthy gut naturally increases from 1 to 3 with growing age (43, 77), demonstrating the age-related dynamics of human microbiota composition. That's why the increased Firmicutes/Bacteroidetes ratio in pediatric patients with MDD was not suitable for serving as biomarkers for clinical childhood depression diagnosis. With the deeply exploration of the gut–brain axis in children with depression, the novel potential non-invasive diagnostic tools, the aforementioned key functional gut bacteria, could be used to discriminate the pediatric patients with depression and healthy children. As in previous studies (16, 17, 23, 78), we found that several genera, such as *Streptococcus*, *Klebsiella*, *Gemmiger*, and *Escherichia/Shigella*, could serve as diagnostic factors for distinguishing between children with MDD and healthy controls. To improve the diagnostic performance, we employed multiple logistic regression analysis to identify the best combinations of the key functional genera (*Bacteroides*, *Streptococcus*, *Faecalibacterium*, *Dorea*, *Romboutsia*, and *Parabacteroides*) that could be used for the diagnosis of depression in childhood (AUC reached 0.987). This represents high diagnostic efficacy, and indicates the potential suitability of this identification method. In the future, it may be possible to diagnose depression in children based on changes in the abundance of microbial biomarkers. However, the diagnostic and therapeutic potential of microbiota-related biomarkers for childhood depression should still consider microbiota-associated confounders such as diet and hygiene.

This study had several limitations. First, because we only enrolled school-aged children, no direct comparison could be made between the gut microbiota of children and adults with depression. Second, this case-control discovery study only served to identify an association between the gut microbiota and depression in childhood, and no causal effects were explored

(79). Longitudinal follow-up validation studies, microbiota-targeted interventional studies, and mechanistic studies using animal models should be undertaken to verify the causal effects of these key functional bacteria depression in children. Third, although we identified several clues linking gut microbiota-derived metabolites and childhood depression in this study, we did not investigate this association further. Additional metabolomics analysis is needed to provide direct evidence for a link between microbiota-associated metabolites and the development of depression in children.

In summary, school-aged children with depression displayed disrupted gut microbiota homeostasis when compared with healthy controls. The altered overall structure of the gut microbiota was characterized by increased richness index values (ACE, Chao 1, and observed species) and altered β-diversity. LEfSe analysis demonstrated that the abundance of several pro-inflammatory bacteria, such as *Prevotella*, *Bifidobacterium*, and *Escherichia/Shigella*, was increased, whereas that of anti-inflammatory bacteria, such as *Bacteroides* and *Faecalibacterium*, was decreased. The combination of *Bacteroides*, *Streptococcus*, *Faecalibacterium*, *Dorea*, *Romboutsia*, and *Parabacteroides* may serve as novel powerful biomarkers for distinguishing between children with depression and healthy children. We further found that the host cytokine profile was altered in child patients with depression, that is, the levels of pro-inflammatory cytokines were increased while those of anti-inflammatory cytokines were decreased. The close correlation identified between the altered fecal microbiota and systemic inflammation in the host suggested that the gut microbiota might potentially play a role in the pathophysiology of depression in childhood. Our findings provide novel insights into the pathogenesis of depression in school-aged children while key functional bacteria in the gut may serve as novel targets for the non-invasive diagnosis and patient-tailored early precise intervention in children with depression.

Data availability statement

The data presented in the study are deposited in the GenBank Sequence Read Archive repository, accession number PRJNA846994.

Ethics statement

These protocols for the study were approved by the Ethics Committee of Lishui Second People's Hospital (Zhejiang, China) and written informed consent was obtained from their guardian before enrollment. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ZL., LZ and QS conceived and designed the experiments. ZL, XY, FC, XL, YC, LS, GLJ, DZ, GZJ, HL, LZ and QS performed the experiments. ZL, LS and XL analyzed the data. ZL, LS and XL wrote the paper and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This present work was funded by the grants of Key R&D Program of Zhejiang (2022C03060), Zhejiang Basic Public Welfare Research Project (LGF20H090016), the Nutrition and Care of Maternal and Child Research Fund Project of Guangzhou Biostime Institute of Nutrition and Care (2019BINCMCF045), the National Natural Science Foundation of China (81771724, 31700800, 81790631), the Research Project of Jinan Microecological Biomedicine Shandong Laboratory (JNL-2022033C), the Taishan Scholar Foundation of Shandong Province (tsqn202103119), the National S&T Major Project of China (2018YFC2000500), and the Foundation of China's State Key Laboratory for Diagnosis and Treatment of Infectious Diseases.

Acknowledgments

The authors thank all the participants who recruited patients in this study.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.964910/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Comparison of the ratio of Firmicutes/Bacteroidetes between childhood MDD patients and the healthy controls. *p < 0.05.

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 04 October 2022
DOI 10.3389/fimmu.2022.1004439

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

RECEIVED 27 July 2022
ACCEPTED 16 September 2022
PUBLISHED 04 October 2022

CITATION
Omaru N, Watanabe T, Kamata K, Minaga K and Kudo M (2022) Activation of NOD1 and NOD2 in the development of liver injury and cancer.
Front. Immunol. 13:1004439.
doi: 10.3389/fimmu.2022.1004439

Activation of NOD1 and NOD2 in the development of liver injury and cancer

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Hepatocytes and liver-resident antigen-presenting cells are exposed to microbe-associated molecular patterns (MAMPs) and microbial metabolites, which reach the liver from the gut *via* the portal vein. MAMPs induce innate immune responses *via* the activation of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain 1 (NOD1), and NOD2. Such proinflammatory cytokine responses mediated by PRRs likely contribute to the development of chronic liver diseases and hepatocellular carcinoma (HCC), as shown by the fact that activation of TLRs and subsequent production of IL-6 and TNF-α is required for the generation of chronic fibroinflammatory responses and hepatocarcinogenesis. Similar to TLRs, NOD1 and NOD2 recognize MAMPs derived from the intestinal bacteria. The association between the activation of NOD1/NOD2 and chronic liver diseases is poorly understood. Given that NOD1 and NOD2 can regulate proinflammatory cytokine responses mediated by TLRs both positively and negatively, it is likely that sensing of MAMPs by NOD1 and NOD2 affects the development of chronic liver diseases, including HCC. Indeed, recent studies have highlighted the importance of NOD1 and NOD2 activation in chronic liver disorders. Here, we summarize the roles of NOD1 and NOD2 in hepatocarcinogenesis and liver injury.

KEYWORDS

NOD1, NOD2, hepatocellular carcinoma, microbiota, microbe-associated molecular patterns, pattern recognition receptor

Introduction

The liver is exposed to various bacterial components and metabolites derived from the intestinal microbiota *via* the portal vein (1). The anatomical relationship between the liver and gastrointestinal tract creates a unique immunological environment, as the liver needs to maintain immunological tolerance to harmful microbe-associated molecular

patterns (MAMPs) of the intestinal microbiota (1, 2). To fulfill this task, the liver contains various types of antigen-presenting cells (APCs), such as Kupffer cells (KCs), dendritic cells (DCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs) (2). These unique types of APCs preferentially induce tolerance to food antigens and allografts through the production of anti-inflammatory cytokines and attenuation of responses to toll-like receptor (TLR) ligands (2). Liver APCs with immunosuppressive functions induce tolerance to gut-derived food antigens and MAMPs; however, the presence of these APCs predisposes individuals to viral infections, leading to inflammation-associated hepatocarcinogenesis (2).

Chronic fibroinflammatory disorders of the liver are classified into chronic hepatitis and non-alcoholic steatohepatitis (NASH) (3, 4). The unique immunosuppressive properties of the liver predispose this organ to attack by microorganisms, including hepatitis virus and intestinal microbiota (5). Indeed, the gut-liver axis plays a critical role in the development of chronic liver diseases, especially NASH, as evidenced by the fact that MAMPs and microbial metabolites promote proinflammatory cytokine responses in the liver through the activation of pattern recognition receptors (PRRs) (6). Thus, gut microbiota and hepatitis virus invading the liver cause persistent inflammation due to proinflammatory cytokine responses when MAMPs are sensed by PRRs. Such persistent inflammation also sets the stage for the development of hepatocellular carcinoma (HCC) through inflammation-associated carcinogenesis (7). Most cases of HCC arise from persistent inflammation, e.g., as a result of viral hepatitis or NASH (8, 9). TLRs and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are major PRRs that detect MAMPs derived from the intestinal microbiota (10–12). Liver APCs and hepatocytes express functional TLRs and NOD receptors to detect MAMPs and produce proinflammatory mediators (13, 14). NOD1 and NOD2 are intracellular receptors that recognize muropeptides derived from bacterial cell walls (10). Although the roles of TLRs in the progression of liver injury and cancer are being actively investigated, it remains largely unknown whether activation of NOD1 and NOD2 is beneficial or harmful in these diseases. Given that impaired sensing of intestinal bacteria by NOD1 and NOD2 is associated with several human diseases, including Crohn's disease and *Helicobacter pylori* infection, it is likely that the progression of liver injury and hepatocarcinogenesis requires activation of NOD1 and NOD2 (10, 11, 15). In this Review, we summarize the recent studies that examined the involvement of NOD1 and NOD2 in hepatocarcinogenesis and liver injury.

Signaling pathways mediated by NOD1 and NOD2

NOD1 and NOD2 are expressed in innate immune cells, such as macrophages, DCs, KCs, LSECs, and hepatocytes (10, 14, 16, 17). NOD1 and NOD2 are intracellular receptors for muropeptides derived from bacterial cell wall components, such as peptidoglycan (PGN) (10, 16). Tripeptide-A-γ-D-glutamyl meso-diaminopimelic acid (Tripeptide-A-iE-DAP) and muramyl dipeptide (MDP) are the minimal motifs recognized by NOD1 and NOD2. Thus, these molecules are widely used as NOD1 and NOD2 ligands (Figures 1, 2) (10, 16). The main outcome of the stimulation of NOD1 and NOD2 is the activation of transcription factors, including nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3), and IRF7 (10, 15, 16). In addition to nuclear translocation of NF-κB and IRFs, sensing of bacterial components by NOD1 and NOD2 leads to activation of mitogen-activated protein kinases (MAPKs) through TGF-β-activated kinase 1 (TAK1) (10, 15, 16). The activation of NF-κB and IRFs by NOD1 and NOD2 depends upon the molecular interaction between NOD receptors and receptor interacting serine/threonine protein kinase 2 (RIPK2) (Figure 1) (10, 16). NF-κB activation caused by the stimulation of NOD1 and NOD2 results in the release of proinflammatory cytokines and chemokines, such as IL-6, TNF-α, and C-C motif chemokine ligand 2 (CCL2), whereas nuclear translocation of IRF3 and IRF7 leads to the production of type I interferons (IFNs) (10, 15, 16). Thus, activation of NOD1 and NOD2 induced by the recognition of components derived from intestinal bacteria results in proinflammatory and type I IFN responses.

RIPK2 is a downstream signaling molecule activated by NOD1 and NOD2, and its activation is tightly regulated by polyubiquitination (18) (Figure 1). Lys (K)63-linked polyubiquitination of RIPK2 is necessary for NF-κB activation. E3 ligases, including cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, Pellino3, TNF-receptor factor 6 (TRAF6), and X-linked inhibitor of apoptosis protein (XIAP), mediate K63-linked polyubiquitination (18). In addition to K63-linked polyubiquitination, RIPK2 undergoes N-terminal methionine (M1)-linked polyubiquitination mediated by the linear ubiquitination chain assembly complex (LUBAC) when RIPK2 interacts with XIAP (18). K63-and/or Met1-linked polyubiquitination modifications are necessary for the nuclear translocation of NF-κB subunits following RIPK2 activation. Polyubiquitination of RIPK2 activates a downstream signaling cascade involving TAK1 and IκB kinase (IKK) complex composed of IKKα, IKKβ, and IKKγ (10, 18). Negative regulators of NF-κB activation suppress RIPK2 polyubiquitination. For example, IRF4 and autophagy-related 16 like 1 (ATG16L1) activated upon sensing of MDP by NOD2, inhibit K63-linked polyubiquitination of RIPK2, thereby suppressing proinflammatory cytokine responses (19–21). In addition, Myb like, SWIRM and MPN domains 1 (MYSM1) and A20 have been shown to dampen NF-κB activation by RIPK2 through the removal of the K63 and M1-linked polyubiquitin chains (22, 23).

Although NOD2 activation causes activation of NF-κB and type I IFN pathways in a RIPK2-dependent manner, sensing of cytosolic single-stranded RNA by NOD2 induces type I IFN

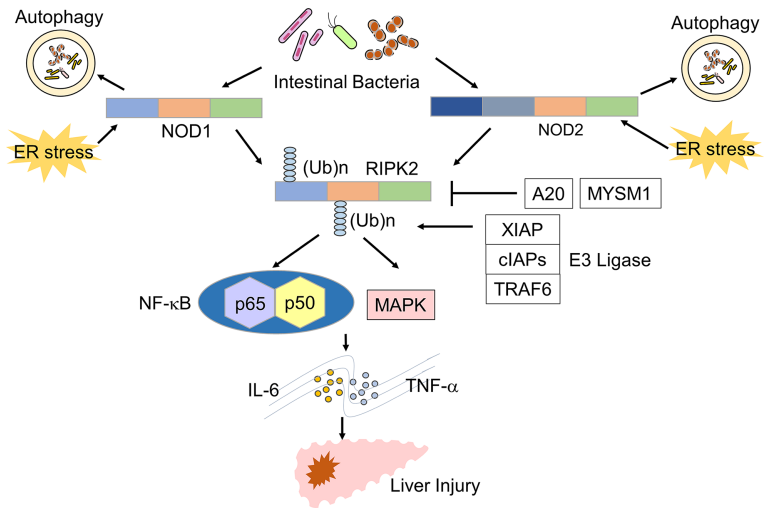

FIGURE 1
Signaling pathways of NOD1 and NOD2 leading to the development of liver injury. Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 detect muropeptides derived from the intestinal bacteria and endoplasmic reticulum (ER) stress. Activation of NOD1 and NOD2 leads to the polyubiquitination of receptor-interacting serine/threonine protein kinase 2 (RIPK2). Polyubiquitination of RIPK2 requires molecular interactions between RIPK2 and E3 ligases, including X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis proteins (cIAPs), and TNF-receptor associated factor 6 (TRAF6). A20 and MYSM1 remove polyubiquitin chains from RIPK2. Activation of RIPK2 induces the production of TNF-α and IL-6 through the nuclear translocation of NF-κB subunits and activation of the mitogen-activated protein kinase (MAPK) pathway and thereby promotes the development of liver injury.

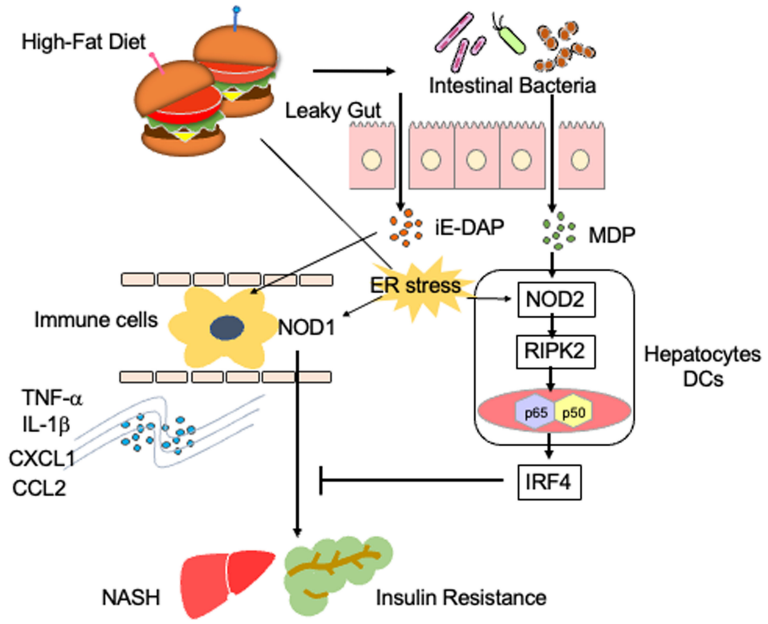

FIGURE 2
Proinflammatory roles played by NOD1 and NOD2 in steatosis. A high-fat diet causes gut leakage and activates NOD1 and NOD2 in gastrointestinal organs. γ-D-glutamyl meso-diaminopimelic acid (iE-DAP) derived from intestinal bacteria activates NOD1 in circulating immune cells. NOD1 activation in circulating immune cells results in the development of NASH and insulin resistance *via* the production of IL-1β and TNF-α. In contrast, muramyl dipeptide (MDP) derived from intestinal bacteria activates NOD2, which is expressed in hepatocytes or dendritic cells (DCs). The activation of NOD2 by MDP induces the expression of IFN regulatory factor 4 (IRF4) and thereby suppresses NOD1-mediated proinflammatory cytokine responses. Endoplasmic reticulum (ER) stress also activates NOD1 and NOD2.

production *via* mitochondrial antiviral signaling protein-dependent and RIPK2-independent mechanisms (24). In addition, sensing of PGN by NOD1 and/or NOD2 leads to the molecular interaction between ATG16L1 and NOD1/NOD2 and thereby induces autophagy without involvement of RIPK2 activation (25).

Activators for NOD1 and NOD2 are not limited to the fragments of bacterial PGN. Recent studies provide evidence that NOD1 and NOD2 function as cytosolic and endoplasmic reticulum (ER) stress sensors (26–29). Endogenous metabolite sphingosine-1-phosphate induces NF-κB activation through direct binding to NOD1 and NOD2 (30). ER-stress inducers, thapsigargin and dithiothreitol, promotes IL-6 production in a NOD1/NOD2-depdendent manner through activation of TRAF2 (29). These new studies support the notion that NOD1 and NOD2 function as sensors not only for microbial components but also for endogenous danger signals to promote and suppress inflammation.

Activation of NOD1 and NOD2 in liver injury

Liver-resident APCs such as DCs, KCs, and LSECs express functional NOD1 and NOD2 (14, 17, 31). NOD2-deficient mice were resistant to the induction of autoimmune hepatitis (AIH) induced by concanavalin A (ConA) compared to the susceptibility of NOD2-intact mice, and this resistance was associated with reduced expression of IFN-γ-in the liver (32). Consistent with this, MDP activation of NOD2 acted synergistically with ConA to induce severe AIH (32). Such synergistic action of MDP and ConA on the development of AIH was accompanied by the expression of IFN-γ and TNF-α (32). Given that hepatocytes and APCs constitutively express NOD2, these results suggest that NOD2 mediates the development of AIH through pro-inflammatory cytokine responses.

Injection of D-galactosamine (D-Gal) in combination with lipopolysaccharide (LPS) is widely used to induce acute liver failure (ALF) (33, 34). Recent studies have highlighted the importance of RIPK2 polyubiquitination in this model. Damagaard et al. reported that pretreatment with MDP increased the severity of ALF induced by D-Gal and LPS through the increase of proinflammatory cytokines such as IL-6 and TNF-α (34). Mice deficient in XIAP, an E3 ligase mediating RIPK2 polyubiquitination, displayed attenuated ALF induced by MDP pre-sensitization in this D-Gal/LPS model, suggesting that polyubiquitination of RIPK2 by XIAP is required for the development of severe ALF (34). Furthermore, *in vitro* studies showed that recruitment of LUBAC to RIPK2 as well as K63-likned polyubiquitination are necessary for the optimal NF-κB activation and subsequent production of IL-6 and TNF-α (34). These results suggest that the NOD2-mediated activation of RIPK2 by K63-linked polyubiquitination and LUBAC recruitment plays a pathogenic role in the development of severe ALF (Figure 1). Conversely, Panda et al. provided evidence that mice deficient in MYSM1, a deubiquitinase of RIPK2, have increased levels of IL-6, TNF-α, and serum liver enzymes upon MDP injection compared with those in mice with intact MYSM1 (23). Thus, MYSM1 attenuates NOD2-mediated liver injury by removing polyubiquitination of RIPK2. These recent studies strongly suggest that activation of NOD2 mediates liver injury through RIPK2 polyubiquitination.

In contrast to the sensitizing action of NOD2, pre-activation of NOD1 by the NOD1 ligand C14-Tri-LAN-Gly markedly inhibited the development of ALF induced by D-Gal/LPS (33). Suppression of ALF by NOD1 activation was associated with enhanced expression of A20 in hepatocytes (33). Given that A20 removes polyubiquitin chains from RIPK2, it is likely that NOD1 suppresses ALF through RIPK2 deubiquitination (22). On the other hand, NOD1 contributed to the development of acute liver injury caused by the exposure to carbon tetrachloride (CCl_4) (35). NOD1-deficent mice were protected from the CCl_4-induced acute liver injury, and this resistance was accompanied by reduced migration of neutrophils into the liver (35). Such discrepancies in the effects of NOD1 on liver pathologies induced by D-Gal/LPS and CCl_4 could be partially explained by differences in the types of immune cells recruited to the liver: the latter model is driven by hepatic infiltration of neutrophils rather than lymphocytes (35).

TLRs are the major PRRs for the detection of MAMPs (12). Myeloid differentiation factor 88 (MyD88) is a downstream signaling molecule for TLRs (12). MyD88-deficient mice were protected from liver damage induced by ConA owing to the downregulation of TNF-α, IL-6, and IFN-γ expression levels (36). Thus, TLR-MyD88 signaling pathways are involved in the development of AIH. We and others showed that NOD2 negatively regulates TLR-mediated proinflammatory cytokine responses (19, 20, 37, 38). As for the molecular mechanisms accounting for the downregulation of TLR-mediated signaling pathways, activation of NOD2 by MDP in DCs leads to the expression of IRF4, which inhibits TLR-mediated signaling pathways by binding to MyD88, TRAF6, and RIPK2 (19, 20, 37, 38). Therefore, it is possible that the development of TLR-dependent liver injury is suppressed by IRF4 induced by the activation of NOD2 with MDP. Indeed, proinflammatory cytokine production induced by the TLR9 ligand CpG in liver plasmacytoid DCs was markedly reduced upon the stimulation with MDP, and accompanied by the induction of IRF4 expression (39). Although no reports have addressed whether NOD2 inhibits the development of liver injury induced by TLRs, the TLR-dependent liver damage may be successfully treated by the activation of NOD2 with MDP.

Activation of NOD1 and NOD2 in steatosis

Activation of NOD1 and NOD2 is involved in the development of metabolic syndromes (26). In animal models, NOD1 has been shown to contribute to the development of insulin resistance and metabolic syndromes caused by the high-fat diet (HFD) (40). Schertzer et al. reported that mice deficient in both NOD1 and NOD2 were protected from hepatic lipid accumulation caused by the HFD (41). Injection of a NOD1 ligand into mice led to adipose tissue inflammation and insulin resistance (41). Moreover, administration of gefitinib, a RIPK2 inhibitor, attenuated metabolic inflammation and insulin resistance caused by NOD1 activation (42). Expression of NOD1 in hematopoietic cells has been highlighted as a molecular mechanism accounting for the development of metabolic inflammation and insulin resistance (Figure 2) (43). Enhanced intestinal leakiness induced by the HFD leads to the accumulation of NOD1 ligands in the serum as a result of increased bacterial translocation (43). NOD1 expressed in circulating hematopoietic cells recognizes NOD1 ligands and induces the production of C-X-C motif chemokine ligand 1 (CXCL1) by macrophages to attract neutrophils into the adipose tissues (43). In line with these findings, HFD-fed mice displayed progressive impairment of insulin signaling, as was evidenced by the impaired activation of AKT in the skeletal muscle (44). Impairment of insulin signaling paralleled the increase in intestinal permeability and accumulation of NOD1 ligands derived from the intestinal bacteria in the serum (44). Thus, NOD1 not only functions as a PRR for intestinal bacterial components but also stimulates the development of insulin resistance and metabolic syndrome, including steatosis.

NOD1-mediated insulin resistance and obesity are negatively regulated by IRF4, which is induced by the activation of NOD2 with MDP (37). Injection of MDP into HFD-fed mice markedly reduced the expression of proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, CXCL1, CXCL9, CXCL10, and CCL2, in white adipose tissue (37). This suppressive effect of MDP on the proinflammatory cytokine and chemokine responses was not seen in mice deficient in IRF4 (37). Thus, NOD1 and NOD2 play, respectively, pathogenic and protective roles in the development of metabolic inflammation (37). In line with this idea, NOD2-deficient mice maintained on the HFD displayed enhanced metabolic inflammation (45, 46). Higher accumulation of T cells and myeloid cells producing IL-6 and TNF-α was observed in the livers of HFD-fed NOD2-deficient mice compared to that in the livers of NOD2-intact mice (45, 46). NOD2 expressed in non-hematopoietic cells, rather than in hematopoietic cells, protects against insulin resistance and metabolic inflammation, because hepatocyte-specific NOD2 deletion resulted in the development of severe steatosis and hepatic fibrosis (47). Indeed, expression levels of the T helper type 1 (Th1) chemokine CXCL9 and profibrogenic cytokine TGF-β1 was enhanced in mice with hepatocyte-specific NOD2 deficiency (47). Furthermore, a bone marrow transplantation experiment revealed that non-hematopoietic expression of RIPK2 is required for the NOD2-mediated protection against insulin resistance and metabolic syndrome (48).

As mentioned earlier, increased metabolic inflammation and insulin resistance are associated with the translocation of gut microbiota into adipose tissue and the liver due to the impaired intestinal barrier. Metabolic inflammation and insulin resistance are driven by the sensing of translocated intestinal microbiota by NOD1, which is downregulated by the activation of NOD2. The ER stress is a major trigger for the development of insulin resistance and obesity (49), and it has been shown to activate NOD1 and NOD2 (26). Therefore, it is possible that insulin resistance and metabolic inflammation can be regulated by the activation of NOD1 and NOD2 through the recognition of intestinal bacteria or *via* the stimulation by the ER stress.

Involvement of NOD1 and NOD2 in hepatocarcinogenesis

Bacterial components and metabolites carried to the liver from the gastrointestinal tract *via* the portal vein include MDP (a NOD2 ligand), iE-DAP (a NOD1 ligand), lipoteichoic acid (LTA, a TLR2 ligand), LPS (a TLR4 ligand), deoxycholic acid (DCA), and short-chain fatty acids (SCFA) (50). Thus, immune responses caused by these microbial components and metabolites are involved in hepatocarcinogenesis (50). Persistent inflammation plays an important role in the development of HCC, as demonstrated by the established notion that hepatitis virus and metabolic syndromes, which cause chronic liver injury, are strong risk factors for hepatocarcinogenesis (7, 9). Chronic liver injury, which leads to compensatory liver regeneration, fibrosis, and cirrhosis, is observed in many cases of HCC (9). A single administration of the carcinogen, diethylnitrosamine (DEN) in combination with repeated injections of CCl_4, has been widely used to create an experimental model of HCC. In this model, repeated liver injuries induced by CCl_4 are exacerbated by DNA damage induced by DEN to mimic inflammation-associated hepatocarcinogenesis. Recent data obtained from the DEN/CCl_4 HCC model supports the view that MAMPs and microbial metabolites entering the liver can be possible triggers of hepatocarcinogenesis.

The pathogenic role of NOD2 in the development of liver injury prompted researchers to examine the involvement of this PRR in hepatocarcinogenesis. Zhou et al. showed that NOD2 promotes hepatocarcinogenesis through proinflammatory cytokines and autophagic responses *via* RIPK2 activation (51).

They found that expression levels of NOD2 and phosphorylated RIPK2 were higher in human HCC tissues than in noncancerous tissues (51). Based on the results of human studies, they also examined whether NOD2 promotes hepatocarcinogenesis in the DEN/CCl_4 model and found that HCC and inflammation were significantly attenuated in mice with hepatocyte-specific NOD2 or RIPK2 knock-out mice (51). Hepatocyte-specific NOD2 or RIPK2 deletion led to decreased activation of two oncogenic transcription factors, signal transducer and activator of transcription 3 (Stat3) and NF-κB, which resulted in diminished expression of proinflammatory cytokines such as IL-6 and TNF-α. Thus, NOD2 activation promotes inflammation-associated hepatocarcinogenesis in a RIPK2-dependent manner.

Obesity and NASH promote the development of HCC (7). Combined treatment with the carcinogen dimethylbenzanthracene (DMBA) and HFD is widely used as an experimental model of NASH-associated HCC (52). DCA is a secondary bile acid synthesized from the primary bile acids by intestinal bacteria. DNA damage may be induced in the liver exposed to DCA (52). Yoshimoto et al. addressed the role of DCA in the development of obesity-associated HCC in this model (52). DCA activates HSCs, which acquire the senescence-associated secretory phenotype (SASP) and produce IL-1β, IL-6, CXCL1, and CXCL9, thereby facilitating the emergence of the tumor microenvironment (52).

Given that NOD2 protects mice from HFD-induced NASH, Gurses et al. investigated whether NOD2-deficient mice are sensitive to NASH-associated liver cancer and showed that upon the treatment with DMBA and consumption of HFD, NOD2-deficient mice gained more weight and bore more HCC tumors than NOD2-intact mice (45). Enhanced activation of Stat3 and infiltration of immune cells were associated with increased hepatocarcinogenesis in NOD2-deficient mice treated with DMBA and HFD (45). Although HSCs with SASP play pivotal roles in obesity-dependent hepatocarcinogenesis (50), the effects of NOD2 activation on HSCs have not yet been explored. In line with the data obtained in obesity-associated HCC, Ma et al. examined the involvement of NOD2 in the model of HCC induced by a combined treatment with DEN and CCl_4 (53). They found that NOD2 acted as a tumor suppressor, as more HCC tumors were seen in the liver of NOD2-deficient mice than in the liver of NOD2-intact mice (53). Expression of NOD2 was significantly decreased in human liver regions affected by HCC compared to that in the non-cancerous tissue (53). In addition, *in vitro* studies in human HCC cell lines revealed that NOD2 is required to enhance sensitivity to sorafenib and lenvatinib through the activation of the adenosine 5′-monophosphate-activated protein kinase (AMPK) pathway (53). As for molecular mechanism, accounting for the NOD2-mediated inhibition of HCC growth, NOD2 induces autophagy-mediated apoptosis of HCC through its interaction with AMPK-α and LKB1. In these models of experimental hepatocarcinogenesis, NOD2 acted not only as a tumor suppressor but also as a chemotherapy enhancer (45, 53).

As mentioned above, data regarding the sensitivity to carcinogen-induced hepatocarcinogenesis in NOD2-deficient mice have been conflicting (51, 53). The reasons why NOD2 has oncogenic activity in the DEN/CCl_4 model remain unknown at present. Differences in cell types expressing NOD2 may explain this discrepancy. In the DEN/CCl_4 model, Zhou et al. observed fewer HCC tumors in mice with hepatocyte-specific NOD2 or RIPK2 deficiency, whereas in mice with NOD2 knockout in both hepatocytes and hematopoietic cells, the number of HCC tumors was increased (51, 53). Therefore, it is possible that NOD2 activation in KCs and DCs protects mice from hepatocarcinogenesis and metabolic syndrome, whereas NOD2 activation in hepatocytes promotes oncogenesis (51). Confirmation of this idea awaits the results of experiments in which mice with NOD2 deficiency specifically in myeloid cells are challenged with DEN/CCl_4. Contrasting data on hepatocarcinogenesis by NOD2 activation may be not surprising. NOD2 activation has been shown to suppress anti-cancer immunity induced by the gut colonization with *Enterococcus hirae* and *Barnesiella intestinihominis* (54, 55). On the other hand, PGN sensing by NOD2 can be deleterious in the intestinal epithelium (54, 55). Given such multifaceted roles by NOD2, it is possible that NOD2 may both positively and negatively regulate the hepatocarcinogenesis.

If activation of NOD2 negatively regulates TLR-mediated chronic inflammation, it is likely that NOD2 attenuates inflammation-associated cancer driven by TLRs (Figure 3). In fact, activation of NOD2 by MDP suppressed colorectal tumorigenesis through IRF4-mediated inhibition of TLR signaling pathways (38). This scenario might also apply to the development of HCC. Recognition of the intestinal microbiota by TLR4 is required to trigger the development of HCC in the DEN/CCl_4 model (56, 57). In another inflammation-associated HCC model, the occurrence of NASH-associated HCC was markedly decreased in TLR2-deficinet mice (50). Thus, activation of TLRs is an indispensable step in hepatocarcinogenesis. TLR-mediated activation of NF-κB and production of proinflammatory cytokines were markedly suppressed in the colonic mucosa of experimental murine colitis upon the activation of NOD2 by MDP (19, 20), which may also protect mice from hepatocarcinogenesis induced by NASH or treatment with DEN/CCl_4. However, to the best of our knowledge, the mechanisms suppressing hepatocarcinogenesis have not been examined with respect to the crosstalk between NOD2 and TLRs.

Although several reports have addressed the role of NOD2 in experimental models of HCC, no study has examined the role of NOD1. However, the effects of NOD1 on cell survival and proliferation have been tested *in vitro* (58). Expression of NOD1 was found to be significantly lower in the HCC tissue than in the non-cancerous parts of the liver (58). NOD1 activation suppressed HCC proliferation through the

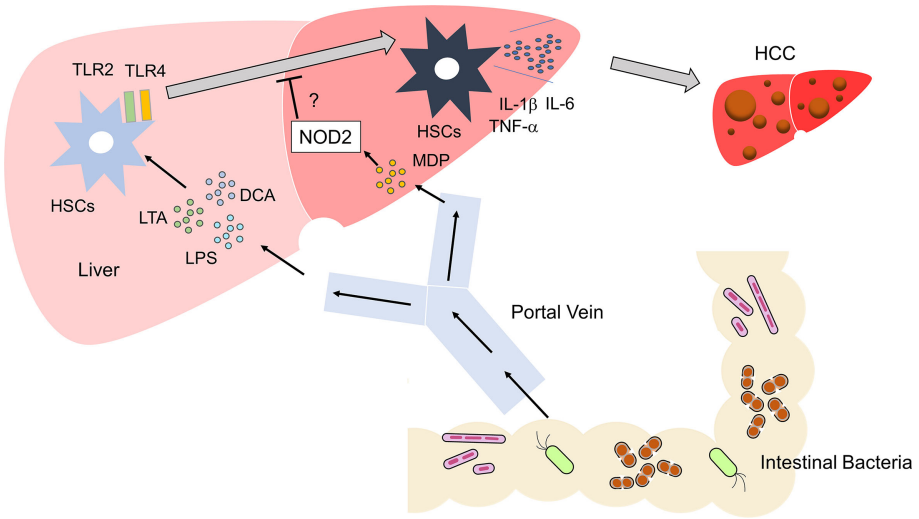

FIGURE 3
Activation of TLRs and NOD2 in the development of liver cancer Microbe-associated molecular patterns (MAMPs), including lipopolysaccharide (LPS) and lipoteichoic acid (LTA), enter the liver *via* the portal vein. These MAMPs activate TLR2 and TLR4 in hepatic satellite cells (HSCs). Metabolites such as deoxycholic acid (DCA) act together with TLR2 and TLR4 ligands to induce differentiation of HSCs with a senescence-associated secretory phenotype (SASP). HSCs with SASP produce large amounts of IL-1β, IL-6, and TNF-α. Differentiation of regular HSCs into those with SASP promotes the development of HCC. Activation of NOD2 by MDP might inhibit the development of HCC through its negative regulation of TLR2 and TLR4.

inhibition of SRC and induction of cell cycle arrest at the G1 phase (58). In addition, overexpression of NOD1 in HepG2 and Huh7 cells resulted in higher sensitivity to sorafenib (58). Despite *in vitro* data alone, these data suggest that NOD1 can suppress the growth of HCC *via* the downregulation of SRC activity and cell cycle progression.

Conclusion

The activation of NOD1 and NOD2 is involved in the development of liver injury and hepatocarcinogenesis. Conflicting data have been reported: NOD2 activation is required for liver injury, whereas NOD1 activation plays both protective and pathogenic roles in the development of hepatitis. Similarly, administration of NOD1 and NOD2 ligands exacerbated and improved steatosis, respectively. The NOD2 signaling pathways are both beneficial and pathogenic in hepatocarcinogenesis. Further elucidation of the molecular mechanisms by which NOD1 and NOD2 activation regulate the development of liver injury and cancer is required for the application of NOD1 and NOD2 ligands as treatments of human diseases. Immune checkpoint inhibitors (ICIs) targeting programmed death-1 (PD-1) or cytotoxic T lymphocyte antigen-4 (CTLA-4) are widely used to treat advanced solid cancers, including HCC (9). However, the efficacy of ICIs alone for HCC is 20%, as determined by the response rate (9). Therefore, the restoration of anti-cancer Th1 immunity by ICIs alone is not sufficient. A novel combination immunotherapy consisting of ICIs and compounds that enhance T cell immunity needs to be established. In this regard, ligands for NOD1 or NOD2 can be promising candidates, because activation of NOD1 and NOD2 efficiently induces Th1 responses (15, 59). In addition to conventional PGN sensors, recent studies highlight roles played by the NOD1 and NOD2 in the maintenance of ER homeostasis. Molecular mechanisms accounting for the development of liver injury and HCC through regulation of autophagy and ER stress by NOD1 and NOD2 need to be addressed in the future studies. In conclusion, the elucidation of the association between NOD1/NOD2-mediated signaling pathways and liver diseases opens new avenues for the development of novel treatments for hepatitis, steatosis, and HCC.

Author contributions

NO and TW drafted the manuscript and prepared figures. MK and KK reviewed the manuscript for intellectual content. KM, TW, and NO were responsible for the revision of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Grants-in-Aid for Scientific Research (22K07996, 22K08090, 21K15987) from the Japan Society for the Promotion of Science, Takeda Science Foundation, Yakult Bio-Science Foundation, SENSHIN Medical Research Foundation, Smoking Research Foundation and a 2022 Kindai University Research Enhancement Grant (KD2208).

Acknowledgments

We thank Ms. Yukiko Ueno for her secretarial assistance.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 01 November 2022
DOI 10.3389/fimmu.2022.1001727

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 23 July 2022
ACCEPTED 10 October 2022
PUBLISHED 01 November 2022

CITATION
Johnson SD, Knight LA, Kumar N, Olwenyi OA, Thurman M, Mehra S, Mohan M and Byrareddy SN (2022) Early treatment with anti-$\alpha_4\beta_7$ antibody facilitates increased gut macrophage maturity in SIV-infected rhesus macaques. *Front. Immunol.* 13:1001727. doi: 10.3389/fimmu.2022.1001727

Early treatment with anti-$\alpha_4\beta_7$ antibody facilitates increased gut macrophage maturity in SIV-infected rhesus macaques

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Despite advances in combination antiretroviral therapy (cART), people living with HIV (PLWH) continue to experience gastrointestinal dysfunction. Infusions of anti-$\alpha_4\beta_7$ monoclonal antibodies (mAbs) have been proposed to increase virologic control during simian immunodeficiency virus (SIV) infection in macaques with mixed results. Recent evidences suggested that therapeutic efficacy of vedolizumab (a humanized anti-$\alpha_4\beta_7$ mAb), during inflammatory bowel diseases depends on microbiome composition, myeloid cell differentiation, and macrophage phenotype. We tested this hypothesis in SIV-infected, anti-$\alpha_4\beta_7$ mAb-treated macaques and provide flow cytometric and microscopic evidence that anti-$\alpha_4\beta_7$ administered to SIV-infected macaques increases the maturity of macrophage phenotypes typically lost in the small intestines during SIV disease progression. Further, this increase in mature macrophage phenotype was associated with tissue viral loads. These phenotypes were also associated with dysbiosis markers in the gut previously identified as predictors of HIV replication and immune activation in PLWH. These findings provide a novel model of anti-$\alpha_4\beta_7$ efficacy offering new avenues for targeting pathogenic mucosal immune response during HIV/SIV infection.

KEYWORDS

simian immunodeficiency virus (SIV), $\alpha_4\beta_7$ integrin, microbiome, butyrate, myeloid cells, macrophage maturation, mucosal immunology, viral reservoir

Introduction

Human immunodeficiency virus (HIV) infections are associated with significant disruption to gut integrity, including microbial dysbiosis, barrier dysfunction, and resultant chronic immune activation (1, 2). These gut pathologies are similar to the symptomology of inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC), during which abdominal pain, diarrhea, constipation, fatigue, and mood disturbances are commonly reported (3, 4). Currently, vedolizumab, a humanized anti-$\alpha_4\beta_7$ integrin monoclonal antibody (mAb) is effectively used to ameliorate IBD symptoms and reduce associated damage by blocking gut homing of inflammatory cells by antagonizing $\alpha_4\beta_7$ integrin binding to its cognate ligand mucosal addressin cell adhesion molecule 1 (MAdCAM-1) on intestinal endothelium (5). Additionally, differences in $\alpha_4\beta_7$ integrin surface expression on circulating immune cells have been demonstrated between disease-resistant natural primate hosts of the simian immunodeficiency virus (SIV) that do not progress to AIDS-like disease and non-natural hosts that do progress, often to fatal disease, with the latter having significantly higher mean $\alpha_4\beta_7$ integrin surface expression (6).

Recently, a primatized anti-$\alpha_4\beta_7$ mAb reagent was developed that could be repeatedly administered *in vivo* to block the higher levels of $\alpha_4\beta_7$ integrin expression in disease-progressing rhesus macaques (RMs) (7). Furthermore, anti-$\alpha_4\beta_7$ mAb was shown to reduce gut viral loads following SIV infection (8, 9). Similarly, anti-$\alpha_4\beta_7$ therapy was found to reduce gut lymphoid aggregates that could serve as potential HIV-1 reservoirs (10). Despite this reduction, other studies have reported conflicting results suggesting that anti-$\alpha_4\beta_7$ therapy does not reduce peripheral viral loads/viral reservoirs in SIV-infected rhesus macaques (11–13). Intriguingly, in a clinical trial involving the administration of anti-$\alpha_4\beta_7$ mAb to HIV-infected individuals revealed that five out of eighteen participants controlled viremia to less than 1,000 copies per mL for the 26 week duration of analytical antiretroviral therapy interruption highlighting the potential utility of anti-$\alpha_4\beta_7$ therapy towards HIV control (14). Likewise, recent findings noted that anti-HIV broadly neutralizing antibodies-treated RMs that were given anti-$\alpha_4\beta_7$ therapy delayed viral rebound compared to those treated with broadly neutralizing antibodies alone (15). Because some studies have demonstrated benefits, understanding why certain individuals/macaques fail or succeed in controlling viremia following treatment interruption could provide crucial insights into future designs of HIV/SIV cure studies.

Although vedolizumab was initially developed to reduce pro-inflammatory lymphocyte trafficking to the gut, thereby reducing local tissue damage, several studies have questioned this proposed mechanism (16). Compared to infliximab (anti-TNF-α mAb), vedolizumab is not associated with changes to total, CD4+, CD8+, central memory, or effector memory lamina propria T cells in rectal biopsies of CD and UC patients at week 14, following three mAb infusions (17). Even though infliximab was associated with decreased T-cell recruitment to the gut, it was less efficacious than vedolizumab in measures of both clinical response and remission (17). However, vedolizumab was associated with a decrease in pro-inflammatory M1 and an increase in anti-inflammatory M2 macrophages. These changes were predictive of remission (17). Interestingly, macrophage turnover in the intestines is predictive of accelerated disease progression in SIV-infected macaques (18, 19). Whether the similarities between IBD and HIV/SIV pathogenesis include macrophage recruitment remains poorly characterized.

In addition to the emerging understanding of myeloid cells as potential mediators of IBD, new studies involving the role of the microbiome are providing further insights into IBD pathogenesis. Individuals with IBDs tend to have a decrease in markers of overall microbiome richness, including α-diversity measuring total operational taxonomic units (OTUs) and Shannon diversity index, which incorporates weighting species abundance evenness (20, 21). Additionally, IBD is associated with an increase in the ratio of Bacteroidetes: Firmicutes (B: F), including reductions in Firmicute butyrate-producing bacteria (BPB) like *Roseburia* and *Faecelibacterium* (22–24). Similarly, HIV infection is also associated with dysbiosis with an expansion of the Bacteroidetes genus *Prevotella* and a decrease in BPB (25, 26). The relative abundance of BPB in people living with HIV has been inversely correlated with markers of microbial translocation, immune activation, and vascular inflammation (25). These findings raise the question of whether microbiome composition could further modulate anti-$\alpha_4\beta_7$ efficacy in SIV infection, possibly contributing to divergent outcomes during therapy.

We characterized gut macrophage maturation dynamics in the duodenum and ascending colon of SIV-infected RMs utilizing both flow cytometry and microscopy techniques and determined tissue viral loads in each compartment prior to and following anti-$\alpha_4\beta_7$ administration. Additionally, we performed 16S rRNA gene sequencing to determine if microbiome composition impacted these immune dynamics. All macaques were infected with SIVmac251 and CD8-depleted to accelerate pathogenesis as part of a more extensive separate study. As expected, without $CD8^+$ cells, the RMs did not control viral replication, reinforcing recent reports that when CD8+ cells are depleted, anti-$\alpha_4\beta_7$ therapy fails at maintaining viral control during SIV-nef-stop infection (27). However, despite these changes, significant decreases in myeloid cell turnover were seen in the small intestine in the anti-$\alpha_4\beta_7$ group compared to IgG-treated controls. These changes were associated with alterations in tissue viral loads and markers of dysbiosis in the fecal microbiome, suggesting that myeloid cell dynamics and microbiome composition may be crucial to the efficacy of anti-

$\alpha_4\beta_7$ mAb treatment during SIV infection. These findings provide a new variable for differential experimental outcomes that has until now remained undefined.

Materials and methods

Animals and ethics statement

A total of nine outbred Indian-origin rhesus macaques (*Macaca mulatta*) (RMs) with a mean age of 6.1 years old (5.1 to 10.0) were procured from the Yerkes National Primate Center of Emory University, Atlanta, Georgia, the USA and the New Iberia Research Center of University of Louisiana, Lafayette, Louisiana, USA and used in this study as summarized in Table S1. All RMs were housed at the Department of Comparative Medicine, University of Nebraska Medical Center (UNMC), Omaha, Nebraska, the USA, in compliance with regulations outlined in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. Animals were pair-housed with visual access to other monkeys in a temperature-controlled (72°F) indoor climate with a 12-hour light/dark cycle. Animals were fed a monkey diet (Purina) twice daily, supplemented with fresh fruits and water being available *ad libitum*. Animals were anesthetized (ketamine 10 mg/kg or telazol 4 mg/kg) prior to all procedures, and meloxicam (0.2 mg/kg) was administered when appropriate. At the end of the study, euthanasia was performed following the guidelines of the American Veterinary Medical Association by a high dose of ketamine-xylazine followed by exsanguination and cardiac perfusion. This study was approved by the UNMC Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) under protocol #15-102-12-FC entitled "Gut Trafficking Cells in SIV Infection".

Study design

Nine RMs were randomly divided into two groups: four in the control group were administered control IgG and five in the experimental group treated with a primatized monoclonal anti-$\alpha_4\beta_7$ IgG. All monkeys were depleted of CD8+ cells by the administration of a rhesus recombinant IgG1 anti-CD8α mAb (MT807R1) Lot: 100LBRX-3206-023-001 (NIH Nonhuman Primate Reagent Resource) four times spanning several days before and after infection (Day -4: 10 mg/kg subcutaneously and on days -1, 3, and 6 administered 5 mg/kg intravenously). On Day 0, all macaques were intravenously inoculated with 1000 $TCID_{50}$ SIVmac251 (Source: Simian Vaccine Evaluation Unit, NIAID, NIH). During acute infection on Day 12, daily intramuscular administration of a combination of anti-retroviral therapy (cART) (20 mg/kg TFV + 40 mg/kg FTC + 2.5 mg/kg DTG) was initiated and continued until Week 14. On day 15, while the controls were administered 50 mg/kg rhesus IgG1 control antibody rhesus IgG1 mAb (DSPR1) Lot: LH15-35 (NIH Nonhuman Primate Reagent Resource), the experimental group monkeys were administered 50 mg/kg rhesus recombinant IgG1 anti-$\alpha_4\beta_7$ mAb (A4B7, Lot: LH17-14-NIH Nonhuman Primate Reagent Resource). Antibody infusions were continued every three weeks until week 23 for a total of eight infusions. Blood was collected from the saphenous vein using K2-EDTA vacutainer tubes (Becton, Dickinson, San Diego, CA, USA) for monitoring plasma viral loads. Feces were collected using fecal loops at baseline and on days 14, 98, and 161 and were snap frozen for future analysis. At necropsy, gut tissues were collected, a portion snap frozen, a portion fixed in 10% formalin, and additional tissue used for immune cell isolation (below) for flow cytometry. An illustration of the study design is available in Figure S1.

Intestinal cell isolation

At necropsy, duodenum and ascending colon were collected, washed with DPBS, and dissected into two compartments: 1) epithelium and lamina propria and 2) muscularis externa and serosa. Tissue-specific immune cells were isolated by mincing, digestion with 10,000 U Collagenase, Type 4 (Worthington Biochemical Corporation, Lakewood, NJ, USA; Product #LS004188) and 25 U DNAse I (Roche Diagnostics, Mannheim, Germany; Product #LS004188) in 10 mL DPBS for two hours, filtration through 100 µm and 40 µm sterile cell strainers (Fisherbrand, Hampton, NH, USA; Product #22-363-549 and 22-363-547), and density gradient centrifugation with 60% and 30% Percoll (Cytiva, Uppsala, Sweden; Product #17089101) layers at 2000 rpm for 30 minutes. Isolated cells were washed with DPBS and resuspended in RPMI.

Flow cytometry

Isolated gut cells were sequentially incubated with Zombie Aqua Fixable Viability Dye (BioLegend, San Diego, CA, USA; Product #423102) to discriminate live/dead cells and incubated with Fc blocker to minimize non-specific binding and then stained with a panel of fluorescent-dye conjugated antibodies (Table S2), and then fixed with 1% PFA. Both compensation and fluorescent minus one (FMO) controls were utilized to assist in gating placement. Event acquisition was performed using a Fortessa x450 flow cytometer (B-D, Mountain View, CA). Analysis was performed with FlowJo 10.6.1 software (Treeland, OR). To determine macrophage maturity, a gating strategy developed by Bujko, et al. was utilized (see Figure S2) (28).

Viral loads

Viral loads were determined as has been described previously (29). In brief, plasma was separated from whole

blood by centrifugation at 1200 rpm for 20 minutes, and RNA was isolated using a QIAamp viral RNA Mini Kit (Qiagen, Germantown, MD, USA; Product #52906). Frozen gut tissue was lysed using a TissueLyser LT (Qiagen, Germantown, MD, USA; Product #69980), and then DNA and RNA were isolated using an AllPrep DNA/RNA Mini Kit (Qiagen, Germantown, MD, USA; Product #80204) according to manufacturer instructions. Copies of SIVgag DNA were normalized to cell number by quantifying genomic RPP30 as previously described to determine copies/cell (30).

16S rRNA sequencing

Fecal samples were later thawed and approximately 100-200 mg of fecal sample was used to isolate DNA using spin column chromatography-based Stool DNA Isolation Kit (Norgen Biotek Corp; Product #27,600) following manufacturer recommendations. DNA was quantified with a GE SimpliNano spectrophotometer and shipped on dry ice to LC Sciences, LLC (Houston, TX, USA) for 16S rRNA sequencing. A library was generated by amplifying the V3 and V4 16S rRNA variable regions and adding sequencing adapters and barcodes after the first cycle. An Illumina cBot system was used to generate clusters for sequencing with an Illumina MiSeq platform. Barcodes were used to separate data, and an in-house script was used to annotate taxa according to RDP, Greengenes, and NCBI 16SMicrobial customized databases for reference. Data output statistics, including clustering into operational taxonomic units (OTU), diversity analysis, species classification, and abundance analysis were performed by LC Sciences, LLC (Houston, TX). FASTQ files were deposited in the NCBI Sequence Read Archive with the BioProject accession number PRJNA870961.

Immunofluorescence microscopy

Formalin-fixed gut tissues collected at necropsy were embedded in paraffin blocks, cut into 5 µm sections using a microtome (Leica Biosystems, Deer Park, IL, USA; Product #RM2235), and placed on positively charged slides (Avantik, Pine Brook, NJ, USA; Product #SL6332). Tissue sections were deparaffinized with xylene and rehydrated with graded ethanol to water. Antigen retrieval was performed by Decloaking Chamber™ NxGen (Biocare Medical, LLC, Pacheco, CA, USA; Product #DC2012). Tissues were washed three times with water, and a blocking buffer (5% normal goat serum + 1% BSA in PBS) was applied for one hour at room temperature to prevent non-specific binding. Sections were incubated overnight with anti-CD163 and anti-CD206 antibodies (Table S3) at 4°C. Tissues were washed with PBS and incubated for one hour with fluorescent secondary antibodies (Table S3) at room temperature. Tissues were finally washed five times with PBS, Prolong™ Gold antifade reagent with DAPI (Invitrogen Waltham, MA, USA; Product #P36935) was added, and coverslips were applied. 20X images were captured using a LIONHEART LX Automated Microscope (BioTek, Santa Clara, CA) using Gen5 3.05 software. Co-localization was performed using the JAKoP plug-in for Image J 1.53e (31).

Statistical analysis

Mann-Whitney tests were implemented when comparing data obtained on the anti-$\alpha_4\beta_7$ with those obtained on the control groups. Multiple t-tests were utilized when comparing myeloid cell phenotypes. Two-way ANOVA, linear regression, and ratio paired T-tests were performed as specified. All statistical analysis was performed with GraphPad Prism 7 for Mac OS X. Statistical significance was determined as $P \leq 0.05$.

Results

Anti-$\alpha_4\beta_7$ therapy is associated with increased macrophage maturity in the duodenum

Contrary to early reports, more recent data has increasingly suggested that the mechanism of action for anti-$\alpha_4\beta_7$ mAbs is more complex than blocking inflammatory lymphocyte infiltration (16). These studies have indicated that myeloid cells, particularly macrophages and DCs, may contribute to the efficacy of vedolizumab (17, 32, 33). To characterize changes in gut myeloid cell differentiation at necropsy, immune cells were isolated from the lamina propria and muscularis externa of the duodenum and ascending colon. The isolated cells were subjected to flow cytometry and a gating strategy was utilized based on a previously described method developed by Bujko, et al., (Figure S2) which was focused on the characterization of gut myeloid cells across developmental stages from monocyte-like macrophages (Mf1), intermediate macrophages (Mf2), to mature (Mf3) macrophages (28). Additionally, this strategy identifies muscularis macrophages (Mf4), determining their frequency as a percent of total macrophages.

Compared to the mean frequency of 63.0% Mf1 macrophages in the duodenum lamina propria of the control group, as seen in Figure 1A, the anti-$\alpha_4\beta_7$ treated group showed a significant reduction (mean 14.6%) (P=0.01). Similarly, there was a trend in reduction of Mf2s in the same tissues with mean values of 16.1% in the control and 3.8% in the anti-$\alpha_4\beta_7$ mAb treated group (NS) (Figure 1A). In contrast, anti-$\alpha_4\beta_7$-treated RMs had higher mean frequencies of the Mf3 macrophages (63.9%) than 20.4% in the control group (P=0.02). Similarly, there was a trend in increased Mf4s with anti-$\alpha_4\beta_7$-treatment (17.7% compared to 0.6%, NS), a minority population in the

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duodenum lamina propria. Similarly, although statistically non-significant, there was a trend in reduced Mf2s and increased Mf3s in the duodenum muscularis externa in the control group as compared to the anti-$\alpha_4\beta_7$-treatment group.

As mentioned, Bujko, et al. developed a gating strategy for analyzing cells isolated from the small intestine (28). We applied this strategy to cells isolated from the ascending colon (28). No significant differences were found in any of the macrophage subtypes in either the lamina propria or muscularis externa Figure 1B. Despite this, some trends existed between the small and large intestinal macrophage subtypes. In all tissue samples, intermediate Mf2s were lower with anti-$\alpha_4\beta_7$ treatment and, when combined, were significantly different with a mean of 6.5% compared to 26.2% with values from the IgG treatment group (P=0.02). Conversely, in most samples, Mf4s were higher with anti-$\alpha_4\beta_7$ treatment. Combined, the anti-$\alpha_4\beta_7$-treatment group had a mean of 15.1% Mf4s compared to 5.8% in the control group (P=0.03). As expected, there was a trend in higher Mf1s and Mf3s in the lamina propria and Mf4s in the muscularis externa.

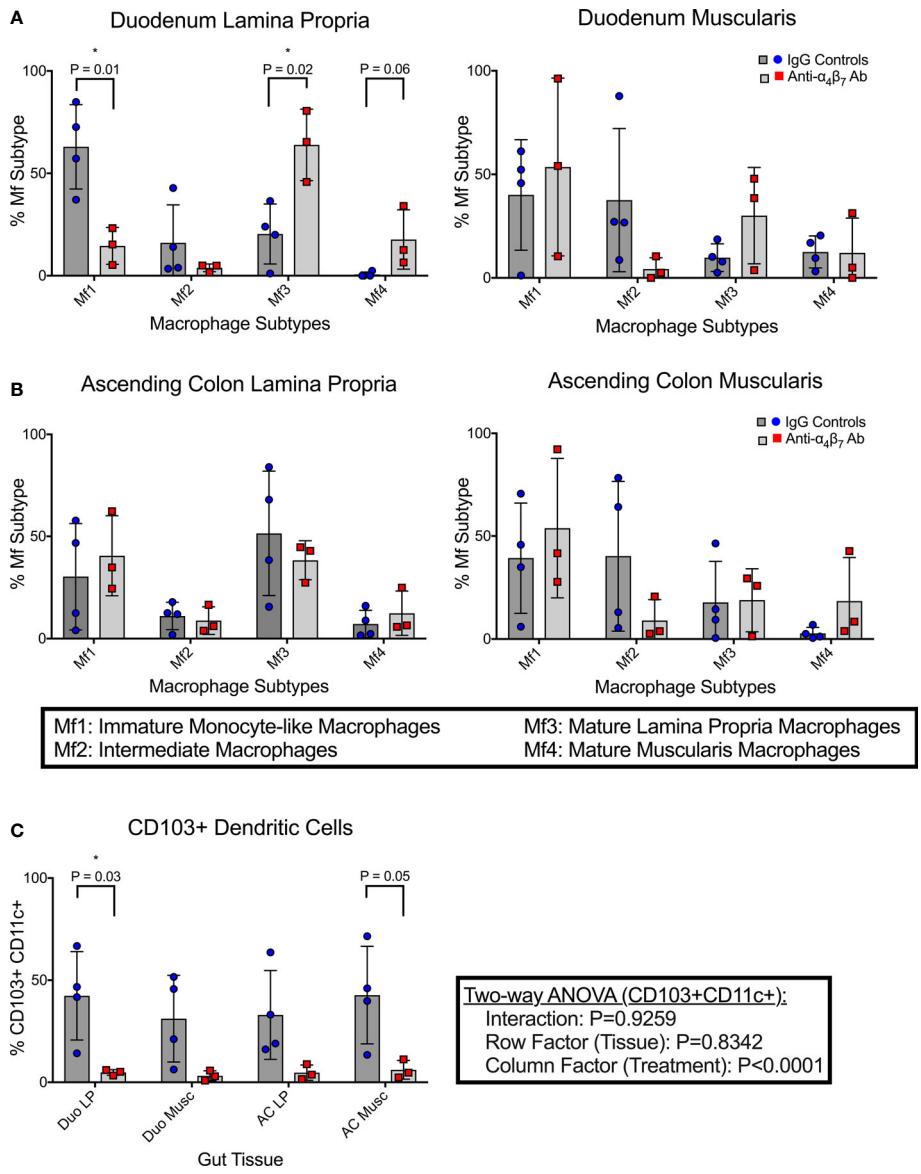

FIGURE 1
Anti-$\alpha_4\beta_7$ therapy is associated with reduced myeloid cell turnover in the small intestine. **(A)** Monocyte-like Mf1s were significantly lower in the duodenal lamina propria of anti-$\alpha_4\beta_7$-treated macaques compared with controls, while mature Mf3 were substantially higher. Similar trends were seen in the muscularis externa. **(B)** This trend was not maintained in the ascending colonic lamina propria. **(C)** CD103 expression on CD11c + cells was quantified in each tissue type and was significantly lower in the duodenal lamina propria of the $\alpha_4\beta_7$-treated group * P < 0.05.

In addition to pro-inflammatory macrophages, localization of dendritic cells (DCs) to the gut has also been coupled to the expression of the β_7 integrin (33). This includes CD103+ conventional DCs responsible for imprinting lymphocytes with gut-homing function by releasing retinoic acid (33). The frequencies of CD103+ expressing CD11c+ dendritic cells were quantified in each histological layer (lamina propria and muscularis externa) of both the duodenum and colon (Figure 1C). In the duodenum lamina propria, anti-$\alpha_4\beta_7$ therapy was associated with a significantly lower CD103+ DCs (4.8%) compared to controls (42.4%) (P=0.03). Similar trends were seen in the duodenum muscularis (3.2%, 28.0%; P=0.08), ascending colon lamina propria (4.7%, 33.0%; P=0.08), and ascending colon muscularis (6.1%, 42.7%; P=0.05), with all tissues having lower CD103+ CD11c+ cells in the anti-$\alpha_4\beta_7$ group. A two-way ANOVA analysis was performed to determine whether the response was dependent on tissue type. No significance was found in the tissue (row factor) or interaction, but the treatment (column factor) was significantly different (P<0.0001), suggesting that the differences were due to treatment and not secondary to the tissue being sampled.

Anti-$\alpha_4\beta_7$ therapy is associated with increased colocalization of CD206 with CD163 in gut tissues

Previously, it has been suggested that intestinal macrophage turnover is associated with peripheral monocyte turnover and is predictive of disease progression in SIV-infected RMs (19). To demonstrate these associations, a gating strategy determining the ratio of CD163+CD206+ double-positive to CD163+CD206- single-positive cells was utilized in conjunction with BrdU/EdU-labeling and confocal microscopy, with the finding that disease progression is associated with a reduction in the ratio of double-positive to single-positive cells during SIV infection (19). To validate our flow cytometry findings, we utilized a similar strategy to determine the co-localization of CD206 with CD163 in gut tissues (duodenum and ascending colon) of each of the macaques obtained at necropsy (Figure 2A, C). In addition to providing further evidence for increased macrophage maturity utilizing different surface markers (Bujko's strategy does not include either CD163 or CD206), this strategy also provided data for the two anti-$\alpha_4\beta_7$-treated RMs with missing flow cytometry data (19). When co-localization of CD206 with CD163 was measured in the duodenum, Mander's coefficient 1 (MC1) was measured providing the ratio of co-localization compared with total CD163 expression. In the IgG-treated controls, the mean MC1 was 0.07 compared with 0.53 in the anti-$\alpha_4\beta_7$-treated group (P=0.0159) (Figure 2B). When the same strategy was performed for the ascending colon, while the control group was 0.35, the anti-$\alpha_4\beta_7$-treated group was 0.53 (P=0.0397) (Figure 2D). In each tissue, the lower MC1 in the control group suggests reduced macrophage maturity compared with the experimental group.

These readings were next compared with macrophage subtype in the duodenum, as expected, Mf1s were negatively associated with MC1 (r=-0.8523, P=0.0148), and Mf3s (r=0.7706, P=0.0426) and Mf4s (r=0.777, P=0.0398) were positively associated with MC1 (Figure 2E). While there was a trend in a negative association with Mf2s, this correlation was not significant. This finding is consistent with Bujko, et al's decision to not use CD206 as a marker for their gating strategy because CD206 is expressed at high levels on Mf2, Mf3, and Mf4 gut macrophages (28).

Anti-$\alpha_4\beta_7$ therapy is associated with changes in tissue viral loads

There were no statistical differences between plasma viral loads during the post-treatment interruption between treated and control animals (Figure S6). However, we found significantly higher DNA viral load in the duodenum of anti-$\alpha_4\beta_7$-treated macaques (geometric mean: 536 copies/10^6 cells) compared to controls (geometric means 106 copies/10^6 cells; P=0.02) as determined by copies of SIVgag compared to the host genomic gene RPP30 (Figure 3A). No difference in DNA viral loads was found in the ascending colon (387 versus 382 copies/10^6 cells). A similar trend was seen in RNA viral loads, with the duodenum of the anti-$\alpha_4\beta_7$ group having a geometric mean of $\log_{10}$ 5.8 copies/100 ng RNA compared with to $\log_{10}$ 4.0 in controls (Figure 3B). Again, no difference was found in the ascending colon ($\log_{10}$ 5.6 copies compared to $\log_{10}$ 5.8 copies). Next, to determine whether there was a relationship between myeloid cells and viral loads, we performed linear regression analysis on the lamina propria, which were the presumed foci of anti-$\alpha_4\beta_7$ activity. When both experimental design groups are combined for the duodenum, RNA viral loads were negatively correlated with Mf1s (r= -0.8758, P= 0.0098), positively correlated with Mf3s (r= 0.9332, P=0.0021), and negatively correlated with $CD103^+$ DCs (-0.8298, P=0.0209) (Figure 3C). Despite few differences between groups in tissue viral load and macrophage maturity phenotype, the two were similarly correlated in the ascending colonic lamina propria with Mf1s negatively (-0.8366, P=0.0190) and Mf3s positively (r=0.771, P=0.0424) correlated (Figure 3D). $CD103^+$ DCs, though were not significantly associated (r=-0.4899, P=0.2644). Thus, in both the small and large intestines, more mature macrophage phenotypes are associated with viral loads but not necessarily CD103+ DCs. Additionally, the duodenum viral load is associated with MC-1 (r=0.7255, P=0.0270) (Figure S5). However, this Pearson's coefficient is lower than that found when comparing Mf3s (r=0.7255 vs. r=0.9332, respectively), suggesting that fully mature Mf3s, but not all $CD163^+CD206^+$ cells, are closely associated with tissue viral loads.

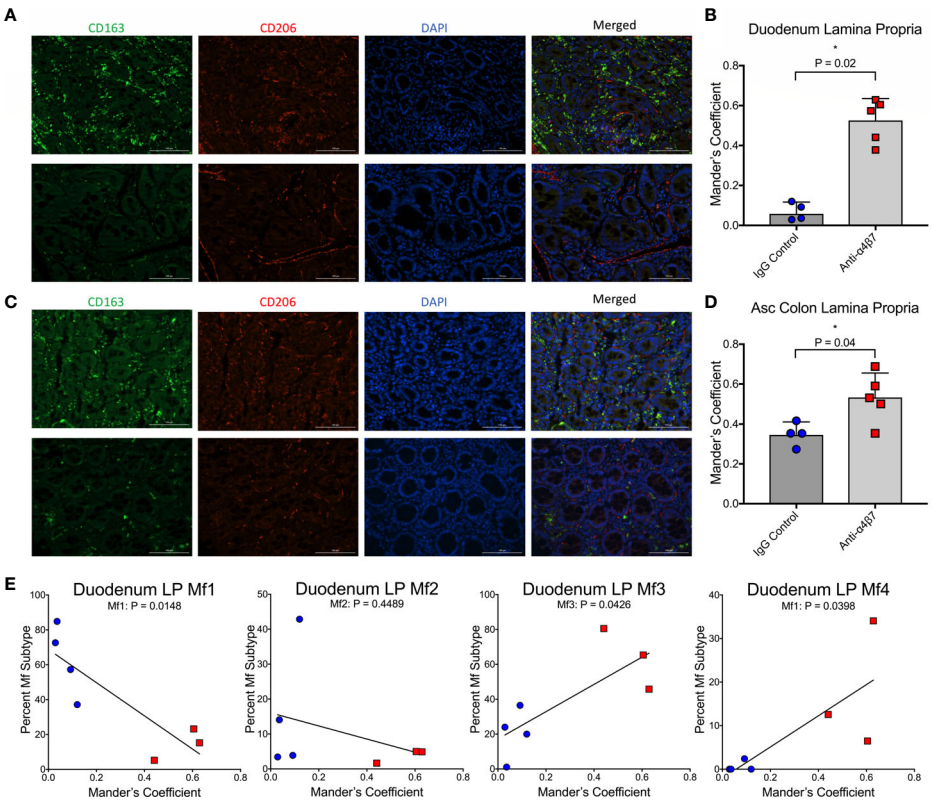

FIGURE 2
Anti-$\alpha_4\beta_7$ therapy is associated with increased CD206+ expression on CD163+ cells. **(A)** Immunofluorescence microscopy was used to compare the expression of CD163 and CD206 in duodenum tissue from anti-$\alpha_4\beta_7$-treated (above) and IgG controls (below). **(B)** Co-localization was quantified with Mander's coefficient 1, showing the ratio of co-localization of CD206 with CD163. **(C)** Immunofluorescence and **(D)** co-localization were also determined for anti-$\alpha_4\beta_7$-treated (above) and IgG controls (below) in the ascending colon. **(E)** Linear regression analysis was used to compare co-localization with duodenal lamina propria macrophage subtypes determined by flow cytometry. * $P < 0.05$.

Acute SIV infection is associated with acute intestinal dysbiosis

Increasingly, the microbial composition has been recognized as a significant modulator of the inflammatory states of mucosal gut myeloid cells (34). Additionally, specific taxa have been linked with vedolizumab efficacy in IBD patients and lymphocyte activation and viral load during HIV infection (25, 35). To determine whether the microbiome may be influencing the efficacy of anti-$\alpha_4\beta_7$ during SIV infection, 16S rRNA gene sequencing was performed on DNA isolated from fecal samples obtained at baseline, acute infection (Day 14), during therapy (Day 98), and after cART interruption (Day 161). Using unweighted principal component analysis, it was clear that acute infection was associated with dysbiosis, with animals in each group having microbial composition divergence at Day 14 compared with the other timepoints (Figure S3a). Additionally, at the phylum level, non-significant differences were seen in the Bacteroidetes: Firmicutes (B: F) ratio, with the anti-$\alpha_4\beta_7$-treated group experiencing an increase in Bacteroidetes (B) and a decrease in Firmicutes (F) resulting in an increased B:F ratio at Day 14 (Figure S3c). Control animals had the opposite trend suggesting that each group experienced divergent dysbiosis before therapeutic intervention. It should be noted that in the present study, all RMs were CD8-depleted to facilitate rapid viremia and accelerated pathogenesis. Whether CD8 depletion also modulates microbiome composition in SIV-infected macaques remains to be fully elucidated. However, CD8 depletion prevents respiratory syncytial virus-associated dysbiosis in mice, reversing losses in the Firmicute families Lachnospiraceae and Lactobacillaceae (36).

For greater granularity in taxonomic changes, we next analyzed the family composition of the microbiome over the time course of the experiment. The data was examined using stacked grouped abundance graphs that demonstrated that samples from Day 14 in each group differed from other timepoints with higher Bray-Curtis dissimilarity in both control and experimental groups (Figure 4A). Other time points in each group clustered together with reduced dissimilarity, indicating that each group partially recovered

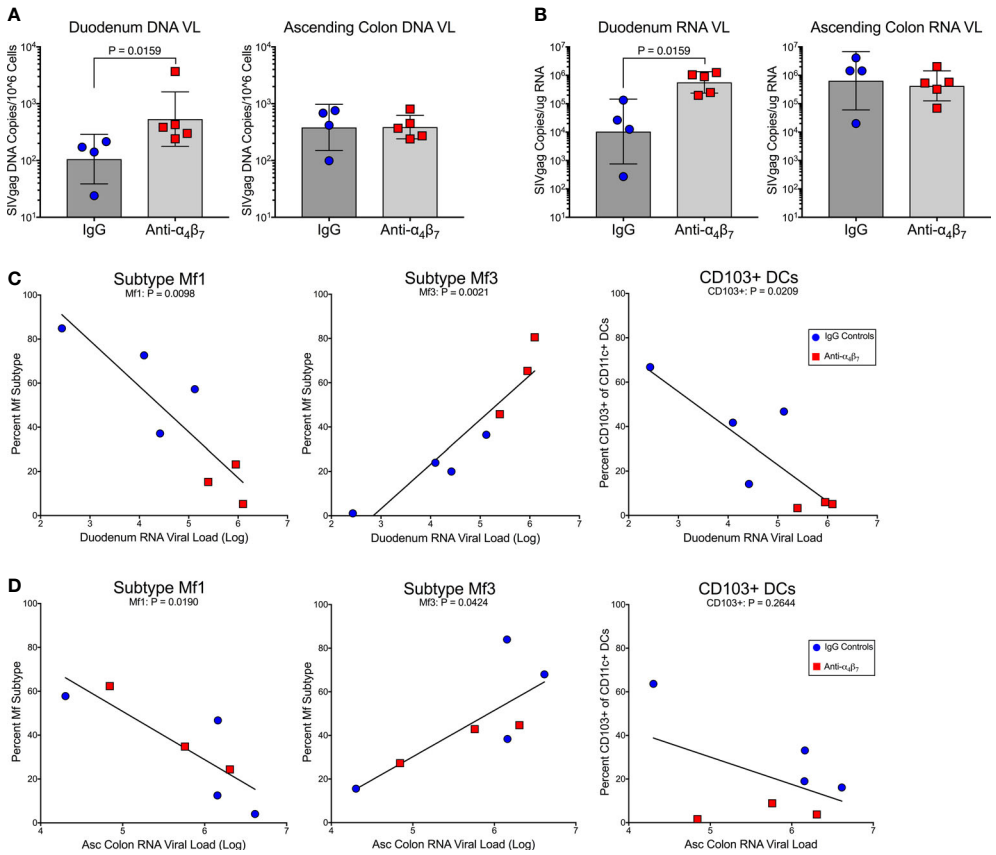

FIGURE 3
Anti-$\alpha_4\beta_7$ therapy was associated with higher small intestine tissue viral loads. **(A)** Tissue RNA viral loads were significantly higher in the duodenum of anti-$\alpha_4\beta_7$-treated macaques compared to controls. **(B)** Tissue DNA viral loads normalized to cell number followed the same trend. **(C)** In the duodenum, viral loads were negatively associated with Mf1s and CD103+ DCs. Viral loads were positively associated with mature Mf3s. **(D)** In the ascending colon, a similar association was found with local viral load and macrophage maturity but not with CD103+ CD11c+ cells.

from acute dysbiosis. A heatmap demonstrates similar dynamics, with data on Day 14 samples considered as outliers in each experimental group (Figure 4B). Critical families from the above analyses were separated to evaluate specific taxonomic changes better, and longitudinal plots were generated (Figure 4C). Prevotellaceae, the most abundant family across all groups, was depleted during acute dysbiosis in the IgG controls, making up only 6.7% in samples from Day 14 compared to 33.3% at baseline. This impact on the relative abundance of Prevotellaceae did not follow a similar trend in the anti-$\alpha_4\beta_7$-treated experimental group. Conversely, Spirochaetaceae were moderately increased in control IgG group from baseline compared to samples on day 14 (7.8% as compared with 17.6%). None of the above differences were statistically significant between groups due to significant inter-individual variations in microbiome composition. However, both groups had an increase in the relative abundance of the family Helicobacteraceae and a decrease in the family Lactobacillaceae, the latter was significantly different (from 5.8% to 0.3%, P=0.003), with every animal showing a decrease in relative abundance during acute dysbiosis by an average of 88%. Aside from the differences in Prevotellaceae, Lachnospiraceae dynamics were also different between the two groups showing a decrease in the anti-$\alpha_4\beta_7$-treated group (10.2% at baseline to 8.3%) but an increase in the control group (6.6% to 7.6%). A final significant difference between the two groups was seen in the numbers of the Veillonelaceae family, which was significantly depleted (P=0.03) in the IgG-treated controls after infection but rebounded (P=0.03).

SIV-associated dysbiosis is partially ameliorated following anti-$\alpha_4\beta_7$ therapy

HIV/SIV-associated dysbiosis has been extensively characterized across human populations and non-human

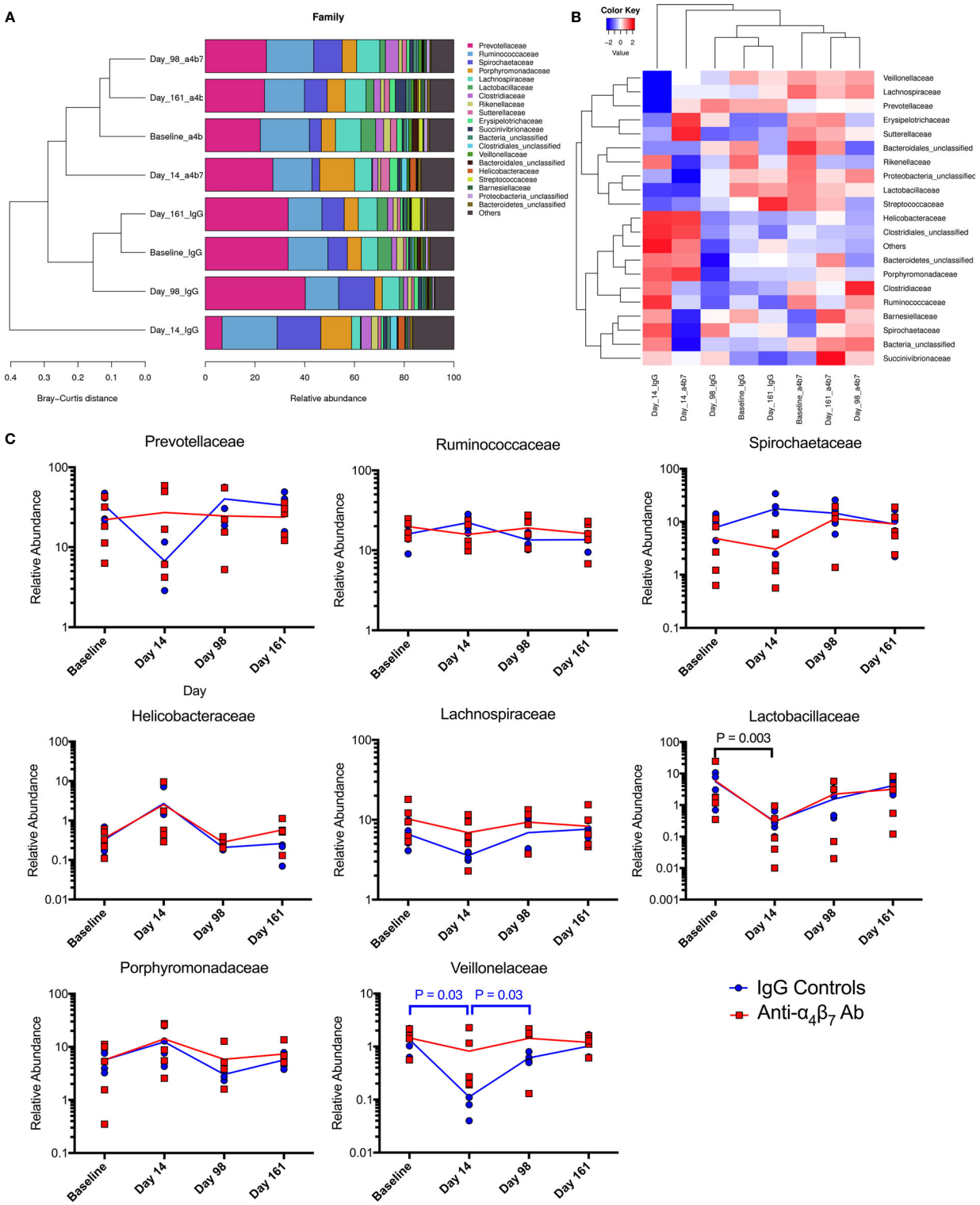

FIGURE 4
Microbiome family composition is disrupted by acute SIV infection: Both **(A)** stacked grouped abundance and **(B)** heatmap demonstrate acute dysbiosis at Day 14 post-infection where family relative abundance does not group within experimental groups. **(C)** Longitudinal changes to family abundance of Prevotellaceae, Ruminococcaceae, Porphyromonadaceae, Spirochaetaceae, Lachnospiraceae, Lactobacillaceae, Helicobacteraceae, and Veillonelaceae.

primate models. Consistently, infection is associated with an increase in the Bacteroidete: Firmicute ratio in fecal samples. These changes are primarily driven by increases in *Prevotella* and loss of BPB (25, 26, 37, 38). Notably, the BPB genus *Roseburia* has specifically been linked to vedolizumab efficacy, as a marker of HIV-associated dysbiosis (35). The ratio of these two bacteria groups has previously been used as a marker of dysbiosis during HIV infection, significantly higher in PLWH (25). Further, this ratio was positively associated with lymphocyte and colonic dendritic cell activation (25). As previously noted, there was a divergent dysbiosis prior to infection between the IgG and anti-$\alpha_4\beta_7$ groups. Because of this difference and for improved graphical representation, the *Prevotella*: *Roseburia* ratio was normalized to acute infection (Day 14), the time point at which the microbiome is significantly disrupted and is unable to recover, even with cART (Figure 5A). At acute infection, both groups trend to show an increase in the *Prevotella*: *Roseburia* ratio, regardless of whether normalization was performed, consistent with previous observations of acute infection-associated dysbiosis in both HIV and SIV infections (25, 38, 39). One fecal sample on Day 98 was not collected from the anti-$\alpha_4\beta_7$-treated group. However, in all four animals from which samples were collected, the values were lower following therapy. When normalized to acute infection ratio, a significant difference was found between the IgG- and anti-$\alpha_4\beta_7$-treated groups (P=0.0286). Even without normalization, the change from acute infection to post-treatment *Prevotella*: *Roseburia* ratio was significant in the anti-$\alpha_4\beta_7$-treated group compared using a ratio paired T-test that showed a decrease from a geometric mean of 29.7 to 6.5 (P=0.0228).

When the Day 161 *Prevotella*: *Roseburia* ratio was compared with macrophage turnover in the duodenal lamina propria, Mf1s were positively associated (r=0.7833, P=0.0372) with a trend in Mf3s (r=-0.704, P=0.0775) (Figure 5B). When the ratio of Mf1:Mf3 was compared with the *Prevotella*: *Roseburia* ratio, the Pearson correlation coefficient was r=0.924 (P=0.0029) (Figure S4b). A similar significant association was found in the Mf1:Mf3 ratio in the ascending colon (r=0.8594, P=0.0132) (Figure S4b). The *Prevotella*: *Roseburia* ratio was also positively associated with CD103+ CD11c+ cells in the duodenum (r=0.8119, P=0.0266) (Figure 5C) and ascending colonic (r=0.9077, P=0.0047) (Figure S4c) lamina propria. Finally, the duodenum viral load was also associated with the *Prevotella*: *Roseburia* ratio (r=-0.7565, P=0.0183) (Figure 5D). A similar non-significant trend was seen in the ascending colon (r=-0.6104, P=0.0809) (Figure S4d). This analysis indicates that increased *Prevotella* and reduced *Roseburia*, two key features of HIV/SIV-associated dysbiosis, are associated with myeloid cell turnover in the duodenum. Further, this dysbiosis was also associated with gut tissue viral loads.

Discussion

Recent interest in determining the mechanism of vedolizumab action has offered new avenues for understanding HIV/SIV

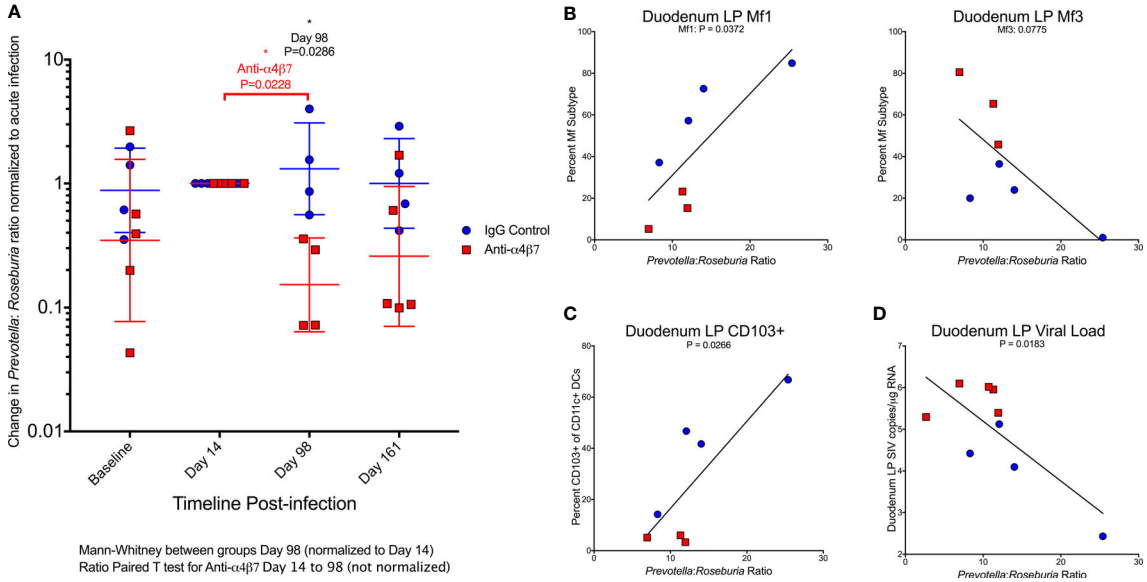

FIGURE 5
Anti-$\alpha_4\beta_7$ therapy is associated with a reduction in the *Prevotella*: *Roseburia* ratio. **(A)** The ratio of *Prevotella*: *Roseburia* was calculated and normalized (for visualization and comparison) for the acute infection dysbiosis timepoint (Day 14) for longitudinal fecal samples utilized for 16S rRNA sequencing. Linear regression was performed with this ratio (not normalized) on Day 161 for **(B)** the duodenal lamina propria macrophage maturity phenotypes, **(C)** CD103+ CD11c+ cells, and **(D)** tissue viral loads.

pathogenesis. Contrary to results from earlier studies, clinical samples and murine models of DSS-induced colitis showed an association with an increase in infiltrating monocytes and a decrease in mature macrophages (40). Additionally, β7-integrin-expressing monocyte infiltration exacerbates DSS-induced colitis in RAG2 mice, which lack mature lymphoid cells, emphasizing the role of monocytes, not lymphocytes, in colitis development in these models (32). Mature lamina propria macrophages express reduced CD14 and have a reduced pro-inflammatory response to lipopolysaccharide stimulation from the microbiome (28, 41). However, during IBD, an influx of peripheral CD14+ monocytes and newly-differentiated M1 macrophages capable of microbial pattern recognition enter this niche and release pro-inflammatory cytokines (42). This signaling results in a self-perpetuating cycle of pro-inflammatory macrophage infiltration. These infiltrating monocytes and pro-inflammatory M1 macrophages in the lamina propria are thought to contribute to barrier dysfunction in IBD thought to be secondary to dysfunction of tight junction proteins and the induction of epithelial apoptosis (42). Unlike infliximab, vedolizumab is associated with significant changes in macrophage populations in human patients with CD or UC, not in lymphocyte populations, including a shift from M1 phenotype dominance to M2 (17). Infliximab, though, by blocking TNF-α, may additionally allow a similar increase in anti-inflammatory macrophage phenotypes (43).

SIV infection is associated with a shift in pro-inflammatory monocyte infiltration to gut tissues replacing mature tissue macrophages, similar to the data from IBD patients (44, 45). In RMs, the frequencies of CD163+ macrophages increased four-fold during SIV infection associated with progression to AIDS (46). Additionally, SIV infection is associated with a shift from mature CD163+CD206+ macrophages in the lamina propria to immature CD163+CD206- macrophages, with changes primarily in the small intestine (19). By applying a different flow cytometry gating approach, we provide evidence for lower percent of Mf1 and higher Mf3 macrophages following anti-$\alpha_4\beta_7$ therapy that is initiated during acute infection, compared with controls. Further, we found that it is the small intestinal lamina propria on which anti-$\alpha_4\beta_7$ mAb seems to exert the most influence, given the fact that we found differences only in CD206 co-localization with CD163 in the colon using microscopy, but not by flow cytometry, and no differences in the muscularis externa macrophage populations. Additionally, it has been hypothesized that longer-lived macrophages are more likely to serve as viral reservoirs in the tissue (19). Although we did not measure this directly, we found tissue viral loads to be closely associated with Mf3s and negatively associated with Mf1s in both tissues. This was confirmed based on CD206 co-localization with CD163, which also correlates with viral loads in the duodenum. Despite this increase in tissue viral load, it should be noted that natural hosts of SIV experience chronic viremia without progressing to AIDS (47). Results of a recent study in which whole gut tissue transcriptomic data were compared between African green monkeys (AGMs) (*Chlorocebus* spp.; non-progressing natural hosts of SIV) and disease-susceptible RMs following SIV infection demonstrated a significantly higher expression of M2 wound-healing macrophage associated genes in AGMs following SIV infection compared with RM gut tissue which had an increase in pro-inflammatory gene expression (48). Whether anti-$\alpha_4\beta_7$ facilitates a similar switch to wound-healing remains an additional possibility requiring further investigation.

While anti-$\alpha_4\beta_7$ alone modulates monocyte trafficking associated with an increase in pro-inflammatory Mf1 macrophages to the lamina propria, we also found the frequency of multiple myeloid cell subsets to be related to the *Prevotella*: *Roseburia* ratio. HIV and SIV are each associated with acute and chronic dysbiosis, even during cART. Prominently, the overall ratio of Bacteroidetes : Firmicutes increases, primarily due to the rise in *Prevotella* at the expense of several Firmicute taxa, a pattern consistent across populations (25, 26, 37, 38, 49, 50). Whether these findings are independent of sexual behavior remains unclear, but similar trends are seen in children and SIV-infected non-human primates (37, 38, 49, 50). In addition to being characteristic of HIV-associated dysbiosis, *Prevotella* has been associated with CD4+ and CD8+ T cell activation in HIV-infected adults (51). *Prevotella* is also negatively related to CD4+ counts in perinatally-infected children and positively associated with plasma IP-10 and soluble CD14 levels, the latter implicating a role in monocyte and/or macrophage activation (37). Further, the inclusion of *Prevotella* in specific pathogen-free mice leads to a significant decrease in the relative abundance of the Firmicutes such as Lachnospiraceae and Ruminococcaceae spp. and exacerbates mucosal inflammation and disease progression in models of colitis in mice suggesting the expansion of *Prevotella* alone may be exacerbating HIV-associated inflammation and dysbiosis (52–54). In contrast to *Prevotella* expansion, several butyrate-producing Firmicute taxa are depleted during HIV pathogenesis, even during cART (25, 26, 37, 38). Most prominently, the relative abundance of colonic *Roseburia* is negatively correlated with plasma viral loads, CD4+ T cell activation, and markers of microbial translocation in PLWH. The colonic ratio of *Prevotella stercorea*: *Roseburia intestinalis* is closely associated with the activation of peripheral CD4+ T cells and colonic DCs, CD4+, and CD8+ T cells in PLWH (25). The short-chain fatty acid butyrate has a pleiotropic role in maintaining gut homeostasis, acting as an energy source for gut epithelial cells, a histone deacetylase inhibitor, and an agonist of GPR41, GPR43, and GPR109A (34, 55). GPR109A agonism specifically facilitates gut macrophages exposed to butyrate to establish their anti-inflammatory phenotype (56). Further, *ex vivo* colonic macrophages cultured with butyrate are imprinted with anti-microbial activity without a concurrent increase in tissue damage (57). Additionally, butyrate-producing bacteria like *Roseburia* have previously been linked to vedolizumab efficacy in IBDs (35), but the results presented herein, we

submit, are the first to implicate the microbiome's role, and the ratio of *Prevotella: Roseburia* specifically, to be closely associated with macrophage phenotype and function following anti-$\alpha_4\beta_7$ therapy during SIV infection.

The data presented herein represents a study significantly different from previous evaluations of anti-$\alpha_4\beta_7$ that offers insight into its potential mechanism during SIV infection. Because of these differences, tissue viral loads, in particular, were higher in anti-$\alpha_4\beta_7$-treated animals compared to controls, while previous reports found the opposite (8, 58). There are several possible reasons for this discrepancy. Computer modeling has shown that anti-$\alpha_4\beta_7$ mAb's facilitation of viral clearance was a prominent mechanism in two of the eight animals in the 2016 study (27). Additionally, the macaques in the current study were depleted of CD8+ T and NK cells by administering an anti-CD8α mAb. When performed in the original 2016 cohort following prolonged virologic control, anti-CD8α administration led to rapid viremia (27). Because CD8+ cells have been demonstrated to play a role in anti-$\alpha_4\beta_7$ efficacy, these findings provide a potential reason for differences in gut tissue viral loads. Finally, diet significantly impacts immune function and viral reservoir (59, 60). Specifically, butyrate produced from the fermentation of dietary fiber can, among other functions, act as an HDAC inhibitor (34). Although HDAC inhibitors have been explored in HIV cure strategies, these studies rarely characterized tissue macrophage reservoirs resistant to cytolysis (61). Whether this mechanism or the immune modulation induced by anti-$\alpha_4\beta_7$ infusions contributed to differences warrants further investigation, as previously suggested (27). Further investigation is needed to confirm changes in the colon with a larger number of animals to see if the macrophage immunophenotypic differences in the duodenum and its correlation with the *Prevotella : Roseburia* ratio is also present in this tissue.

In addition to immunologic differences, the differences in virus tropism may have also played a role in gut tissue viral load compared with previous studies. Recently, the expression of HIV restriction factor SERINC5 was shown to increase in the process of macrophage differentiation (62). When incorporated into HIV virions, SERINC5 inhibits fusion with target cells, but Nef expression reduces SERINC5 incorporation. Further, HIV-1 ΔNef has a reduced ability to infect mature macrophages. In comparison, infection of HIV-1 ΔNef was non-statistically increased in monocytes (62). If anti-$\alpha_4\beta_7$ reduces the abundance of monocytes, the lack of functional Nef at infection in the original study may partially explain differences in gut viral loads (8). This is particularly true if longer-lived, mature macrophages serve as reservoirs (19). Even without the nef-stop mutation, SIVmac239 has comparatively lower macrophage tropism than dual tropic SIVmac251 used in this study (63, 64). Additionally, it is known that $\alpha_4\beta_7$ becomes incorporated into SIV/HIV, and when comparisons of $\alpha_4\beta_7$ integrin incorporations were made in diverse HIV and SIV strains, it was found that SIVmac251 had the highest levels of incorporation of the 14 total viruses examined (65). Such $\alpha_4\beta_7$ incorporation into virions has been attributed to increased trafficking to the gut facilitating trans-infection of cells close to MADCAM-1 (65). Regardless of the SIV viral stock used, rapid SIV diversification *in vivo* means that reduced myeloid turnover likely enhances the viral reservoir, thereby increasing tissue viral loads when control monkeys replace macrophages with uninfected monocytes during cART suppression. However, it should be noted that earlier anti-$\alpha_4\beta_7$ administration studies have been inconsistent, with some unable to replicate viral control and others showing increased time to viral rebound when co-administered with neutralizing antibodies (11, 12, 66, 67). While macrophage turnover is rapid during SIV infection, complete suppression may be necessary prior to anti-$\alpha_4\beta_7$ therapy to reduce tissue viral loads (19). Future studies need to determine if macrophage turnover and therefore reduced maturity during cART can help limit the tissue macrophage reservoir, thereby explaining discrepancies between our study and previous findings. If true, a delay in initiating anti-$\alpha_4\beta_7$ administration may improve tissue virologic outcomes.

Although our findings are statistically significant, the interpretation of our data has limitations beyond differences in study design compared with previous similar studies. First, the study utilized for this analysis was designed to test a different hypothesis and was meant to be preliminary. Because of this design, the sample size was small, and two samples were missing for our flow cytometry analysis, thus reducing their statistical power. Next, cART was also interrupted several weeks before necropsy, making translation to IBDs or cART-suppressed PLWH impossible since ongoing viral replication may influence macrophage maturity. Unlike previous studies determining macrophage and monocyte turnover during SIV infection, we did not utilize BrdU to track recently divided cells and instead relied on newly developed flow cytometry techniques and previously validated microscopic markers to determine gut macrophage maturity (19, 28). Finally, the microbiome analysis was performed on fecal samples instead of gut tissue, which are usually only partially correlated (68, 69). Additionally, several factors, including local viral replication, may modulate the microbiome, further complicating interpretation. Therefore, future additional studies incorporating these limitations may address the precise mechanisms behind these observations, and further studies are warranted in this direction.

Conclusions

The *in vivo* administration of a primatized anti-$\alpha_4\beta_7$ mAbs during SIV suppression has yielded inconsistent results on virologic control suggesting the involvement of a complex mechanism and possible co-factors that may be responsible for differential efficacy. First, we found that increased macrophage maturity phenotypes were associated with tissue viral loads, a difference from earlier studies that administered anti-$\alpha_4\beta_7$ after

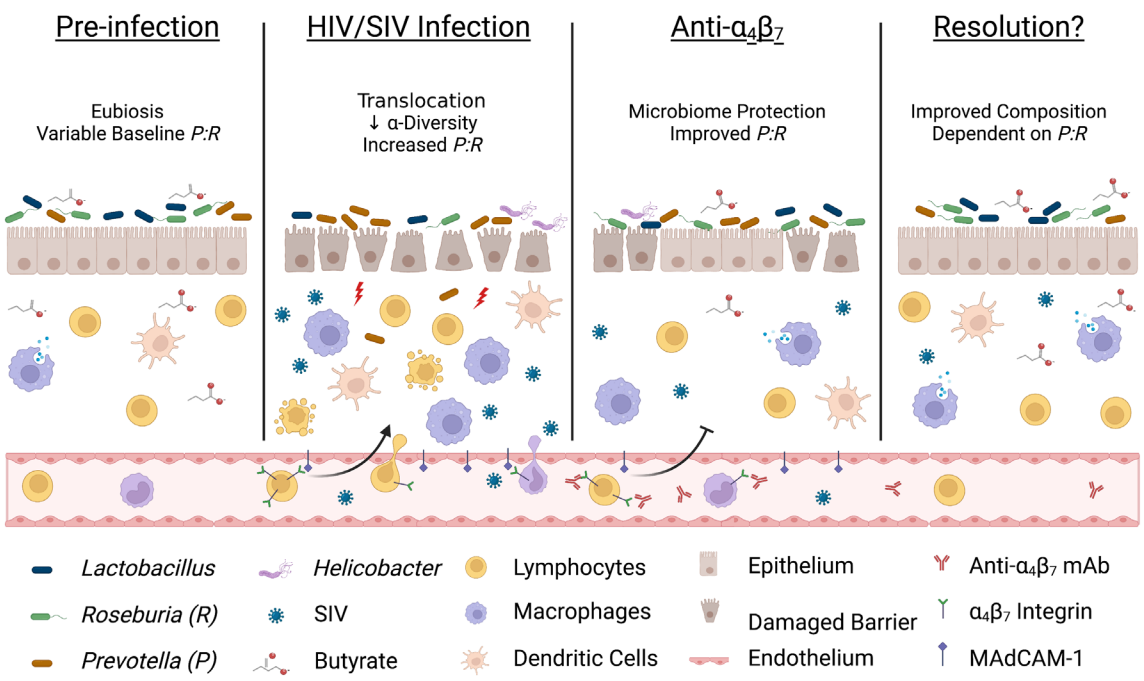

FIGURE 6
Acute infection dysbiosis is an independent factor in anti-$\alpha_4\beta_7$ therapy. Several lines of evidence suggest that starting microbial composition can impact the efficacy of anti-$\alpha_4\beta_7$ therapy. Early during HIV/SIV infection, butyrate-producing bacteria like *Roseburia* are rapidly replaced with *Prevotella* resulting in dysbiosis and an increase in microbial translocation exacerbating pro-inflammatory processes, including trafficking of circulating immune cells. Administration of anti-$\alpha_4\beta_7$ mAbs facilitates the resolution of inflammation by modulating immune trafficking, including myeloid cells, thereby allowing a reduction in the *Prevotella*: *Roseburia* ratio (*P:R*). However, myeloid cell phenotype, maturation, and function are modulated by butyrate-producing bacteria so if pre-infection levels are low, re-establishment may not be sufficient for efficacy necessitating new approaches to influence gut immune inflammatory milieu Image created with Biorender.com.

infection. This may indicate that the timing of anti-$\alpha_4\beta_7$ administration with regard to tissue viral suppression is an essential determinant of efficacy. Second, we found that dysbiosis markers are associated with the relative impact of anti-$\alpha_4\beta_7$ on macrophage maturity. Low dietary fiber consumption in humans is associated with reduced butyrate production and increased risk of IBD (59, 60), and the relative abundance of BPB has been shown to impact the efficacy of vedolizumab in humans (35). Based on the data presented herein, we propose that important shifts in microbiome composition during HIV/SIV infections like increases in *Prevotella* spp. and depletion of BPB like *Roseburia* spp. may be key independent factors in anti-$\alpha_4\beta_7$-treated SIV-infected macaques (Figure 6). Future studies aiming to modulate gut immune function in HIV/SIV should include assays like 16S rRNA sequencing of colon/fecal samples to characterize microbiome dynamics at baseline, acute dysbiosis, during, and following therapeutic intervention. Further, factors impacting gut microbial diversity and composition, such as animal source, age, co-housing, antibiotic use, and diet, should be considered when interpreting such study results.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. FASTQ files generated during 16S rRNA sequencing are deposited in the NCBI Sequence Read Archive with the BioProject accession number PRJNA870961.

Ethics statement

The animal study was reviewed and approved by University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC).

Author contributions

SB designed the study and SJ, LK, participated in research design. SJ and LK performed animal protocols. OO designed antibody panel and ran flow cytometer. SJ performed flow cytometry analysis. SJ and

LK performed microbiome assays. LK and MT determined viral loads. NK performed microscopy and co-localization quantification and wrote microscopy methods. SJ performed data analysis and wrote the manuscript, which all authors reviewed. SB designed, acquired funding, supervised the entire study and edited the manuscript. MM and SM edited the manuscript, provided funding support and interpretation of the data. All authors contributed to the article and approved the submitted version.

Funding

National Institutes of Health grant R21MH113455 (SB). National Institutes of Health grant R01 AI129745 (SB). National Institute of Health Grant R01AI134245 (SM & SB). National Institute of Drugs of Abuse Grant R01 DA052845 (MM & SB).

Acknowledgments

We Thank Dr. Aftab Ansari for critical reading of the manuscript. We thank UNMC comparative medicine Veterinary staff and SB's current and previous lab members for animal related experimental support Figures 6, S1 were created with Biorender.com.

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/fimmu.2022.1001727/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 07 November 2022
DOI 10.3389/fimmu.2022.977528

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

RECEIVED 24 June 2022
ACCEPTED 19 October 2022
PUBLISHED 07 November 2022

CITATION
Wang X, Li L, Bai M, Zhao J, Sun X, Gao Y, Yu H, Chen X and Zhang C (2022) Dietary supplementation with *Tolypocladium sinense* mycelium prevents dyslipidemia inflammation in high fat diet mice by modulation of gut microbiota in mice. *Front. Immunol.* 13:977528. doi: 10.3389/fimmu.2022.977528

Dietary supplementation with *Tolypocladium sinense* mycelium prevents dyslipidemia inflammation in high fat diet mice by modulation of gut microbiota in mice

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Obesity is a risk factor for many serious health problems, associated with inflammation, hyperlipidemia, and gut dysbiosis. Prevention of obesity is especially important for human health. *Tolypocladium sinense* is one of the fungi isolated from Chinese caterpillar fungus, which is a traditional Chinese medicine with putative gut microbiota modulation effects. Here, we established a high-fat diet (HFD)-induced hyperlipidemia mice model, which was supplemented with lyophilized *T. sinense* mycelium (TSP) daily to evaluate its anti-obesity effects. The results indicated that TSP supplementation can effectively alleviate the inflammatory response and oxidative stress levels caused by obesity. TSP significantly prevented obesity and suppressed dyslipidemia by regulating the expression of lipid metabolism genes in the liver. TSP is also effective in preventing the HFD-induced decline in short-chain fatty acid (SCFA) content. Gut microbiota profiling showed that TSP supplementation reversed HFD diet-induced bacterial abundance and also altered the metabolic pathways of functional microorganisms, as revealed by KEGG analysis. It is noteworthy that, correlation analysis reveals the up-regulated gut microbiota (*Lactobacillus* and *Prevotella_9*) are closely correlated with lipid metabolism parameters, gene expression of liver lipid metabolism and inflammatory. Additionally, the role of TSP in the regulation of lipid metabolism was reconfirmed by fecal microbiota transplantation. To sum up, our results provide the evidence that TSP may be used as prebiotic agents to prevent obesity by altering the gut microbiota, alleviating the inflammatory response and regulating gene expression of liver lipid metabolism.

KEYWORDS

***Tolypocladium sinense*, gut microbiome, dyslipidemia, inflammation, obesity**

Introduction

Non-communicable Disease Risk Factor Collaboration reported that the global rate of the prevalence of the age-standardized obesity increased approximately 2-3 times in 2014 compared with that in 1975. Approximately 1.9 billion people in the world are overweight, and among them, 600 million are obese. Obesity has become a serious hazard to human health, it can induce diabetes, non-alcoholic fatty liver disease, hypertension and certain (1, 2). Multiple factors contribute to the development of obesity, including energy consumption, high fat intake and the microbiome (3). Many reports reveal that gut microbiota acts an important modulator in the diet and metabolic syndrome is caused by obesity (4, 5). Diet is a significant factor altering the diversification and metabolism of the gut microbiota, consequently inducing or preventing obesity (6, 7).

The over consumption of food in the host and consequent increase in energy intake is the main cause of obesity; the intestinal flora is involved in the regulation of nutrient absorption and energy balance. The results of some basic studies showed that the intestinal permeability of obese mice is significantly enhanced, the diversity of the intestinal flora is reduced, Bacteroidetes decrease by approximately 50%, and the number of Firmicutes increased in proportion, as compared with lean mice (8, 9). The results of a clinical research show that Bacteroides ferment dietary fibers to produce short chain fatty acids, while Firmicutes obtain energy from food and store it in the form of adipose tissue (10). Studies in animal models suggest that certain gut microbes can prevent diet-induced obesity. Indeed, several probiotics have been used in clinical trials to reduce lipid levels in obesity-regulated subjects, achieving good results (11–13).

Chinese caterpillar fungus is a traditional Chinese medicinal mushroom, which contain a wide range of immuno-modulatory and bioactive compound with many medical effects, such as anti-aging, anti-bacteria, anti-cancer, expanding blood vessels, improving arteriosclerosis, hepatoprotective and hypolipidemic (14). *Tolypocladium sinense* is one of the fungi isolated from Chinese caterpillar fungus. The research and application of *T. sinense* mainly focus on the culture conditions and the preliminary pharmacological analysis of its chemical components (15, 16). Fang (17) carried out pharmacological experiments on the mycelium culture of *T. sinense* in mice. The results showed that it possesses sedative effects, anti-inflammatory activity, hypoxia tolerance, organ expansion and androgen like promotion. In the acute toxicity test, the dose of 80 g (maximum allowable volume) per mouse was administered once by gavage, and no adverse effects were found. Gao (18) reported that the mycelium extract and polysaccharide extract of *T. sinense* possess scavenging effects on DPPH free radicals. The test results showed that *T. sinense* has potential application and development prospects as antioxidant and anti-tumorigenic.

At present, the research on the pharmacological value of *T. sinense* is not complete, since its role in preventing obesity and its ability to change the gut microbiota composition is still unclear. Therefore, the purpose of our study was to determine the effects of *T. sinense* mycelium (TSP) in the prevention of hyperlipidemia and to understand its potential lipid-lowering mechanism. This study could provide a theoretical basis for the development of prebiotic agents to prevent obesity from a Chinese traditional edible fungus.

Materials and methods

Materials and reagents

Serum biochemical detection index kit such as total cholesterol (TC), triglyceride (TG) and ELISA detection kit were purchased from Nanjing Jiancheng Institute of Bioengineering (Jiangsu, China). Blood glucose assay kit was obtained from Jiangsu Yuyue Medical Equipment & Supply Co., Ltd. (Jiangsu, China). All other chemical reagents were analytical grade.

Preparation and identification of *Tolypocladium sinense* fungus powder

Natural fresh Chinese caterpillar fungus was collected in the plateau area at an altitude of 4000 ~ 4500 m in the Naqu, Tibet Autonomous Region. The fungus was thoroughly rinsed with tap water. Then it was submerged in 10% bleach water for 20 min and rinsed with sterile distilled water. The fruiting bodies of Chinese caterpillar fungus were cut into small pieces of 2 ~ 5 mm and cultured on a separation medium (10 g/L peptone, 100 g/L glucose, 3 g/L yeast extract, 0.5 g/L $MgSO_4$, 1 g/L KH_2PO_4, 100 U/L penicillin, and 20 g/L agar) at 26 °C. After the grow of the mycelium, the tip was collected and inoculated on fresh medium. The separation and passages were repeated several times until the colonies with consistent morphology were obtained (18, 19). The strain identification was performed as follows: the mycelial DNA was extracted, the whole genome was used as the template, and the universal primers ITS1 and ITS4 as primers (Table S1) were used for PCR amplification. The amplified products were sequenced and analyzed by Shanghai Sangon Biotech Co., Ltd. The strain screened by morphological observation and sequencing identification was *T. sinense*. After the identification of the strain, the metabolites of the bacterial mycelium were analyzed by Beijing BioMarker Technology Co., Ltd. (Supplemental Methods and Figure S1). In total, we detected 1652 metabolites from *T. sinense* fungus mycelium, and most of them belong to Organic acids, Nucleic acids, Glycerophospholipids, Fatty Acyls, Organoheterocyclic compounds, Carbohydrates, Polyketides,

Organic oxygen compounds and Sterol Lipids. Pick up the cultured colonies, connect with 5% seed culture medium for culture, shake at 26 °C for 4-5 days (150 r·min^{-1}), centrifuge the obtained fermentation culture medium at 4000 r · min^{-1} for 10 min, take the precipitation and freeze-dry to obtain the dried mycelium powder.

Animals and diet

Six-week-old male C57BL/6 mice weighing 20.0 ± 1.0g were provided by the experimental animal center of Qiqihar Medical University (SYXK (HEI) 2016-001). The mice were then randomly divided into the following groups (n = 8 per group): NC group, in which the mice were fed with a standard diet (total calories: 4.3 kcal/g, 10 kcal% fat); HFD group, in which the mice were fed with a high-fat diet (total calories: 6.1 kcal/g, 60 kcal% fat); TSP group, in which the mice were fed with a high-fat diet supplemented with *T. sinense* mycelium (400 mg·kg^{-1}·day^{-1}). Animal weight and food intake were recorded weekly during the study. Fresh feces were collected in a separate sterile EP tube after 10 weeks and stored at − 80°C for subsequent microbiota analysis. The mice were sacrificed after fasting overnight. Liver tissue, fat pad and blood samples were collected. Serum was obtained by centrifugation (1200 g, 15 min) and stored at -80°C for further study. Serological analysis and histology were described in the supplementary data.

TSP treatment for antibiotic-treated mice

The male C57BL/6J mice aged 6 weeks (20.0 ± 2.0g) were fed with the NC-diet and treated with antibiotics (0.5 g/L vancomycin, 1.0 g/L ampicillin, 1 g/L metronidazole, 1 g/L zincomycin sulfate) to establish pseudo germ-free mice, mixed antibiotics diluted daily with distilled water for drinking (20). After 14 days of antibiotics treatment, the microbiota-depleted mice were randomly allocated into three groups, MTNC, MTHFD and MTTSP (n=12/group) which were transplanted with the microbiota from mice fed with NC, HFD, and TSP (treated for 10 weeks) respectively. In detail, every 200 mg of pollution-free feces was added into 5 mL PBS/DTT sterile solution to a 5 mL sterile EP tube, which was shaken and rotated for 2 min in anaerobic state (20). The impurities were removed by 100 μm sterile filter for three times. After 7 days, 4 mice were randomly selected to collect fresh feces to detect colonization by high-throughput sequencing (16SrDNA v3-v4). The results are shown in the supplementary data (Figure S2). Then half of the mice in each microbiota transplanted group fed with the NC diet and the other half fed with HFD for 15 days. Then, blood, tissues and feces were collected for analyses.

Quantitative real-time PCR

Total RNA from hepatic tissue was isolated using Biozol reagent (Invitrogen Carlsbad, CA, USA) by a method previously described (21), and the concentration was determined by NanoDrop spectrophotometer (BioTeke, Beijing, China). cDNA was synthesized using a reverse transcriptase Kit (manufacturer) according to the manufacturer's instructions. SYBR Green real-time (TransGen Biotech, Beijing, China) was used for quantitative PCR in real time. The primer sequences used in this study are listed in Table S1 of the supplementary data. The quantification of the target genes was performed using the $2^{-\triangle\triangle}$Ct method (22) using β-actin as the reference gene and the NC group as control.

Short chain fatty acids analysis

The concentration of short chain fatty acids (SCFAs) was measured by gas chromatography (GC) as previously described with some modification (23, 24). Feces were collected from each rat, 2 g into were placed into a sterile centrifuge tube, and 1 ml methanol solution was added. The tube was left standing for 10 min, then it was shaken and well mixed to form a fecal suspension. Then, a concentrated sulfuric acid was used to adjust its pH to 2 ~ 3, the tube was left standing for 5 min, and then it was shaken and mixed several times. Next, the tube was centrifuged at 5000 r·min^{-1} for 20 min, the supernatant was collected and centrifuged at 5000 r·min^{-1} for 5 min, and the supernatant was collected and placed into the gas chromatograph for the analysis. An Agilent kit was used to determine the content of short chain fatty acids. The gas chromatograph 7890a used in this analysis was equipped with a flame ionization detector. The da-ffap column (30 m × 0.320 mm × 0.25 μ m) was used to separate short chain fatty acids. The parameters of the gas chromatograph were the following: temperature of injection port, 250°C; nitrogen as carrier gas, with purity ≥ 99.99%; carrier gas flow rate, 30 mL·min^{-1} injection mode, split injection; split ratio, 50:1; injection volume, 1 μL; detector temperature 250°C; temperature rise procedure, 80°C, 10°C·min^{-1}, 180°C.

Gut microbiota analysis

Genomic DNA was extracted using the MOBIO PowerSoil® DNA Isolation Kit (MOBIO, UnitedStates), and the concentration was determined by NanoDrop spectrophotometer (BioTeke, Beijing, China). A total of 10 ng DNA template was used for PCR amplification according to the sequence of 16SrDNA v3-v4 region with specific primers 338F/806R. Truseq© DNA PCR-Free

Sample Preparation Kit was used to construct the library. The constructed library was quantified by qubit and qPCR. After the library was qualified, the sequencing was carried out on Illumina Novaseq 6000 platform according to the manufacturer's instruction. The sequencing was completed by Beijing Bio Marker Technology Co., Ltd. Usearch software (25) was used to cluster the reads at 97.0% similarity level and OUT was obtained. SILVA was used as the reference database, using naive Bayesian classifier combined with comparison method to annotate the feature sequence. The species classification information corresponding to each feature was obtained, then the community composition of each sample at each level (phylum, class, order, family, genus, species) was counted, and the species abundance at different classification levels was generated by the QIIME software. Then, the community structure map of each taxonomic level of the sample was drawn by R software (Version 3.4.1). Non-Metric Multi-Dimensional Scaling(NMDS);adopts Bray Curtis algorithm; Lefse (26) (line discriminant analysis (LDA) effect size) was used to find biomarkers with statistical differences between different groups.

Serologic and hepatic index analysis

The concentrations of total triglyceride (TG), cholesterol (CHO), low density lipoprotein (LDL-C), non-esterified fatty acid (NEFA), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) in serum and hepatic carried out in strict accordance with the instructions of the kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histological analysis

The hepatic of mice in each group were dissected and extracted and fixed with 4% paraformaldehyde. After the fixation was in good condition, they were trimmed, dehydrated, embedded, sliced, stained, sealed, sliced, stained with Hematoxylin eosin (HE), and the structure of liver tissue was observed and analyzed under optical microscope, as it was previously described (27).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software. Statistical differences among different groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer *post hoc* test. Other statistical tests for significance were performed using R software (Version 3.4.1) for windows. Results were expressed as mean± SD. A value of $p < 0.05$ was considered statistically significant.

Results

TSP supplementation alleviated HFD-induced weight gain and fat accumulation in mice

Our preliminary animal experiment was performed using TSP at the doses of 100, 200, and 400 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$. The medium dose and high dose exerted a significant prevention of the abnormal lipid metabolism compared to HFD group ($p< 0.05$; Table S2), while the low dose had no significant effect compared to the HFD group. The effect of the high dose was more remarkable than that of the medium dose. Therefore, 400 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ of TSP supplementation was the dose used in this study.

During the 10-week experimental period (Figure 1A), the average body weight of the NC mice group at week 10 was 29.27 ± 2.89 g, and that of the HFD mice group was 40.28 ± 1.73 g ($p < 0.05$ versus the NC group, Figure 1B). The increase in the body weight of the TSP group was significantly suppressed compared with the HFD group ($p< 0.05$). Consistently, the body weight gain, liver weight and adipose tissue weight was lower in the TSP group than that in HFD group (Figures 1C–E). No differences were observed in the daily food intake among the three groups (Figure 1F).

Additionally, HFD induced hepatic fat accumulation and dyslipidemia could also be prevented by TSP supplemented, as indicated by the serum levels of total cholesterol (TC), triglycerides (TG), non-esterified fatty acids (NEFA) and low-density lipoprotein-cholesterol (LDL-C) in the HFD-fed mice sharply increased when compared with the NC group ($p< 0.05$, Figures 2A–D). In addition, the HFD-fed mice were characterized by higher levels of TC, TG, NEFA and total bile acid (TBA) in the liver ($p< 0.05$, Figures 2E–H). TSP supplementation significantly prevented these adverse changes expect TBA in the HFD-fed mice. H&E staining showed less ballooning degeneration in the TSP group than in the HFD group Figure 2I).

To explore the mechanism of TSP in lipid metabolism, we examined the expression of genes related to lipid metabolism in the liver by qRT-PCR (Figure 3). Compared with the NC group, the expression of ACC, HMGCR, LXRα and SREBP-1c was significantly higher and the expression of AMPK and PPARα was significantly lower expression in the HFD group ($p<0.05$). Compared with the HFD group, TSP supplementation significantly decreased ACC, HMGCR, LXRα and SREBP-1c expression in the liver and enhanced AMPK and PPARα expression ($p < 0.05$). TSP supplementation did not affect expression of CD36, CYP7A1, FAS, Ldlγ, LXRβ or PPARγ (Figure S3).

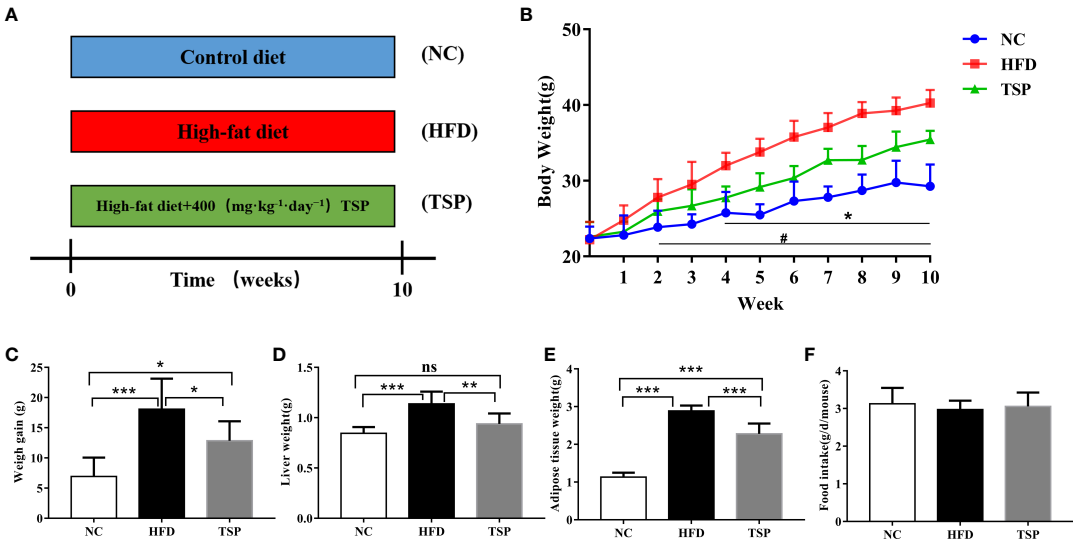

FIGURE 1
Effects of TSP consumption on the **(A)**the experimental protocol used in this study, n=8, **(B)** body weight $^{\#}p < 0.05$, NC compare with HFD, $^{*}p < 0.05$, TSP compare with HFD, **(C)** body weight gain, **(D)** liver weight **(E)** adipose tissue weight, **(F)** food intake. Data are expressed as means ± SD ($n = 8$). $^{\#/*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, ns, no significant $p > 0.05$.

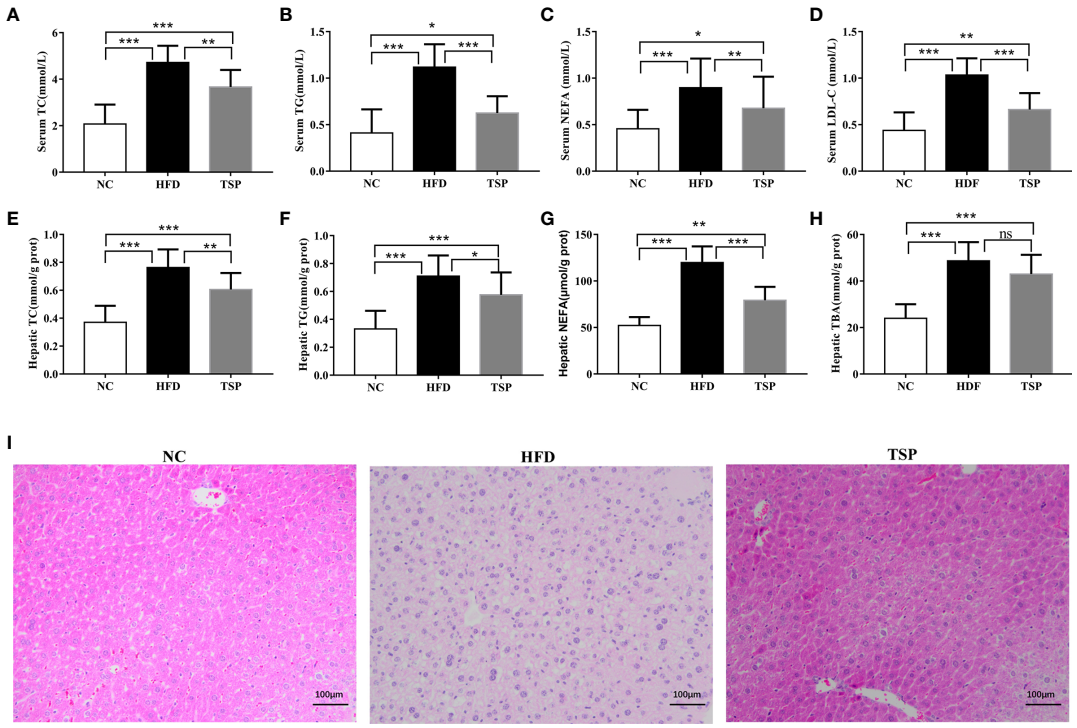

FIGURE 2
Effects of TSP supplementation on the serum and hepatic **(A, E)** total cholesterol (TC), **(B, F)** triglyceride (TG), **(C, G)** non-esterified fatty acid levels (NEFA), **(D)** low-density lipoprotein cholesterol (LDL-C) in serum, **(H)** Hepatic total bile acid (TBA),**(I)** H&E staining of mouse livers. Values are expressed as mean ± SD in each group (n = 8), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, ns, no significant $p > 0.05$.

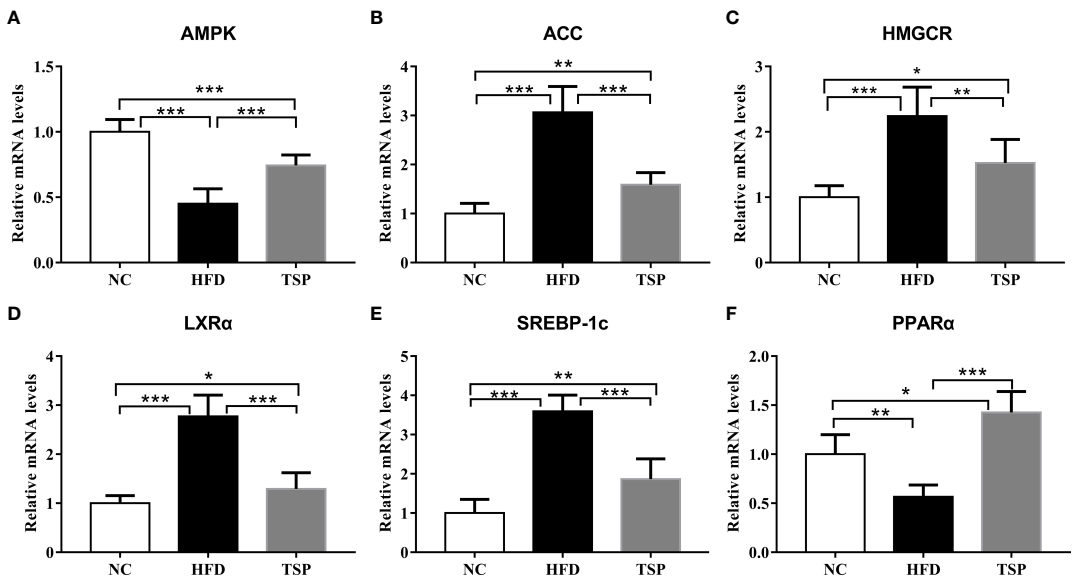

FIGURE 3
Effect of TSP on mRNA expression levels of hepatic metabolic regulators. **(A)** AMPK, **(B)** ACC, **(C)** HMGCR, **(D)** LXRα, **(E)** SREBP-1c, **(F)** PPARα. Data are expressed as means ± SD (n = 8), *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Intake of TSP notably alleviate systematic inflammation and improve antioxidant ability in high fat diet-fed mice

The levels of TNF-α, IL-6 and IL-1β in both serum and liver were higher in the HFD group compared to those in the NC group. The level of serum LPS showed the similar trends (Figures 4A–C). TSP supplementation was able to significantly reduce serum and hepatic TNF-α, IL-6, IL-1β and LPS ($p< 0.05$). Thus, TSP supplementation significantly alleviated systemic inflammation. In addition, the activity of the antioxidative enzymes (SOD, GSH-Px) and MDA level were measured in the serum and hepatic to evaluate the influence of TSP on the antioxidant ability (Figures 4D–I). Compared with the NC group, mice in the HFD group showed higher MDA level in serum, while the GSH-Px activity were lower in HFD group ($p < 0.05$). TSP supplementation was able to significantly reduce serum MDA level and improve SOD and GSH-Px activity ($p< 0.05$). The activity of SOD and MDA level in the liver showed the same tendency. The activity of GSH-Px has no significant differences between HFD and TSP in liver ($p > 0.05$).

TSP supplementation increased short chain fatty acids contents in the feces

Compared with the NC group, the content of acetate, propionate, butyrate, valerate and total short chain fatty acids was decreased by 34.54%, 49.58%, 33.02%, 11.11% and 35.16%, respectively, in the HFD group ($p < 0.05$, Table 1). However, compared with the HFD group, an increased short chain fatty acids level was observed by TSP supplementation in the TSP group ($p < 0.05$).

TSP modulated composition and function of gut microbiota at different taxonomic levels

The gut microbiota composition was analyzed by Illumina MiSeq platform.After quality filtering, the 24 samples (n = 8 for each group) resulted in a total of 1,501,889 clean reads, and at least 58,512 clean reads were generated per sample. Alpha diversity reflected the community richness and microbial evenness. Changes in alpha diversity due to the TSP treatment are shown in Figure 5A. The results showed that the ACE index, Chao 1 index, Shannon index and PD-whole-tree index of the HFD group were significantly lower than those in the NC group ($p < 0.05$), indicating that the HFD induced a lower microbiota community diversity. TSP treatment ineffectively increased microbial richness and diversity. The Beta diversity analysis using UPGMA clustering (Figure S4) and NMDS on the Bray-Curtis algorithm (Figure 5B) showed that the NC group clustered separately from the HFD and TSP groups. The results of PERMANOVA showed a significant difference among NC, HFD, and TSP groups ($p< 0.001$, R^2 = 0.414, Stress=0.1481).

The relative abundance at phylum, family, and genus level was compared among groups to identify specific changes in the

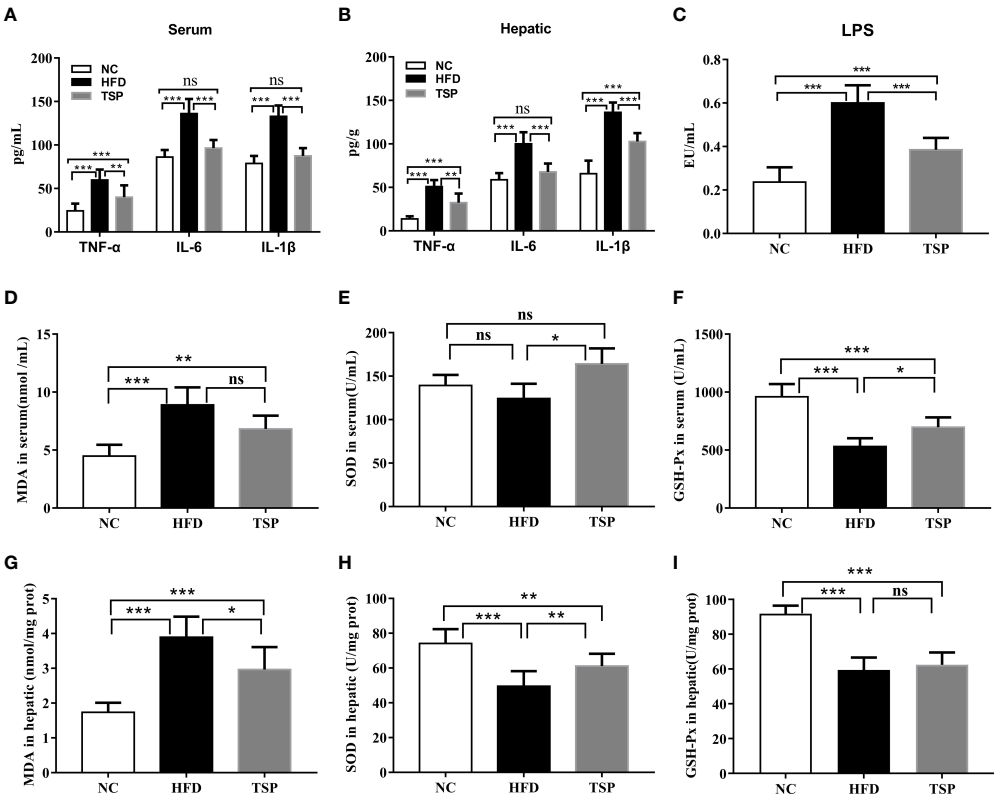

FIGURE 4
Effect of TSP on inflammation in serum **(A)** and in hepatic, **(B)** Effect of TSP on LPS, **(C)** MDA level and antioxidative enzymes (SOD, GSH-Px) in serum, **(D–F)** and in hepatic**(G-I)**. Data are expressed as means ± SD (n = 8), *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, ns, no significant $p > 0.05$.

gut microbiota due to TSP supplementation. At the phylum level (Figure 5C), HFD induced a much lower relative abundance of Bacteroidetes than the NC group, which increased after TSP treatment ($p < 0.05$). The ratio of Firmicutes to Bacteroidetes (F/B ratio) in the HFD group was higher than that in the NC group ($p < 0.05$). TSP supplementation fully prevented HFD-induced increase in the F/B ratio, a hallmark of obesity, which is a common indicator for gut microbiota balance. Besides, the HFD group showed a much higher relative abundance of Patescibacteria than NC group, with no difference in the relative abundance of Proteobacteria between the two groups ($p > 0.05$). While, the relative abundance of Proteobacteria and Patescibacteria significantly decreased in the TSP group compared with HFD alone ($p < 0.01$).

At the family level (Figure 5D and Table S3), compare with the NC group, the HFD group showed an increase in the abundance of Peptostreptococcaceae and Saccharimonadaceae ($p < 0.05$), while TSP supplementation decreased these two genera compared with their abundance in the HFD group. HFD induced a much lower abundance of Lactobacillaceae, Muribaculaceae and Prevotellaceae and the three genera significantly increased after TSP supplementation ($p < 0.05$). The mice in the TSP group showed a lower Ruminococcaceae and Clostridiaceae_1 compared with the mice in the HFD group ($p< 0.05$).

TABLE 1 Effect of TSP supplementation on the concentrations of acetate, propionate, butyrate, valerate and total SCFAs in the feces.

SCFAs(μmol/g)	NC	HFD	TSP
Acetate	37.69 ± 4.45a	24.67 ± 4.76b	40.21 ± 5.77a
Propionate	2.38 ± 0.31a	1.20 ± 0.43b	2.56 ± 0.22a
Butyrate	3.21 ± 0.74a	2.15 ± 0.50b	4.29 ± 0.84c
Valerate	0.18 ± 0.04a	0.16 ± 0.04b	0.20 ± 0.05a
Total SCFAs	43.46 ± 4.29a	28.18 ± 4.81b	47.26 ± 6.21a

Significant differences ($p < 0.05$) are indicated using different letters (a, b, c)

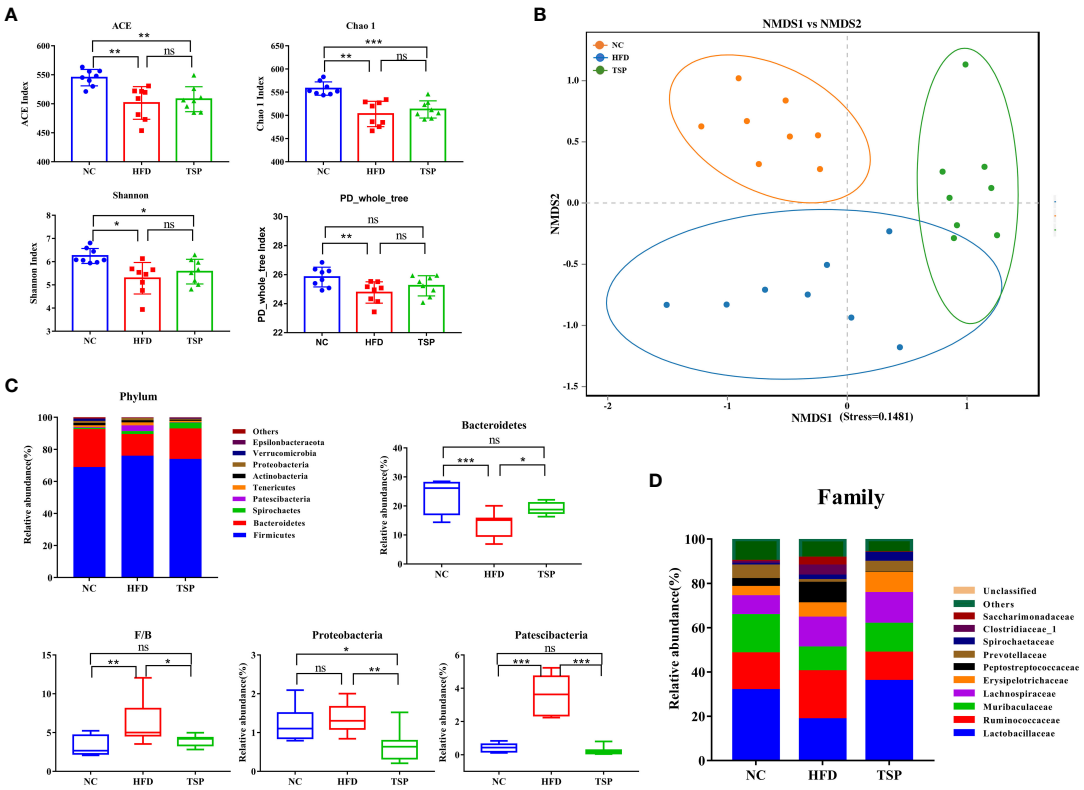

FIGURE 5
Effect of TSP supplementation on diversity and structure of the gut microbiota. **(A)** Alpha diversity analysis of ACE, Chao1, Shannon and PD-whole-tree index **(B)** Non-metric multidimensional scaling (NMDS) result based on Bray Curtis algorithm. **(C)** significantly changes ($p < 0.05$) of the composition of the gut microbiota at phylum taxa level. **(D)** Changes of the composition of the gut microbiota at family taxa level. Data are expressed as mean ± SD, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, ns, no significant $p > 0.05$.

LEfSe (LDA sore > 3.5) was used to recognize the specific altered bacterial phenotypes at each phylogenetic level (Figures 6A, B) to further explore the difference in the gut microbiota among NC, HFD and TSP group. A total of 61 bacteria significantly changed among the NC group, HFD group and TSP group; they respectively showed 15, 33 and 13 dominant microorganisms. At the genus level, the main microbiota in the NC group were *Prevotellaceae_NK3B31_group*, *Alloprevotella* and *Faecalibaculum*. The result showed eleven discriminative features in the HFD group, and the main microbiota were *Romboutsia*, *Ruminococcaceae_UCG-014*, *Clostridium_sensu_stricto_1*, *Candidatus_Saccharimonas* and *Lachnospiraceae_NK4A136_group*. Moreover, *Lactobacillus*, *Allobaculum*, *uncultured_bacterium_f_Lachnospiraceae* and *Prevotella_9* were the main microbiota in the TSP group.

At the genus level, the microbiota with significant differences between groups were screened using Mann Whitney U test by pairwise comparison (Figure 6C). Collectively, the HFD group showed an increased level of *Ruminococcaceae_UCG-014*, *Romboutsia*, *Lachnospiraceae_NK4A136_group*, *Candidatus_Saccharimonas* and *uncultured_bacterium_f_Ruminococcaceae* compared with the NC group ($p < 0.05$). Therefore, TSP supplementation reduced the abundance of the above gut microbiota except *Lachnospiraceae_NK4A136_group* compared with HFD ($p < 0.05$), and TSP also effectively increased the relative abundance of *Lactabacillus*, *Prevotella_9* and *Allobaculum* which have a much lower abundance by HFD induced ($p < 0.05$).

Effects of TSP supplementation on the functional change of microbial communities

PICRUSt analysis was carried out to explore the functional change of microbiota communities, and the comparison of top 6 metabolism category in each group is shown in Figure 7A. Compared with the NC group, HFD group decreased the carbohydrate metabolism, lipid metabolism and energy metabolism ($p< 0.05$), while TSP supplementation increased

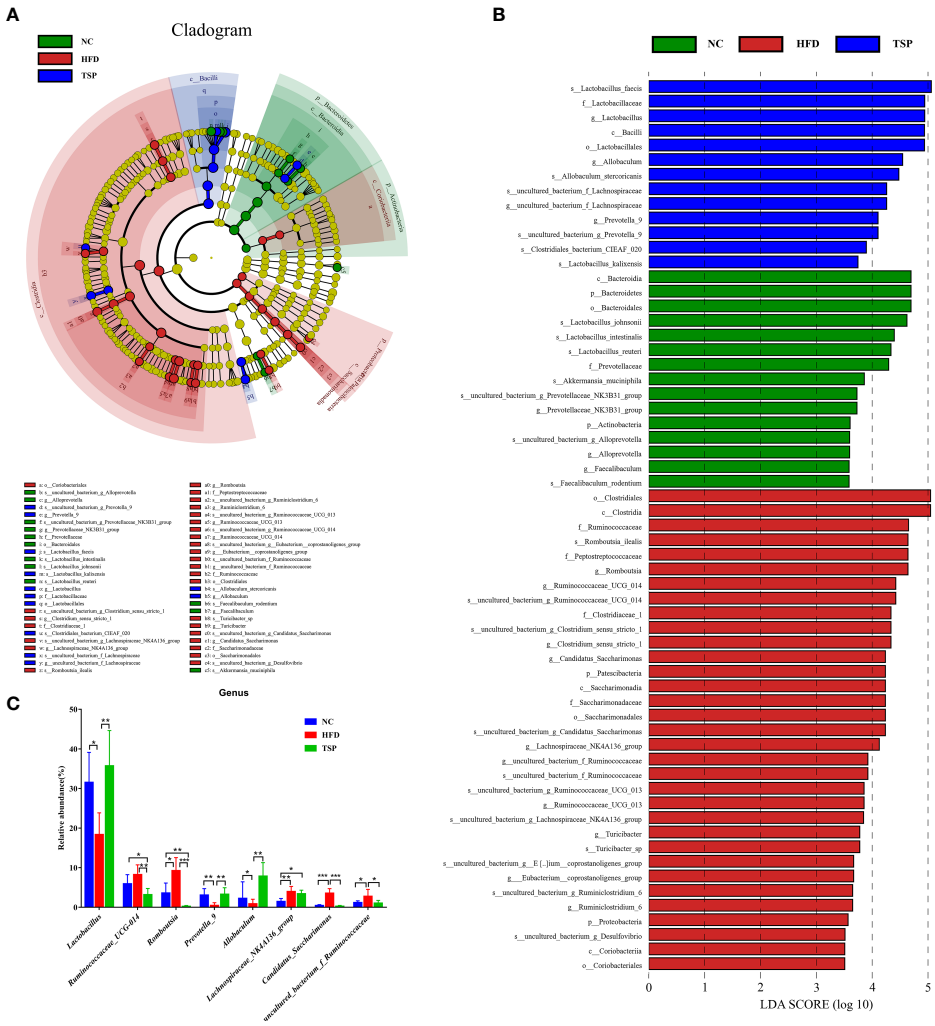

FIGURE 6
TSP supplementation induced gut microbial changes in mice. **(A)** Linear Discriminant Analysis Effect Size (LEfSe) analysis of key genera of gut microbiota in mice, **(B)** and the LDA score, **(C)** significantly changes ($p < 0.05$) among top 15 taxa of the composition of the gut microbiota at genus taxa level, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

the proportion of carbohydrate metabolism, lipid metabolism, cofactors and vitamin metabolism and energy metabolism compared with the HFD group ($p< 0.05$).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway showed a significant difference ($p< 0.05$) in the predictive function between the HFD group and TSP group (Figure 7B). Compare with HFD, carbohydrate metabolism such as amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, fructose and mannose metabolism and galactose metabolism were significantly increased in the TSP mice ($p<0.05$). Lipid metabolism such as fatty acid biosynthesis and degradation, glycerophospholipid metabolism as well as biosynthesis of unsaturated fatty acids was increased in the TSP group ($p< 0.05$). Moreover, the amino acid metabolism pathway such as alanine, aspartate and glutamate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, arginine biosynthesis, valine, leucine and isoleucine biosynthesis and arginine, proline metabolism and histidine metabolism were decreased in the TSP group ($p < 0.05$). Only two functions of the gut microbiota in the amino acid metabolism pathway were increased in the TSP group than in the HFD group ($p< 0.05$). The functions related to cofactors and vitamin metabolism were increased in the TSP group, mainly involving porphyrin and chlorophyll metabolism, thiamine metabolism and folate biosynthesis than in the HFD group ($p< 0.05$). It is worth noting that the lipopolysaccharide biosynthesis belonging to glycan biosynthesis was decreased in TSP mice compared with HFD mice ($p< 0.05$).

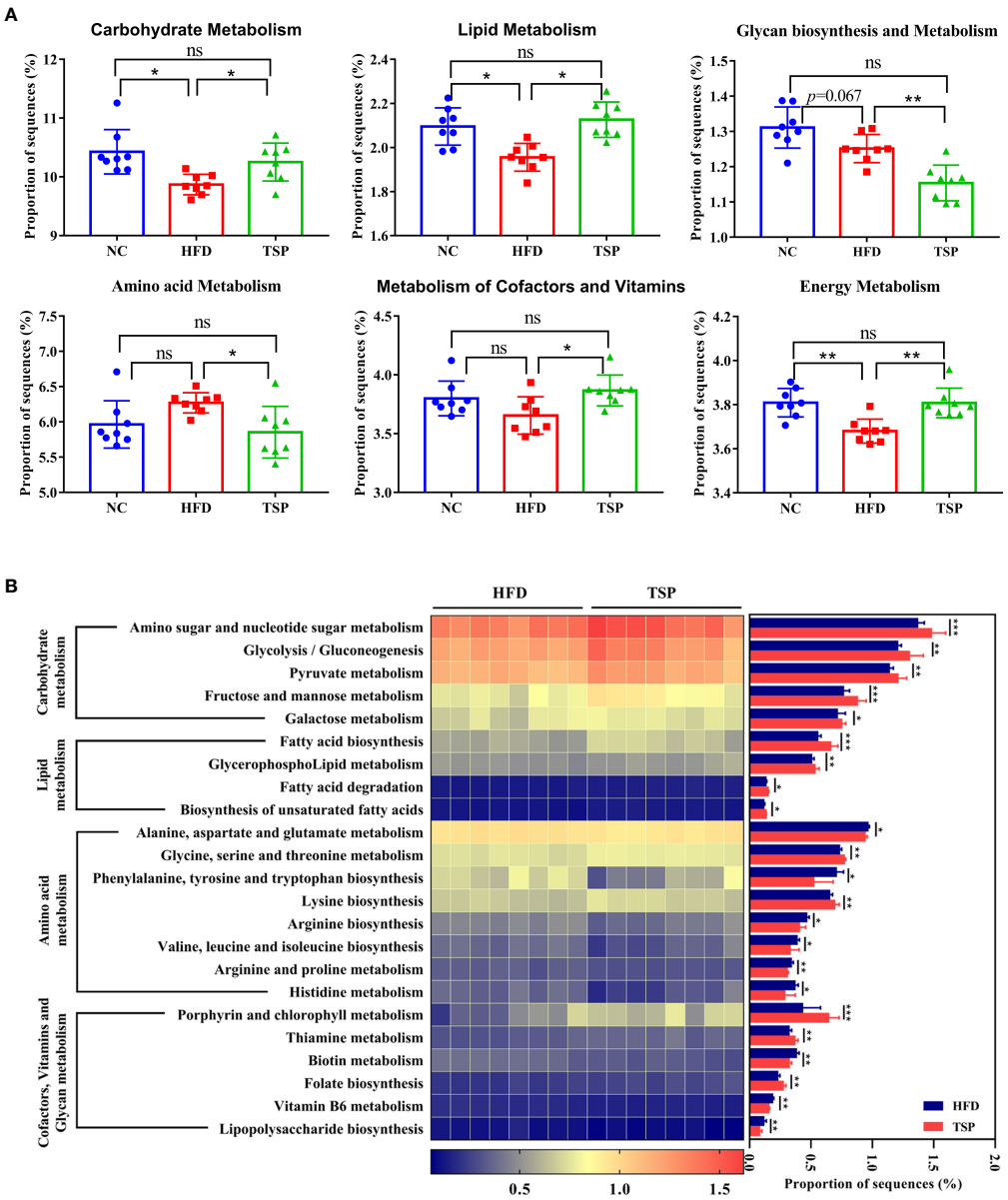

FIGURE 7
TSP supplementation induced function microbial changes in mice. **(A)** Abundances of top 6 KEGG pathways in level-2 of the functional prediction by PICRUSt, **(B)** Functional profiles with significant different between HFD and TSP treated groups. n = 8, *p < 0.05, **p < 0.01, and ***p < 0.001, ns, no significant p > 0.05.

Possible relationships between reshaped gut microbiotas and biochemical changes

The Spearman's correlation analysis revealed between the abundance of significantly differential bacteria at genus level identified above and parameters associated with obesity (Figure 8). We found that *Prevotella_9* and *Lactococcus* both showed a significant negatively correlated with parameters of lipid metabolism except body weight gain, liver weight, NEFA, IL-1β in serum and TC, NEFA, HMGCR expression in liver, and significant positive correlation with the expression of PPARα, AMPK. *Allobaculum* has the same correlation trend. *Candidatus_Saccharimonas* and *Romboutisia* both showed a significant positive correlation with parameters of lipid metabolism except TC, IL-1β

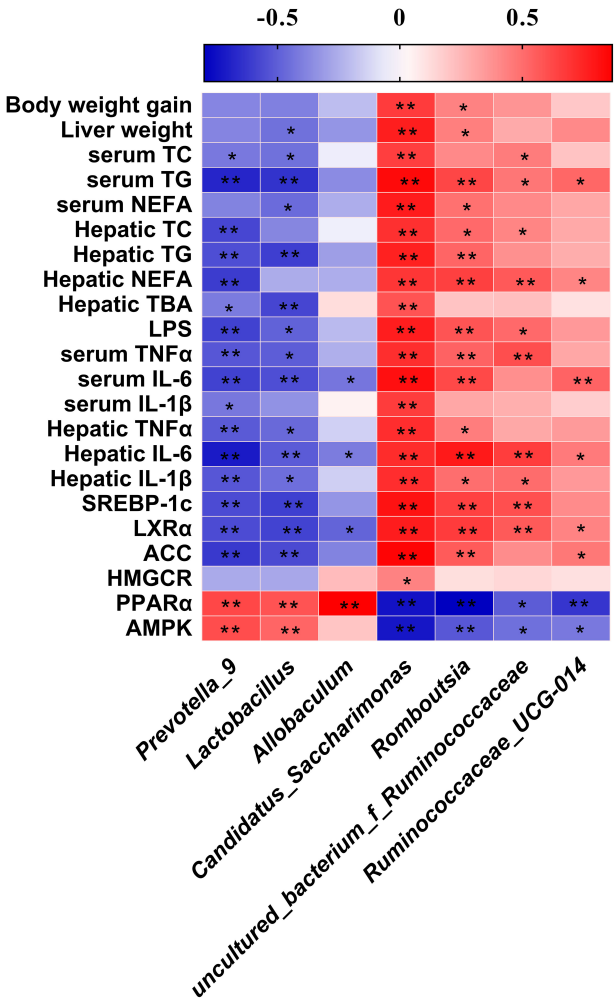

FIGURE 8
Heatmap of Spearman's correlation between the gut microbiota and obesity-related indices. The intensity of the colors represented the degree of association (red, positive correlation; blue, negative correlation). Significant correlations are marked by *$p < 0.05$; **$p < 0.01$.

inserum and TBA, HMGCR expression in liver. The genus *uncultured_bacterium_f_Ruminococcaceae* and *Ruminococcaceae_UCG-014* had the same correlation trend.

Microbiota transplantation from TSP-supplementation mice exerts an anti-obese Effect in HFD-fed mice

The fecal bacteria from NC-, HFD-, or TSP-fed mice (ten weeks) were transplanted to pseudo germ-free mice to explore whether TSP supplementation could attenuate hyperlipidemia in HFD-diet mice by altering gut microbiota (Figure 9). As revealed in Figure 9A, after microbiota transplantation, the weight of body, liver and adipose tissue and the indexes of lipid metabolism in serum (TC, TG, NEFA, and LDL) in mice fed with NC-diet have no significant different ($p > 0.05$).

It had different results in microbiota-transplanted mice fed with HFD (Figure 9B). Compared with MTHFD group, the weight of body and adipose tissue in MTNC and MTTSP mice was significantly decreased ($p < 0.05$). Furthermore, MTNC group mice significantly reduced the serum content of TG and LDL compared to MTHFD group. However, there was no significant different in the liver weight and serum levels of TC and NEFA among the three groups ($p > 0.05$). The detailed experimental scheme is shown in Figure 9C.

Then, the gut microbiota phylotypes of microbiota-transplanted mice fed with HFD-diet were further measured by sequencing the bacterial 16S rRNA. As revealed in Figure 10A, no significant different was observed in alpha

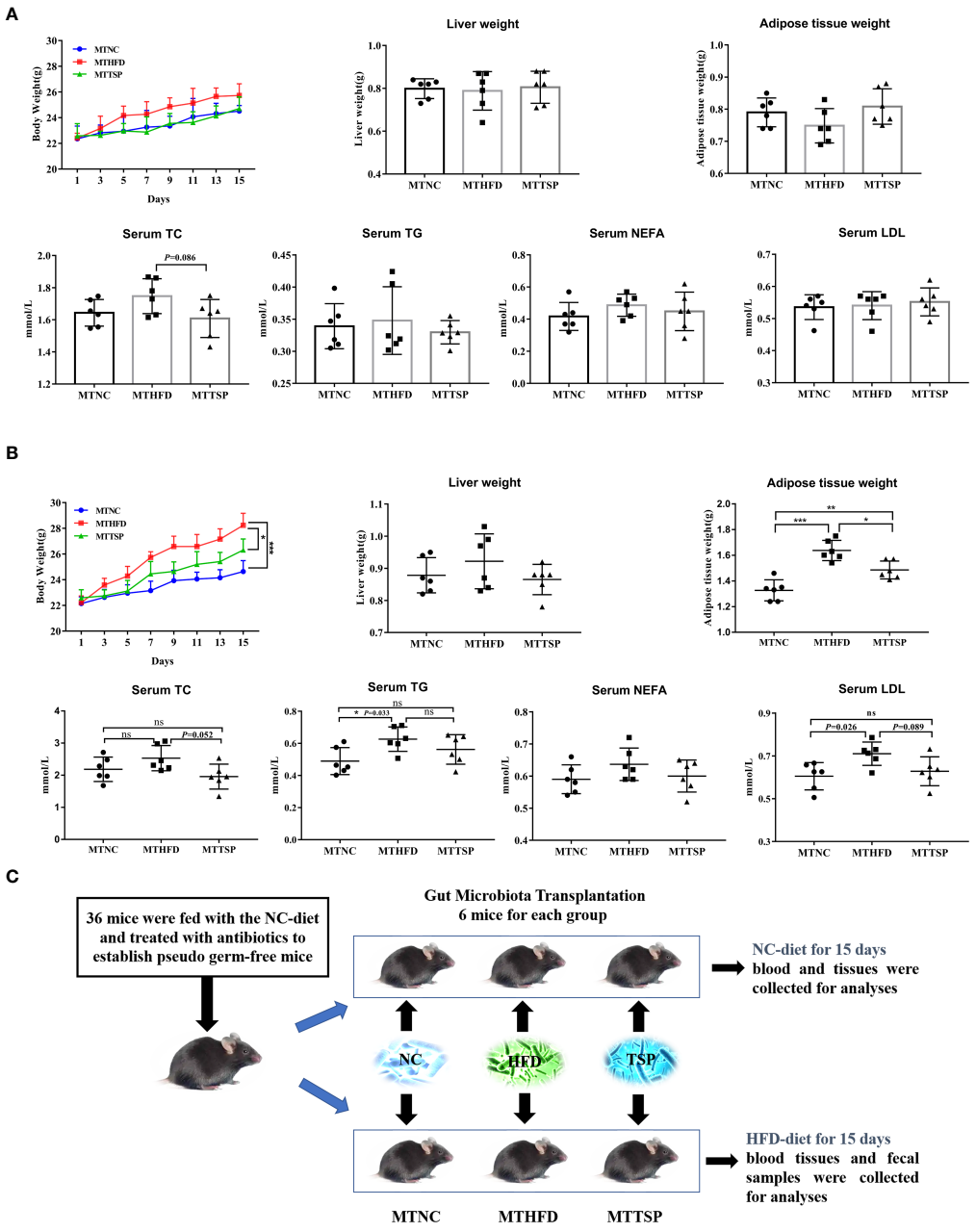

FIGURE 9
Microbiota Transplantation from TSP- supplementation Mice Exerts an Anti-Obese Effect in HFD-Fed Mice. **(A)** Microbiota-transplanted mice fed with NC-diet; **(B)** microbiota-transplanted mice fed with HFD-diet; **(C)** The experiment design of microbiota transplantation. Data are presented as mean ± SEM, differences were denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no significant $p > 0.05$.

diversity among all groups. Furthermore, we analyzed β diversity which indicate the gut microbiota structural changes by using the NMDS on the Bray-Curtis algorithm (Figure 10B) showed significant different among the three groups ($p< 0.01$, $R^2 = 0.26$, Stress=0.1576). At the phylum level, the alterations in the relative abundances of Bacteroidetes and Patescibacteria in MTHFD mice showed the same trends as it of HFD-fed mice (Figure 10C). Meanwhile, the relative abundance of Peptostreptococcaceae at the family level in MTNC and MTTSP mice tended to decrease relative to the MTHFD mice ($p < 0.05$), whereas the relative abundance of Saccharimonadaceae in MTTSP group significantly decreased compared with MTNC and MTHFD group ($p < 0.05$, Figure 10D). Additionally, the relative abundance of

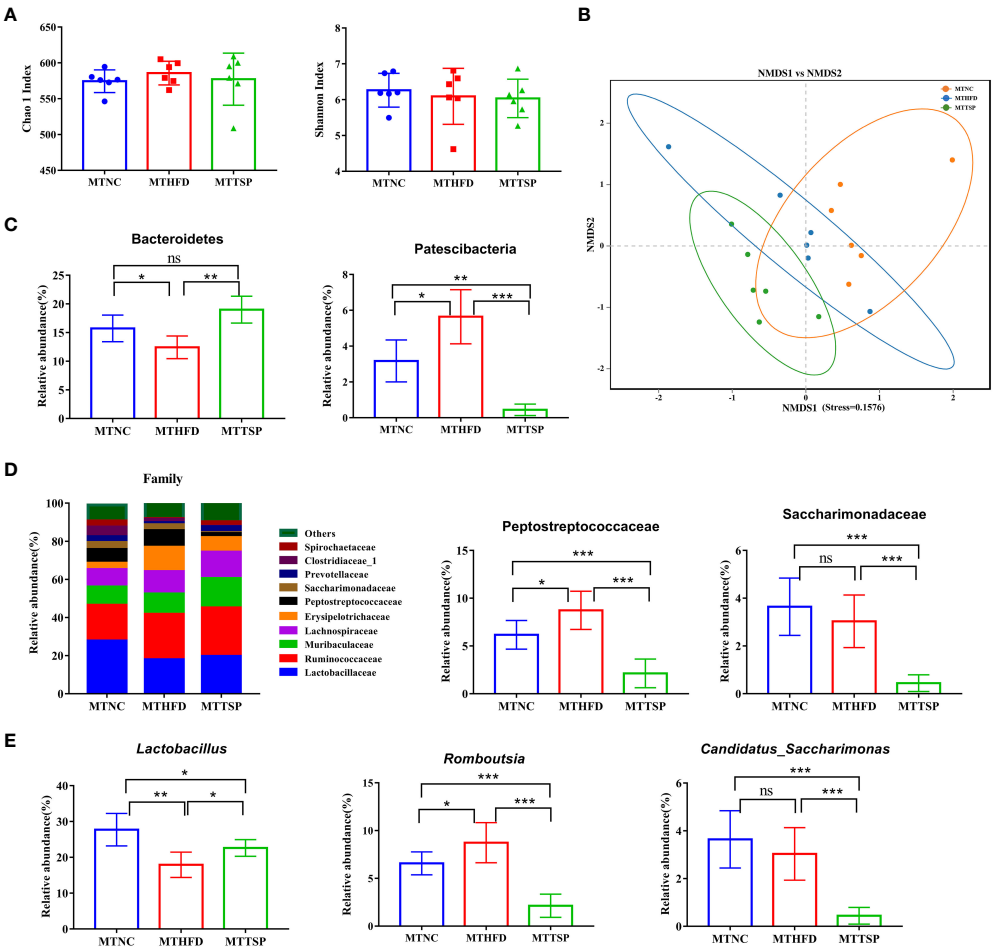

FIGURE 10
Gut microbiota in response to microbiota transplantation from NC (MTNC), HFD (MTHFD), and TSP (MTTSP) groups (n = 6), **(A)** Indexes of Chao 1 and Shannon in α-diversity analysis, **(B)** NMDS plot analysis from each sample, **(C)** significantly changes of the composition of the gut microbiota at phylum taxa level ($p < 0.05$), **(D)** microbiota compositions at the family level, and **(E)** significantly changes of the composition of the gut microbiota at the genus level ($p < 0.05$). Data are expressed as mean ± SD, *p **p ***p/span>, ns, no significant, $p > 0.05$.

Lactabacillus and *Romboutsia* at the genus level was significantly changed among the three groups ($p< 0.05$, Figure 10E), and they also showed the same trends as it of HFD-fed mice. The relative abundance of *Candidatus_Saccharimonas* in MTTSP group was significantly reduced compared with MTNC and MTHFD group ($p < 0.01$). Overall, these data indicate that mechanism of TSP to inhibit the occurrence of lipid metabolism disorder and obesity may be realized by regulating intestinal microbiome.

Discussion

Tolypocldium sinenis is an entomogenous fungus isolated from the mycelial tissue of the sclerotia and cotyledon of Chinese caterpillar fungus. Some gene sequencing results of TSP are compared with the gene sequencing results of *Cordyceps sinensis* (accession number AF291749) in the gene database, and the similarity is 99% (28). Relevant studies showed that TSP and *C. sinensis* have basically the same pharmacological effects, indicating that TSP has a potential pharmacological value. At present, some reports on the bacteriostatic, anti-inflammatory, antioxidative stress and anti-tumor effects of TSP are available, but the effect of TSP on preventing obesity and on the change of the intestinal microbiota has not been studied. The regulation of the composition of the gut microbiota is a promising approach to prevent the development of obesity and related metabolic disorders. This study was the first showing that the dietary supplementation TSP prevented HFD-induced obesity and hyperlipemia. The potential mechanism could reduce systemic inflammation and by regulating the composition and potential function of the gut microbiota.

As a global epidemic, obesity increases the risk of a variety of chronic diseases, reduces life expectancy and brings a serious personal and socio-economic burden (29–31). Different theoretical explanations in obesity research are available. One of the important reasons is the change of people's diet, since the high-fat diet is an important factor in obesity. The excessive weight gain leads to the abnormal increase in blood lipid and blood glucose levels (32). Our results also revealed that HFD feeding promoted an evident increase in body weight, liver weight, serum lipid levels, and fat vacuoles in hepatocytes in mice when compared with these parameters in the NC group, which was in agreement with previously published reports (33, 34). TSP supplementation reduced the accumulation of abdominal adipose tissue induced by HFD and effectively prevented the increase in body weight and liver weight in mice. Moreover, TSP decreased the levels of TC, TG and NFFA in serum and liver, and prevented the increase of serum LDL-C and TBA in liver, indicating its potential preventive effect on the development of fatty liver disease induced by HFD. However, our results revealed that food consumption and energy intake of mice supplemented with TSP were similar to those in the HFD group, indicating that the role of TSP in preventing obesity was not related to the reduction of appetite.

The genes related to liver lipid/cholesterol synthesis and metabolism were measured by qRT-PCR to further clarify how TSP supplementation prevented HFD feeding-induced liver lipid metabolism disorder. The results revealed that TSP supplement down-regulated the expression of ACC, HMGCR, LXRα and SREBP-1c, which involved in hepatic lipid/cholesterol synthesis and metabolism (35), and up-regulated the expression of AMPK and PPARα, that involved in fatty acid oxidation (36, 37), compared with their expression in the HFD group. HMGCR catalyzes 3-hydroxyl in the process of cholesterol synthesis 3-methylglutaryl CoA is the rate limiting enzyme for the conversion of 3-methylglutaryl CoA to mevalonate (37). Therefore, the inhibition of the activity of HMGCR in the liver reduces the synthesis of cholesterol in the body, thus regulating the disorder of lipid metabolism. SREBPs promotes the regulation of cholesterol and adipose formation by a strict transcription and post-translational regulation. SREBP1c is an important subtype of SREBPs that positively regulates cholesterol and fatty acid syntheses and uptake in the hepatocytes (38). PPARα is one of the PPAR family proteins, and play an important role in lipid metabolism, glucose homeostasis and anti-inflammatory effects, and upregulation the mRNA level of PPARα expression could promote fatty acid catabolism and reduce fat mass (7, 39, 40). PPARα enhances the antioxidant function of hepatocytes by regulating the levels of SOD, ALT and AST (41). In addition, PPARα reduces serum cholesterol and LDL levels by regulating cholesterol 7A-hydroxylase, sterol 12a-hydroxylase, increases the level of high-density lipoprotein (HDL), hydrolyzes very low-density lipoprotein (VLDL), and delays the progression of coronary atherosclerosis (42). Thus, TSP supplementation might partially contribute to the regulation of genes of the lipid metabolism involved genes in liver, preventing adipose tissue deposition and improved hyperlipidemia in HFD mice.

Studies showed that chronic obesity is closely related to low-grade inflammation. Obesity-induced inflammation is called metabolic inflammation, which is different from the classical inflammation because the metabolic inflammation belongs to the chronic and low-grade inflammation (43). Mice fed with long-term HFD have hyperglycemia, hyperlipidemia, as well as increased systemic chronic inflammation and proinflammatory factors (44–46). Our results showed that HFD feeding promoted the occurrence of inflammation in the serum and liver tissue, and the daily supplement of TSP effectively inhibited the inflammatory factors (TNF-α, IL-6, and IL-1β). Some studies reported that oxidative stress is a part of the inflammatory response, which activates the cascade of inflammatory signals to promote the occurrence of inflammation. Furthermore, the oxygen free radicals produced by inflammatory activated immune cells further aggravate the oxidative stress response (47). Obese people are often accompanied by impaired mitochondrial function, such as decreased mitochondrial density and ATP synthesis (48). It is reported that mitochondrial dysfunction precedes hepatic steatosis and insulin resistance in obese rodent model (49),. The increased level of circulating fatty acids in obese individuals leads to the excessive accumulation of lipids in cells, the damage of the mitochondrial function and the increase in the content of ROS. Excessive ROS not only damages the ability of mitochondrial ATP synthesis and oxidative phosphorylation, but also interferes with the replication of mitochondrial DNA and RNA, affecting the structure and function of mitochondria, and leading to mitochondrial dysfunction and further increase in the production of ROS (50). Relevant studies confirmed that the mycelial extract and mycelial polysaccharide of TSP have sedative, anti-inflammatory and antioxidant effects (16, 18, 51), which might be due to the effect of cyclosporin A rich in TSP. Cyclosporin A is widely used as an immunosuppressant to avoid rejection of organs after transplantation, and has a certain antifungal and anti-inflammatory effects (52, 53). Our study found that TSP supplementation effectively inhibited the increase of MDA level in the serum and liver caused by HFD, as well as it significantly increased the SOD and GSH-Px activity.

Different dietary structure can modify and change the composition and structure of the intestinal flora, so as to change the physiological metabolism of the host. As a bioreactor of human food, the intestinal microorganisms are related to many physiological effects and diseases of the host, especially obesity (54, 55). More and more studies showed that intestinal microorganisms can affect host metabolism through immune, endocrine and intestinal brain axis (56–58), so as to

regulate human energy absorption, lipid metabolism and inflammatory response. For example, the gut microbiota metabolizes complex carbohydrates and plant polysaccharides to produce short chain fatty acids, which are an important substrate providing energy for the human body and microorganisms (59). It mainly includes acetic acid, butyric acid, and propionic acid. In addition to being a direct energy supplier, short chain fatty acids also play a role in metabolic regulation after entering the tissues and organs by blood circulation, working as signal molecules by stimulating the release of saturated hormone peptides (60) and glucagon peptides (61, 62). The intestine by activating nutrient receptors to reduce physical inflammation and brain signal transmission. Previous studies showed that dietary short chain fatty acid supplementation is effective in preventing obesity and dyslipidemia in HFD-fed mice (63, 64). TSP supplementation partially restored the HFD-induced decrease in SCFAs, especially the content of butyrate was significantly higher than that of the blank control, while acetate and propionate went back to normal levels. Butyric acid strengthens the intestinal barrier by affecting the length of small intestinal villi and mucosal thickening to control the occurrence of metabolic diseases (65).

Since the gut microbiota serves as a pivotal mediator in the regulation of host energy absorption, appetite and consumption (66), our study also found that the alpha diversity of gut microbiota was significantly lower in the HFD group than that in the NC group. Generally, dietary supplementation or weight loss contribute to the recovery of gut microbial diversity (67). Our results showed that although TSP supplementation inhibited weight gain, it did not restore gut microbial diversity, which might be related to its pharmacological properties. The efficacy of TSP is associated to immune regulation, anti-tumor and antioxidant effect, as well as bacteriostasis (68). Therefore, TSP supplementation might inhibit some non-probiotics and reduce the gut microbial diversity. Our study demonstrated that TSP supplementation could alter the gut microbiota structure and composition revealed by NMDS and hierarchical cluster analysis. TSP supplementation did not affect the relative abundance of Firmicutes, but it significantly enhanced the relative abundance of Bacteroidetes, thus significantly decreasing the F/B ratio. The higher F/B ratio in the intestines of obese individuals could promote obesity of the host by absorbing energy from food (69). Wu et al (70). suggested that reducing the HFD-induced increase of the F/B ratio together with the reduction of the inflammatory markers IL-2, IL-6 and TNF-a in mice serum could be obtained by promoting the growth of Bacteroidetes. TSP also significantly inhibited the proliferation of Proteobacteria and Patescibacteria. Studies suggested that the proportion of Bacteroidetes in the gut microbiota of NAFLD patients was lower than non-obese people, accompanied by an increase in Actinomycetes, Firmicutes and Proteobacteria and the abnormal increase of Proteobacteria in gut microbiota reflects the imbalance of microecology or the instability of gut microbiota structure (71, 72). The specific families reported as increased by HFD include Lactobacillaceae, Ruminococcaceae, Lachnospiraceae and Clostridiaceae (70). Our results showed that daily supplementation of TSP effectively and significantly reduced the relative abundance Ruminococcaceae and Clostridiaceae compared with the HFD mice. On the contrary, TSP mice showed a higher and significant relative abundance of Lactobacillaceae than HFD mice. *Lactobacillus* is traditional probiotics that play an important role in the balance of the human intestinal microecology. Some studies reported that the increase in *Lactobacillus* effectively reduces serum cholesterol level and body fat (73), and it also prevents chronic inflammation and the worsening of insulin resistance and are recognized as beneficial bacteria (74, 75). The correlation analysis showed that *Lactobacillus* was significantly negatively correlated with inflammatory factors and lipid metabolism in our study. The comprehensive analysis revealed that the HFD group showed a higher abundant of *Romboutsia* and *Candidatus_Saccharimonas* which positively correlated with obesity and obesity-related physiological markers. However, TSP group mice had significantly lower abundance of bacteria linked to obesity than HFD-fed mice. *Romboutsia* are linked with obese like features and they are highly abundant in obese mice (76–78). The spearman correlation analysis also found that *Romboutsia* were positively correlated with serum and liver inflammatory factors in our study. In some ways, TSP supplementation also effectively increased the relative abundance of the two species *Prevotella_9* and *Allobaculum*. The abundance of genera *Prevotella* is associated with carbohydrate intake (79). A recent study further revealed that individuals with a high abundance of *Prevotella* were more likely to lose weight than those with *Bacteroides*, when these individuals go on a diet (80). To summarize our discovery, our work demonstrated that the beneficial bacteria that protects the body increased after TSP supplementation and it protects the balance of the gut microbiota from being destroyed by high-fat diet, inhibiting the growth of bacteria that promote obesity, and improving the relative abundance of beneficial bacteria.

The change of microbial composition is always accompanied with significant functional alteration and changes in the gut microbiota that inevitably lead to changes in the host metabolism (81, 82). Therefore, in this study, the functional abilities of the microbial communities were analyzed by PICRUSt. The reduced lipid accumulation in the liver of TSP mice might be caused by the functions related to carbohydrate and lipid metabolism in microbiota. TSP effectively enhanced the carbohydrate and lipid metabolism ability, suggesting that the microbiome in TSP mice might consume more dietary lipid and more carbohydrates. These findings are also consistent with the more profound downregulation of lipogenic gene expressions by TSP treatment. Combining with the existing results, our hypothesis was that the effects of TSP on

attenuating HFD-induced lipid metabolism disorders were mediated, at least in part, by the modulation of the gut microbiota. We confirmed this hypothesis with the fecal bacteria transplant experiment, that gut microbiota might be required for TSP to carry out its anti-obese and prevent hyperlipidemia effects on HFD-fed mice.

Conclusion

In summary, TSP supplementation confers protective effects against HFD-induced obese and hyperlipidemia by altering the gut microbiota, alleviate the inflammatory response and regulating gene expression of liver lipid metabolism. It raises the possibility that TSP poses great therapeutic potential in treating obesity and its complications. Therefore, the development of TSP act as daily health food has a good prospect.

Data availability statement

The datasets presented in this study can be found in National Microbiology Data Center (https://nmdc.cn/), with the Accession number: NMDCX0000149

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of the Qiqihar Medical University

Author contributions

CZ, XW, HY and XC designed the experiments. XW, and MB performed the animal experiments. XW and LL conducted gut microbiota analysis. XW, LL, JZ, YG, and XS measured biochemical data and conducted immunohistochemical analysis. XW and LL wrote the draft of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the Qiqihar Medical University, grant number QMSI2017B-06, QMSI2017B-13 and QMSI2019L-27, by Natural Science Foundation of Heilongjiang Province, grant number LH2020H129, by Health commission of Heilongjiang Province, grant number 20210101060181 and by Graduate Innovation Fund of Qiqihar Medical University, grant number QYYCX2022-05.

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/fimmu.2022.977528/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 10 November 2022
DOI 10.3389/fmicb.2022.1050271

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

RECEIVED 21 September 2022
ACCEPTED 25 October 2022
PUBLISHED 10 November 2022

CITATION
Fonseca S, Carvalho AL, Miquel-Clopés A, Jones EJ, Juodeikis R, Stentz R and Carding SR (2022) Extracellular vesicles produced by the human gut commensal bacterium *Bacteroides thetaiotaomicron* elicit anti-inflammatory responses from innate immune cells.
Front. Microbiol. 13:1050271.
doi: 10.3389/fmicb.2022.1050271

Extracellular vesicles produced by the human gut commensal bacterium *Bacteroides thetaiotaomicron* elicit anti-inflammatory responses from innate immune cells

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Bacterial extracellular vesicles (BEVs) produced by gut commensal bacteria have been proposed to play an important role in maintaining host homeostasis *via* interactions with the immune system. Details of the mediators and pathways of BEV-immune cell interactions are however incomplete. In this study, we provide evidence for the anti-inflammatory and immunomodulatory properties of extracellular vesicles produced by the prominent human gut commensal bacterium *Bacteroides thetaiotaomicron* (Bt BEVs) and identify the molecular mechanisms underlying their interaction with innate immune cells. Administration of Bt BEVs to mice treated with colitis-inducing dextran sodium sulfate (DSS) ameliorates the symptoms of intestinal inflammation, improving survival rate and reducing weight loss and disease activity index scores, in association with upregulation of IL-10 production in colonic tissue and in splenocytes. Pre-treatment (conditioning) of murine bone marrow derived monocytes (BMDM) with Bt BEVs resulted in higher ratio of IL-10/TNFα production after an LPS challenge when compared to LPS pre-conditioned or non-conditioned BMDM. Using the THP-1 monocytic cell line the interactions between Bt BEVs and monocytes/macrophages were shown to be mediated primarily by TLR2. Histone (H3K4me1) methylation analysis showed that Bt BEVs induced epigenetic reprogramming which persisted after infectious challenge, as revealed by increased levels of H3K4me1 in Bt BEV-conditioned LPS-challenged BMDM. Collectively, our findings highlight the important role of Bt BEVs in maintaining host immune homeostasis and raise the promising possibility of considering their use in immune therapies.

KEYWORDS

extracellular vesicles, Bacteroides, anti-inflammatory response, innate immune tolerance, BMDM, THP-1 cells, TLR2, IL-10

Introduction

The ecosystem of the human gastrointestinal tract (GIT) is shaped by complex interactions between resident microbes (the microbiota), the epithelium and immune cells. Host–microbe interactions have traditionally been analyzed from the perspective of pathogenic relationships, but it has become evident that commensal microbes also exert important beneficial effects on the host. The ability of immune cells to discriminate between pathogens and commensal bacteria is therefore essential to maintain immune homeostasis and preserve host health, by simultaneously providing protection against pathogens and tolerance toward symbiotic microbiota (Bron et al., 2011).

Innate immunity plays an important role in intestinal protection and is the first line of host defense against infection comprising physical, chemical, and cellular barriers. Various stimuli and conserved microbe-associated molecular pattern (MAMPs) molecules can activate and modulate innate immunity and inflammatory responses with enhanced or decreased production of pro-inflammatory mediators and cytokines depending on the type and dose of the ligand recognized by individual pattern recognition receptors (PPRs; Ifrim et al., 2014). Bacterial lipopolysaccharides (LPS), one of the major triggers of inflammatory response *via* interactions with toll-like receptor 4 (TLR4), can induce a state of tolerance in macrophages and monocytes after repeated or prolonged exposure, resulting in reduced pro-inflammatory cytokine production (Novakovic et al., 2016; Seeley and Ghosh, 2017). The innate immune system can be also activated by sterile endogenous dietary substances from Western-type diets that can contribute to various chronic inflammatory diseases (Bekkering et al., 2014; Christ and Latz, 2019). Innate immune activation leads to modifications in the chromatin state of the innate immune cells, with epigenetic changes persisting even after the cells return to homeostasis, altering their long-term responsiveness to re-infection (Netea et al., 2020). Enhanced inflammatory responses in trained innate immune cells and diminished activation in tolerized innate immune cells are based on epigenetic reprogramming events, including DNA methylation and histone modifications that up- or down-regulate the transcription of inflammatory genes (Netea et al., 2020). Identifying the receptors, signaling pathways and epigenetic modifications that induce and maintain immune tolerance is therefore important for understanding how immune tolerance contributes to a state of controlled inflammation with potential benefits for autoimmune conditions and chronic inflammation diseases without contributing to immunodeficiency.

All bacteria naturally produce and release nano-sized, non-replicative extracellular vesicles (BEVs) with roles in response to stress, quorum sensing, biofilm formation, and interspecies and interkingdom communication (Schwechheimer and Kuehn, 2015). BEVs contain various cargo including enzymes, signaling molecules, and metabolites (Bryant et al., 2017). BEVs generated by Gram-negative pathogens contain toxins and virulence factors that can breach host defenses facilitating invasion and infection by parental cells (Kaparakis-Liaskos and Ferrero, 2015). By contrast, the role of BEVs produced by commensal microbes, and in particular the vast numbers residing in the GIT, is less clear, although recent studies have identified a potential role in host-microbe communication and in maintaining immune homeostasis *via* interactions with dendritic cells (Durant et al., 2020). BEVs generated by prominent members of the microbiota such as *Bacteroides thetaiotaomicron* (Bt) can cross the epithelial barrier of the intestine and access underlying lamina propria cells and, *via* the vasculature, other organs and tissues. They mediate bacteria-host interactions which modulate the physiology of various host cells including those of the innate and adaptive immune system (Stentz et al., 2018; Jones et al., 2020; Gul et al., 2022). BEVs produced by the pathobiont *Bacteroides fragilis* have been implicated in immune homeostasis as they can mediate anti-inflammatory effects by TLR2-dependent activation of dendritic cells and the production of IL-10 by regulatory T cells (Shen et al., 2012). The inbuilt adjuvanticity and immune-potentiation properties of Bt-derived BEVs has also been exploited in drug delivery formulations and in mucosal vaccines for respiratory viruses (Carvalho et al., 2019a,b), supporting their ability to modulate host immune cell function.

Based upon the biophysical and immunological properties of BEVs generated by commensal bacteria, we have investigated the potential of Bt-derived BEVs to act *via* their interactions with innate immune cells as modulators of the immune tolerance and the inflammation response using well-established *in vivo* and *in vitro* models. Identifying the interactions between BEVs and host innate immune cells is an important step toward considering their use in immunotherapy.

Materials and methods

Isolation and characterization of Bt BEVs

Bacteroides thetaiotaomicron VPI-5482 was grown in 500 ml of Bacteroides Defined Medium (BDM4; Supplementary Table S1) at 37°C in an anaerobic cabinet. For BEVs preparations, cells were harvested after 16 h at an approximate OD_{600nm} of 2.5 corresponding to early stationary phase. BEVs were isolated following a method adapted from Stentz et al. (2022). Briefly, Bt cultures (500 ml) were centrifuged at 6000 g for 50 min at 4°C and the supernatants filtered through polyethersulfone (PES) membranes (0.22 μm pore-size; Sartorius) to remove debris and cells. Supernatants were concentrated by cross-flow ultrafiltration (100 kDa molecular weight cut-off, Vivaspin 50R, Sartorius), the retentate was rinsed once with 500 ml of PBS (pH 7.4) and concentrated to 1 ml. Further purification of BEVs was performed by fractionation of the suspension by size-exclusion chromatography using qEVoriginal 35 nm columns (Izon) according to manufacturer's instructions. Fractions containing BEVs were combined and filter-sterilized through a 0.22 μm PES membrane (Sartorius) and the suspensions were stored at

4°C. Absence of viable microorganisms was confirmed by plate count and absence of LPS was confirmed by Limulus Amebocyte Lysate (LAL) test.

The size and concentration of the isolated Bt BEV suspension was determined using nanoparticle tracking analysis and the ZetaView PMX-220 TWIN instrument according to manufacturer's instructions (Particle Metrix). Aliquots of BEV suspensions were diluted 1,000-to 20,000-fold in particle-free water for analysis. Size distribution video data were acquired using the following settings: temperature: 25°C; frames: 60; duration: 2 s; cycles: 2; positions: 11; camera sensitivity: 80 and shutter value: 100. The ZetaView NTA software (version 8.05.12) was used with the following post acquisition settings: minimum brightness: 20; max area: 2,000; min area: 5 and trace length: 30.

Animal studies

Specific-pathogen-free (SPF) C57BL/6 male mice were bred and maintained in the Disease Modeling Unit at the University of East Anglia (United Kingdom). Animals were housed in individually ventilated cages and exposed to a 12 h light/dark cycle with free access to drinking water and standard laboratory chow diet. Animal experiments were conducted in full accordance with the Animal Scientific Procedures Act 1986 under UK Home Office (HMO) approval and HMO project license 70/8232.

Acute colitis mouse model

The dextran sulfate sodium (DSS) induced mouse model of acute colitis was used to investigate the therapeutic potential of BEVs on intestinal inflammation. SPF C57BL/6 mice, 8 weeks old, were divided into two groups and administered with either PBS ($n=8$) or BEVs ($n=6$). Experimental colitis was induced by administration of 2.25% w/v DSS (36,000–50,000 Da, MP Biomedicals) in drinking water *ad libitum* for 5 days. From day 5 until the end of the experiment, DSS was replaced by fresh water. PBS and BEVs were administered by oral gavage (100 µl at 10^{11} BEVs/ml) on days 5, 7, and 9, and on day 11 mice were euthanized by cervical dislocation after exposure to rising concentrations of CO_2. Distal colon tissue samples (1 cm) and spleens were collected for cytokine production analysis. The extent of colitis was evaluated using survival rate and a disease activity index (Supplementary Table S2) comprising daily body weights, stool consistency, tissue and content appearance, and bleeding assessments.

Each distal colon tissue sample was cut open longitudinally and incubated in flat-bottomed 24-well plates with 0.5 ml per well of complete RPMI medium (RPMI-1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biosera) and 1% Pen/Strep (Sigma-Aldrich)) for 24 h in a CO_2 incubator. Spleens were macerated in a cell strainer at 70 µm, washed with complete RPMI medium and incubated with Ammonium-Chloride-Potassium (ACK) lysis buffer (Gibco) for 10 min to lyse red blood cells. Splenocyte count was adjusted to 5×10^6 cells/ml and incubated in flat-bottomed 96-well plates with 0.2 ml per well of complete RPMI medium, with or without restimulation with 10^9 BEVs/ml, for 72 h at 37°C and 5% CO_2 in a humidified incubator. Supernatants from colon and splenocyte cultures were then centrifuged and store at −80°C prior to cytokine analysis.

Murine bone marrow-derived monocyte cultures

SPF C57BL/6 mice, 13–16 weeks old, were euthanized by cervical dislocation after exposure to rising concentrations of CO_2. Femurs were immediately removed and placed into cold sterile PBS. Bone marrow cell suspensions were isolated by flushing the femurs and tibias with RPMI-1640 supplemented with 10% heat-inactivated FBS and 1% Pen/Strep under sterile conditions. Debris was removed by passing the suspension through a 70 µm cell strainer. Cells were washed with complete RPMI media and concentration was adjusted to 6×10^6 cells/ml. Cells were seeded on flat-bottomed 12-well plates (1.2 ml/well) and incubated at 37°C and 5% CO_2 in a humidified incubator.

BMDM—Bt BEVs co-culture

Bone marrow-derived monocyte (BMDM) cells (1.2 ml at 6×10^6 cells/ml) were incubated in flat-bottomed 12-well plates in complete RPMI medium for 24 h at 37°C and 5% CO_2 in a humidified incubator, in the presence of either different concentrations of Bt BEVs (3×10^9, 3×10^7, and 3×10^5 BEVs/ml), LPS from *E. coli* (10 ng/ml; Sigma-Aldrich) as a positive control or PBS as negative control. After 24 h, supernatants were collected and stored at −20°C prior to cytokine measurements. Cells were washed with warm PBS and maintained in complete RPMI medium for 5 days at 37°C and 5% CO_2 with fresh media added on day 3. Cells were then incubated with LPS (10 ng/ml) to mimic an infectious challenge, or PBS as negative control. After 24 h, supernatants were collected and stored at −20°C for subsequent cytokine measurement. Cells were washed with warm PBS and detached from the wells by gently scraping after 1 h incubation with ice-cold Macrophage Detachment Solution (PromoCell) at 4°C. Cells were then washed with PBS containing 0.5 mM EDTA and stored at −80°C in FBS with 10% DMSO (Sigma-Aldrich) for histone methylation analysis. All incubations were performed in triplicate in two independent experiments.

Cytokine measurements

The production of IL-10 by colon tissue and splenocytes, and TNFα, IL-6, and IL-10 produced by BMDM after 24 h of

conditioning and after 24 h of challenge was measured by ELISA (Invitrogen) according to the manufacturer's instructions. The results were recorded as pg of each cytokine per mL of supernatant.

THP1-Blue cell assay

THP1-Blue NF-κB cells (Invivogen) were derived from the human THP-1 monocyte cell line by stable integration of an NF-κB-inducible secreted alkaline phosphatase (SEAP) reporter construct. THP1-Blue cells were cultivated in RPMI-1640 supplemented with 10% heat-inactivated FBS, 1% Pen/Strep and 100 µg/ml Normocin (Invivogen) at 37°C and 5% CO_2 in a humidified incubator. To maintain selection pressure during cell subculturing, 10 µg/ml blasticidin was added to the growth medium every other passage.

To establish the threshold dose of BEVs that allows for the quantification of THP-1 cells activation, cells were seeded in flat-bottomed 96-well plates at a density of 5×10^5 cells/ml and incubated for 24 h at 37°C and 5% CO_2 in a humidified incubator in the presence of different concentrations of Bt BEVs (from 3×10^9 to 3×10^6 BEVs/ml) using LPS (10 ng/ml) as a positive control and BDM4 and PBS as negative controls.

To identify pattern recognition receptors (PRRs) involved in BEV-mediated THP1-Blue cell activation, cultures were incubated with different inhibitors of TLR2 (PAb-hTLR2 (5 µg/ml), Invivogen), TLR4 (PAb-hTLR4 (5 µg/ml), Invivogen), NOD1 (ML130 (5 µg/ml), Abcam), and NOD2 (GSK717 (5 µg/ml), Merck) prior to the addition of BEVs (3×10^8/ml). Heat-killed *Listeria monocytogenes* (HKLM, 10^7 cells/ml, Invivogen), LPS (10 ng/ml, Sigma-Aldrich), lauroyl-g-D-glutamyl-meso-diaminopimelic acid (DAP, 1 µg/ml, Invivogen) and N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP, 10 µg/ml, Invivogen) were used as specific ligands for each inhibitor, respectively. Subsequently, 20 µl of the cell suspension was added to wells of 96-well plate, mixed with 180 µl of Quanti-Blue (Invivogen) colorimetric assay reagent and incubated for 1 h at 37°C to allow color development. NF-κB-inducible SEAP levels were quantified by absorbance reading at 620 nm. All incubations were performed in triplicate in three independent experiments.

THP1-Blue cell—Bt BEVs co-culture

To investigate phenotypic changes in THP1-Blue cells after exposure to with Bt BEVs, the cells (1.2 ml at 10^6 cells/ml) were incubated on flat-bottomed 12-well plates in complete RPMI medium for 24 h at 37°C and 5% CO_2 in a humidified incubator, in the presence of different concentrations of Bt BEVs (5×10^8 BEVs/ml, 5×10^6 BEVs/ml, and 5×10^4 BEVs/ml), using LPS from *E. coli* (10 ng/ml; Sigma-Aldrich) as a reference and positive control and PBS as a negative control. Cells were washed with warm PBS and detached from the wells by gently scraping after 1 h incubation with ice-cold Macrophage Detachment Solution at 4°C. Cells were then washed with PBS containing 0.5 mM EDTA and collected for flow cytometry analysis.

Flow cytometry

THP1-Blue cells were stained with Zombie Aqua Fixable Viability kit (BioLegend) following the manufacturer's protocol. Cells were washed with Cell Staining Buffer (BioLegend) before blocking Fc receptors by incubation with Human TruStain FcX (BioLegend) for 10 min at 21°C. Cells were then surface stained for 20 min at 4°C in the dark using APC/Fire 750 anti-human CD14 Clone 63D3 (BioLegend) and flow cytometry performed on BD LSRFortessa Cell Analyzer (BD Biosciences) with the data analyzed using FlowJo software 10.8.1 (BD Biosciences).

Histone methylation

Histone proteins from bone marrow-derived cells were extracted using the EpiQuik Total Histone Extraction Kit (Epigentek) according to the manufacturer's instructions and quantified by measuring absorbance at 280 nm. The level of histone 3 lysine 4 mono-methylation (H3K4me1) was quantified using the ELISA-based colorimetric kit EpiQuik Global Mono Methyl Histone H3 K4 Quantification (Epigentek) and results were expressed as ng of H3K4me1 per µg of total protein.

Statistical analysis

Data were subjected to One-way ANOVA or Two-way ANOVA followed by Tukey's multiple comparison *post-hoc* test or Dunnett's multiple comparison *post-hoc* test using GraphPad Prism 5 software. Statistically significant differences between two mean values were established by a $p < 0.05$. Data are presented as the mean ± standard deviation.

Results

Anti-inflammatory effect of Bt BEVs *in vivo*

To investigate the ability of Bt BEVs to influence inflammatory responses *in vivo* we used DSS-induced murine colitis as a model of acute inflammation (Figure 1A). This is a well-established lymphocyte-independent model of intestinal inflammation in which the clinical severity can be quantified, providing a reliable method to study the contribution of the innate immune system to inflammatory responses in the host.

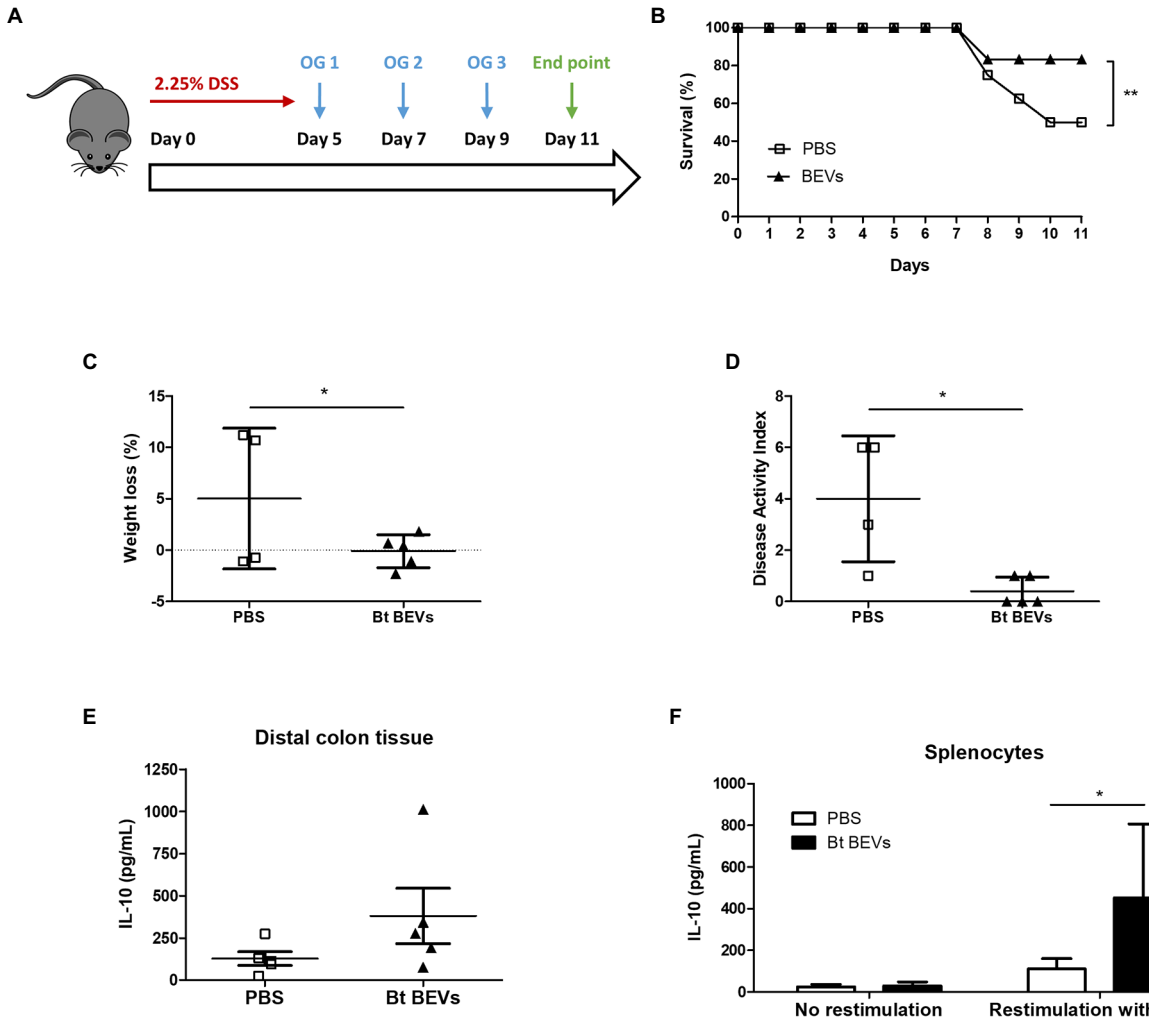

FIGURE 1
Bt BEVs ameliorate DSS-induced colitis in mice. **(A)** Mice were provided with drinking water containing 2.25% DSS (w/v) for 5days. On days 5, 7, and 9 mice were orally administered with either PBS or Bt BEVs (100μl at 10^{11} BEV/ml). **(B)** Survival rates. **(C)** Percent weight loss at day 11. **(D)** Disease Activity Index (DAI) at day 11. **(E)** IL-10 production in distal colon tissue. **(F)** IL-10 production by splenocytes cultured in complete media alone or in media containing Bt BEVs. Graphs depict mean±SD values. *p<0.05, **p<0.01.

Mice orally administered with Bt BEVs exhibited a significantly higher ($p < 0.01$) survival rate throughout the experiment compared to mice that received vehicle (PBS) only (Figure 1B). Oral administration of Bt BEVs also contributed to a significant decrease ($p < 0.05$) in weight loss and disease activity index scores (Figures 1C,D). Analysis of cytokine production by freshly excised and cultured colonic tissue showed that production of the anti-inflammatory cytokine IL-10 was higher in distal colon tissue from mice previously administered with Bt BEVs (Figure 1E). When splenocytes from mice orally gavaged with Bt BEVs were restimulated *ex vivo* with Bt BEVs, IL-10 production was also significantly increased ($p < 0.05$). By comparison, there was no difference in IL-10 production from non-stimulated splenocytes across all experimental groups (Figure 1F).

Bt BEVs modulation of cytokine production in BMDM

Bone marrow mononuclear cells cultured under conditions that favor the growth of monocytes and macrophages were used to examine further the potential interaction of Bt BEVs with innate immune cells. Specifically, we investigated how Bt BEVs influence murine bone marrow-derived monocytes (BMDM) in response to an inflammatory stimulus. Cytokine production in BEV-conditioned BMDM was measured before and after an infection-like challenge with LPS (Figure 2A). We observed significantly increased amounts ($p < 0.001$) of IL-10 in BMDM cultures after incubation for 24h with 3×10^9 BEVs/ml. No significant changes in IL-10 production were detected in BMDM containing lower concentrations of Bt BEVs or with LPS and

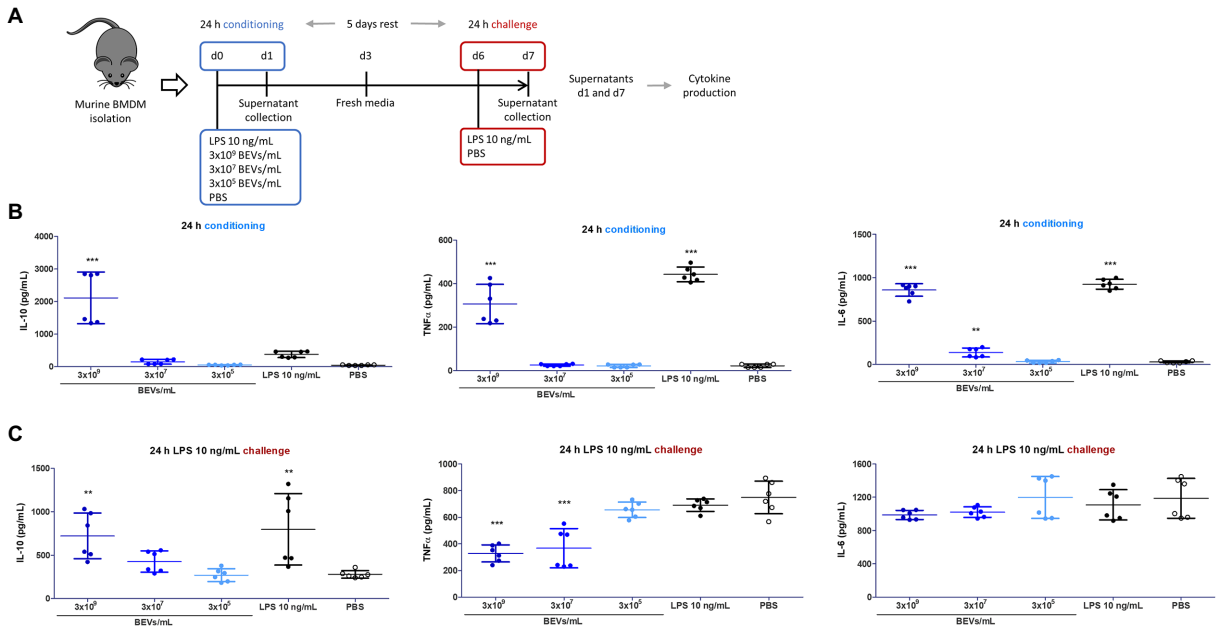

FIGURE 2
Bt BEVs modulate the production of anti- and pro-inflammatory cytokines by murine bone marrow derived macrophages (BMDM). **(A)** Experimental plan. **(B)** Cytokine production by BMDM 24h after conditioning with Bt BEVs and LPS was assessed by ELISA. **(C)** Cytokine production by Bt BEV- and LPS-conditioned BMDM 24h after exposure to LPS (10ng/ml) determined by ELISA. Non-conditioned BMDM (PBS) were used as the reference group for statistical analysis. Graphs depict mean±SD values. **p<0.01; ***p<0.001.

PBS. The concentrations of the pro-inflammatory cytokines TNFα and IL-6 were significantly higher ($p < 0.001$) in BMDM incubated with 3×10^9 BEVs/ml when compared to PBS alone. However, these levels were lower than in LPS-conditioned BMDM cultures (Figure 2B). Analysis of cytokine production in BEV-conditioned BMDM cultures after an LPS challenge revealed opposite trends when comparing anti- and pro-inflammatory activity. While IL-10 production was directly associated with the Bt BEVs concentration used to condition BMDM, the levels of pro-inflammatory cytokines showed an inverse relationship with the Bt BEV conditioning dose, with TNFα production significantly decreased ($p < 0.001$) when BMDM were incubated with BEVs at 3×10^7 BEVs/ml or higher concentrations (Figure 2C). Collectively, these data show that Bt BEVs exert in a dose-dependent manner anti-inflammatory responses from monocytes/macrophages, which was particularly noticeable in a re-infection (LPS challenge) scenario.

Molecular basis of BEV-monocyte interactions and the involvement of specific pattern recognition receptors

The human monocytic cell line THP-1 that expresses an NF-κB inducible secreted alkaline phosphatase (SEAP) reporter construct (THP1-Blue) was used to identify the receptors and pathways in monocytes involved in the anti-inflammatory effect of Bt BEVs observed in both the *in vivo* and *in vitro* model systems. To investigate if Bt BEVs can activate THP-1 cells and to establish the activation threshold, cells (5×10^5 cells/ml) were incubated with different concentrations of Bt BEVs (3×10^6 BEVs/ml to 3×10^9 BEVs/ml) using a chemically defined Bt media (BDM4) and PBS or LPS (10 ng/ml) as negative and positive controls, respectively. NF-κB activation in THP-1 cells in response to Bt BEVs was dose-dependent (Figure 3A) with no detectable activation seen in cultures containing BDM4 or PBS alone. Based upon the level of activation induced by 3×10^8 BEVs/ml being equivalent ($p > 0.05$) to that of LPS (10 ng/ml), we established this as an optimal Bt BEVs concentration for subsequent inhibition assays. Specific inhibitors of key extracellular and intracellular PRRs were used in THP1-BEV co-cultures to identify those contributing to NF-κB activation in THP-1 cells. In a series of optimization experiments, the optimal concentration of each inhibitor was established by titration and their specificity at that concentration was confirmed with individual PRR-specific ligands (Supplementary Figure S1). The most potent inhibition of NF-κB activation (~60%) was seen in cultures containing PAb-hTLR2 (5 μg/ml), an antibody that specifically inhibits TLR2 activation (Figure 3B; Supplementary Figure S1), consistent with TLR2 activation mediating interactions between Bt BEVs and monocyte/macrophages (Figure 3B). By contrast, no significant inhibition of NF-κB activation was seen using inhibitors of TLR4, NOD1, or NOD2 (Figure 3B).

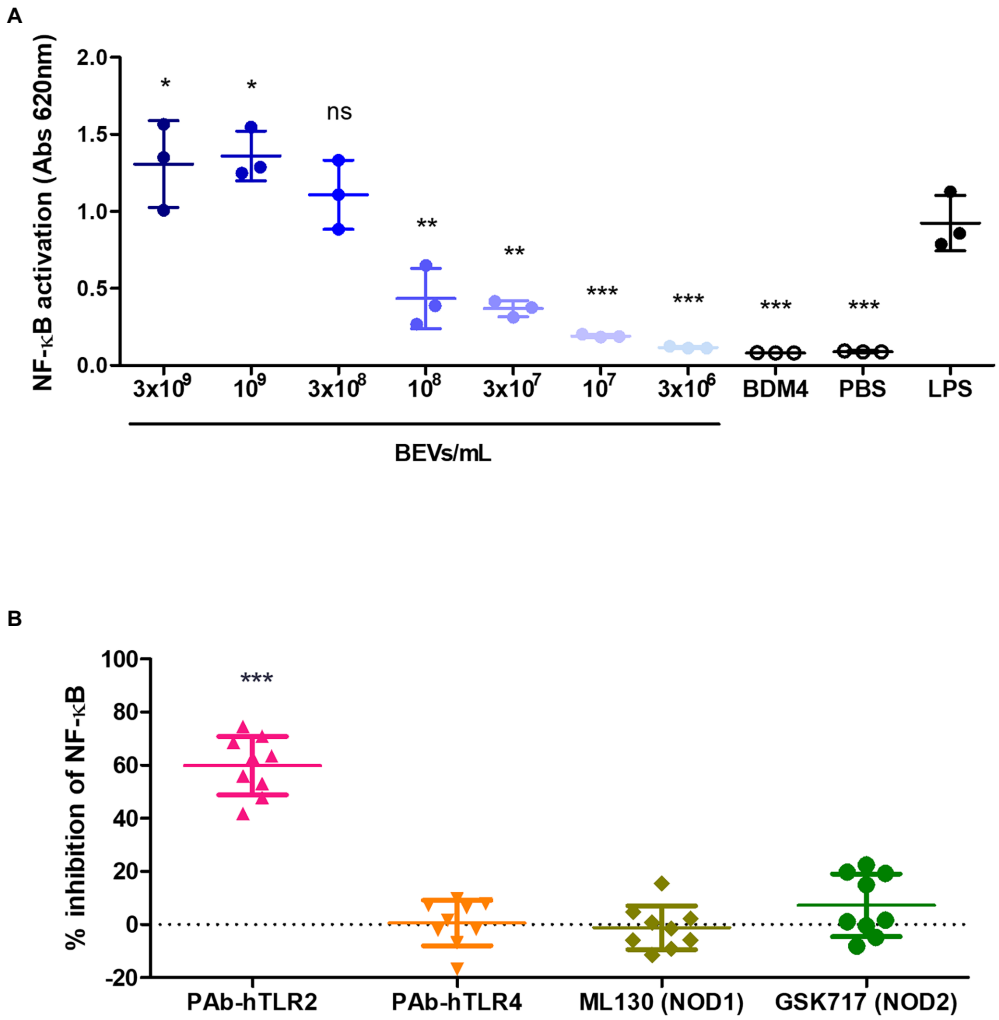

FIGURE 3
Mediators of Bt BEV-monocyte/macrophage interaction identified using THP1-Blue monocytes. **(A)** THP-1 cells were incubated with a range of Bt BEV concentrations or with Bt growth media (BDM4), PBS or LPS for 24h prior to assessing level of NF-kB activation. Statistical analysis was performed using LPS as the reference group. **(B)** THP-1 cells were pre-incubated with optimal concentrations of TLR2, TLR4, NOD1 or NOD2 inhibitors prior to the addition of Bt BEVs and subsequent assessment of NF-kB activation. Graphs depict mean±SD values. ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Phenotypic changes in monocytes following stimulation with Bt BEVs

We investigated the phenotypic changes in THP-1 cells after exposure to Bt BEVs by measuring levels of CD14 expression using flow cytometry. $CD14^+$ cells were gated based on a fluorescence minus one (FMO) strategy. CD14 expression is a marker of activation related to pro-inflammatory monocytes, is a co-receptor for TLR4 responsivity to LPS, and contributes to the activation of other PRRs including TLR2. Among THP-1 cells cultured in media alone approximately 22% were $CD14^+$ (Figure 4). In cultures containing the highest concentration of Bt BEVs (5×10^8 BEV/mL equivalent to a ratio of 500 BEVs,THP-1) the proportion of $CD14^+$ cells more than doubled to approximately 51% and was equivalent to the levels seen in cultures containing LPS (~53%; Figure 4). By contrast, THP-1 cells stimulated with lower concentrations of Bt BEVs showed no significant changes in the proportion of $CD14^+$ monocytes when compared to cells incubated with PBS (Figure 4).

Effect of Bt BEVs on histone modifications in BMDM

Inflammatory processes and innate immunity are tightly regulated by epigenetic mechanisms with genomic DNA methylation and modification of histones influencing the function of innate immune cells (Saeed et al., 2014; Fraschilla et al., 2022). To determine if Bt BEVs can induce epigenetic changes, and in particular histone methylation, we measured levels of H3K4me1 in

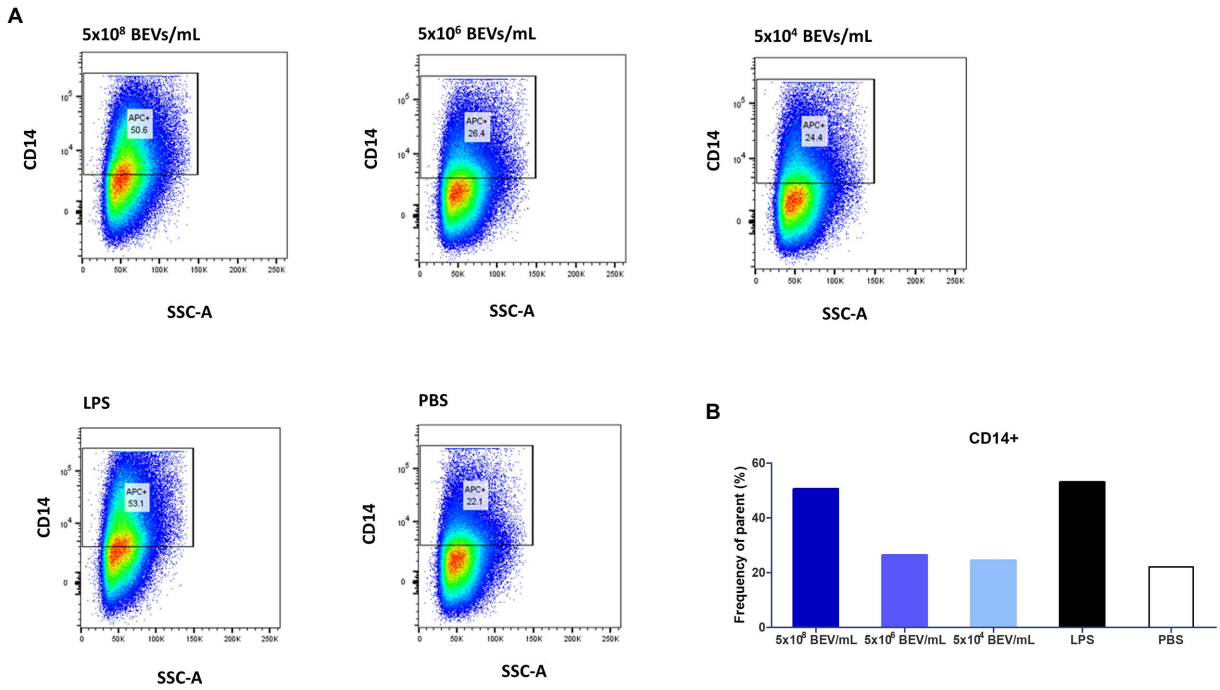

FIGURE 4
Bt BEVs elicit phenotypic changes in THP1-Blue NF-κB cells (10^6 cells/ml). **(A)** THP-1 cells were incubated with different concentrations of Bt BEVs for 24h and analyzed for CD14 expression by flow cytometry. Gating strategy was based on scatterplots of CD14 vs. side scatter signal. **(B)** The bar graph represents THP-1 cells with high expression of CD14 as percentage frequency of parent.

BMDM, which indicates the mono-methylation at the 4th lysine residue of histone H3 protein and is an enhancer signature (Figure 5A). Varying levels of H3K4me1 were detected in BMDM after culture with Bt BEVs, with the highest levels seen in cultures containing 3×10^9 BEV/ml, comparable to those in BMDM cultured in media alone (PBS) and higher than that in cultures containing LPS (Figure 5B). By comparison, lower levels of H3K4me1 were seen in cultures containing fewer Bt BEVs (3×10^5 and 3×10^7 BEV/ml) with the differences being significant ($p < 0.05$) for BMDM cultured with 3×10^7 BEV/ml. These data demonstrate that Bt BEVs altered the histone methylation status in innate immune cells.

Discussion

BEVs produced by gut bacteria can cross the intestinal epithelium, gaining access to host cells (Stentz et al., 2018; Jones et al., 2020) and contribute to immune homeostasis *via* interactions with innate immune cells (Maerz et al., 2018; Diaz-Garrido et al., 2019; Durant et al., 2020). However, the details and nature of this BEV-immune cell crosstalk is incomplete. In this study, we have provided *in vitro* and *in vivo* evidence for the anti-inflammatory and immunomodulatory properties of BEVs produced by the major human gut commensal bacterium Bt and identified the molecular basis of their interaction with monocytes and macrophages. We recognize that different types of particles could be copurified with BEVs due to technical limitations but their effect is taken into consideration and appropriate controls were utilized wherever possible to aid data interpretation (Juodeikis and Carding, 2022).

Oral administration of Bt BEVs ameliorates DSS-induced colitis in mice, underlining their potential as a treatment for non-infectious autoimmune pathologies. Similar protective effects have been reported in DSS models after treatment with fresh and lyophilized cultures of Bt (Delday et al., 2019) and other *Bacteroides* species (Hudcovic et al., 2009; Chiu et al., 2014; Chang et al., 2017), although the mediators of these effects and the possibility that it includes BEVs were not investigated. The size, stability, and non-replicative status of Bt BEVs makes them good candidates for therapeutic interventions compared to whole bacteria. Conditions involving chronic inflammation of the gut are associated with dysregulation of mucosal innate immune response (Xu et al., 2014), increased NF-κB activation (Schreiber et al., 1998) and increased levels of pro-inflammatory cytokines such as TNF-α and IL-6 (Atreya et al., 2000; Komatsu et al., 2001). In this context, the significance of BEV-elicited IL-10 from BMDM is implied from its role in the prevention of inflammatory bowel disease (Mazmanian et al., 2008; Hansen et al., 2009). Mice lacking IL-10 or IL-10 receptor genes spontaneously develop intestinal inflammation (Kuhn et al., 1993) with IL-10 acting by suppressing antigen presentation by downregulating MHC class II expression (Koppelman et al., 1997) and inhibiting pro-inflammatory cytokine synthesis by blocking the activation of the inhibitor of

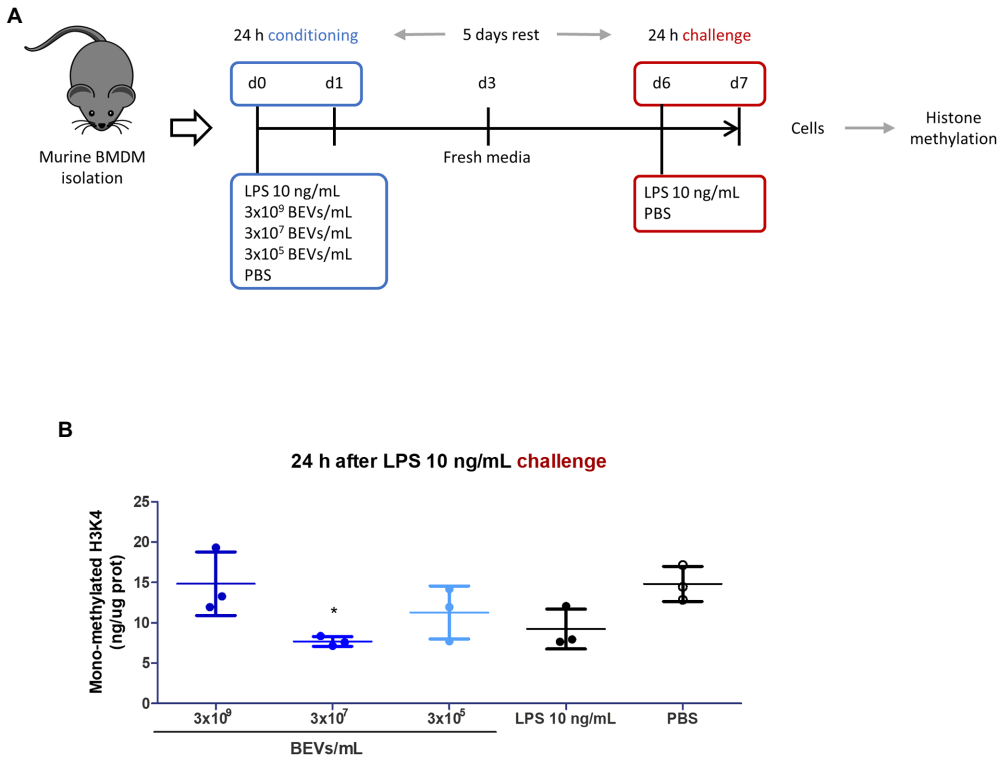

FIGURE 5
Histone mono-methylation (H3K4me1) as an epigenetic signature of Bt BEV modulation of innate immunity. **(A)** Experimental plan. **(B)** Methylation of histone proteins from Bt BEV- or LPS-conditioned BMDM 24h after exposure to LPS (10ng/ml) was quantified using an ELISA-based colorimetric kit and results were expressed as ng of H3K4me1 per µg of total protein. Non-conditioned BMDM (PBS) were used as the reference group for statistical analysis. Graph depicts mean±SD values. *$p<0.05$.

NF-κB kinase (IKK) and dysregulating NF-κB (Schottelius et al., 1999). The beneficial effects of BEVs or Bt (Li et al., 2021) in DSS-colitis are associated with increased production of IL-10 in serum, colonic tissue and by peripheral splenocytes which can promote a non-inflammatory status by counteracting pro-inflammatory responses. The contribution of Bt BEVs to the maintenance of immune homeostasis by promoting IL-10 production by innate immune cells is also implied by our previous study in which we reported the absence of Bt BEV-elicited IL-10 production by innate immune cells isolated from patients with inflammatory bowel disease (Durant et al., 2020). It is interesting to note that lower levels of *Bacteroides* spp. are present in the gut microbiota of inflammatory bowel disease patients (Zhou and Zhi, 2016). While the present study focused on BEVs, we cannot exclude the possibility for other cell-associated or secreted constituents of Bt to contribute to immunoregulatory responses *in vivo*. Indeed, administration of live or dead (freeze dried) Bt to IL-10r-deficient mice protects the animals from developing colitis (Delday et al., 2019) which may involve different bacterial mediators and multiple interactions with host cells. Nevertheless, Bt and BEV-elicited IL-10 production by host immune cells appears to be central to their protective effects.

In BMDM cultures pre-conditioned with BEVs prior to an infection-like challenge with LPS, high doses of Bt BEVs significantly upregulated the production of IL-10. Although Bt BEVs also increased the production of pro-inflammatory cytokine TNFα in BMDM, the levels were significantly lower than those achieved with LPS. This could be explained by the inhibitory effect of IL-10 on pro-inflammatory cytokine synthesis. This anti-inflammatory effect was more evident in a subsequent LPS-challenge. Pre-conditioning by Bt BEVs altered the cytokine profile of BMDM in a dose-dependent manner, especially in the case of the IL-10/TNFα ratio. High doses of Bt BEV pre-conditioning produced a high IL-10/TNFα ratio, indicatory of a homeostatic or tolerance like status and an attenuated inflammatory response to LPS stimulation. This phenomenon resembles that of endotoxin tolerance which is characterized by upregulated IL-10 and downregulated TNFα production leading to an immune hyporesponsiveness (Biswas and Lopez-Collazo, 2009; Gu et al., 2022). Interestingly, IL-6 levels were not affected by BEV conditioning which was also noted in a related study investigating the immunomodulatory effect of different *Bacteroides* species on murine bone marrow-derived dendritic cells (BMDC; Steimle et al., 2019). In this study, IL-6 secretion was also not reduced in *Bacteroides*-primed and *E. coli*-challenged BMDC, and priming of BMDC with *Bacteroides* resulted in decreased TNFα expression after *E. coli* challenge in contrast to non-primed BMDCs. Since IL-6 is a pleiotropic cytokine capable

of acting as a defense mechanism in acute inflammation, and conversely exhibits a pro-inflammatory profile in chronic inflammation (Scheller et al., 2011), further studies are required to determine the significance of IL-6 production in BMD-innate immune cells conditioned with BEVs. It is also interesting to note the contrasting impacts of intact *Bacteroides* cells versus their BEVs on cytokine production. Whereas we have identified and confirmed IL-10 production as a signature of BEV interaction with human (Durant et al., 2020) and murine innate immune cells, this signature is not as evident using intact *Bacteroides* cells as a stimulus, suggesting that commensal gut bacteria can utilize different means including both cell-associated and secreted mediators to communicate with and influence host immune cells.

The molecular basis of Bt BEV-monocyte interactions was established using the human monocytic reporter cell line THP1-Blue NF-κB. TLR2 activation was shown to mediate Bt BEV-elicited NF-κB activation, whereas TLR4, NOD1, and NOD2 made no significant contribution. In a previous study (Gul et al., 2022), we reported the influence of TLR2 in Bt BEV-host communication *via* the TLR2/TLR4 adaptor protein TIRAP (toll-interleukin-1 receptor domain-containing adaptor protein), although TLR4 alone also showed some involvement. This apparent discrepancy is most likely explained by our prior use of a complex bacteria growth media (Brain-Heart Infusion, BHI) containing animal tissue and cellular lipids which may function as TLR4 ligands. This factor was excluded in this study by the use of a chemically defined media (BDM4). TLR2 recognizes several microbial products from both Gram-positive and Gram-negative bacteria, including lipoproteins and peptidoglycans, and forms heterodimers with either TLR1 or TLR6 for downstream signaling *via* NF-κB pathway (Akira and Takeda, 2004). The polysaccharide A (PSA) antigen expressed in BEVs from the closely related commensal *Bacteroides fragilis* also interact with dendritic cells in a TLR2-dependent manner (Round et al., 2011; Shen et al., 2012). It has been recently reported the presence of serine-dipeptide lipids in Bt BEVs (Sartorio et al., 2022), which can also act as TLR2 ligands (Clark et al., 2013; Nemati et al., 2017). Further detailed biochemical characterization of BEV-associated lipoproteins is required to identify the ligands triggering TLR2 signaling pathways in innate immune cells. TLR2 signaling has been reported to have a protective role in inflammatory conditions (Lowe et al., 2010; Brun et al., 2013) and colorectal cancer (Sittipo et al., 2018), and to promote immune homeostasis by inhibiting the expression of pro-inflammatory cytokines and enhancing IL-10 production (Chang et al., 2017). These findings align with our proposal that the anti-inflammatory effect elicited by Bt BEVs is associated with modulations in the host innate immune system through the IL-10 signaling pathway, triggered by BEV-TLR2 interactions.

TLR activation depends on different co-receptors such as CD14, which is widely used as a marker of activation related to pro-inflammatory and classical monocytes (Lotz et al., 2004). Although generally characterized as a co-receptor for the TLR4 responsivity to LPS, CD14 also contributes to the activation of other PRRs including TLR2 (van Bergenhenegouwen et al., 2013). CD14 binds to triacylated lipopeptides, typically present in Gram-negative bacteria including *Bacteroides*, to enhance their recognition by the TLR2/TLR1 heterodimer (Jin et al., 2007). The highest proportion of $CD14^{+}$ THP-1 cells were seen after stimulation with high concentrations of Bt BEVs, with levels equivalent to those stimulated with LPS, which suggests the functional involvement of CD14 in TLR2-mediated innate immune response induced by Bt BEVs.

The functional phenotype of immune cells is highly dependent on the establishment of unique epigenetic profiles that integrate microenvironmental cues into the genome to establish specific transcriptional programs (Calle-Fabregat et al., 2020). Among key epigenetic markers is the acquisition of histone 3 lysine 4 methylation (H3K4me1) in short lived monocytes and macrophages (van der Meer et al., 2015; Netea et al., 2016) and in long-lived myeloid bone marrow progenitors (Kaufmann et al., 2018; Mitroulis et al., 2018). The highest levels of H3K4me1 in Bt BEV-conditioned LPS-challenged BMDM, were evident in monocyte/macrophages incubated with high concentrations of Bt BEVs. Unexpectedly, these levels of H3K4me1 were higher than in LPS-conditioned BMDM and comparable to those found in non-conditioned BMDM. This seems to contradict our cytokine production results, since the repressed pro-inflammatory cytokine expression prompted by Bt BEV conditioning would be expected to correlate with close chromatin and low H3K4me1 levels. However, the IL-10 genomic locus of monocytes is poised for activation with open chromatin already at the steady state (Northrup and Zhao, 2011; Tamassia et al., 2013) and the presence of H3K4me1 is associated with IL-10 gene enhancers (Taubert, 2017), which could explain the increased levels of H3K4me1 found in BMDM conditioned with Bt BEVs. However, we cannot confirm this since our approach comprised global histone modifications. To further investigate the role of H3K4me1 and other relevant modifications involved in immune tolerance chromatin immunoprecipitation sequencing (ChIP-seq) could be used to localize these histone modifications throughout the genome.

Conclusion

We have shown that BEVs from the major gut commensal bacterium Bt elicit anti-inflammatory and immunomodulatory properties in innate immune cells, consistent with promoting and maintaining host immune homeostasis. Bt BEVs alleviated acute intestinal inflammation in DSS-treated mice, in association with increased IL-10 production. This was confirmed *in vitro* with increased IL-10 and decreased TNFα production in BEV-conditioned and LPS-challenged BMDM cultures. BEV-mediated monocyte activation and cytokine production was mediated by TLR2 interactions and resulted in stable epigenetic changes reflected by increased levels of H3K4me1. These findings

provide the rationale and basis for investigating the potential of Bt BEVs as an immune therapy.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal experiments were conducted in full accordance with the Animal Scientific Procedures Act 1986 under UK Home Office (HMO) approval and HMO project license 70/8232.

Author contributions

SF and SC conceived and designed the experiments and wrote the manuscript. SF carried out the experimental work with contribution from AC, AM-C, EJ, RJ, and RS. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the BBSRC Institute Strategic Programme Grant Gut Microbes and Health BB/R012490/1 and its constituent projects BBS/E/F/000PR10353 (Theme 1, Determinants of microbe-host responses in the gut across life) and BBS/E/F/000PR10355 (Theme 2, Changes in gut microbe-host interactions and their impact beyond the gut) and Quadram Institute Bioscience Proof-of-Concept Research Grant Fund project number 43134-000-A.

Acknowledgments

The authors gratefully acknowledge the support of the Biotechnology and Biological Sciences Research Council (BBSRC).

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/fmicb.2022.1050271/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 18 November 2022
DOI 10.3389/fimmu.2022.1010368

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 03 August 2022
ACCEPTED 26 October 2022
PUBLISHED 18 November 2022

CITATION
Li Y, Zhu M, Liu Y, Luo B, Cui J, Huang L, Chen K and Liu Y (2022) The oral microbiota and cardiometabolic health: A comprehensive review and emerging insights.
Front. Immunol. 13:1010368.
doi: 10.3389/fimmu.2022.1010368

The oral microbiota and cardiometabolic health: A comprehensive review and emerging insights

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There is mounting evidence demonstrating that oral dysbiosis causes periodontal disease and promotes the development of cardiovascular disease. The advancement of omics techniques has driven the optimization of oral microbiota species analysis and has provided a deeper understanding of oral pathogenic bacteria. A bi-directional relationship exists between the oral microbiota and the host, and oral-gut microbiota transfer is known to alter the composition of the gut microbiota and may cause local metabolic disorders. Furthermore, cardiovascular health can also be highly affected by oral microbiota functions and metabolites, including short-chain fatty acids (SCFAs), nitric oxide (NO), hydrogen sulfide (H_2S), and some lipid metabolites. Studies have found that trimethylamine oxide (TMAO) may have adverse effects on cardiovascular health, whereas SCFAs, NO, and H_2S have cardioprotective effects. SCFAs and H_2S exert varying oral and cardiovascular effects, however reports on this specific topic remain controversial. Previous evidences are accustomed to summarizing the functions of oral microbiota in the context of periodontitis. The direct relationship between oral microbiota and cardiovascular diseases is insufficient. By systematically summarizing the methods associated with oral microbiota transplantation (OMT), this review facilitates an investigation into the causal links between oral microbiota and cardiovascular disease. The concomitant development of omics, bioinformatics, bacterial culture techniques, and microbiota transplantation techniques is required to gain a deeper understanding of the relationship between oral microbiota and cardiovascular disease occurrence.

KEYWORDS

oral microbiome, cardiovascular disease, microbial metabolites, oral microbiota transplantation, periodontal disease

1 Introduction

With the advancement of multi-omics and bioinformatics, the microbe-host interactions in the human body have begun to gain an increasing amount of attention. There is mounting evidence to suggest that the commensal microbiota plays a crucial role in human health and disease development, including cardiovascular disease (1, 2). The oral microbiota is the second largest microbial community present in the human body (3). The ecological niches in the oral cavity are divided into the saliva, tongue, dental surface, gingiva, buccal mucosa, palate, and subgingival/supragingival sites, with variations in microbiota species and activity, as well as varying susceptibility to diseases across the different niches. The oral microbiota is dominated by *Streptococcus*, belonging to the *Firmicutes* phylum (36.7%) (4), which produces an abundance of primary and secondary metabolites, and is associated with the occurrence of systemic diseases (5). Studies have shown that age-related variations have little effect on the oral microbiota when compared to other habitats in the bodies of healthy populations (6, 7). Furthermore, new evidence suggests that oral microbiota are involved in the preliminary digestion of food in the oral cavity and produce a variety of metabolites (8, 9). Oral microbiota sampling is also highly convenient, and the tongue-coating morphology and dental plaques can be observed under direct vision. These advantages have therefore enabled the potentially rapid clinical translation of research on the oral microbiota.

The oral microbiota has immense potential and value with regard to research on cardiovascular disease, specifically atherosclerosis (10, 11). The bi-directional interaction between periodontal diseases and oral microbiota, as well as the interaction between periodontal diseases and cardiovascular disease, has long been investigated (12, 13). Periodontal diseases are associated with transparent pathogens of cardiovascular disease and are associated with hypertension, heart failure, atherosclerosis, and coronary heart disease (14–16). Aggressive treatment of periodontal disease can significantly reduce the risk of cardiovascular disease development (17).

Therefore, the oral microbiota may have a substantial impact on systemic disease. This review seeks to answer the following questions based on existing evidence: (1) How does the oral microbiota affect the progression of cardiovascular disease? (2) Does cardiovascular disease have a reciprocal effect on the oral microbiota? (3) What are the metabolites produced by the oral microbiota that affect cardiometabolic health? How do these metabolites regulate inflammation, oxidative stress, or vascular function? (4) Can the novel techniques and methods currently under investigation (such as oral microbiota transplantation [OMT]) be applied to research on oral microbiota? Our findings will potentially serve as a reference for future investigations on the relationship between oral microbiota and cardiovascular disease occurrence.

2 Oral dysbiosis and phenotypes of cardiovascular disease: Bi-directional causality

2.1 Periodontal diseases: The role of oral dysbiosis in cardiovascular disease

It has been reported that the presence of periodontal disease and dental plaque may exacerbate cardiovascular disease (18). Research conducted on the correlation between oral dysbiosis and cardiovascular disease is usually based on the effects of periodontal diseases (19, 20). Therefore, understanding the mechanism of these three comorbidities could be helpful and necessary.

Oral dysbiosis is a key feature of periodontitis and research has indicated that gram-negative bacterial populations are significantly increased in patients with periodontitis (21). The progression of periodontal inflammation is accompanied by community dysbiosis. 16S pyrosequencing and metagenomic sequencing results have indicated that the oral α-diversity was higher in patients with periodontitis when compared to healthy people (21–24) and this same trend has been observed in other systemic diseases; however, the correlation between oral α-diversity and cardiovascular diseases has still not been well researched (25). Studies have reported that specific key pathogens are significantly increased in patients with periodontal disease and atherosclerosis, such as *Streptococcus mutans* and *Porphyromonas gingivalis* (26, 27). Clinical studies have reported that the density of the oral microbiota (in saliva, as well as in supra- and subgingival sites) was positively correlated with the severity of periodontal parameters, the number of periodontal pathogens, and severity of abdominal aortic aneurysm. These findings have been supported by serological and immunological studies, in which immunoglobulin G antibodies targeting *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were detected in patient oral tissues (28).

Certain oral commensal bacteria that are found in coronary plaques are also present in non-cardiac organs, such as *Campylobacter rectus, Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia* and *Prevotella nigrescens* (29). It is yet to be established if these bacteria specifically influence the formation of atherosclerotic plaques or if it is an oportunistic infection. The pathogenic mechanism of periodontitis may help us understand the relationship between oral microbiota and cardiovascular diseases. It has been reported that cardiovascular disease may be caused by periodontal disease *via* mechanisms such as inflammatory response, oxidative stress, immune response, and platelet aggregation (19, 30). Variations in the manifestations of dysbiosis have also been observed between patients of different sexes and between patients with or without dental caries/missing teeth (31, 32). Studies have found that

patterns of oral dysbiosis may induce the host's immune response (30); however certain pathogens may synergistically induce immunosuppression through an association with signaling pathways. For example, pathogens may inhibit T helper 1 (TH1) cell-mediated immunity using complement Toll-like receptor regulation, which may disrupt functional receptor interactions (33), thereby aggravating the cardiovascular disease. Periodontal disease is an important marker of oral dysbiosis; however, it may not be the sole cause of cardiovascular disease. Oral dysbiosis leads to a host immune response (30), which exacerbates cardiovascular disease. Animal models of periodontitis include different approaches, one is surgical approach to periodontitis (34), and the others are associated with the oral microbiota (inoculation with microbial pathogens (35, 36) and lipopolysaccharide (LPS) injection (37)). Excluding the effect of surgical approach (34), oral microbiota or LPS could still promote systemic inflammation (38).

The relationship between oral microbiota and periodontal diseases is constantly evolving (39). A new hypothesis has recently emerged in microbial research suggesting that the onset of inflammatory or immune response may not be induced by a single type of pathogenic bacteria but by changes in the overall microbiota, and this idea has challenged the concept of oral pathobionts. Disrupted homeostasis may be associated with a series of upstream and downstream bacteria, rather than a specific low-abundance species (39). For example, the "red complex" bacteria (*Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*) (40) were initially considered a major etiological factor of periodontitis; however, this perspective has now been challenged (41). This is because previous studies on oral microbiota using *in vitro* cultures may have overestimated the importance of bacterial species that are prone to growth, such as Gram-negative bacteria. However, more recent studies have found a significant increase in the abundance of Gram-positive anaerobic bacteria in the oral cavity of diseased individuals, sometimes even surpassing the abundance of Gram-negative bacteria (42). Omics research (43, 44) has verified that the pathogenesis of periodontitis involves a synergy and dysbiosis of multiple microorganisms, and is referred to as the polymicrobial synergy and dysbiosis model (45). Interestingly, the abundance of bacteria was negatively correlated with the relative abundance of *Porphyromonas ginglivlis*. In the low-abundance state, these typical pathogens promote the overall increase of the bacterial load, which can be indicated as a delicate ecological balance between mutualistic and antagonistic interactions in the microbiome (21).

2.2 The oral microbiota affects cardiometabolic health

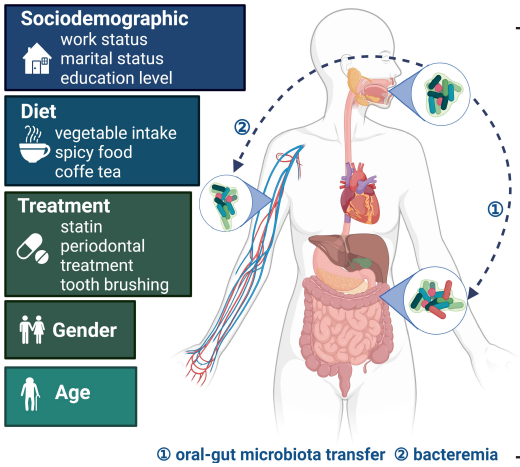
Oral dysbiosis is thought to be closely linked to cardiovascular disease and various species including, *Streptococcus mutans* and *Porphyromonas gingivalis*, which have been shown to increase with the occurrence of periodontal disease and systemic inflammation (26, 27). A number of other species, namely; *Treponema denticola, Tannerella forsythia, Prevotella intermedia, Prevotella nigrescens, Actictinobacillus actinomycetemcomitan, Campylobacter rectus, Parvimonas micra, Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia, Eubacterium timidum, Eubacterium brachy*, and *Eubacterium saphenum*, have also been found to be associated with oral dysbiosis and cardiovascular disease (29, 30, 46) (Figure 1). As most of these reports were made after conducting cross-sectional studies, researchers were unable to determine the causal relationship between oral dysbiosis and periodontal disease occurrence (39).

The oral microbiota induces inflammatory and immune responses in oral tissues (49, 50), which in turn affects cardiometabolic health and promotes the onset of cardiovascular disease. The microbial invasion of the bloodstream (51) and alterations in gut microbiota caused by oral-gut microbiota transfer (52, 53) may also exacerbate systemic inflammation. Oral dysbiosis ultimately manifests as systemic inflammation, immunoreaction, oxidative stress, and thrombosis. Systemic inflammation may also disrupt the balance of the oral microbiota, as the relationship is bi-directional (54). This paper primarily focuses on discussing the potential upstream mechanisms of the abovementioned pathological outcomes (55).

2.2.1 Crosstalk between the microbiota and local environment

Oral microbiota participates in the inflammation and immune regulation of local environment (56). Oral dysbiosis induces the recruitment of neutrophils and macrophages, which not only prevents further destruction of connective tissues by the microbiota, but also stimulates the immune responses of cells such as dendritic and gamma delta cells, thus inducing the release of pro-inflammatory mediators (tumor necrosis factor α (TNF-α); interleukin-1β (IL-1β); interleukin-17 (IL-17)), and regulates the function of T helper cells (21). It has been reported that these inflammatory states are positively correlated with the oral microbiota load, thereby creating a vicious cycle (21).

An elevated concentration of bacterial surface molecules, such as LPS or bacterial flagellins, stimulates the production of inflammatory mediators and cytokines, thereby promoting inflammation and immunoreaction (57, 58). The mechanisms of this stimulation may involve the activation of inflammatory pathways such as the matrix metalloproteinase 9 (MMP9) and Nuclear factor kappa-B (NF-κB) and Basic Helix-Loop-Helix ARNT Like 1 (BMAL1) pathways (27, 59). Under the effects of TNF-α, interleukin 6 (IL-6), and transforming growth factor β (TGFβ), epithelial and immune cells trigger the production of reactive oxygen species (ROS), reactive nitrogen species, and



Disease	Spices
Systemic inflammation	*Porphyromonas endodontalis* *Eubacterium brachy* *Eubacterium saphenum* *Corynebacterium matruchotii*
Abdominal aortic aneurysm	*Aggregatibacter actinomycetemcomitans*
atherosclerosis	*Campylobacter rectus,* *Porphyromonas gingivalis* *Porphyromonas endodontalis* *Prevotella intermedia,* *Prevotella nigrescens*
Peripheral arterial disease	*Streptococcus mutans* *Porphyromonas gingivalis* *Tannerella forsythia,* *Prevotella intermedia* *Treponema denticola* *Aggregatibacter actinomycetemcomitans*
Hypertension	*Porphyromonas gingivalis* *Neisseria subflava*

FIGURE 1
Oral microbiota and bacterial species related to cardiovascular disease occurrence The oral microbiota is influenced by multiple factors. Pathogens gain entry into systemic tissues *via* oral-gut microbiota transfer and bacteremia, thereby endangering cardiovascular health. The table presents the relationship between the microbiota and specific diseases. Red and blue indicate microorganisms with adverse and protective effects, respectively. Oral microbiota and cardiovascular disease common influencing factors are on the left (7, 18, 47, 48).

matrix metalloproteinases, which activate the NF-κB pathway (56).

These mechanisms may be involved in the formation of atherosclerotic lesions. In mice, *Porphyromonas gingivalis* infection induces the accumulation of macrophages and inflammatory mediators (such as CD40, interferon-γ (IFN-γ), IL-1β, IL-6, and TNF-α) in atherosclerotic lesions; however, the abovementioned inflammatory responses were shown to be milder in mice with congenital immunodeficiency (60). Animal experiments have demonstrated that *Porphyromonas gingivalis* and other bacteria can cause abnormal endothelial relaxation, and thus aggravate atherosclerosis and hypertension; however, periodontal treatment may improve endothelial function (16). In addition to *Porphyromonas gingivalis*, other pathogenic bacteria can trigger destructive inflammation involving both innate and adaptive immune factors (61). Host oral tissue immunoreaction further induces inflammation, thereby causing irreversible downstream pathological changes. Mice with oral mucosal damage that underwent OMT from healthy mice exhibited a reconstruction of the epithelium and tongue papillae, decreased leukocyte infiltration in the oral epithelium, and amelioration of oral mucositis, thereby demonstrating the causal effects of the oral microbiota on local tissues (62).

The oral microbiota also interacts with mucus such as saliva. Moreover, mucus rinses microorganisms off from the inner epithelial surface, while also establishing a protective barrier between the microbiota and the oral epithelium (63). Oral mucus is mainly composed of mucins, which are densely glycosylated polymers that can form three-dimensional structures (64). The oral soft tissues and teeth are coated by a thin film predominantly composed of mucins such as MUC5B and MUC7. Mucins contain glycans that serve as the main energy source for the oral microbiota. Many microorganisms contain genes encoding the relevant enzymes that break down and digest these glycans. Mucus also affects the competition between the microbiota, and hence the viability of bacterial species. For example, previous studies using an artificial model of salivary mucins reported that mucins promote the co-existence of two competing bacterial species, *Streptococcus mutans* and *Streptococcus sanguinis*. Furthermore, other studies have confirmed that mucins prevent the formation of biofilms by pathogens, including *Streptococcus mutans* (65, 66). MUC5B affects intraspecific interactions by promoting the production of bacterial proteomes. For example, *Streptococcus gordonii* cultured with MUC5B promoted the production of six novel biofilm cell proteins and three planktonic proteins, thereby eliciting specific responses in the bacterial biofilm cell proteome (67). These findings highlight the important roles oral environment on the microbiota. (Figure 2).

2.2.2 Bacteremia

Dysbiosis involves microbial invasion of the bloodstream. It is usually transient due to the rapid immune response that follows. Due to the unstable duration and nature of bacteremia (68), it is hard to confirm that oral microbiota is directly involved in the formation of atherosclerotic plaques (69). Similar findings have been reported in gut microbiota research, but a clear link between dysbiosis and cardiovascular disease occurrence has not yet been established (70). Bacteremia may result from local tissue barrier

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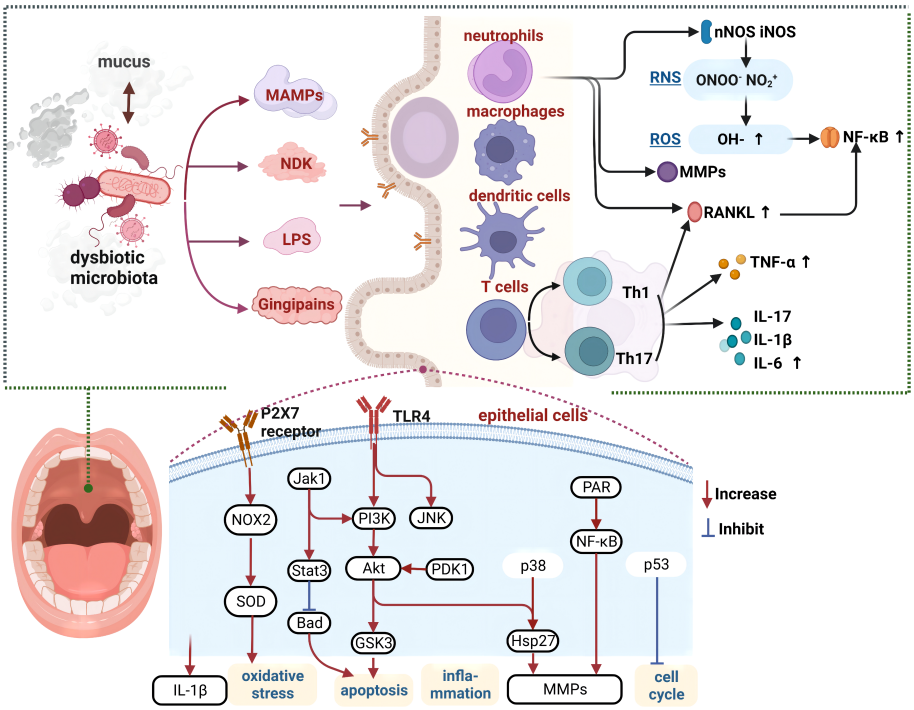

FIGURE 2
Crosstalk mechanisms between the oral microbiota and local tissues Crosstalk of the oral microbiota with saliva and oral epithelial tissues, causing epithelial cell apoptosis, immune cell proliferation, and inflammation, which may further exacerbate dysbiosis. Various cytokines act on vascular endothelial cells to facilitate the onset of cardiovascular disease. MAMPs, microbe-associated molecular patterns; NDK, nucleoside-diphosphate-kinases; LPS, lipopolysaccharide; TLR, toll-like receptors; MMPs, matrix metalloproteinases; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor-α; MMPs, matrix metalloproteinases; Th, T helper; IL, interleukins.

damage. Although the bacterial species found in atherosclerotic plaque formation are common in the oral cavity, they are few (71) (Figure 1). At the species level, *Streptococcus* spp., "red complex" bacteria, *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum*, and *Prevotella intermedia* have been detected in atherosclerotic plaques (10, 11, 72). The antibodies induced by *Porphyromonas gingivalis* are commonly found in saliva and subgingival sites (60, 72). The cross-reactivity of bacterial antibodies (predominantly against *Firmicutes, Pseudomonadota*, and *Bacteroidetes*) with human heat shock protein 60 in host endothelial cells can elicit autoimmune responses, thereby activating a series of cascade reactions and promoting atherosclerosis (73). Bacterial surface proteins, such as glycosyltransferase, the sialic acid-binding human serum albumin (Hsa), and the co-aggregation proteins CshA and CshB, play a key role in host endothelial cell invasion and bacteremia development (51). Some bacterial surface proteins bind to host pattern recognition receptors. For example, bacterial surface proteins in the bloodstream that bind to nucleotide-binding oligomerization domain 2 receptors in the brain can regulate metabolism, body temperature, and appetite (74). In addition, bacterial structures, such as the flagellum, may destroy tissue barriers by acting as virulence factors, thereby increasing inflammation (75).

The microbiota dysbiosis could promote bone marrow activity and increase risk of cardiovascular diseases. Cytokines and bacterial by-products are known to increase marrow myelopoiesis and glycolysis *via* the hematopoietic-arterial axis or inflamed periodontal tissues which aggravate arterial inflammation (76).

2.2.3 Oral-gut microbiota transfer

Studies have found that there may be an overlap of approximately 45% between the oral and gut microbiotas (77), and the oral microbiota may lead to gut dysbiosis *via* oral-gut microbiota transfer or through other pathways (Figure 1). Similarities can be detected between the microbial colonization of the oral and gut microbiotas (78). Furthermore, certain genetic variations in the host are associated with both types of microbiotas, as demonstrated by the identification of five genetic loci that were significantly associated with the oral microbiota, three of which were also significantly associated with the gut microbiota (79). The oral microbiota is mainly composed of five main phyla (*Protebacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteriota*) (7), of which *Proteobacteria;*

Neisseria, *Firmicutes and Streptococcus* are the most extensively studied. However, studies have found that the dominant genus in the oral cavity may differ between people in different countries (Chinese population was dominated by *Neisseria* (7), Canadian population was dominated by *Veillonella* (47), Qatari population was dominated by *Prevotella* (48)), which may be related to sociodemographics. The gut microbiota generally consists of six main phyla (*Firmicutes, Bacteroidetes, Actinobacteria, Pseudomonadota, Fusobacteriota*, and *Verrucomicrobiota*), of which Firmicutes and Bacteroidetes are the dominant phyla (80). Hitherto, the modes of oral-gut interactions remain poorly understood.

The majority of existing studies involve orally administering mice with oral microbiota and observing changes in the composition of gut microbiota. A previous study reported that an oral administration of *Porphyromonas gingivalis* triggers a clear floristic separation in gut microbiota, with a significant increase and decrease in the proportions of Bacteroidetes and Firmicutes, respectively. This was accompanied by a decreased mRNA expression of tight junction proteins (TJPs) in the small intestines and a downregulated genetic expression of TJP-1 and occludin, which are involved in intestinal permeability, thus leading to increased intestinal permeability and impaired barrier function (53). In addition to altering immunomodulation and gut barrier function, the oral administration of *Porphyromonas gingivalis* can affect host metabolism. Mounting evidence suggests that alterations in gut microbiota underlie the pathology of metabolic diseases *via* gut metabolite profile modulation (53, 61). The oral administration of *Porphyromonas gingivalis* in C57BL/6 mice decreased and increased the relative abundances of *Bacteroidetes* and *Deferribacterota* in the gut microbiota, respectively. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed significant decreases in the activation of pathways related to amino and nucleotide sugar metabolisms, chaperones and folding catalysts, glycosyltransferases, limonene, and pinene degradation, as well as folate biosynthesis (81). Interestingly, the oral administration of *Porphyromonas gingivalis* had opposite effects on the relative abundances of gut *Bacteroidetes* and *Firmicutes* in both studies. Moreover, the proportions of gut *Bacteroidetes* and *Firmicutes* are key biomarkers in patients with cardiovascular disease (such as hypertension, coronary heart disease, and stroke), which also decrease and increase the relative abundances of *Bacteroidetes* and *Firmicutes*, respectively (82–84). In a large-scale study of salivary and fecal microbiota in individuals from five countries, bioinformatic analysis revealed that 10% of the oral microbiota are transferred to and subsequently colonize the gut (52). It has been found that the species transfer of opportunistic pathogens is more frequent among diseased individuals; however, the presence of *Fusobacterium nucleatum* subspecies may facilitate this transfer, and therefore aggravate disease severity (85).

OMT can be employed to further investigate the effect of oral microbiota on the composition of gut microbiota. Following the oral transplantation of *Fusobacterium nucleatum* in healthy mice, an elevated conversion of protein 1 light chain 3-I (LC3-I) to protein 1 light chain 3-II (LC3-II) was observed in the colorectal tissue (LC3-II is an important molecular marker of autophagy), and the administration of antibiotics such as metronidazole eliminated this phenomenon. In addition, *Fusobacterium nucleatum* transplantation led to changes in fecal microbiota composition, as demonstrated by an increased abundance of fecal *Fusobacterium nucleatum* (85). Thus, we can assume that the gut microbiota affects the progression of oral mucositis, whereas OMT reduces the malignant reduction of oral and gut bacteria and regulates the gene expression of lingual tissues, and hence OMT has potential therapeutic significance (62). The abovementioned two studies illustrate the causality behind oral-gut microbiota transfer. However, there is ongoing debate as to whether the effects of the oral microbiota are predominantly pathogenic or therapeutic, and further investigations are needed on cardiovascular models.

2.3 Host genotype affects the oral microbiota

Metagenome-genome-wide association studies have revealed that host genes promote the growth of specific oral bacteria. Leucine zipper motif isoform 2 (APPL2) and glucose transporter 9 (SLC2A9) gene polymorphisms have been shown to affect the abundances of multiple oral bacteria and fecal *Bifidobacterium animalis*. These metabolism-related genes are closely associated with obesity and insulin resistance; further, the mechanisms by which these gene polymorphisms affect bacterial abundance may involve specific oral bacterial growth regulation through host microRNAs (79, 86). Loci CAMTA1 (intron variant)/VAMP3 (rs1616122) ($p<5\times10^{-6}$) (87) and loci VAMP8 (rs1561198) ($p<5\times10^{-8}$) (88) may be replicated in the genetic risk locus of cardiovascular diseases and periodontitis. The VAMP8 function is related to membrane vesicular trafficking and corrupting host immune defense (88). The long non-coding RNA ANRIL (antisense noncoding RNA in the INK4 locus) regulates glucose and fatty acid metabolism and is associated with periodontitis (89).

In addition, epigenetic mechanisms can affect the microbiota (90). Obesity, insulin resistance, and angiogenic responses are all cardiovascular risk factors that are closely related to epigenetic mechanisms, which may promote pathogenesis. In a pathological state, host immunodeficiency may shift the balance towards dysbiosis, thereby transforming commensals into pro-inflammatory pathobionts (39).

3 The oral microbiota is involved in the formation of products related to energy metabolism

Previous studies have mostly focused on the identification of microbial communities that are related to cardiovascular event

occurrence, and less on oral microbiota metabolites. The oral cavity is also responsible for the metabolism of energy substances, and hence the effects of metabolites produced by the oral microbiota on the host should not be overlooked (91). As with the gut microbiota, the oral microbiota contains a large number (approximately 1839) of biosynthetic gene clusters that produce a variety of metabolites *via* a wide range of mechanisms (78). Currently, bacteriocins and sactipeptides are the most popular secondary metabolites in oral microbiota research, although their actions have not been linked to cardiovascular disease occurrence. This paper mainly introduces the metabolites that are closely associated with cardiovascular disease occurrence and examines the differences between oral and gut microbiotas.

3.1 Short-chain fatty acids

SCFAs are key metabolites produced by the microbiota that are involved in the host's inflammatory response, lipid metabolism pathway, and gluconeogenesis (92, 93). Pyruvate is produced by the microbiota *via* glycolysis and the pentose phosphate pathway, and then converted *via* other branch pathways into SCFAs, such as acetic, propionic, butyric, and isobutyric acids (94). Amino acids can also be metabolized to produce small amounts of SCFAs (91). Oral bacteria can utilize carbohydrate-active enzymes for the degradation of carbohydrates into SCFAs, which then support their own energy metabolism (95). The proteases and peptidases produced by the microbiota break down proteins in food, and the resulting peptides and amino acids are converted into SCFAs after deamination (96, 97). Therefore, different dietary habits, especially sugar intake, can immensely affect the oral microbiota (96–98). Bacteria that are capable of utilizing sugars to produce SCFAs include *Streptococcus, Actinomyces, Lactobacillus*, *Propionibacterium*, and *Prevotella* (91, 99).

There is conflicting evidence surrounding the local and systemic effects of SCFAs in the oral cavity. On the one hand, whilst breaking down carbohydrates to produce SCFAs, the oral microbiota also generates lactic and acetic acids, thereby causing SCFA-producing bacteria to act as a double-edged sword. Lactic and acetic acids reduce the local pH, leading to dysbiosis development, which exacerbates periodontitis and dental caries (98, 100). Lactic acid may also promote immune cell activation and damage oral epithelial cells, leading to persistent local inflammation (101). On the other hand, there is an ongoing debate as to whether SCFAs exert protective or destructive effects on the oral cavity. Due to the differences in host tissues, gut SCFAs may reduce the occurrence of intestinal epithelial cell apoptosis and autophagy *via* the phosphatidylinositide 3-kinases/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway, which leads to the protection of the local mucosal barrier (102, 103); nevertheless, oral SCFAs have been found to alter the expressions of connexins and adhesion proteins, thereby impairing oral epithelial cell function (104). Pathogenic bacteria that cause tumor proliferation and metastasis (105) generally exhibit glycolysis and acid production functions, which decrease the pH and cause dysbiosis. Examples of such bacteria include *Bifidobacterium longum, Bifidobacterium dentium, Streptococcus mutans*, and *Scardovia wiggsiae* (98, 106, 107).

SCFAs exhibit anti-inflammatory effects in plasma. Mice supplemented with 1% butyrate for 10 weeks displayed a 50% reduction in the area of aortic plaques compared with those in the control group; this suggests that butyrate may have an anti-inflammatory function (84). SCFAs are also known to inhibit the NF-κB and Akt signaling pathways, thereby reducing plasma cytokine (TNF-α, IL-12, and INF-γ) levels to achieve anti-inflammatory effects and increase peroxisome proliferator-activated receptor-γ (PPAR-γ) pathway expression to improve insulin sensitivity (108, 109). Furthermore, SCFAs suppress histone deacetylases (HDACs) and bind with specific G protein-coupled receptors (GPRs) to achieve cardiovascular protective effects (110). SCFAs that act as HDAC inhibitors include valproic acid and sodium butyrate; moreover, the reversible lysine acetylation process is closely associated with myocardial infarct size reduction, myocardial hypertrophy, and cardiac fibrosis suppressions, as well as angiogenesis promotion (111, 112). In spontaneously hypertensive rats, HDAC activation was found to promote hypertension and myocardial hypertrophy occurrence, whereas valproic acid administration led to the reversion of inflammation and hypertension reversions (113, 114). Among the GPRs, GPR43 is expressed in the heart and binds with a wide range of SCFAs from formic to valeric acid, to enhance insulin sensitivity, energy expenditure, and anti-inflammatory effects (115). Furthermore, Olfactory receptor78 (Olfr78) and GPR41, which are expressed in the kidneys, can facilitate blood pressure reduction in response to propionate administration (116). More specifically, Olfr78 is expressed in the juxtaglomerular apparatus to mediate renin secretion (117), and GPR41 is expressed in the smooth muscle cells of renal blood vessels to reduce vascular resistance (116, 118). Therefore, SCFAs are promising research target metabolites, and the extent to which they are involved in cardiovascular disease processes warrants further exploration (Figure 3).

3.2 Nitric oxide

Nitric oxide (NO) is an important gaseous signaling molecule involved in endo- and exogenous metabolic pathways (119), and the oral microbiota are a key NO source for exogenous metabolism. When there is insufficient NO synthesis mediated by endogenous nitric oxide synthase (NOS), the nitrite (NO_2^-) produced by the oral microbiota serves as an important NO reservoir in the bloodstream and tissues. Thus, the absence of

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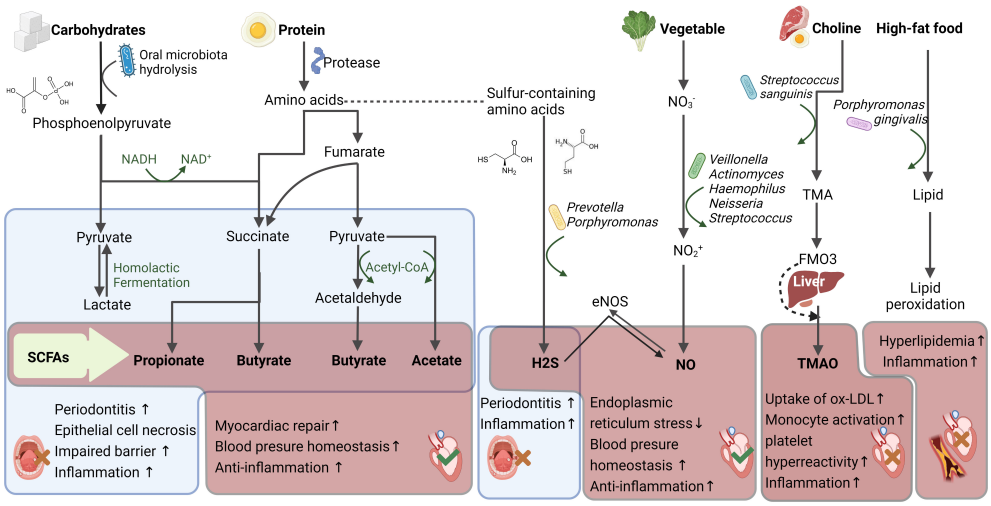

FIGURE 3
Oral microbiota metabolites and its impact upon cardiovascular health. The oral microbiota produce short chain fatty acids (SCFAs), hydrogen sulfide (H_2S), nitric oxide (NO), trimethylamine oxide (TMAO), lipids and other metabolites from the digestion of different foods, which affect oral and cardiovascular health. The red box displays the cardiovascular effects of the metabolites and the blue displays the oral effects. Red wrong number refers to harmful, while green right number refers to beneficial. DANH/NAD+, Nicotinamide adenine dinucleotide; ox-LDL, Oxidized-Low-Density Lipoprotein Cholesterol; Flavin-containing monooxygenase 3, FMAO3; Trimethylamine, TMA.

specific nitrate (NO_3^-)-reducing bacteria or alterations in oral microbiota structure may disrupt the NO_3^--NO_2^--NO pathway, leading to NO insufficiency in the body (120). Dietary NO_3^- is mainly sourced from green leafy vegetables and can be reduced by the oral microbiota to NO_2^- and NO, *via* bacterial NO_3^- reductases. As humans lack these enzymes, this process cannot be accomplished by the host alone. Dietary NO_3^- is recycled in the human body through enterosalivary NO_3^- circulation. NO_3^- is first absorbed in the proximal digestive tract along with food; approximately 25% of NO_3^- is actively concentrated in the salivary glands, such that NO_3^- concentration in saliva is 20 times higher than that in plasma (121, 122). Following concentration in the salivary glands, salivary NO_3^- is reduced *via* the action of NOS and the anaerobic pathway to form NO_2^-, which re-enters the body through mastication. Subsequently, NO_2^- is converted to nitrous acid in the digestive tract, followed by the formation of NO and NO donors, as well as a series of secondary nitrosation and nitrification products from reactions facilitated by the low gastric pH (123), or further reduction by nitrite reductases released by the gut microbiota (124). The genera *Veillonella, Actinomyces, Haemophilus, Neisseria, Streptococcus* have been reported to exhibit NO_3^- reduction function (125, 126).

Both NO_2^- and NO have strong NO signaling effects that can stimulate the circulatory system to promote systemic health (127). NO_2^- metabolism is activated by hypoxia, low pH, and reactions with metalloproteins (128, 129). NO is a key molecule in the oxidative stress pathway with vasoactive and endothelial protective effects. It can reduce blood pressure, ameliorate atherosclerosis, protect against ischemia-reperfusion injury, improve platelet aggregation, and exert anti-inflammatory effects (129). NO deficiency is closely associated with cardiovascular disease onset, and therefore NO can serve as a predictor of cardiovascular events (130). The level of NO produced by the oral microbiota affects the cardiovascular disease course. Using a murine periodontitis model, a previous study found that infection with *Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia* for 16 weeks led to significant plasma NO level reduction, NO-related pathway (tetrahydrobiopterin/neuronal nitric oxide synthase/Nuclear factor (erythroid-derived 2)-like 2 (BH4/nNOS/NRF2)) inhibition in the colon and plasma, and atherosclerotic plaque area increase. Therefore, oral dysbiosis triggered a reduction in NO synthesis and bioavailability, which resulted in impaired vascular function (131). In $ApoE^{-/-}$ mice colonized with *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia*, and *Fusobacterium nucleatum* for 24 weeks, a significant decrease in plasma NO levels was observed, which was accompanied by significant increases in the levels of inflammatory factors such as IL-1β, IL-13, IL-4, lymphotactin, and the regulated chemokine (upon on activation normal T cell expressed and secreted factor (RANTES) (132)). Furthermore, the mice developed bacteremia, inflammatory response, and atherosclerosis. These findings indicate that NO, which is mediated by the oral and gut microbiotas, also plays a crucial role in the pathogenesis of atherosclerosis (Figure 3).

3.3 Hydrogen sulfide

The oral environment is rich in sulfur-containing amino acids (such as cysteine, and methionine) that can be metabolized *via* proteolytic bacteria (including *Prevotella* and *Porphyromonas*) to produce hydrogen sulfide (H_2S). H_2S is considered the third most important endogenous gaseous signaling molecule, after CO and NO, and plays a physiological role in life processes. Its metabolic process in the body mainly involves the use of L-cysteine and L-homocysteine as substrates and is completed under enzymatic catalysis. A paradox also exists for H_2S; the presence of H_2S in the oral cavity may lead to halitosis (133), enhance oral inflammation (134), and even increase the risk of oral cancer (135). However, in plasma, H_2S triggers strong anti-oxidation, anti-inflammation, as well as insulin resistance improvement, and thus can regulate several cardiovascular functions (136). Furthermore, H_2S may exert more beneficial effects by interacting with NO. For example, H_2S can activate endothelial NOS *via* the phosphorylation of Ser1177, which significantly increases the bioavailability of NO and NO-mediated cytoprotective signaling (137).

H_2S is produced by a minority of oral bacteria, and hence remains at low levels in healthy individuals; however, an elevated oral microbial load associated with the oral disease can significantly increase overall H_2S levels (138), which amplifies the inflammatory response, thereby leading to periodontal disease onset. One possible explanation for the amplified inflammatory response is that H_2S can trigger the release of IL-1β and IL-18, which are inflammatory cytokines. A previous study demonstrated that the dose-dependent activation of the cyclooxygenase-2 (COX-2), Akt, and extracellular regulated protein kinases1/2 (ERK1/2) pathways by H_2S can promote the proliferation of oral cancer cells (135). It is currently unclear whether the H_2S produced by the oral microbiota can affect plasma H_2S concentration and the related metabolic pathways. However, existing studies have confirmed that the oral administration of H_2S may have a beneficial effect on cardiovascular metabolism (139, 140). Dietary supplementation with garlic oil (a natural source of H_2S) can help to increase renal mRNA expression, H_2S-generating enzyme activity, NO bioavailability, and plasma SCFA levels. Moreover, garlic oil supplementation during lactation and pregnancy reportedly confers protection against hypertension in adult offspring (141). In addition, the oral administration of sulfur-containing products can restore the circulating levels of sulfides, and H_2S therapy has been found to restore adiponectin levels and suppress high-fat diet (HFD)-induced cardiac endoplasmic reticulum stress. It has been reported that intraperitoneal injection of Na_2S improves survival through attenuation of inflammasome-mediated adverse remodeling (142). Furthermore, plasma and myocardial H_2S levels play important roles in the pathophysiology of diabetic cardiomyopathy (143). However, oral bacteria produce very low concentrations of H_2S, and there is no evidence of simultaneous changes in H_2S metabolism in oral and cardiac tissues (Figure 3).

3.4 Lipid metabolites

A HFD induces significant changes in the oral microbiota (144); in addition, it causes lipid regulator activity elevation or atherosclerosis-promoting metabolite production through specific bacterial populations. For example, trimethylamine oxide (TMAO) accelerates cardiac remodeling, stimulates the renin-angiotensin system, increases oxidative stress, and accelerates endothelial dysfunction, which can promote the development of cardiovascular diseases such as heart failure, hypertension, coronary heart disease, and arrhythmia. The gut microbiota has been thought to be the main source of TMAO (145). Specifically, the gut microbiota converts choline and carnitine from ingested meat and eggs into trimethylamine (TMA), which passively diffuses into the bloodstream through the intestinal wall and enters *via* the portal vein into the liver, where it is oxidized by flavin-containing monooxygenases into TMAO. In the oral cavity, TMAO can also be produced by *Streptococcus sanguinis*, which has been shown to enhance the role of the gut microbiota in TMA-accelerated atherosclerosis (146). Furthermore, oral dysbiosis may exacerbate dyslipidemia and *Porphyromonas gingivalis* has been found to have a significant proteolytic effect on (6)lipoproteins and is involved in the aggravation of lipid peroxidation (147) (Figure 3).

4 Oral microbiota transplantation facilitates research on systemic diseases

Three issues are still heavily debated: 1) the causal relationship between oral microbiota and cardiovascular diseases; 2) the pathological mechanism of oral dysbiosis rather than periodontal disease; 3) the therapeutic effect of oral microbiota. In terms of treatment, dietary supplements, such as arginine, can also substantially affect the composition and metabolic output of oral microbial communities and are known to be involved in NO regulation (148, 149). Microbiota sequencing suggests that brushing the teeth could not only remove dental plaque but may also have a positive effect on the oral chemical environment and the metabolism of the oral microbial population (150). The combined analysis of multi-omics and the support of experimental techniques are improving the current situation.

OMT and animal models that mimic the oral dysbiosis of humans could be used to reconstruct the oral microbiota in mice and observe changes in cardiovascular disease phenotypes. It has been reported that oral microbiota may influence the composition of the intestinal microbiota and induce intestinal injury after

transplantation. Oral *Fusobacterium nucleatum* infection is reportedly an exacerbating factor of colon cancer and affects the efficacy of radiotherapy (85). In terms of treatment, OMT ameliorates oral mucositis, which manifests as a remodeling of the oral mucosal epithelium and lingual papillae, a decrease in the leukocyte count, and an increase in the number of proliferating oral epithelial cells (62). Standardized sampling strategies (151)and sterilization of oral microbiota (152) are the basis of OMT. At present, few animal-based studies have employed the OMT technique; nevertheless, a consensus has not been reached with regard to its protocol, and thus further investigation is warranted. Based on the literature, we have compiled a standardized OMT protocol for the mechanism of cardiovascular disease in the future (62, 85, 153) (Figure 4).

Sample preparation: Aimed at human systemic diseases, sampling sites include saliva, supragingival plaque, subgingival plaque, tongue coating, etc (151). In animal experiments, due to the limitation of the oral region, the whole oral cavity is generally collected (152).

Recipient mice preparation: It's not mandatory to deplete the oral microbiota of recipient mice. To deplete specific bacteria, fluoroquinolones are generally more effective against gram-negative bacteria compared to gram-positive bacteria (152). Metronidazole can remove gram-negative bacteria (85).

5 Discussion

In 2021, *Nature* specifically launched research on special topics in oral health, and repeatedly proposed the potential research value of the oral microbiota and its relationship with chronic diseases (154, 155). There is mounting evidence to support the crucial role of oral microbiota in cardiometabolic health and diseases. However, the effects of the oral microbiota on oral and cardiovascular health remain paradoxical. A more conclusive finding among the numerous studies is that oral *Porphyromonas gingivalis* and *Porphyromonas endodontalis* can trigger oral and systemic inflammation as well as immunoreaction in the host. Under conditions of oral dysbiosis, chronic inflammation and persistent infection may cause a buildup of immunological memory in immune cells, which elicits an overreaction of the immune system to inflammatory and bacterial signals, thereby creating a mutually reinforcing vicious cycle. The oral microbiota causes local barrier damage and bacteremia, which have been previously demonstrated in the gut microbiota. However, the oral microbiota is upstream to the digestive tract, and therefore can affect the gut microbiota *via* microbial transplantation, thereby further aggravating the cardiovascular disease. This may be a pathogenic mechanism that is unique to the oral microbiota. Furthermore, the oral microbiota is involved in various forms of energy metabolism. Carbohydrates and proteins are metabolized by the oral microbiota to produce SCFAs. Plant-based dietary NO_3^- are metabolized to produce NO, which has vasoactive effects. Aromatic and sulfur-containing amino acids produce indole and H_2S, respectively; moreover, H_2S causes vascular smooth muscle relaxation and therefore confers cardiovascular protection. The oral microbiota also intervenes in the HFD-induced elevation of blood lipids. Hence, the oral microbiota is a catalyst for the development of cardiovascular disease induced by poor dietary habits.

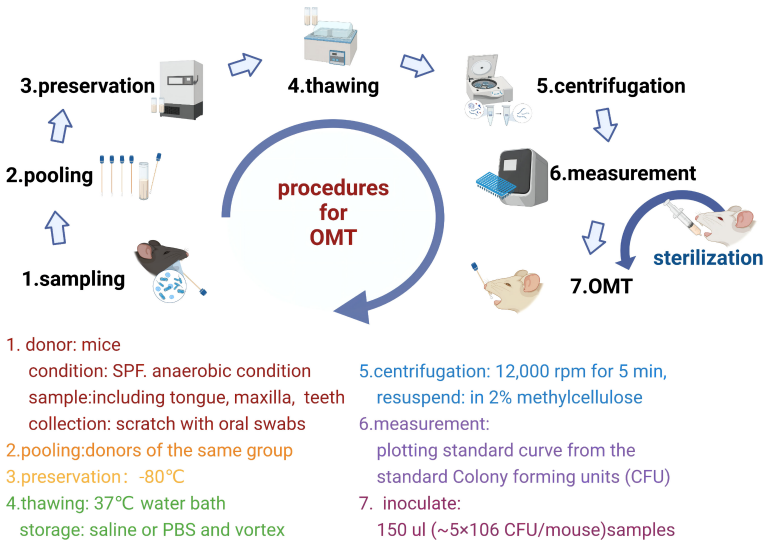

FIGURE 4
Flowchart of a standardized oral microbiota transplantation protocol.

The metabolites of the oral microbiota have not yet been fully explored. Although it is known that microbiota structure, LPS, and metabolites directly affect an individual's health, there is no consensus concerning which of the factors is the predominant pathogenic factor. The effects and mechanisms of secondary gut microbiota metabolites (such as bile acids and TMAO) on cardiovascular disease occurrence have been verified, although these effects have not been detected using secondary oral microbiota metabolites. Despite ample research demonstrating the potential significance of oral microbiota, no study has reported a direct link between oral microbiota and cardiovascular disease occurrence. Numerous questions remain unanswered concerning the abovementioned relationship from the perspective of microbiota metabolites or composition. Many microbes in the oral cavity are dependent on commensals, and thus cannot be cultured alone, which may pose obstacles to understanding the functions of the microbiota. To overcome this limitation and evaluate the effects of the oral microbiota on cardiovascular disease occurrence, a future direction of research should be aimed at developing culture-free deep metagenomic sequencing and single-cell sequencing techniques. Interestingly, gut microbiota research may provide numerous ideas and methods for reference in oral microbiota research. Studies have shown that the oral microbiota can be modulated using mouthwash (156) or vegetable-derived nitrate (157, 158) hence carrying out cardioprotective effects, while tooth brushing and periodontal therapy can somewhat ameliorate the severity of cardiovascular diseases (159–162). However, there is currently a lack of targeted therapy for the oral microbiota, and dietary interventions are still in the preliminary stage.

Traditional Chinese medicine theorizes that the human tongue coating can assist in the diagnosis and treatment of diseases. This clinical practice has been verified at the microbiome level, and the presence of *Campylobacter concisus* in the tongue coating can be used to guide the early diagnosis of gastric cancer (163). There is a traditional Chinese medicinal theory that "the tongue is the window of the heart". Thus, an accurate analysis of the composition and function of the oral microbiota will contribute to the diagnosis and treatment of cardiovascular disease. This may be one of the most perceptive insights for oral microbiota research derived from traditional Chinese medicine and may yield brilliant results in the future.

Author contributions

YWL and MZ performed the reference collection, conducted the reference analysis, and wrote the manuscript, thus are considered as co-first authors. YL contributed to the topic conception, manuscript revision, and decision to submit for publication. YFL, JC and BL contributed to reference analysis and helped in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (82022076).

Acknowledgments

We would like to acknowledge the support of colleagues/students who have contributed to the work relevant to this article and thank Editage (www.editage.cn) for English language 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.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 07 December 2022
DOI 10.3389/fimmu.2022.1009304

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 01 August 2022
ACCEPTED 09 November 2022
PUBLISHED 07 December 2022

CITATION
Saghari M, Gal P, Grievink HW, Klaassen ES, Itano A, McHale D and Moerland M (2022) Impact of oral administration of single strain *Lactococcus lactis* spp. *cremoris* on immune responses to keyhole limpet hemocyanin immunization and gut microbiota: A randomized placebo-controlled trial in healthy volunteers. *Front. Immunol.* 13:1009304. doi: 10.3389/fimmu.2022.1009304

Impact of oral administration of single strain *Lactococcus lactis* spp. *cremoris* on immune responses to keyhole limpet hemocyanin immunization and gut microbiota: A randomized placebo-controlled trial in healthy volunteers

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Introduction: *Lactococcus lactis* spp. *cremoris* has been associated with promising immunomodulatory results in preclinical trials. The aim of this study was to investigate the pharmacodynamic (PD) effects of three monoclonal microbial formulations of *L. lactis* spp. *cremoris* (EDP1066) on the immune response to keyhole limpet hemocyanin (KLH). Potential effects on the gut microbiota were also investigated.

Methods: The trial was registered on Netherlands Trial Register (trial ID NL7519, https://trialsearch.who.int). Eighty-one healthy subjects (median 28, range 18–59 years) were randomized to 28 days of enteric-coated capsules at five doses (n = 13) ($1.5 * 10^{12}$ total cells daily), freeze-dried powder at one dose (n = 12) ($3.0 * 10^{11}$ total cells daily) or five doses (n = 12), minitablets at one dose (n = 12) or five doses (n = 12), or placebo (n = 20) prior to KLH immunization. Antibody responses and circulating regulatory T cells (Tregs) were measured after KLH immunization, and skin responses were evaluated after a KLH rechallenge by laser speckle contrast imaging and multispectral imaging. *Ex vivo* lymphocyte (phytohemagglutinin) and monocyte (lipopolysaccharide (LPS)) cytokine release assays were explored in the minitablet-treated groups only. The prevalence of *L. lactis* spp. *cremoris* in the gastrointestinal tract and the impact on the fecal microbiota were assessed by qPCR and 16S rRNA sequencing, respectively.

Results: Repeated-measures analysis of covariances revealed no significant treatment effects on the antibody responses to KLH, number of Tregs, or KLH

10.3389/fimmu.2022.1009304

skin rechallenge outcomes. *Ex vivo* LPS-driven cytokine responses in whole blood were lower in the low dose minitablet group compared to placebo: tumor necrosis factor (estimated difference (ED) from placebo: –44.2%, 95% confidence interval (CI) –65.3% to –10.3%), interleukin (IL)-1β (ED –41.4%, 95% CI –63.5% to –5.8%), and IL-6 (ED –39.2%, 95% CI –56.8% to –14.5%). The fecal presence of *L. lactis* spp. *cremoris* increased during treatment by all EDP1066 formulations and normalized 5 days after the last dose. Microbiome α-diversity did not change by the treatments compared to placebo.

Discussion: The EDP1066 formulations did not affect the immune response to KLH immunization in healthy individuals. However, exposure to *L. lactis* spp. *cremoris* in minitablet formulation impacted *ex vivo* whole blood LPS cytokine response. The clinical impact of these effects awaits further investigations.

Netherlands Trial Register: trialsearch.who.int, trial ID NL7519.

KEYWORDS

EDP1066, *Lactococcus lactis* spp. *cremoris*, gastrointestinal microbiome, keyhole limpet hemocyanin, late-phase skin reaction, delayed-type hypersensitivity, autoimmune disease

Introduction

Over the past decades, evidence has emerged for an interplay between the systemic immune system and the intestinal microbiome (1–3). The epithelium of the intestinal wall contains immune cells throughout, including in aggregated lymphoid nodules (Peyer's patches), and the lamina propria and linked mesenteric lymph nodes (1, 4). Regional specialization of the gut immune network has been thoroughly studied in mice with differences found in antigenic composition, leukocyte populations, and gut-associated lymphoid tissue (GALT) (1). Although less evident, similar observations have been made in humans. The mucosa of the intestinal wall is also home to an abundance of microorganisms, and the composition and distribution of the microbial populations are dependent on the location within the gastrointestinal (GI) tract (1). Alterations in either the intestinal immune system or the gut microbiome can lead to various ailments such as celiac disease and inflammatory bowel disease (1, 5–7). Importantly, there is a growing body of evidence that hypothesizes that the effects of intestinal dysbiosis are not limited to local immunity and can also modify the immune response more distally as observed in systemic lupus erythematosus (8), rheumatoid arthritis (9), psoriasis (10), and more (11, 12). Altering the intestinal microbiota in these patient populations with intestinal dysbiosis, therefore, seems a plausible approach to evoke systemic immune modulation and consequently treat diseases associated with dysregulated immune responses. This hypothesis has been tested in more recent trials with orally administered probiotics (live microorganisms, when administered in adequate amounts, confer a health benefit on the host) (13, 14), prebiotics (non-digestible carbohydrates used as nutrients for probiotics), and/or synbiotics (blend of probiotics and prebiotics), which seem to have beneficial effects on dysregulated systemic immune responses (15–19), with some exceptions (15). Intake of certain probiotics has also been found to increase the responses to certain vaccinations (e.g., influenza) in humans depending on the choice, strain, dose, etc., of probiotics and vaccine type, dose, timing, and route (20). Interestingly, oral probiotics have also been demonstrated to be effective for the treatment of topical skin conditions, such as atopic dermatitis, acne, and rosacea (21), indicating induction of immune regulators. How oral administration of probiotic bacteria can modulate systemic immune responses and T cell-mediated inflammation in remote skin tissue is however unclear. Furthermore, studies using microbial strain mixtures suggest different immunomodulatory effects or even antagonism between species when compared with single-strain microbes, complicating the understanding of the underlying mechanisms (22–25).

One such single-strain microbial intervention is EDP1066, prepared from *Lactococcus lactis* spp. *cremoris* identified from powders used in dairy product manufacturing. Preclinical data of EDP1066 on both *in vitro* immune cell cultures and *in vivo* murine immune challenge and disease models show promising results; however, these data are not currently available in the

public domain. In separate independent research, *L. lactis* spp. *cremoris* restored T-cell impairment in aged mice (26), and coadministration of *L. lactis* spp. *cremoris* with *Lactobacillus paracasei* spp. *paracasei* relieved atopic dermatitis symptoms, decreased serum IgE concentration, and rebalanced the population of Th1/Th2 cells in an atopic dermatitis mouse model (27).

Keyhole limpet hemocyanin (KLH) is a metalloprotein derived from the hemolymph of the marine mollusk, *Megathura crenulata*, which can be found in the Pacific coastal waters of California and Mexico. As the human body is unfamiliar with KLH, an *in vivo* immune response to this protein can be used to "mimic" an immune response to a pathogen or allergen in healthy volunteers (such as KLH-specific antibody formation and increased T-cell response after intradermal KLH rechallenge), providing essential information on proof-of-pharmacology during early-phase drug development (28–34). KLH was clinically introduced in 1967 to study the immunocompetence of humans (35) and since then is proven to be safe and widely used in clinical trials (28–31, 36–41).

The primary aim of the present study was to characterize the pharmacodynamic (PD) effects of EDP1066 on the systemic immune response to an intramuscular immunization with KLH and secondary to evaluate the effects on a subsequent KLH skin rechallenge. Because the exposure sites within the GI tract for ingested microbes may depend on the formulation and therefore be important for the immunomodulatory effect (1, 42), we also aimed at comparing different EDP1066 formulations (enteric-coated capsules, free freeze-dried powder, and minitablets) having different expected peak exposure sites. Furthermore, EDP1066 effects on numbers of circulating regulatory T cells (Tregs) were evaluated, and the *ex vivo* immunomodulatory activity of EDP1066 was explored by whole blood stimulation with the Toll-like receptor 4 ligand lipopolysaccharide (LPS) and phytohemagglutinin (PHA) for monocyte and lymphocyte stimulation, respectively. Finally, we aimed at assessing the impact of EDP1066 on the fecal microbiota, next to routine safety and tolerability assessments.

Materials and methods

Ethics

The independent Medical Ethics Committee "Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek" (Assen, the Netherlands) approved the study prior to any clinical study activity. All subjects provided written informed consent before participation. The trial was registered on the Netherlands Trial Register, currently available for consultation at the International Clinical Trial Registry Platform (trial ID NL7519, https://trialsearch.who.int).

Subjects

Healthy male and female participants were recruited *via* media advertisements and from the subjects' database of the Centre for Human Drug Research, Leiden, the Netherlands. Enrolled participants were 18 to 60 years of age with a body mass index between 18 and 35 kg/m^2 (2) and without previous exposure to KLH. Health status was verified by recording a detailed medical history, a complete physical examination, vital signs, a 12-lead electrocardiogram (ECG), and laboratory testing (including hepatic and renal panels, complete blood count, fecal calprotectin, virology, and urinalysis). Subjects were excluded in case of any disease associated with immune (e.g., active infection, auto-immune disease, primary or acquired immune deficiency, and clinically profound allergies) or GI system impairment (e.g., short bowel syndrome, diarrhea, inflammatory bowel disease, irritable bowel syndrome, and celiac disease) or use of prescription medication within 4 weeks prior to the first dose. Other exclusion criteria were antibiotic treatment within 42 days prior to initial dosing and during the course of the study and the use of probiotic capsules within 14 days of screening and during the course of the study.

Dose selection and regimen

All EDP1066 and placebo formulations were manufactured and provided by Evelo Biosciences Inc. (Cambridge, MA, USA). The doses tested were based on the results of a separate first-in-man study (43). The highest dose tested contained 1.5 * 10^{12} total cells per dose, approximately five times the predicted therapeutic dose level, calculated from allometric scaling of the preclinically efficacious dose level based on conversion between mouse and human gut surface area. This dose was well tolerated in humans. Three different formulations of the investigational drug were investigated: enteric-coated capsules containing EDP1066 freeze-dried powder, EDP1066 as free freeze-dried powder, and non-coated capsules containing enteric-coated EDP1066 minitablets. For each EDP1066 formulation, matching placebo formulations were used in order to preserve the blinding. The three placebo formulations contained similar excipients as their active treatment counterparts, without the EDP1066 microbes. The excipients present in the three EDP1066/placebo formulations (e.g., microcrystalline cellulose, magnesium stearate, mannitol, citric acid, and sodium hydroxide) are widely used in drug product manufacturing, and none of the excipients were expected to elicit immune system modulation.

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Study design and treatments

This was a phase 1, randomized, placebo-controlled, double-blind, multiple-dose study in 80 healthy volunteers performed at the Centre for Human Drug Research (CHDR), Leiden, the Netherlands, based on the principles of the Declaration of Helsinki. An overview of the study design is shown in Table 1. Participants were randomized to one out of the five groups of EDP1066 or placebo (12:4 per group) in a consecutive order starting with the lowest number. The randomization code was computer-generated by a study-independent statistician and was only made available for data analysis after study completion. One group received EDP1066 freeze-dried powder in enteric-coated capsules, supplied as $1.5 * 10^{11}$ total cells per capsule, administered orally at a dose of 10 capsules daily (5× Capsules). Two other groups received EDP1066 as free freeze-dried powder with an achlorhydria regimen administered orally at a dose of $3.0 * 10^{11}$ (1× Powder) and $1.5 * 10^{12}$ (5× Powder) total cells daily. The achlorhydria regimen consisted of omeprazole 40 mg and aluminum hydroxide/magnesium hydroxide 200/400 mg administration 3 h prior to each EDP1066 dose. Both drugs increase the gastric pH (44–46) and were expected to improve the transition of EDP1066 through the stomach and into the duodenum. Omeprazole and aluminum hydroxide/magnesium hydroxide are not known to induce immune system modulation. Another two groups received non-coated capsules containing enteric-coated EDP1066 minitablets, supplied as $1.5 * 10^{11}$ total cells per capsule, administered orally at a dose of 2 (1× Minitablets) and 10 (5× Minitablets) capsules daily. Participants were dosed once daily for 28 consecutive days. Compliance was confirmed by the supervised administration of the study treatment during the in-clinic period. Administration at home was recorded by an electronic diary. Intramuscular KLH immunization was performed in the left deltoid muscle after the completion of the third administration of EDP1066/placebo. KLH immunization was administered as 0.1 mg of Immucothel® adsorbed in 0.9 mg of aluminum hydroxide (Alhydrogel®) into 0.5 ml of NaCl 0.9% as previously described (47). All subjects were administered KLH (0.001 mg of Immucothel®) and saline in 0.1 ml of NaCl 0.9% intradermally in the left and right ventral forearms, respectively, 23 days after KLH immunization. The skin challenge response was quantified prior to and 2 days after intradermal KLH administration. These are similar intervals between assessments as in our previous studies, which also detail the methodology (29, 32, 36, 38, 41, 47, 48). To account for ambient and environmental factors, the responses observed at the intradermal KLH administration site were corrected against the intradermal saline administration site on the contralateral forearm. A follow-up visit 5 days after the last EDP1066/placebo dose and a study discharge visit 12 days after the last EDP1066/placebo dose were included in order to assess EDP1066 stool persistence and prevalence and EDP1066 effects on the gut microbiome.

Humoral immunity to keyhole limpet hemocyanin

The humoral response to KLH immunization was measured by anti-KLH IgM and IgG serum titers. Serum samples for the analysis of anti-KLH IgM and IgG were obtained in non-additive tubes by venipuncture at the time points indicated in Table 1. Samples were centrifuged at 2,000 *g* for 10 min with a temperature of 2°C–8°C, and the serum was aliquoted. The aliquots were stored at a temperature of −40°C until shipment and analysis. Samples were assessed by quantitative enzyme-linked immunosorbent assay (ELISA) for anti-KLH IgM and IgG as previously described (ELISA developed in-house by Ardena Bioanalytical Laboratory (Assen, the Netherlands)) (47). For the analysis of human antibodies raised against KLH, no reference material was available for the preparation of calibration standards and quality checks. Quantitative

TABLE 1 Study timeline.

	Treatment									FU
Timepoint	**D -1**	**D 1**	**D 3**	**D 5**	**D 10**	**D 17**	**D 26**	**D 28**	**D 33**	**D 40**
Activity										
EDP1066 / placebo administration		←——————————Once daily——————————→								
KLH immunization			X							
Anti-KLH IgM and IgG			X		X	X	X			X
Tregs + *ex vivo* stimulation assays		X	X	X	X		X			X
Intradermal KLH administration							X			
Intradermal KLH readout (LSCI, MI)								X		
Fecal EDP1066 concentration	X						X		X	
Fecal microbiome	X								X	X
Admission	←——————————→									

X indicates performed activity.
D, day; KLH, keyhole limpet hemocyanin; Tregs, regulatory T cells; LSCI, laser speckle contrast imaging; MI, multispectral imaging; FU, follow up.

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measurement of human anti-KLH IgG and IgM (in μg/ml) using a standard curve was not an option. Therefore, the mean optical density of baseline samples was set to 1.00, and relative ratios were calculated for all subsequent samples.

Cutaneous blood perfusion and erythema

Cutaneous blood perfusion quantification was performed with laser speckle contrast imaging (LSCI) (PeriCam PSI System, Perimed AB, Järfälla, Sweden), and erythema quantification was performed with multispectral imaging (Antera 3D®, Miravex, Dublin, Ireland) as previously described (47). Circular regions of interest at the intradermal injection sites were defined. Cutaneous blood perfusion (indicated as basal flow) was quantitatively assessed and expressed in arbitrary units (AUs). The homogeneity of cutaneous blood perfusion in the region of interest (indicated as flare), expressed as values that are +1 standard deviation (SD) from the mean basal flow within the region, was also quantitatively assessed and expressed in AUs. Erythema was quantified using the average redness and CIELab a* Antera 3D® software modalities expressed as AUs. The average redness modality displays the distribution of redness using an internal software algorithm, and the CIELab a* value, which is part of the CIELab color space, expresses color as a numerical value on a green–red color scale.

Circulating regulatory T cells and *ex vivo* stimulation assays

The percentage of circulating Tregs was evaluated by flow cytometry. Venous blood was collected in sodium heparin tubes by venipuncture at the time points indicated in Table 1. Red blood cell (RBC) lysis was performed on heparinized whole blood using RBC lysis buffer (Thermo Fisher, Waltham, MA, USA). Leukocytes were stained with fluorochrome-labeled antibodies CD4-VioBlue, CD25-APC, and CD127-PE; propidium iodide was used as viability dye (all Miltenyi Biotec, Bergisch-Gladbach, Germany). Samples were analyzed on a MACSQuant 16 analyzer (Miltenyi Biotec) using FlowLogic software (Inivai, Mentone, VIC, Australia). Tregs were defined as $CD4^+CD25^+CD127^-$; see Figure S1 for the gating strategy. *Ex vivo* lymphocyte and monocyte cytokine release assays were incorporated later in the study to examine NF-κB-driven responses and only performed in the minitablet-treated groups in which the most optimal immunomodulatory results were expected based on preclinical data. Sodium heparinized whole blood was incubated with 10 μg/ml of PHA (Sigma-Aldrich, Deisenhofen, Germany) or 2 ng/ml of LPS (strain O111:B4 from *Escherichia coli*, Sigma-Aldrich) for 24 h at 37°C, 5% CO_2. After 24 h, the supernatant was collected, and cytokines were measured using qualified ELISA-based assays by Ardena Bioanalytical Laboratory. Interferon gamma (IFN-γ) and IL-2 were measured in the PHA-stimulated samples; tumor necrosis factor (TNF), IFN-γ, IL-1β, IL-6, and IL-8 were measured in the LPS-stimulated samples.

EDP1066 stool persistence and gut microbiome

Fecal concentrations of EDP1066 for stool persistence and prevalence and the gut microbiome were measured by Diversigen Inc. (Houston, TX, USA) using validated bioanalytical assay methods. In short, fecal microbial DNA was extracted based on the Zymo Research (Irvine, CA, USA) fecal DNA extraction methodology. EDP1066-specific primers and probes had been developed to enable the detection of the *L. lactis* spp. *cremoris* strain. The fecal samples were analyzed using a qPCR with a lower limit of quantification of 5.0 copies/5 ng DNA. For gut microbiome analyses, extracted DNA was prepared for Illumina sequencing *via* PCR amplification of the variable region 4 of the bacterial 16S rRNA gene. After PCR purification using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA), sample-specific barcodes using Illumina Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) were appended to the PCR products during a second PCR. The PCR products were purified for a second time, and lastly, the PCR products were equimolarly pooled and sequenced on the Illumina MiSeq platform using the MiSeq v3 sequencing kit.

Safety and tolerability

Safety and tolerability were monitored by physical examination, assessment of vital signs, laboratory parameters (i.e., full blood count, biochemistry, serology, immunophenotyping, circulating cytokines, fecal calprotectin, and urinalysis), and ECG data from 12-lead ECGs at regular intervals. Subjects were monitored continuously for adverse events (AEs). Participants were also asked to daily complete the Bristol Stool Scale (BSS) and questions regarding defecation patterns using an electronic diary app in order to obtain insight into the participants' stool patterns at the time of fecal sample collection.

Statistics

The sample size was based on previously performed power calculations on KLH challenge endpoints (47). In order to detect a 75% inhibition of the KLH-specific antibody response, cutaneous blood perfusion response (LSCI), and erythema response (multispectral imaging), a sample size of 12 per group was required using a parallel study design, with an α of

0.05 and a power of 80%. It was deemed appropriate to pool the placebo-treated participants for analyses in order to increase the statistical power. Demographic and baseline variables were summarized by treatment. PD endpoints measured at multiple time points after baseline were analyzed with a mixed-effects repeated-measures model with fixed factors treatment, time and treatment by time, random factor subject, and the baseline value as covariates. Endpoints with one post-dose measurement were analyzed with a linear model with treatment as a fixed factor. Anti-KLH antibody parameters were analyzed without baseline as a covariate. Skin rechallenge endpoints were analyzed with an analysis of covariance with treatment as a fixed factor and the baseline and the change from baseline (CFB) of the saline-injected control added as covariates. Anti-KLH IgM and IgG titers and *ex vivo* monocyte cytokine release assays required log transformation. The general treatment effect and specific contrasts were reported as the estimated difference (ED) with a 95% confidence interval (CI) and graphically as ED with 95% CI, as least squares mean (LSM) with 95% CI, or as mean with SD. Fecal EDP1066 concentration was reported graphically as median with range. Fecal microbiome endpoints were analyzed using Python (Python Software Foundation, Wilmington, DE, USA) by Diversigen Inc. Read count and relative abundance tables were calculated at the genus level and retrieved using custom Python scripts and the One Codex Python library, an in-house curated database of bacterial marker genes including 16S ribosomal RNA. The relative abundances of all microorganisms at the genus level were calculated to present the occurrence of the *Lactococcus* genus relative to all microbial DNA in the samples. Diversity trend analysis was performed using the Shannon diversity index. The Shannon diversity index was calculated for all samples using the One Codex Python library. Results were aggregated and plotted using custom Python scripts. To determine whether some genera were more or less abundant in placebo *vs.* EDP1066 treated individuals, read count tables were fed to ANCOM, a statistical framework for the analysis of microbiomes. Fecal microbiome diversity was reported graphically as median with an interquartile range.

Results

Baseline characteristics

The study was conducted between February 2019 and January 2020. Ninety-five subjects were enrolled in the study of which 81 were treated (Figure 1). A total of 76 subjects completed the treatment and the follow-up period. Five subjects did not complete the study. One subject was withdrawn due to a possible hypersensitivity reaction to EDP1066. Due to very limited EDP1066 exposure (two doses) and collected data, it was decided to replace this subject. The withdrawal in the other four was unrelated to the study drug or procedures (emergency dental procedure (one), tetanus vaccination and antibiotics treatment (one), and consent withdrawal (two)). The baseline characteristics of all treatment groups are presented in Table 2. Treatment compliance was 99.4% in subjects who completed the treatment and follow-up period (range number of days EDP1066 intake 26–28 days). Nine subjects missed one dosing day, and two subjects missed two dosing days.

Humoral immunity to keyhole limpet hemocyanin and cutaneous blood perfusion and erythema

No statistically significant treatment or formulation effects were observed on the humoral KLH challenge outcomes. Observations closest to the desired treatment effect were lower anti-KLH IgG (Figure 2, ED −16.8%, 95% CI −35.5% to 7.3%, p = 0.15) and IgM (Figure 2, ED −16.8%, 95% CI −31.8% to

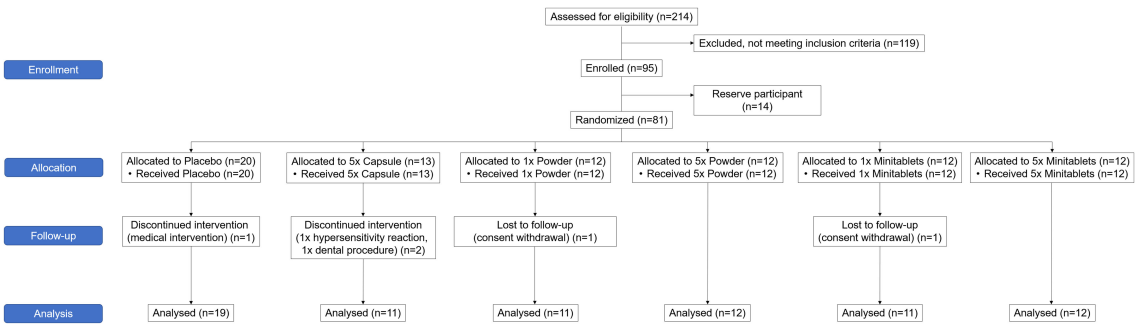

FIGURE 1
CONSORT 2010 flow diagram of the progress through the phases of the trial.

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TABLE 2 Baseline characteristics.

EDP1066 FORMULATION	Enteric-coated capsules	Free powder		Minitablets		Placebo
DAILY DOSE	**$1.5*10^{12}$ (5x) total cells**	**$3.0*10^{11}$ (1x) total cells**	**$1.5*10^{12}$ (5x) total cells**	**$3.0*10^{11}$ (1x) total cells**	**$1.5*10^{12}$ (5x) total cells**	
	n=13	**n=12**	**n=12**	**n=12**	**n=12**	**n=20**
DEMOGRPAHICS						
Age (years)	30 (18-59)	26 (19-58)	29 (20-59)	25 (18-56)	51 (22-56)	26 (19-59)
BMI (kg/m^2)	24.5 (3.8)	23.7 (2.7)	26.0 (4.4)	21.9 (3.0)	22.0 (2.6)	24.3 (3.7)
Male gender (n)	9 (69.2%)	6 (50.0%)	8 (66.7%)	6 (50.0%)	6 (50.0%)	10 (50.0%)
VITAL SIGNS						
Systolic blood pressure (mmHg)	117 (13)	111 (9)	118 (11)	109 (13)	110 (10)	110 (8)
Diastolic blood pressure (mmHg)	68 (10)	65 (9)	69 (9)	64 (10)	66 (8)	64 (6)
Heart rate (bpm)	60 (12)	56 (9)	59 (8)	62 (8)	61 (5)	56 (7)
Temperature (°C)	36.4 (0.3)	36.5 (0.3)	36.5 (0.3)	36.6 (0.3)	36.4 (0.5)	36.5 (0.5)
LABORATORY TESTS						
Leucocytes ($*10^9$/L)	7.47 (2.00)	7.00 (1.50)	7.55 (1.79)	7.27 (2.42)	7.35 (1.34)	6.71 (1.53)
Lymphocytes ($*10^9$/L)	2.25 (0.49)	2.22 (0.55)	2.46 (0.50)	2.38 (0.81)	2.55 (0.73)	2.35 (0.77)
Thrombocytes ($*10^9$/L)	276.7 (61.3)	254.8 (49.3)	251.9 (35.4)	230.8 (48.7)	253.5 (64.0)	259.3 (46.4)
ALT (IU/L)	21.4 (6.7)	24.4 (8.9)	24.6 (10.4)	20.6 (13.0)	25.6 (11.3)	19.8 (7.7)
AST (IU/L)	20.3 (3.3)	22.4 (7.4)	23.9 (9.2)	20.1 (4.6)	25.7 (5.2)	20.4 (5.9)
CRP (mg/L)	1.05 (1.34)	1.56 (2.36)	1.41 (1.43)	1.58 (2.05)	0.53 (0.50)	1.61 (1.70)
Fecal calprotectin (µg/g)	34.0 (36.2)	11.0 (12.9)	9.0 (11.9)	17.4 (14.6)	16.8 (11.9)	18.0 (15.3)

Parameters are shown as mean (standard deviation), age as median (range), and male gender as count (percentage).
ALT, alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein; BMI, body mass index.

1.4%, p = 0.07) levels in the 5× Minitablets group compared to placebo, not reaching a level of statistical significance. No statistically significant treatment or formulation effects were observed on the KLH skin rechallenge outcomes (Figure 3).

Circulating regulatory T cells and *ex vivo* stimulation assays

There were no consistent EDP1066-dependent changes in the percentage of circulating Tregs over all groups, though Tregs were significantly increased in subjects treated with 5× Powder (Figure 4, ED 0.55%, 95% CI 0.14%–0.96%, p < 0.01) compared to placebo. EDP1066 slightly impacted LPS-driven cytokine release in whole blood cultures. Overall, all cytokines (IFN-γ, IL-1β, IL-6, IL-8, and TNF) in supernatants from LPS-stimulated whole blood cultures were lower in the 1× and 5× Minitablets groups compared to placebo. Furthermore, a statistically significant decreased TNF (Figure 4, ED −44.2%, 95% CI −65.3% to −10.3%, p < 0.05), IL-1β (Figure 4, ED −41.4%, 95% CI −63.5% to −5.8%, p < 0.05), and IL-6 (Figure 4, ED −39.2%, 95% CI −56.8% to −14.5%, p < 0.01) release were observed in the 1× Minitablet group compared to placebo. There were no consistent findings in supernatants of PHA-stimulated whole blood samples over the biomarkers and groups.

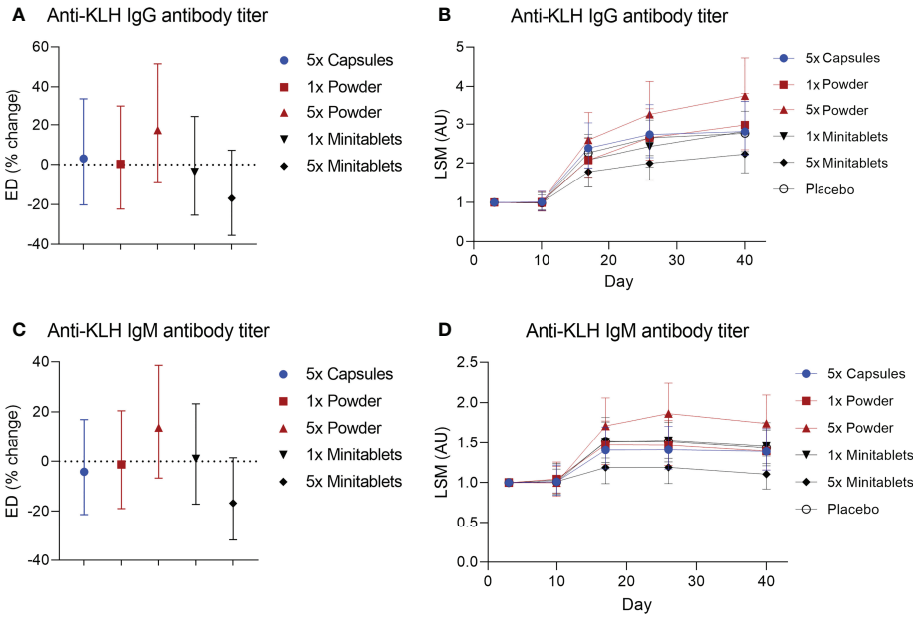

FIGURE 2
Anti-keyhole limpet hemocyanin **(A, B)** IgG and **(C, D)** IgM antibody titers by EDP1066 treatment group. Data are shown as estimated difference with 95% confidence interval expressed as percentage of placebo in panels **(A, C)** and as least square means with 95% confidence interval in panels **(B, D)** The estimated difference was calculated with a mixed-effects repeated-measures model with fixed factors treatment, time and treatment by time, and random factor subject as covariate. KLH, keyhole limpet hemocyanin; ED, estimated difference; LSM, least square means.

EDP1066 stool persistence and gut microbiome

L. lactis spp. *cremoris* was detected in all actively treated groups in 64% to 73% of subjects on study day 26. Levels returned toward baseline 5 days after the last EDP1066 dose (Figure 5). Dosing by 5× Capsules formulation resulted in the detection of fecal *L. lactis* spp. *cremoris* in all subjects on study day 26 (Figure 5). *Lactococcus* genera were represented only in trace amounts in all samples (Figure S2). The maximum number of *Lactococcus* reads detected in any of the subjects was approximately 500, which corresponds to 0.6% of the total classified 16S reads. These results suggest that EDP1066 did not colonize the gut of any of the participants. Microbiome diversity (Shannon diversity index) was comparable among time points and treatment groups, albeit some changes could be observed on individual levels for a subset of the participants (data not shown). Overall microbiome diversity seemed to be slightly lower in EDP1066-treated samples; however, many of these differences probably occured due to the small sample size when calculations were performed for individual groups. When Shannon diversity indices were aggregated across all the groups, the mean Shannon diversity was very stable between time points and treatment groups (Figure 6). The 10 most abundant genera were very stable between EDP1066- and placebo-treated subjects (Figure S3). There was some variation in relative abundance, but no large or consistent shifts were seen across all groups. Variation was most likely due to individual differences in microbiome composition between subjects and not dependent on treatment.

Safety and tolerability

Overall, no major safety concerns were observed during the study. No serious adverse events occurred. Most AEs were related to the GI tract (93 AEs in 46 subjects) with no distinction between EDP1066 and placebo treatment (Table S1). One subject was withdrawn from further treatment after the second EDP1066 dose due to a possible hypersensitivity reaction to EDP1066 consisting of a mild burning sensation and itch of the throat lasting approximately 6 h. No abnormalities were found upon physical examination and additional vital sign measurements. Due to the mild and limited nature of the AEs, no further diagnostics were conducted. No earlier hypersensitivity AEs after EDP1066 administration had been reported. The subject also did not report any allergies to cheese or other dairy products. Allergic reactions to excipients used in the 5× Capsule formulation (microcrystalline cellulose, magnesium stearate, and colloidal silicon dioxide) have been reported before; however, these are very rare (49–51). Placebo-treated subjects had slightly fewer AEs (75%) compared to EDP1066-treated subjects (83.3% to 91.7%).

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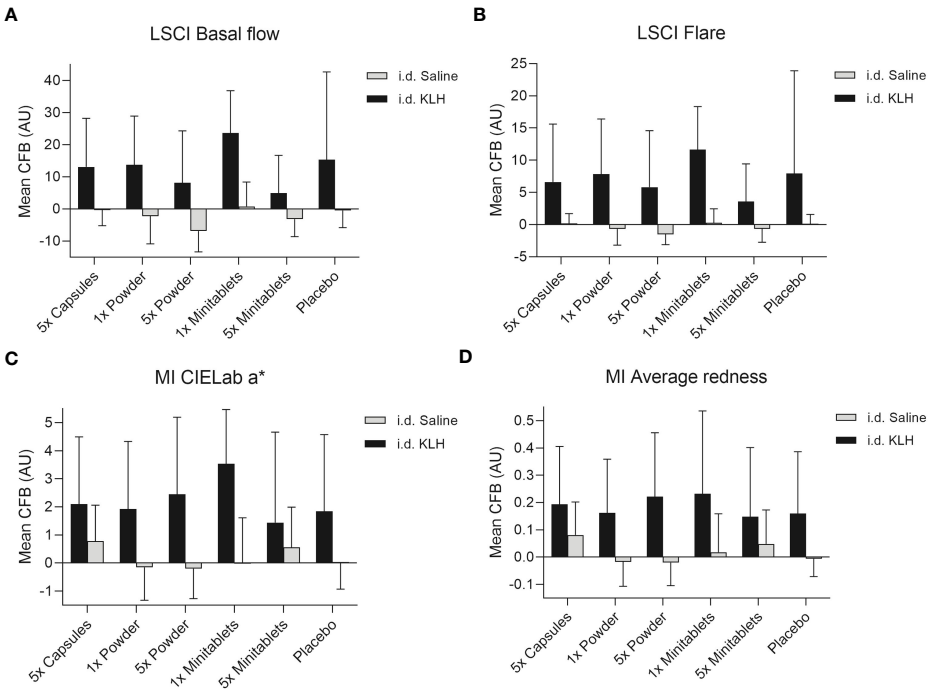

FIGURE 3
Cutaneous blood perfusion by **(A)** LSCI basal flow and **(B)** LSCI flare, erythema by **(C)** CIELab a* (multispectral imaging), and **(D)** average redness (multispectral imaging) after intradermal KLH and saline administration by three EDP1066 formulations of *Lactococcus lactis* spp. *cremoris*: i) enteric-coated capsules, ii) freeze-dried powder (dose 1× and 5×), and iii) minitablets (dose 1× and 5×). The average redness modality displays the distribution of redness using an internal software algorithm and the CIELab a* value, which is part of the CIELab color space, expresses color as a numerical value on a green–red color scale. Data are shown as mean change from baseline (CFB) with standard deviation. LSCI, laser speckle contrast imaging; MI, multispectral imaging; KLH, keyhole limpet hemocyanin; CFB, change from baseline; AU, arbitrary unit; i.d., intradermal.

No clinically significant changes were observed in laboratory parameters, vital signs, ECG recordings, and the BSS and feces questionnaire.

Discussion

In this study, we showed that daily EDP1066 treatment in encapsulated, powdered, and minitablet formulations and daily doses up to 1.5 * 10^{12} total cells, five times the expected therapeutic dose, did not result in consistent significant effects on KLH challenge responses and LPS- and PHA-driven cytokine release in whole blood cultures. We demonstrated that *L. lactis* spp. *cremoris* was detected in the fecal samples and increased during the 28-day treatment period for all EDP1066 formulations tested. However, the fecal levels returned to baseline levels 12 days after the end of treatment, indicating no prolonged persistence. Overall, EDP1066 was considered safe and well-tolerated. To the best of our knowledge, the current trial is the first to investigate the effects of orally administered *L. lactis* spp. *cremoris* in high doses on systemic immune responses and the gut microbiome.

EDP1066 did not show a consistent immunomodulatory effect on KLH-driven responses in the present study. Though no statistical significance was reached, decreased anti-KLH antibody titers and cutaneous blood perfusion and erythema were observed in the 5× Minitablets group compared to placebo. Although circulating Tregs as a percentage of $CD4^+$ T cells were significantly increased in subjects treated with 5× Powder compared to placebo, it should be noted that these percentages remained within the general range of Tregs in the CD4 population as reported in the literature (5%–10%) (52, 53). The PD results observed in this study are in contrast with preclinical data where EDP1066 induced IL-10 production in *in vitro* human dendritic cell (DC) cultures, without significant induction of pro-inflammatory cytokines (unpublished data), and EDP1066 significantly reduced KLH- and ovalbumin-induced ear inflammation in mice and improved intestinal pathology and weight loss in an acute dextran sulfate sodium-induced colitis mouse model (unpublished data). Also in contrast to our results, other preclinical trials reported that *L. lactis* spp. *cremoris* restored T-cell impairment in aged mice (26) and that coadministration of *L. lactis* spp. *cremoris* with *L. paracasei* spp. *paracasei* showed promising results in an atopic

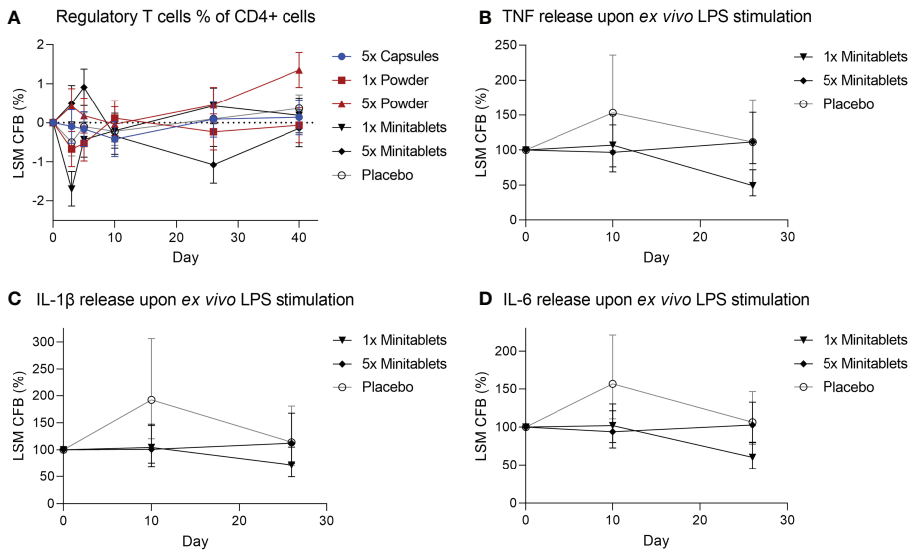

FIGURE 4
(A) Circulating regulatory T cells as percentage of $CD4^+$ T cells from heparinized blood. Monocyte cytokine release assay of **(B)** tumor necrosis factor, **(C)** interleukin-1β, and **(D)** interleukin-6 release from whole blood cultures after *ex vivo* lipopolysaccharide stimulation. X-axis represents number of days after initial EDP1066 dose. Data are shown as least squares mean change from baseline (CFB) with 95% confidence interval. LSM, least squares mean; CFB, change from baseline; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL = interleukin.

dermatitis mouse model (27). Probiotics in general have been shown to be effective in (the prevention of) multiple diseases (20, 25). Multiple studies have reported enhanced responses to influenza vaccination after the intake of probiotics (54–58). Another study showed an enhanced response to hepatitis A vaccination after probiotic intake (59). Single strains of both *Lactobacillus rhamnosus GG* and *Lactobacillus helveticus R52* have been shown to reduce the risk of developing antibiotic-associated diarrhea (25). *L. rhamnosus GG* single-strain treatment was also effective in the prevention of necrotizing enterocolitis (25). Furthermore, *Bifidobacterium animalis* spp. *lactis Bb12* prevented upper respiratory tract infections, indicating distally evoked immune system effects (25).

EDP1066 treatment suppressed KLH-driven increases in LPS-driven cytokine release *ex vivo* in both the 1× and the 5× Minitablets groups, reaching statistical significance for IL-1β, IL-6, and TNF-α

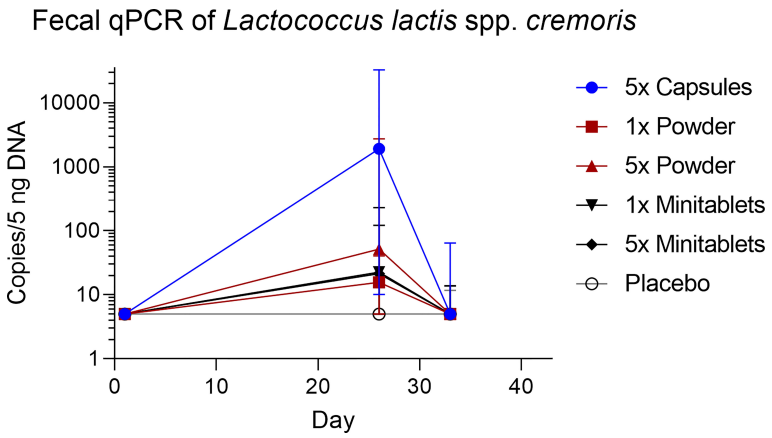

FIGURE 5
Fecal concentration of *Lactococcus lactis* spp. *cremoris* measured by quantitative polymerase chain reaction. Data are shown as median with range. qPCR, quantitative polymerase chain reaction.

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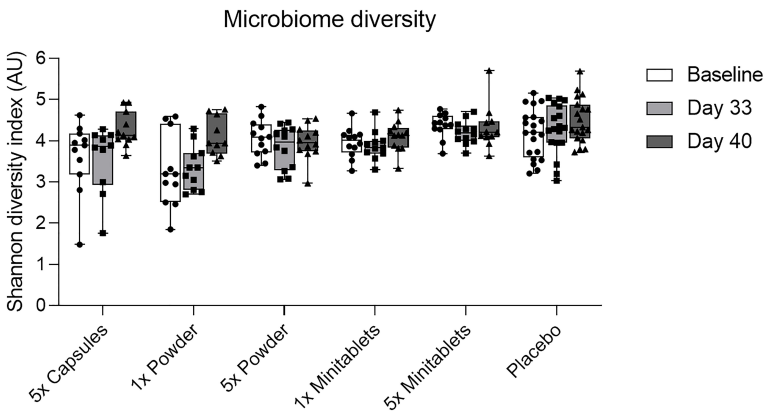

FIGURE 6
Fecal microbiome diversity calculated using the Shannon diversity index by treatment group per time point. Data are shown as median with interquartile range. AU, arbitrary unit.

in the 1× Minitablets group, which may indicate innate immune system inhibition (60). The observed increase in LPS-driven cytokine release by monocytes in placebo-treated subjects may be attributed to KLH immunization, priming the innate immune response for subsequent stimulation. Although KLH is primarily recognized as an agent that induces cell-mediated responses, there is evidence that KLH immunization and rechallenge most likely cause a mixed reaction of innate, late-phase skin reaction and delayed-type hypersensitivity (61). Furthermore, similar to LPS, KLH induces innate immunity *via* the activation of NF-κB (60).

In the present study, we evaluated the immunomodulatory activity of EDP1066 as powder formulation (free and in enteric-coated capsules) and as minitablets in non-coated capsules. The minitablets in non-coated capsule formulation were expected to achieve the highest concentration of relatively intact EDP1066 bacteria in the duodenum. Non-coated capsules were used to ease the intake of relatively large numbers of enteric-coated minitablets and to preserve the blinding. Based on *in vitro* experiments, the minitablet formulation was predicted to release in the proximal small intestine (unpublished data). Duodenal EDP1066 exposure was hypothesized to be important for the immunomodulatory effect, as immune cell subsets are found at the highest concentrations in the duodenum and jejunum, particularly the $CD103^{+}CD11b^{+}$ DCs, which are thought to play distinct roles in intestinal immune homeostasis (1). The small intestine is the most likely point where luminal contents can access GALT and have a pronounced immune-regulatory effect (1). However, we did not observe any differences between the formulations on the humoral KLH challenge and subsequent skin KLH rechallenge, circulating Tregs, gut microbiome, and safety and tolerability outcomes. We did observe increased fecal detection of EDP1066 in the capsule formulation compared to powder and minitablet formulations. This can possibly be explained by the fact that the enteric-coated capsules dissolve lower in the GI tract leading to postponed EDP1066 release and higher EDP1066 exposure toward the end of the GI tract.

The current human trial did not confirm previous findings from preclinical trials that oral administration of EDP1066 had immunomodulatory effects as measured on antibody response to KLH immunization or skin immune responses to KLH re-challenge. There are several potential explanations for the suboptimal translation of EDP1066 activity between mice and humans. Firstly, it was impossible to do conventional allometric scaling between mice and humans. Other than for most medicinal products, the exposure to EDP1066 was considered to remain restricted to the GI tract. This was hypothesized to be sufficient, since the mechanism of action of EDP1066 only requires local interaction with cells of the GI mucosa, driving subsequent systemic effects. Under these conditions, assumptions of traditional allometric scaling may not hold true. For this reason, relative GI mucosal surface area and stool mass are key parameters for allometric scaling. The relative GI mucosal surface area has been estimated as a function of body mass to the ¾ power (62). As the dose selection rationale was mainly hypothetical, the actual EDP1066 doses administered might have been too low to exert significant PD effects. For practical reasons, the administration of higher EDP1066 doses was not explored since this would require a daily intake of >10 capsules. Secondly, differences in diet and also differences in microbial composition are likely to introduce highly variable individual responses to microbial exposure. As *L. lactis* spp. *cremoris* is used by the dairy industry, it is likely that participants have developed at least some intestinal tolerance to this microbe (63), possibly explaining the differences observed between the current trial and preclinical data. Furthermore, apart from the well-known immunological differences between rodents and humans, EDP1066 activity is dependent on relatively unknown

physiological systems or principles such as the GI microbiome and the interplay between the local and systemic immune systems. The exact molecular target and target location for EDP1066 are unknown, as are the exact EDP1066 components required for biological effect, further complicating inter-species translation. As intestinal dysbiosis can cause altered local as well as systemic immune system changes, we hypothesized that live EDP1066 would be required to interact with the gut microbiome and local immune system. However, based on the results observed in this study, we cannot exclude the possibility that dead EDP1066 could potentially also provoke immune system responses. Finally, the *in vitro* prediction of the release criteria of the enteric-coated capsules and minitablets may underestimate the time to release, suggesting that the true release of EDP1066 was in the distal small intestine or colon rather than in the proximal small intestine.

In conclusion, oral EDP1066 treatment for healthy volunteers did not consistently result in significant immune modulation. Future clinical studies should build onto the insights obtained in this study and further investigate formulation versus local release and dose–effect relationships, which will ultimately be beneficial not only for EDP1066 but also for the field of therapeutic human commensals in general.

Data availability statement

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors wrote the manuscript. AI, DM, MM, PG, and MS designed the research. MM, PG, and MS performed the research. HG and EK analyzed the data. All authors approved the final version of the manuscript.

Funding

Evelo Biosciences Inc. (Cambridge, MA, USA) was the sponsor for the study and had no other role than funding all study-related activities.

Conflict of interest

AI and DM are employed by the sponsor.

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1009304/full#supplementary-material

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TYPE Original Research
PUBLISHED 08 December 2022
DOI 10.3389/fimmu.2022.1066036

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

RECEIVED 10 October 2022
ACCEPTED 17 November 2022
PUBLISHED 08 December 2022

CITATION
Blázquez-Bondia C, Parera M, Català-Moll F, Casadellà M, Elizalde-Torrent A, Aguiló M, Espadaler-Mazo J, Santos JR, Paredes R and Noguera-Julian M (2022) Probiotic effects on immunity and microbiome in HIV-1 discordant patients.
Front. Immunol. 13:1066036.
doi: 10.3389/fimmu.2022.1066036

Probiotic effects on immunity and microbiome in HIV-1 discordant patients

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Background: Some HIV-1 infected patients are unable to completely recover normal CD4+ T-cell (CD4+) counts after achieving HIV-1 suppression with combined Antiretroviral Therapy (cART), hence being classified as immuno-discordant. The human microbiome plays a crucial role in maintaining immune homeostasis and is a potential target towards immune reconstitution.

Setting: RECOVER (NCT03542786) was a double-blind placebo-controlled clinical trial designed to evaluate if the novel probiotic i3.1 (AB-Biotics, Sant Cugat del Vallès, Spain) was able to improve immune reconstitution in HIV-1 infected immuno-discordant patients with stable cART and CD4+ counts <500 cells/mm3. The mixture consisted of two strains of *L. plantarum* and one of *P. acidilactici*, given with or without a fiber-based prebiotic.

Methods: 71 patients were randomized 1:2:2 to Placebo, Probiotic or probiotic + prebiotic (Synbiotic), and were followed over 6 months + 3-month washout period, in which changes on systemic immune status and gut microbiome were evaluated. Primary endpoints were safety and tolerability of the investigational product. Secondary endpoints were changes on CD4+ and CD8+ T-cell (CD8+) counts, inflammation markers and faecal microbiome structure, defined by alpha diversity (Gene Richness), beta diversity (Bray-Curtis) and functional profile. Comparisons across/within groups were performed using standard/paired Wilcoxon test, respectively.

Results: Adverse event (AE) incidence was similar among groups (53%, 33%, and 55% in the Placebo, Probiotic and Synbiotic groups, respectively, the most common being grade 1 digestive AEs: flatulence, bloating and diarrhoea. Two grade 3 AEs were reported, all in the Synbiotic group: abdominal distension (possibly related) and malignant lung neoplasm (unrelated), and 1 grade 4 AE in

10.3389/fimmu.2022.1066036

the Placebo: hepatocarcinoma (unrelated). Synbiotic exposure was associated with a higher increase in CD4+/CD8+ T-cell (CD4/CD8) ratio at 6 months vs baseline (median=0.76(IQR=0.51) vs 0.72(0. 45), median change= 0.04 (IQR=0.19), p = 0.03). At month 9, the Synbiotic group had a significant increase in CD4/CD8 ratio (0.827(0.55) vs 0.825(0.53), median change = 0.04(IQR=0.15), p= 0.02) relative to baseline, and higher CD4+ counts (447 (157) vs. 342(73) counts/ml, p = 0.03), and lower sCD14 values (2.16(0.67) vs 3.18(0.8), p = 0.008) than Placebo. No effect in immune parameters was observed in the Probiotic arm. None of the two interventions modified microbial gene richness (alpha diversity). However, intervention as categorical variable was associated with slight but significant effect on Bray-Curtis distance variance (Adonis R2 = 0.02, p = 0.005). Additionally, at month 6, Synbiotic intervention was associated with lower pathway abundances vs Placebo of Assimilatory Sulphate Reduction ($8.79{\cdot}10^{-6}$ ($1.25{\cdot}10^{-5}$) vs. $1.61{\cdot}10^{-5}$ ($2.77{\cdot}10^{-5}$), p = 0.03) and biosynthesis of methionine ($2.3{\cdot}10^{-5}$ ($3.17{\cdot}10^{-5}$) vs. $4{\cdot}10^{-5}$ ($5.66{\cdot}10^{-5}$), p = 0.03) and cysteine ($1.83{\cdot}10^{-5}$ ($2.56{\cdot}10^{-5}$) vs. $3.3{\cdot}10^{-5}$ ($4.62{\cdot}10^{-5)}$, p = 0.03). At month 6, probiotic detection in faeces was associated with significant decreases in C Reactive Protein (CRP) vs baseline (11.1(22) vs. 19.2(66), median change= -2.7 (13.2) ug/ml, p = 0.04) and lower IL-6 values (0.58(1.13) vs. 1.17(1.59) ug/ml, p = 0.02) when compared with samples with no detectable probiotic. No detection of the probiotic was associated with higher CD4/CD8 ratio at month 6 vs baseline (0.718(0.57) vs. 0.58(0.4), median change = 0.4(0.2), p = 0.02). After washout, probiotic non-detection was also associated with a significant increase in CD4+ counts (457(153) vs. 416(142), median change = 45(75), counts/ml, p = 0.005) and CD4/CD8 ratio (0.67(0.5) vs 0.59(0.49), median change = 0.04 (0.18), p = 0.02).

Conclusion: A synbiotic intervention with *L. plantarum* and *P. acidilactici* was safe and led to small increases in CD4/CD8 ratio and minor reductions in sCD14 of uncertain clinical significance. A probiotic with the same composition was also safe but did not achieve any impact on immune parameters or faecal microbiome composition.

KEYWORDS

probiotics, prebiotics, synbiotics, HIV, immune reconstitution

Introduction

One of the key aspects of HIV infection is a fast and widespread destruction of CD4+ T-lymphocytes (1), which becomes more exacerbated in the latest stages of infection (2, 3). Additionally, the virus presents the ability to establish reservoirs in which it can remain dormant mostly in high-CCR5 memory CD4+ T-cells (4, 5), where it remains integrated in the host genome (3–5). The ability to maintain a latency state has made it impossible to achieve a complete remission, although it can be life-long supressed in most patients with combined Antiretroviral Therapy (cART) (6). Nevertheless, even when the virus remains supressed, an important fraction of HIV-infected people will become immunodiscordant, as they fail to fully recover CD4+ counts and immune function (7–9) especially those who failed to receive cART on the early stages of infection.

This lack of recovery stems from the virus early replication site and its reservoir sanctuary: the Gut-Associated Lymphoid Tissue (GALT) (10), where the biggest population of high CCR5 + CD4+ T-cells resides. Depletion of such cells in the gut is coupled with a decrease in Treg numbers (11), but more slowly than its CD4+ counterpart, lowering the ratio between Treg and effector CD4+ cells, especially the Th17 subtypes (11, 12). A relative decrease of the Th17/Treg ratio has been correlated to a higher chance of disease progression (13). Th17 have also been

shown to maintain gut barrier integrity, stimulating tight junction expression in epithelial cells (14), as well as modulating bacterial populations in the mucosa by secreting antimicrobial peptides (15). Hence, the skew in the Treg/Th17 ratio compromises the gut barrier integrity, and creates a feedback loop where dysbiosis and gut inflammation cause a leakage of bacterial compounds into the bloodstream (16), which increases residual systemic inflammation (17) and leads to further T-cell exhaustion and senescence (18), which further promotes dysbiosis and gut barrier. This vicious circle ends up causing immune exhaustion and may hinder any attempt of immune reconstitution.

While many approaches have been considered to recover immunity and gut integrity, modulation of the gut microbiome awakened great interest recently. It is now known that some bacterial species directly affect the immunologic makeup of the gut barrier (19) by modulating tryptophan to kynurenine catabolism (17). This pathway is thought to promote Treg differentiation (17) and has been shown to increase with presence of some *Proteobacteria* (20) and decrease with *Lactobacillus species* (20, 21). For this, probiotics have been widely tested with promising results for wide variety of ailments, both from the gut itself such as Inflammatory Bowel Disease (IBD) (22), diarrhoea, both HIV-induced (23) and by other pathogens (24) and even outside of the gut, such as allergies and upper respiratory infections (25).

Recently, the gut microbiome has awakened a great interest in the scope of HIV clinical management, as immune recovery has been shown to be affected by the state of the gut mucosa (26). Cross-sectional studies have found microbiome signatures correlated to immune reconstitution such as higher Prevotella/Bacteroides ratio and enrichment of *Faecalibacterium prausnitzii and Coprococcus comes* (27) or increased abundance of *Fusobacterium* negatively linked to immune recovery (28). Consequently, probiotic interventions have have become an interesting therapeutic target, as they promote tolerogenicity (29), displace pathogenic strains, and reduce inflammation (30), which could reduce T cell depletion and senescence, opening a way to improve immune reconstitution after viral suppression. However, many different combinations of probiotic strains and prebiotic substrates have been tested with mixed results (30, 31).

In this study, we performed a randomized double-blinded trial to test the safety, tolerability and effectiveness of a probiotic consisting of two strains of *Lactobacillus plantarum* and one of *Pediococcus acidilactici*, combined with prebiotic fibers over the course of 6 months, followed by a 3-month long washout period in immunodiscordant (<500 counts/ml) HIV patients with stable cART. The primary endpoints consisted of safety and tolerability. The secondary endpoints were changes in CD4+, CD8+ counts, CD4/CD8 ratio, inflammation, and gut permeability markers, as well as changes in the gut microbiome taxonomical and functional composition after 6 months of intervention + 3 of washout.

Materials and methods

Ethics statement

The study was reviewed and approved by the Institutional Review Board of the Hospital Universitari Germans Trias i Pujol (reference PI-13-046). All participants provided written informed consent in accordance with the World Medical Association Declaration of Helsinki, Fortaleza and Brazil, October 2013 and personal data was managed according to Spanish data protection law (LOPD 15/1999). The study concept, design, patient information and results were discussed with the FLSida Community Advisory Committee, in accordance with AB-Biotics internal QC auditing. All available information can be found in the protocol (Supplementary Methods), and the study is registered in clinicaltrials.gov, accession: NCT03542786.

Cohort description

This study took place in a two-year span between 2017 and 2019 and was designed as a masked randomized, placebo-controlled, double-blinded three-arm study in a cohort of 100 HIV+ patients with the following inclusion criteria: 18 years of age or older, chronic HIV infection with stable Anti-Retroviral Treatment (cART) ongoing for longer than a year prior to the start, peripheral CD4+ counts lower than 500 cells/ml in plasma, <50 HIV copies/ml in plasma for at least 6 months before the start, no antibiotic treatments at least 1 month before start, lack of severe AIDS-defining diseases and no pregnancy. An additional filter was later implemented, in which only those patients with at least 2 samples along the trial would be selected for further analysis, to preserve the longitudinal approach of the study.

After inclusion, patients were randomly assigned to one of the three following groups: Placebo, Probiotic or Synbiotic in a 1:2:2 ratio and matched by 3rd cART drug class: Integrase Strand Transfer Inhibitors (INSTI), Non-nucleoside reverse transcriptase inhibitors (NNRTI) or Protease Inhibitors (PI), and CD4+ nadir higher or lower than 200 cells/ml at the time of screening.

All recruited patients followed a 6-month treatment followed by a 3-month washout periods, with check-ups at months 0,1,3,6 and 9. Every follow-up visit consisted of a sample collection of both blood and faeces (except at the 1st month checkout, where only stool was collected), a physical examination and a questionnaire about quality of life, and self-reported treatment adherence since last check-up. During the treatment period, all participants received different formulation depending on whether they received a prebiotic + probiotic (Synbiotic), probiotic alone (Probiotic) or a placebo (Placebo), which was administered orally as dissolved powder sachets, daily.

Treatment formulations

The probiotic used in this study consisted of a mix of 3 *Lactobacillales* strains: *L. plantarum* (strains CECT7484 and CECT7485) and *P. acidilactici* (strain CECT7483). In the Synbiotic group, the probiotic was co-administered with two different mixes of vegetal fibers consisting of pectin, inulin, oat, acacia, maltodextrin polydextrose and Partially Hidrolyzed Guar Gum (PHGG) that were alternatively combined with the probiotic every other week (the exact formulation can be found in the protocol). The Probiotic and Placebo groups received excipient-containing envelopes that were identical to those of the Synbiotic group to preserve the double-blind. The exact composition and manufacturing process can be found in Supplementary Methods.

T-cell and inflammation marker quantification

Blood samples were collected in fasting conditions at the same time as stool samples, when possible. A fraction of these samples was used as whole blood to perform peripheral CD4+ and CD8+ counts by flow cytometry at the Germans Trias i Pujol Hospital. Soluble markers of microbial translocation in plasma (sCD14 and LBP) and inflammation markers (IL-6, D-Dimer and CRP) were quantified using sCD14 and LBP DuoSet ELISA development system (pg/mL), R&D systems (Minneapolis, MN), Human IL6- High Sensitivity ELISA, Invitrogen (Waltham, Massachusetts, USA) and RayBio Human D-Dimer or CRP Elisa Kit, RayBiotech (Peachtree Corners, GA, USA), respectively.

Faecal DNA extraction, library preparation and sequencing

Stool samples were collected by the patient or nursing staff according to the GUT (DNA Stabilized-frozen Inc., Ottawa, Ontario, Canada) extraction kit. Samples were stored at -80°C till processing. Faecal DNA was extracted using the PowerSoil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA, USA), which was then fragmented into 300 bp clone-sized libraries using Nextera-XT Illumina kit (Illumina, Inc. San Diego, CA, USA) and sequenced in an Illumina HiSeq sequencer (Illumina, Inc. San Diego, CA, USA) with a sequencing depth target of 20 million reads.

Sequence filtering and quality control

Raw.*fastq* files were first processed for quality control. Read Quality filtering and trimming was performed with trimmomatic (32), with a 30-nt sliding window approach, trimming when the average phred score dropped below 20. Trimmed reads were then aligned against the human Hg19 genomic database using bowtie2 to remove any human DNA contamination.

Taxonomy annotation

Taxonomy assessment was performed with Metaphlan3 (33), performing a marker gene-based quantification, using the CHOCOPhlan 201901.1 database. The obtained data was packaged into an R phyloseq structure.

Gene function and pathway diversity analysis

Parallel to taxonomic analysis, gene function and metabolic pathway quantification was performed from the raw, quality-filtered sequencing data using HUMAnN3.0 (33). The software was run with its standard configuration and built-in databases. Results from all samples were combined into a unique table using HUMAnN3 inner script *merge_tables.sh* and clustered into Metacyc pathway abundances.

Alpha and beta diversity metrics

In this study, ecological alpha diversity was studies as gene richness. To obtain it, the post-QC sequencing data was aligned against the Integrated Gene Catalog (IGC) database (34) using Bowtie2 (35). The output was sampled at different numbers of reads to obtain rarefaction curves, from which the minimum sampling threshold was defined as the 95th percentile of maximum per-sample coverages, which equalled $2{\cdot}10^7$ reads. Samples with max coverages below this threshold were discarded, and those above were subsampled to said value to remove coverage biases.

For beta diversity, the taxonomy tables were used to construct pairwise Bray-Curtis distance matrixes between samples. The obtain matrices were then projected into NMDS coordinates using the function MetaMDS from the R vegan (36) package using their default configuration.

Statistical testing

All statistical testing was performed using R 4.0.2. Since most of the quantitative variables tested departed from normality, assessed by Shapiro-wilk test, comparisons across group were performed using Wilcoxon Rank-Sum test, while within-group, longitudinal comparisons were performed using Wilcoxon rank-sum matched-pairs test, with Benjamini-Hochberg correction. In the case of gene pathways, Kruskal-Wallis tests between groups were performed to filter out those pathways which changed in any timepoint other than basal, before proceeding to the pairwise group/timepoint comparisons using a $p < 0.05$ cut-off. Statistical results are reported as median (IQR), and longitudinal tests included median change (IQR). The number of patients per group for each statistical comparison performed in this study can be found in Supplementary Tables 3, 4, for categorical and longitudinal comparisons, respectively.

In addition, we studied the changes in time for numerical variables (diversity, inflammation, T-cells, and pathways) with Linear Mixed Models (LMM) using the Lme4 package. Models were built in two different approaches: one where the dataset was split by group, in which the LMM would be built for each using time as the only fixed effect, in order to study the magnitude of the change for each group and its significance, and another where an LMM was used with the entire dataset with both group and time as fixed effects, to assess the difference in change in slope between groups and its significance *via* two-way ANOVA. In all cases, the patients individual IDs were inputted as the random variable.

Bray Curtis distances were compared between groups using PERMANOVA. T-cell counts, inflammation and gut permeability markers were correlated with the NMDS coordinates using Spearman correlation.

Results

Cohort description and follow-up

From the original goal of 100 patients recruited of which 92 passed all the inclusion criteria, 89 patients were successfully randomised for the study. At study ending, 53 patients had complete follow-up and 36 had incomplete follow-up, of which 18 provided samples for at least two timepoints. Thus, 71 patients were finally selected for analysis, resulting in a proportion of 18 patients in the Placebo, 21 in the Probiotic and 32 in the Synbiotic groups (Figure 1). The self-reported mean adherences to the treatment showed no significant differences between groups (96% for the Placebo, 89.7% for Probiotic and 96.6% for the Synbiotic group). Demographic and clinical variables (Table 1, Supplementary Table 1) were tested with the corresponding statistical method depending on their normality according to a previous Shapiro test.

Safety and adverse events

The proportion of patients who suffered at least one Adverse Event (AE) during the study remained comparable between all arms, with no significant differences found: 9 patients with AE (50%) in the Placebo, 7 (33%) in the Probiotic and 17 (53%) in the Synbiotic groups (Supplementary Table 2). Most AEs consisted of severity grade 1 and 2, being the gastrointestinal conditions the most frequent, especially flatulence, dyspepsia, and diarrhoea, although other conditions were reported, but could not be associated with treatment group assignation. Two instances of severe grade 3 AEs were reported: abdominal distension (possibly related) and malignant lung neoplasm (unrelated), cancer (unrelated), while one grade 4 event (hepatocarcinoma, unrelated) was reported in the Placebo group.

Synbiotic formulation does not affect CD4+ and CD8+ counts but correlates with increased CD4/CD8 ratio and reduced inflammation

CD4/CD8 ratio showed a slight albeit significant increase in the Synbiotic group at month 6 respect to baseline (median=0.76 (IQR=0.51) vs 0.72 (0.45), median change= 0.04 (0.19), $p = 0.03$). At month 9, the Synbiotic group still had increased CD4/CD8 vs baseline (0.827 (0.55) vs 0.825 (0.53), median change = 0.04 (0.15), p= 0.02), CD4+ was higher (447 (157) vs 342 (73) counts/ml, $p = 0.03$), and sCD14 was lower (2.16 (0.67) vs 3.18 (0.8) $p = 0.008$) than Placebo (Figure 2).

Analysis with LMMs mirrored most of these previous findings, a significant increasing trend was found for both CRP (ANOVA $p = 0.049$, slope = 4.5) and CD4/CD8 ratio ($p = 0.002$, slope = 0.012) in the Synbiotic group (Supplementary Figure 2).

Finally, CD8+ counts appeared to be impacted by overall microbiome structure, as it was positively correlated with the 2nd coordinate of the NMDS (Supplementary Figure 1) and, as a result, CD4/CD8 ratio was negatively correlated (Supplementary Table 5)

Intervention doesn't correlate with faecal microbiome changes

No significant differences in gene richness were detected either between groups or longitudinally within groups (Figure 3A). Beta diversity did not increase either, as no changes in Bray Curtis distance vs each patient's respective baseline could be observed (Figure 3B). NMDS showed no significant clustering based on Bray-Curtis distance and the intervention variable only explained 1.8% of the variance (Adonis R2 = 0.02, P=0.0051; Figure 3C).

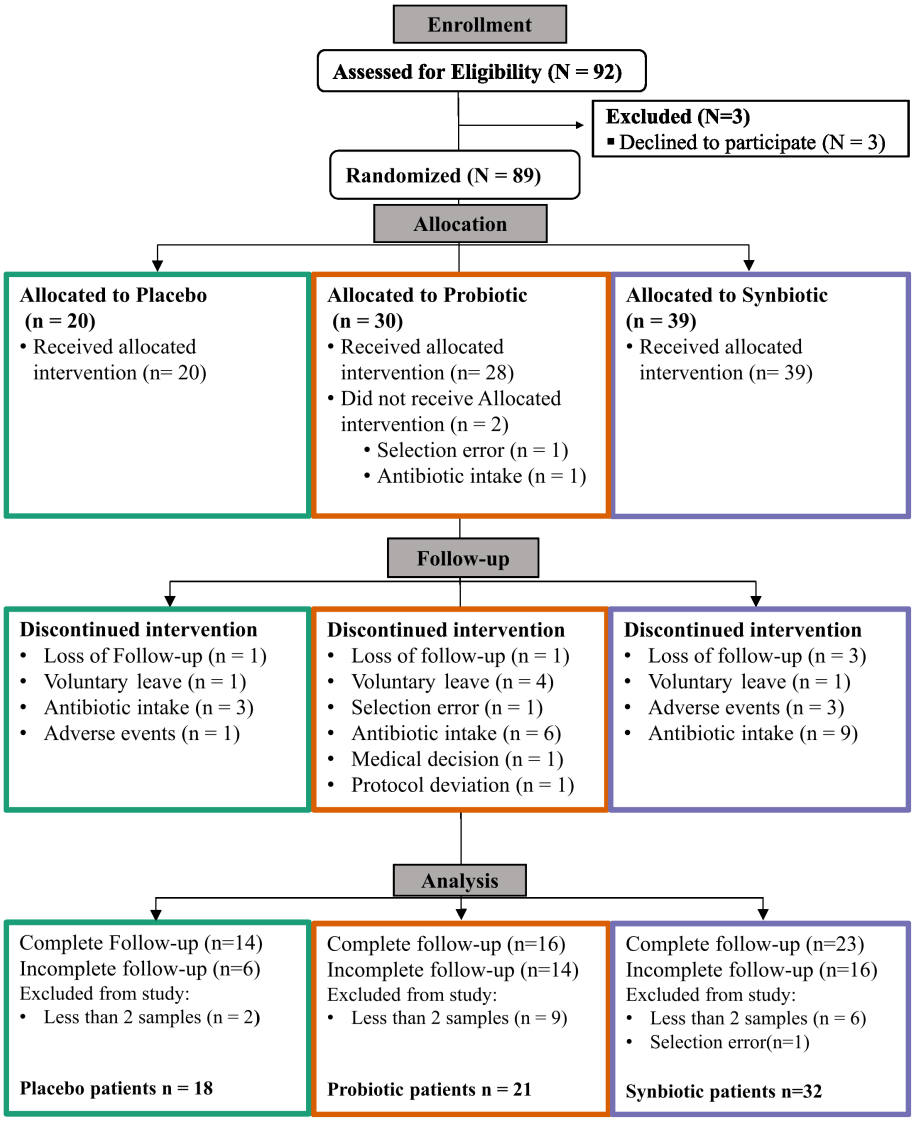

FIGURE 1
CONSORT (Consolidated Standards of Reporting Trials) Flow diagram.

Synbiotic supplementation affects sulphate assimilation on the gut

An exploratory analysis between groups identified 3 biochemical pathways with significant changes in relative abundance at month 6 (Kruskal-Wallis $p < 0.01$). Those pathways were consistently lower in the Synbiotic group respective to the Placebo: L-methionine biosynthesis (Met) ($2.3{\cdot}10^{-5}$ ($3.17{\cdot}10^{-5}$) vs $4{\cdot}10^{-5}$ ($5.66{\cdot}10^{-5}$), $p = 0.03$), Assimilatory Sulphate Reduction (ASR) (median = $8.79{\cdot}10^{-6}$ (IQR=$1.25{\cdot}10^{-5}$) vs $1.61{\cdot}10^{-5}$ ($2.77{\cdot}10^{-5}$), $p = 0.03$), and cysteine biosynthesis (Cys) ($1.83{\cdot}10^{-5}$ ($2.56{\cdot}10^{-5}$) vs $3.3{\cdot}10^{-5}$ ($4.62{\cdot}10^{-5}$), $p = 0.03$). At month 9, all three pathways increased in the Synbiotic group vs month 6 (Met: $3.8{\cdot}10^{-5}$ ($4.2{\cdot}10^{-5}$) vs $2.3{\cdot}10^{-5}$ ($3.17{\cdot}10^{-5}$), median change = $1.4{\cdot}10^{-5}$ ($3.12{\cdot}10^{-5}$), $p = 0.03$; ASR: $1.5{\cdot}10^{-5}$ ($1.7{\cdot}10^{-5}$) vs $8.79{\cdot}10^{-6}$ ($1.3{\cdot}10^{-5}$) median change = $5.6{\cdot}10^{-6}$ ($1.3{\cdot}10^{-5}$); $p = 0.03$; Cys: $3.1{\cdot}10^{-5}$ ($3.2{\cdot}10^{-5}$) vs $1.8{\cdot}10^{-5}$ ($2.6{\cdot}10^{-5}$), median change= $1.13{\cdot}10^{-5}$ ($2.42{\cdot}10^{-5}$), $p = 0.03$), but no significant changes were found in the other groups (Figure 4).

A more in-depth approach with LMMs suggested a significant decreasing trend in the relative abundances of all three pathways over time in the Synbiotic group (Met: $p = 0.003$, slope = $-3.12{\cdot}10^{-6}$; ASR: $p = 0.003$, slope = $-1.59{\cdot}10^{-6}$; Cys: $p = 0.003$, slope = $-2.53{\cdot}10^{-6}$) (Supplementary Figure 4). Additionally, a test of fixed effect interaction found a significant effect of intervention over the magnitude of change over time of pathway relative abundance (ANOVA = 0.008, Supplementary Figure 6A).

TABLE 1 Study population description of demographic variables and clinical markers at baseline: Age, height, LBP, and weight represented as mean-(sd), as they followed a continuous normal distribution (Shapiro test pval < 0.05).

		[ALL] *N = 71*	Placebo *N = 18*	Probiotic *N = 21*	Synbiotic *N = 32*	p.overall	N
Age		49.9 (9.36)	52.8 (10.4)	49.1 (10.4)	48.8 (7.88)	0.316	71
CD4+ nadir		124 [74.5;236]	140 [81.8;212]	109 [74.0;186]	157 [70.0;261]	0.395	71
Gender:	F	10 (14.1%)	1 (5.56%)	5 (23.8%)	4 (12.5%)	0.310	71
	M	61 (85.9%)	17 (94.4%)	16 (76.2%)	28 (87.5%)		
BMI		24.4 [22.1;25.6]	24.8 [22.6;26.3]	24.4 [22.7;25.3]	24.1 [22.2;25.4]	0.778	69
Weight (Kg)		72.8 (11.0)	75.2 (12.6)	72.8 (12.3)	71.3 (9.08)	0.491	69
Height (cm)		172 (7.13)	174 (9.26)	171 (6.98)	171 (5.71)	0.336	71
Third drug class	INSTI	47 (66.2%)	9 (50.0%)	16 (76.2%)	22 (68.8%)	0.282	71
	NNRTI	18 (25.4%)	6 (33.3%)	5 (23.8%)	7 (21.9%)		
	PI	6 (8.45%)	3 (16.7%)	0 (0.00%)	3 (9.38%)		
LBP (µg/mL)		5.99 (2.17)	5.71 (2.31)	6.12 (2.54)	6.06 (1.86)	0.815	70
sCD14 (µg/mL)		2.48 [2.01;2.92]	2.62 [2.03;2.95]	2.62 [2.03;3.25]	2.36 [1.90;2.61]	0.087	70
IL6 (pg/ml)		0.95 [0.65;1.72]	0.87 [0.59;2.41]	0.74 [0.64;1.67]	1.02 [0.68;1.42]	0.934	70
CRP (µg/mL)		18.1 [10.0;64.5]	17.5 [9.14;48.0]	15.3 [13.4;56.9]	20.3 [10.4;65.6]	0.901	70
D-Dimer (µg/mL)		2.76 [2.14;3.69]	2.67 [2.02;3.96]	2.56 [1.97;3.48]	2.76 [2.39;4.02]	0.290	70
CD4+ (counts/mL)		397 [337;466]	358 [344;427]	403 [318;466]	422 [338;476]	0.259	68
CD8+ (counts/mL)		696 [507;870]	734 [603;819]	690 [507;877]	657 [500;865]	0.583	68
CD4/CD8		0.55 [0.42;0.83]	0.49 [0.42;0.61]	0.56 [0.40;0.67]	0.57 [0.45;0.88]	0.333	68

Age, weight, height, and LBP and were tested by ANOVA, categorical variables were compared using Fisher's exact test, while non-normal continuous variables (BMI, CD4 + nadir, years with cART, diagnosis, and other clinical variables) were tested by Kruskal-Wallis. Third drug row refers to the class of the 3rd ARV drug they received INSTI, Integrase Strand Transfer Inhibitor; NNRTI, Non-nucleoside reverse transcriptase inhibitors. All other variables represented as either count (percentage), or in the case of non-normally distributed as median-[Q1;Q3].

Presence of probiotic strains in faeces does not relate to changes in faecal microbiome

To better understand the lack of microbiome changes and the uncertain clinical changes, we tested how well the probiotic strains could maintain their presence in the gut. Only 6 (28.6%) patients in the Probiotic and 10 (31.3%) in the Synbiotic groups had detectable levels of either *L. plantarum* or *P. acidilactici* in at least two stool samples at months 1, 3 or 6, although in these cases, probiotic species median relative abundance started declining after the 3rd month of treatment, long before the wash-out phase. In the remaining patients, probiotic species were detected in only one of the timepoints in 5(23.8%) and 13 (40%) of the Probiotic and Synbiotic, respectively. None of the probiotic strains was detected in any sample obtained at baseline, month 9 or in the Placebo group at any time point (Figure 5A).

To better filter any potential probiotic strain-specific effect, as well as uncover potential factors that may affect probiotic engraftment, a new variable was defined. Two groups were created that separated patients with or without detectable levels of *P. acidilactici* in at least 2 stool samples belonging to different time points, excluding those patients in the Placebo group. *P. acidilactici* was chosen because it had presence in all samples where *L. plantarum* was detectable, but not the other way around (Figure 5B).

Microbiome analysis under this new stratification found no significant changes in alpha diversity as gene richness (Figure 6A) or beta diversity as Bray-Curtis distance from baseline (Figure 6B) over time between groups with (Present) and without (Absent) probiotic detection. This new variable only explained a 3.6% of the variance of Bray-Curtis distances between samples (Adonis R2 = 0.0362, p = 0.001). No clear group clustering could be observed under this new definition (Figure 6C)

Detection of probiotic in faeces associates with decreased inflammation (CRP, IL-6) while non-detection associates with increased CD4/CD8 ratio

At month 6, the Present group was associated with a significant decrease of CRP (median=11.1 (IQR=22) vs 19.2 (66), median change= -2.7 (13.2) ug/ml, p = 0.04), while the Absent group related to an increase in CD4/CD8 ratio (0.72 (0.57) vs 0.56 (0.4), median change = 0.4 (0.2) p=0.015) vs baseline. IL-6 differed between both groups at month 6, being lower in Present than Absent group (0.58 (1.13) vs 1.17 (1.59) ug/ml, p = 0.02). At month 9, an increase vs baseline was observed for CD4/CD8 ratio (0.67 (0.5) vs 0.59 ± (0.49), median change = 0.04 (0.18), p = 0.02), and CD4+ counts (457 (153) vs 416 (142), median change = 45

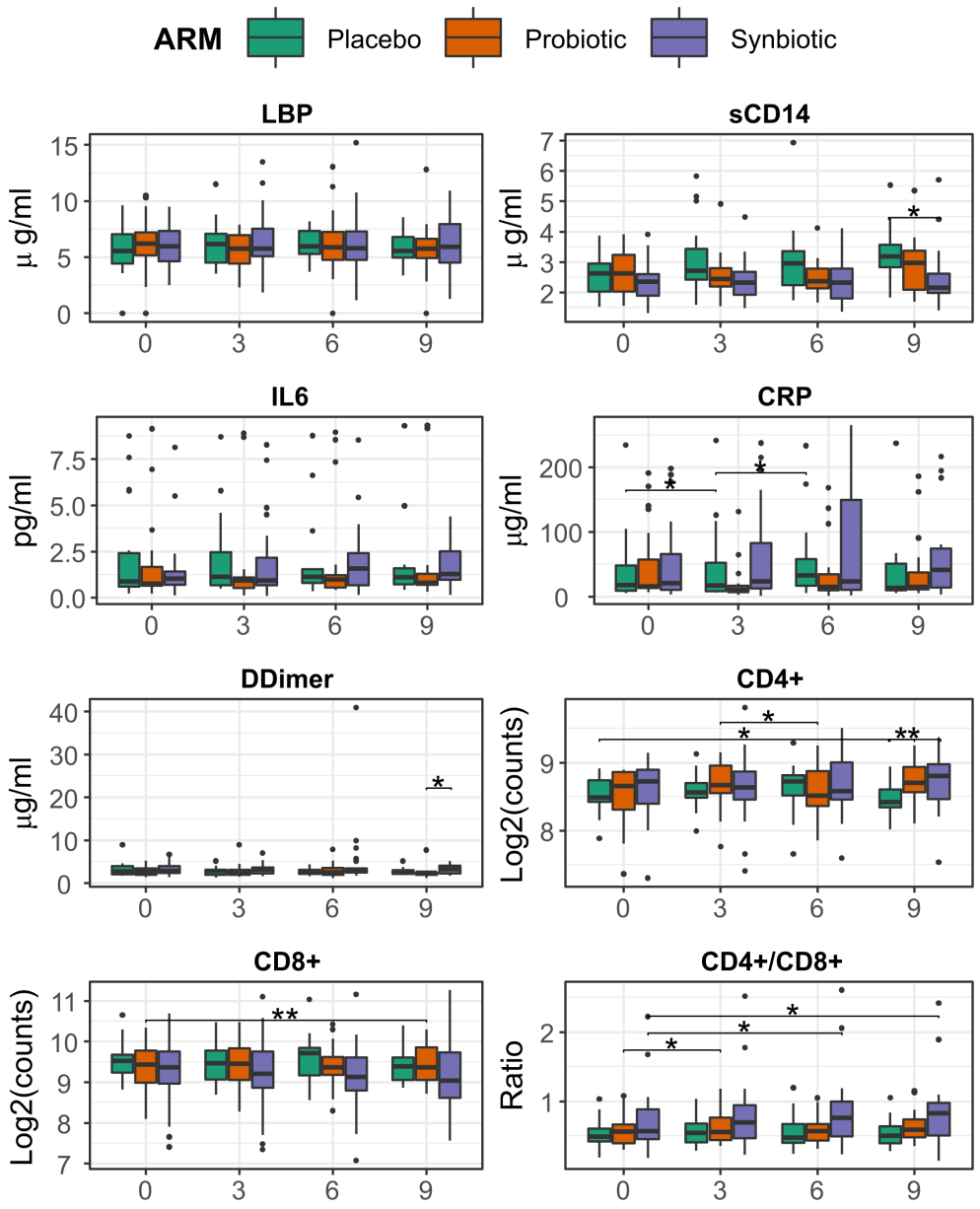

FIGURE 2
Evolution of T-cell counts, ratio, inflammation, and bacterial translocation markers between months 0 and 6, among treatment group. Comparisons within group and between time points were performed by Wilcoxon test, in its paired form for longitudinal differences and unpaired for cross-group comparisons. Significance was coded as follows: *($p < 0.05$), **($p < 0.01$).

(75), counts/ml, p = 0.005) in Absent but not in Present group (Figure 7).

Analysis with LMM found little overall effect on immune status and inflammation, with a slight increase of CD4/CD8 ratio (p = 0.008, slope = 0.012) and D-Dimer (p = 0.047, slope = 0.236) in the Absent group (Supplementary Figure 3A). However, these trends changed when stratifying by treatment group, especially for CRP, which increased in the Synbiotic arm (p = 0.013, slope = 7.4) (Supplementary Figure 3C). Also, CD4/CD8 ratio increase was specific to the subjects in Absent group who were in the Synbiotic arm (p = 0.005, slope = 0.015).

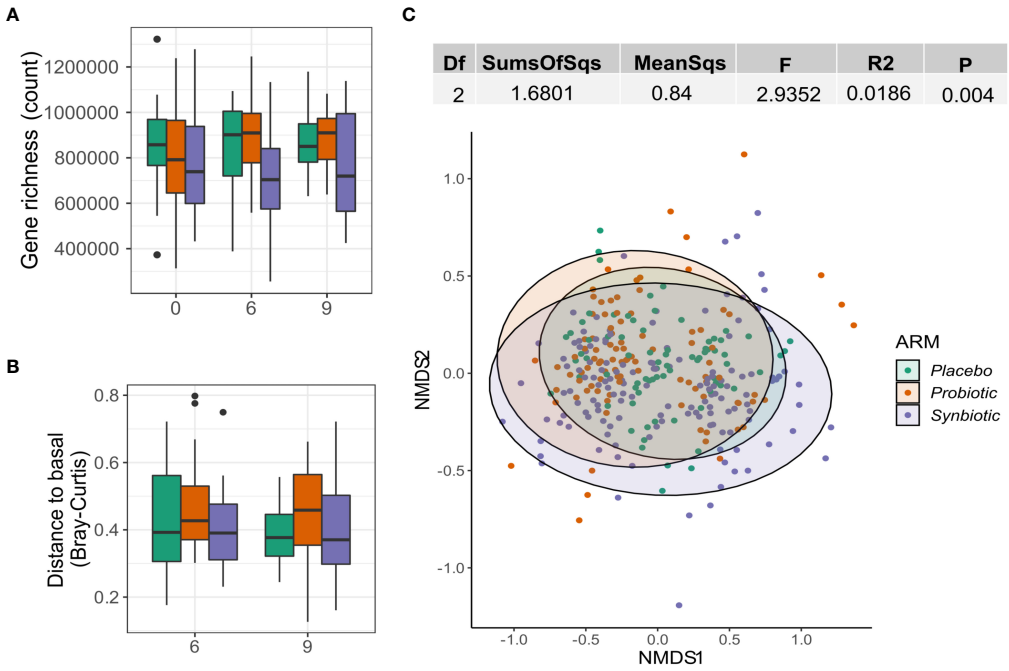

FIGURE 3
Effect of probiotics on the gut microbiome. **(A)** Gene richness counts for each intervention arm and month. Comparisons between group at each timepoint were performed using Wilcoxon test, while the paired version was used for within-group comparisons between months. **(B)** Distribution of Bray Curtis-Distances from baseline for each month and intervention arm. **(C)** NMDS showing sample ordination according to Bray-Curtis distance. Group distance dissimilarity was tested using ADONIS.

Probiotic presence in faeces is not coupled with lower abundances of ASR pathways

Sulphate assimilation pathways seemed slightly reduced in the probiotic Present vs Absent group, but the differences were statistically non-significant (Figure 8). Analysis with LMMs found no significant trends for any of the three previously studied pathways neither in the Present nor in the Absent group. Interestingly, after stratifying by intervention, pathway relative abundances did not change over time in the Present patients within the Probiotic group but increased in the Absent group (Met: $p = 0.026$, slope = $2.13{\cdot}10^{-6}$; ASR: $p=0.024$, slope = $1.11{\cdot}10^{-6}$; Cys: $p = 0.021$, slope = $1.256{\cdot}10^{-6}$), while the Synbiotic group showed a relative abundance decline in both Present and Absent groups (Supplementary Figure 5). Fixed effect interaction tests on unified models found no significant effect of presence/absence in any of the pathways, with and without stratification (Supplementary Figure 6).

Discussion

We assessed the efficacy and safety of long-term probiotic supplementation on immunodiscordant (CD4+ counts < 500) patients with supressed HIV after long, stable cART in a double-blind, randomized trial. We found that Synbiotic intervention with strains of *L. plantarum* and *P. acidilactici* along with prebiotic fibers is safe and associated with slight increases of CD4+ counts, CD4/CD8 ratio, and a decrease of the gut leakiness, as measured with sCD14 which is a proxy for bacterial endotoxin entering the bloodstream. However, the clinical significance of such improvements is uncertain, especially as they manifested 3 months after the intervention stopped. A more in-depth analysis suggests that the presence of the probiotic strains in stool was associated with lower levels of proinflammatory cytokines (IL-6 and CRP), while improvements in CD4/CD8 ratio appeared to be linked to the prebiotic fibers. The lowering in proinflammatory cytokines seems in agreement with previous reports using the same probiotic composition in animal models of IBD (37). This could point to a trade-off between immune activation and modulation by the prebiotic and probiotic, respectively. Of note, the reduction in inflammation markers happened during the intervention period, when no changes in gut permeability markers could be observed, suggesting that the immunomodulatory activity of the probiotics may be independent of the gut barrier status. Some gut barrier-unrelated anti-inflammatory mechanisms have been described, as Kawashima, Tadaomi et al. (38) found, lactic acid bacteria can

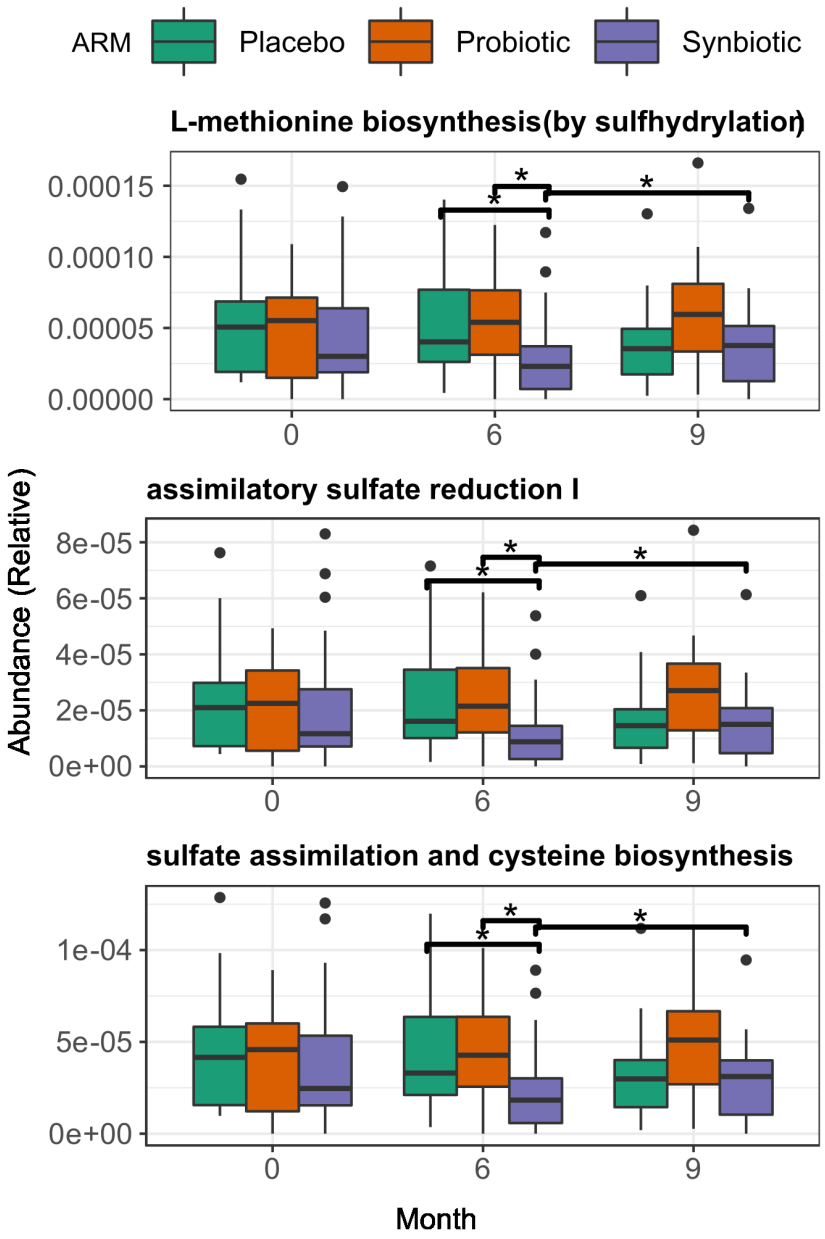

FIGURE 4
Changes in gene function at both start and end of the treatment period, among treatment group. Across group comparisons were performed with regular Wilcoxon test and longitudinal comparisons were performed using matched-samples Wilcoxon test. Significance was coded as follows: *($p < 0.05$).

induce IgA secretion to the gut lumen, which is a known immunomodulatory agent (39, 40).

The health-promoting properties of lactic acid bacteria have been widely reported *in vivo* and *in vitro* (41, 42). Nevertheless, the actual implantation and ecological viability of such strains is a multidimensional problem where host related factors and inherent ecological features of the host microbiome (such as normal ecological succession across the intestine) (43). Within the gastrointestinal (GI) tract, some lactobacilli show a nomadic behaviour (44) and tend to stay most commonly on the upper GI hence they tend to be underrepresented and transient in stool samples (45). In turn, implantation itself is not a strict requirement to generate change in the gut ecosystem or the host's physiology (46). In this study, *P. acidilactici* was more consistently found than *Lactobacillus plantarum* in faeces but, unlike the later, the former has been reported as having good

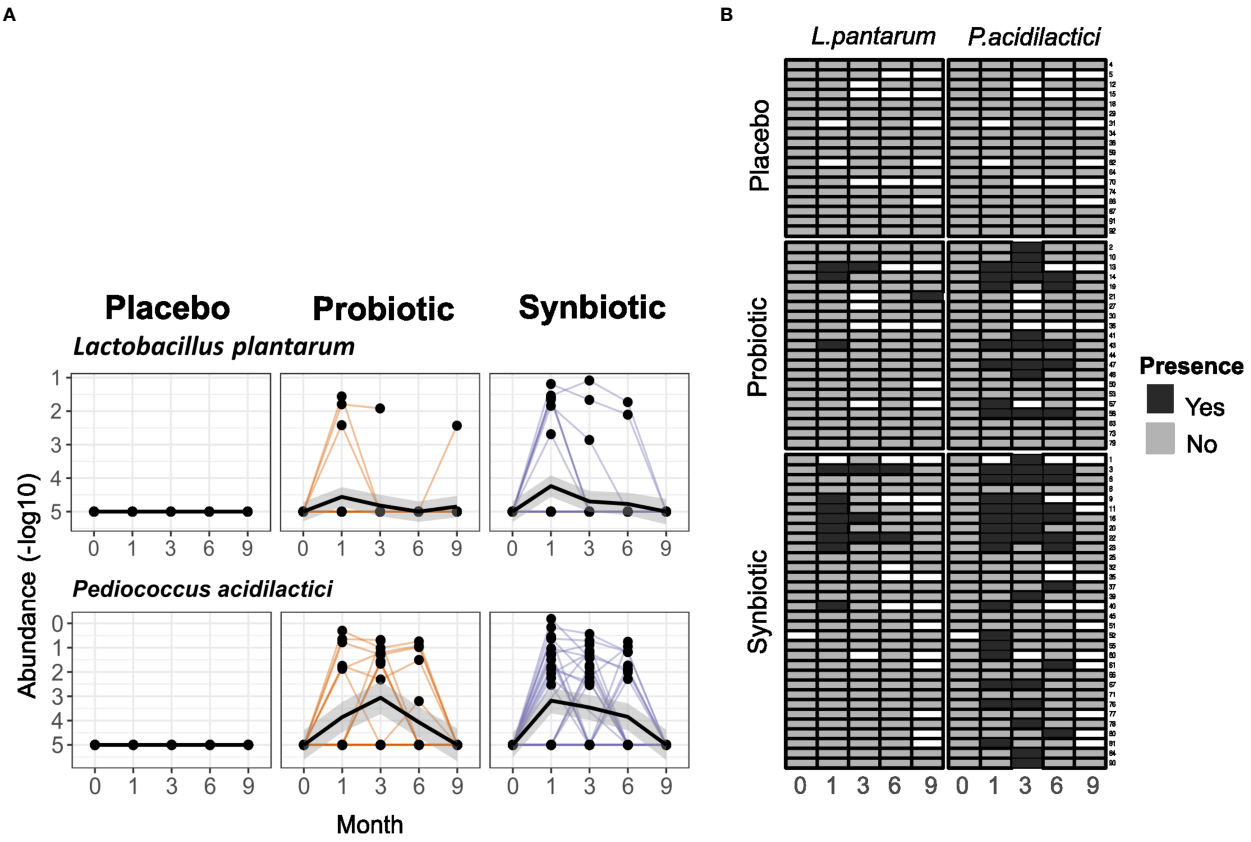

FIGURE 5
Detection of the probiotic strains in stool samples. **(A)** Relative abundances (in -log10) of *P. acidilactici* and *L. plantarum* in faeces for each of the intervention arms. **(B)** Heatmap representing presence/absence of each arm in each sample. Rows represent single patients and columns represent the month each sample belongs to. White cells represent lack of sample.

adhesion to the lower GI and higher presence in stool (47). Noteworthy, such differences may be strain-specific and not generalizable to other formulations (43). Hence, assessing the effectiveness of the probiotic strains in this study has been problematic, due to the low proportion of patients having a detectable presence of those, but also to the spatial and temporal complexity that characterizes the gut microbiome.

Additionally, we found that synbiotic intake was linked to decreases in the Assimilatory Sulphate Reduction (ASR) and sulphur-containing amino acids biosynthesis pathways. ASR, unlike the Dissimilatory Sulphate Reduction (DSR), takes sulphate without producing hydrogen sulphide as a final product, which has been shown to impair butyrate oxidation, the primary source of energy of enterocytes, and has been linked to gut inflammation and Ulcerative Colitis (48, 49). DSR is exclusive of anaerobic bacteria that undergo sulphate respiration, while ASR is more ubiquitous (50). Intake of Fructose and Glucose Oligosaccharides (FOS and GOS respectively) has been extensively used to modulate the microbiome, and has been linked to metabolic changes, especially of short chain fatty acids (51), but little is known about their relationship with sulphate metabolism. The fact that the Synbiotic group showed a consistent decline in such pathways could point to metabolic modulation and a population shift by the prebiotic fibers in the small intestine, although such changes may have been partially represented in the faecal samples.

Previous studies also found a lack of change in neither peripheral blood T-cells and/or gut permeability markers with probiotics only. Serrano-Villar et al. (52) found a significant decrease in inflammation markers (IL-6 and CRP) but no differential improvement in either circulatory T-cells nor sCD14 after 48 weeks, using a synbiotic formulation of *Saccharomyces boulardii* with various additives. However, this study was performed on late presenter, cART-naïve individuals, while our study was conducted on immunodiscordant patients with stable cART.

Previous studies using HIV infected immune discordant cohort reported diverse results. Presti et al. (53) tested a probiotic treatment consisting of different strains of

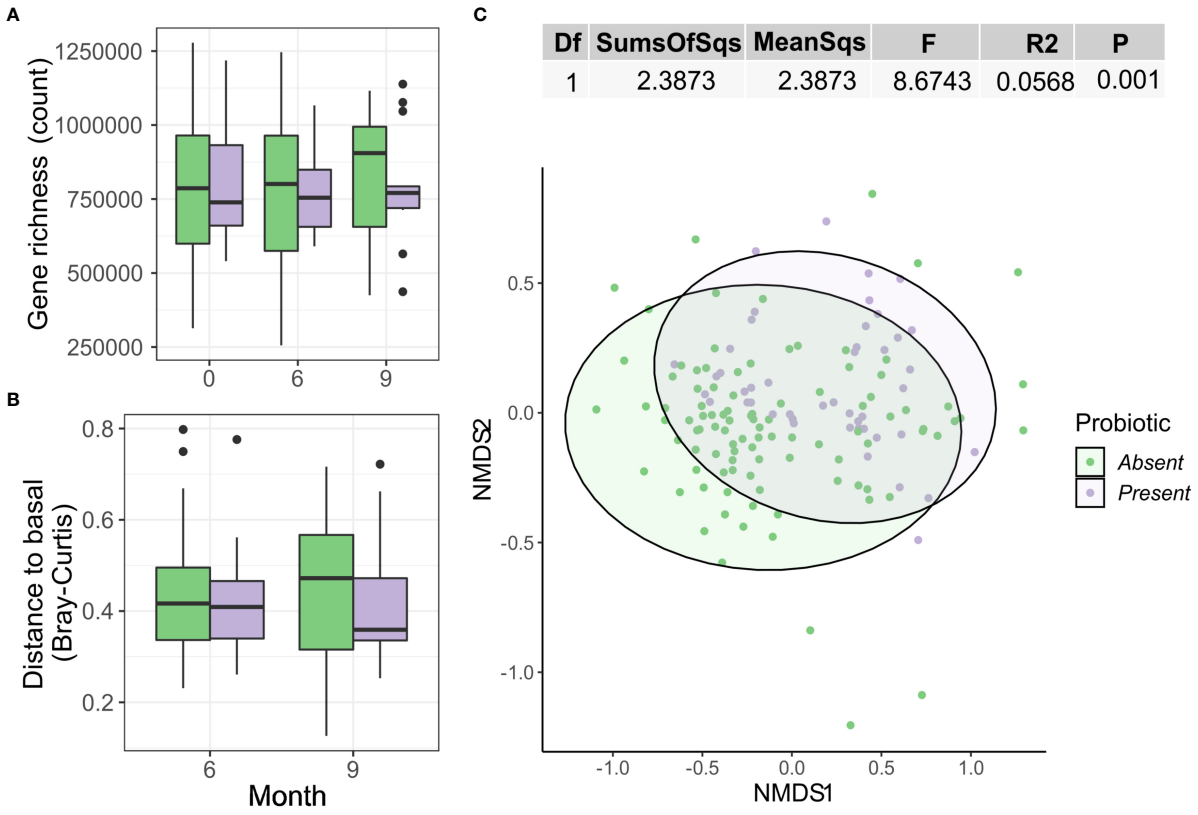

FIGURE 6
Differences by presence or absence of the probiotic strains on the gut microbiome. **(A)** Gene richness counts for each group arm and month. Comparisons between group at each timepoint were used simple Wilcoxon test, while the paired version was used for within-group comparisons between months. **(B)** Distribution of Bray Curtis-Distances from baseline for each month and group. **(C)** NMDS showing sample ordination according to Bray-Curtis distance. Group distance dissimilarity was tested using ADONIS.

streptococcus, bifidobacteria, and lactobacilli, without prebiotics, over 12 weeks, finding no differences in gut integrity (sCD14) and inflammation markers (D-Dimer, IP-10) but a significant decrease in *Proteobacteria*. Stiksrud et al. (54) found a decrease in inflammation (IL-6 and D-Dimer) but no differences in bacterial translocation markers and CD4+ counts after 8 weeks of intervention with formulation of skimmed milk, enriched with various species of *Lactobacillus* and Bifidobacterium vs a placebo of skimmed-milk only and a control groups. Geng et al. (55) reported an improvement in gut integrity (D-Dimer, DAO) and an enhanced CD4+ recovery in immune discordant patients using pre-digested protein supplementation. Despite the differences from the previously described studies (whether from study design or probiotic formulation), our study found improvement in inflammation and translocation markers but not an overall improvement in immune reconstitution. In addition, we could assess probiotic-related gut microbiome at species-level resolution of the microbiome, coupled with functional evidence and a washout period which adds robustness to any finding of potential signal of treatment effect.

Several limitations in this study should be considered. First, since patients were randomized by class of third antiretroviral drug, same-class ARV drgus might have different effects of the gut microbiome. Additionally, the cohort selected for this study was composed by immunodiscordant individuals, whose low CD4+ counts after viral suppression made them prone to infections that required antibiotic treatment, causing many dropouts. However, no significant biases were created by dropouts among the groups, although final sample size (n=71) was clearly smaller than original target (n=100) and may have been underpowered to detect some effects. Importantly, faecal samples hold an inherent bias and may not be representative of the actual gut microbiome composition, especially from the upper GI. Also, diet and concomitant treatments may affect faecal microbiome composition. While we found low rates of concomitant medications and these were balanced among groups, dietary information was not available, although extreme diets were excluded. This may affect our capability to detect any correlation between clinical outcomes and

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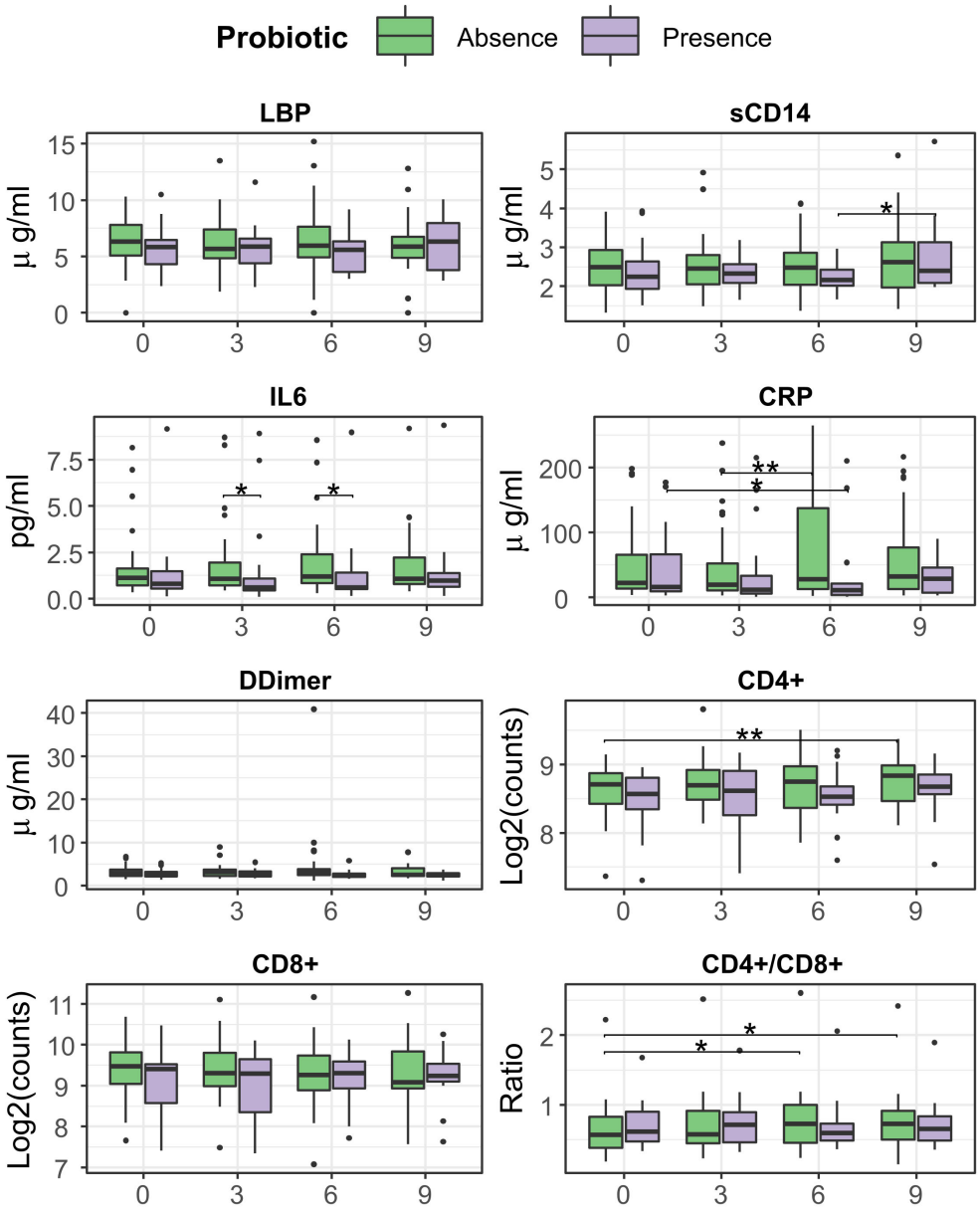

FIGURE 7
Evolution of T-cell counts, ratio, inflammation, and bacterial translocation markers between months 0 and 6, separated presence/absence of probiotic strains. Comparisons within group and between time points were performed by Wilcoxon test, in its paired form for longitudinal differences and unpaired for cross-group comparisons. Significance was coded as follows: *($p < 0.05$), **($p < 0.01$).

microbiome features. Finally, the fact that treatment intake was self-reported, could have led to many patients not taking the treatment but reporting otherwise during the 6 months follow-up, thus overestimating the actual intake of the probiotics. All these shortcomings add up to the limited effect of probiotics in immune reconstitution and affect the capability to translate research results from into clinical practice and warrant further research.

Conclusions

A synbiotic intervention with *L. plantarum* and *P. acidilactici* was safe and well tolerated. Synbiotic intervention led to small increases in CD4/CD8 ratio and minor reductions in sCD14 after 6 months and continued 3 months after discontinuing the intervention, but such changes are of uncertain clinical significance. A probiotic with the same composition but without

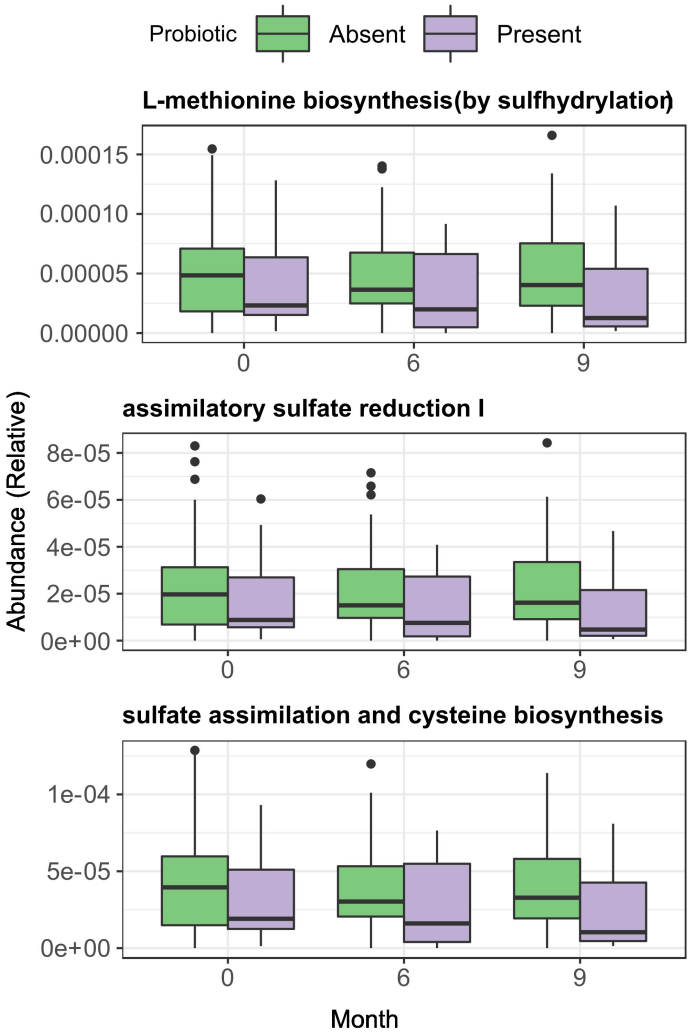

FIGURE 8
Longitudinal and across-group comparisons of Assimilatory Sulphate Reduction and sulphur amino acid pathways across intervention groups. Comparisons were performed across between groups by Wilcoxon test and longitudinally within-group with paired Wilcoxon test.

prebiotics was also safe but did not achieve any impact on immune parameters or faecal microbiome composition.

Data availability statement

The data presented in the study are deposited in the EBI-ENA repository, accession number PRJEB56022.

Ethics statement

The study was reviewed and approved by the Institutional Review Board of the Hospital Universitari Germans Trias i Pujol (reference PI-13-046). All participants provided written informed consent in accordance with the World Medical Association Declaration of Helsinki, Fortaleza and Brazil, October 2013 and personal data was managed according to Spanish data protection law (LOPD 15/1999). The study concept, design, patient information and results were discussed with the FLSida Community Advisory Committee, in accordance with AB-Biotics internal QC auditing. All available information can be found in the protocol (Supplementary Methods), and the study is registered in clinicaltrials.gov, accession: NCT03542786. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RP, JE-M, JRS, and MN-J conceived and designed the study. RP and JRS recruited the study participants and performed their clinical evaluations. MA performed study monitoring. MP, AE-T, and MC performed sample processing for sequencing and inflammation markers experiments, under the supervision of MN-J and RP. CB and FC-M performed the bioinformatic and statistical analyses with the supervision of RP, MN-J, CB, RP, and MN-J wrote the paper, which was reviewed, edited, and approved by all authors.

Funding

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement N° 847943, and the Spanish Ministry of Economy and Competitiveness (MINECO), reference RTC-2016-5223-1. This research was supported by CIBER -Consorcio Centro de Investigación Biomédica en Red- (CB 2021), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea – NextGenerationEU.

Acknowledgments

We thank all the enrolled participants who contributed to this study, the staff from Fundació Lluita contra la Sida (FLS), IrsiCaixa and Hospital Germans Trias I Pujol. We also thank AB-Biotics for their non-financial support and feedback, and the staff from MISTRAL.

Conflict of interest

JE-M and MA are full-time employees of AB-BIOTICS SA, the company owning IP rights for the probiotic strains used in this study. MN-J is co-founder and shareholder of Nano1Health SL, outside the scope of the submitted work.

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1066036/full#supplementary-material

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 15 December 2022
DOI 10.3389/fmicb.2022.1015130

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

RECEIVED 09 August 2022
ACCEPTED 28 November 2022
PUBLISHED 15 December 2022

CITATION
Zhou B, Dong C, Zhao B, Lin K, Tian Y, Zhang R, Zhu L, Xu H and Yang L (2022) *Bacteroides fragilis* participates in the therapeutic effect of methotrexate on arthritis through metabolite regulation. *Front. Microbiol.* 13:1015130. doi: 10.3389/fmicb.2022.1015130

Bacteroides fragilis participates in the therapeutic effect of methotrexate on arthritis through metabolite regulation

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Methotrexate (MTX) is a preferred disease-modifying anti-rheumatic drug in the management of rheumatoid arthritis (RA). However, the toxicity and inefficiency of MTX limit its clinical application. Gut microbiota has been implicated in the side effects and efficacy of MTX. In this study, the analysis of the gut microbiota in RA patients revealed that the abundances of intestinal *Bacteroides fragilis* was reduced after MTX treatment. We observed that MTX has no obvious therapeutic effect in the absence of *B. fragilis*, while transplantation of *B. fragilis* restored the efficacy of MTX in antibiotics-pretreated collagen-induced arthritis (CIA) mice. In addition, *B. fragilis* gavage was accompanied by an increase in butyrate. Supplementation of butyrate restored the response to MTX in gut microbiota-deficient mice, to a similar level achieved by *B. fragilis* gavage. These results show that gut microbiota-regulated butyrate plays an essential role in the efficacy of MTX, which will provide new strategies to improve the effectiveness of methotrexate in RA treatment.

KEYWORDS

methotrexate, rheumatoid arthritis, gut microbiota, *B. fragilis*, butyrate

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, cartilage and bone damage, and is associated with progressive disability, systemic complications as well as early death (Mcinnes and Schett, 2011). With an incidence of 0.5–1%, RA has been one of the most common chronic inflammatory diseases and adds a series of burdens to individuals, families and society (Smolen et al., 2016). Disease-modifying antirheumatic drugs (DMARDs) are widely

used as first-line RA drugs due to their clinical efficacy and cost effectiveness. As one of DMARDs, methotrexate (MTX) has been commonly used in the treatment of RA since the 1980s (Weinblatt et al., 1985; Bedoui et al., 2019). Whereas, the clinical application of MTX is still limited by adverse events and unsatisfactory therapeutic effects. More than 75% of patients with low-dose MTX treatment suffer several common side effects, such as gastrointestinal toxicities, hepatotoxicity and so on (Iannone et al., 2016; Wang et al., 2018). In addition, about one-third of patients do not respond to MTX (Weinblatt et al., 1994; Saevarsdottir et al., 2011). Although several strategies have been used to manage side effects of MTX, such as the treatment with folic acid and changing the way of administration, the results are not ideal because of the decreased efficacy of MTX or other adverse effects (Tishler et al., 1988; Morgan et al., 1994; Wang et al., 2018). Therefore, there is still an urgent need to alleviate the side effects and to improve the efficacy of MTX.

The gut microbiota is closely related to the development of cancers and autoimmune diseases (Kamada et al., 2013; Garrett, 2015; Zitvogel et al., 2017; Yachida et al., 2019). In addition to this, there is accumulating evidence that the gut microbiota plays a crucial role in the toxicity and efficacy of various drugs (Kang et al., 2013; Alexander et al., 2017; Weersma et al., 2020). Studies demonstrate that cyclophosphamide causes changes in the composition of gut microbiota in mice and promote the transfer of some gram-negative bacteria to secondary lymphoid organs (Viaud et al., 2013). The bacteria stimulate a specific subset immune cells and enhanced immune responses. Moreover, cyclophosphamide has no effect on sterile mice, suggesting that the gut microbiota help define the anticancer effects of cyclophosphamide (Viaud et al., 2013). Similarly, two studies have shown that gut microbiota can modulate responses to PD-1-based immunotherapy in mice and in patients, confirming the importance of gut microbiota in the efficacy of drugs (Matson et al., 2018; Routy et al., 2018). In our previous study, we observed that MTX treatment led to alteration in the diversity and composition of the gut microbiota, with significantly decreased abundance in *Bacteroides fragilis* (*B. fragilis*), (Zhou et al., 2018) *which* is a prominent human commensal. It was shown that *B. fragilis* can inhibit T cell-mediated inflammation and prevent intestinal inflammatory diseases, such as colitis (Wexler and Goodman, 2017). The immunomodulatory molecule polysaccharide A (PSA), a component of the *B. fragilis*, induces an anti-inflammatory immune response mediated by IL-10 produced by T cells in intestinal tissue (Mazmanian et al., 2008; Round and Mazmanian, 2010). In addition to PSA, *B. fragilis* can regulate immune cells through short-chain fatty acids (SCFAs) (Su et al., 2020). Our previous data suggested that *B. fragilis* ameliorated MTX-induced mucositis by modulating macrophage polarization. Since the regulatory effect of *B. fragilis* in MTX-induced gastrointestinal toxicities has been known (Zhou et al., 2018), it is of interest to investigate the role of *B. fragilis* in the efficacy of MTX in RA.

In this study, we found that RA patients who were treated with MTX exhibited lower abundances of *B. fragilis*. Then we established a collagen-induced arthritis (CIA) model and utilized antibiotics to remove *B. fragilis* in mice. *B. fragilis*-deficient CIA mice were lack of response to MTX treatment. Meanwhile, supplementation with *B. fragilis* restored the efficacy of MTX in antibiotic-treated mice. We observed that *B. fragilis* stimulated the production of immunomodulatory M2 macrophages. In addition, we found that *B. fragilis* supplementation led to elevated production of butyrate and that butyrate restored the therapeutic effect of MTX in gut microbiota-deficient CIA mice. These data suggest that *B. fragilis* is critical for the therapeutic effect of MTX in RA.

Materials and methods

Patient fecal samples

Stool samples were collected from 21 RA patients with MTX treatment at day 0 and day 30 in the Affiliated Hospital of Zunyi Medical University. Patients were provided a feces collection tube to collect stool sample at home. The samples were sent to the lab within 24 h after collection. Stool bacterial DNA was isolated using the Stool DNA Isolation Kit (Foregene, Chengdu, China). The DNA and the rest of the sample was stored at −80°C. All human studies were approved by the Ethics Committee of Affiliated Hospital of Zunyi Medical University. Written informed consents were received from all patients prior to inclusion in the study.

16S rRNA amplicon sequencing and data analysis

The fecal samples were collected from CIA mice on day 0 and day 30 after MTX treatment. All samples were stored at −80°C. Stool bacterial DNA was extracted using the Stool DNA Isolation Kit (Foregene, Chengdu, China). One nanogram of purified fecal DNA was used for PCR amplification. Amplicons spanning the variable region 4 (V4) of the 16S rRNA gene were generated by using the following primers: forward, 5′-GTGCCAGCMGCCGCGGTAA-3′; reverse, 5′-GGACTACHVGGGTWTCTAAT-3′. The PCR products were then sequenced on an Illumina Hi-seq sequencer at Novogene (Novogene, Beijing, China). Paired-end reads from the original DNA fragments were merged by using FLASH (Magoc and Salzberg, 2011). Paired-end reads was assigned to each sample according to the unique barcodes. Sequences were analyzed using QIIME software package (quantitative insights into microbial ecology) (Caporaso et al., 2010). Sequences

with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). Taxonomical classification was performed using the RDP-classifier. The alpha diversity (such as ACE) for each subsample was calculated in Mothur. The unpaired, two-tailed t test was used to calculate differences between means (GraphPad Software). Principal component analysis (PCA) and principal coordinate analysis (PCoA) clustering were conducted using R. The linear discriminant analysis (LDA) with effect size (LEfSe) method of analysis was used to compare abundances of all bacterial clades using the Kruskal–Wallis test at a pre-defined α of 0.05. Significantly different taxa resulting from the comparisons of abundances between groups were used as input for LDA.

Mice and generation of CIA model

Male 8-week-old DBA/1j mice were purchased from Beijing Vital River Laboratories Animal Technology Co. Ltd. All mice were maintained in a pathogen-free animal facility. All experimental procedures and animal care were approved by the Animal Care Committee of Sichuan University and were performed in accordance with the relevant ethical guidelines (Guidelines for Ethical Review of laboratory Animal Welfare No. GB/T 35892-2018). The method for the generation of CIA model was described previously (Zhou et al., 2019).

Treatment of CIA mice

In this study, we performed four experiments to explore the relationship between gut microbiota and MTX efficacy in CIA mice. Mice in MTX-treated group were intraperitoneally (i.p.) injected with 1 mg/kg of MTX (Sigma-Aldrich, USA) every 3 days for 30 days. Control mice received PBS only. In the second and third experiments, 1mg/ml or a combination of antibiotics (Abs) (1 mg/ml ampicillin + 5 mg/ml streptomycin + 1 mg/ml metronidazole) were added in sterile drinking water of antibiotics-treated group on day −7 to day 0, The solutions and bottles were changed every 3 days. In Abs + MTX + *B. fragilis* and Abs + MTX + *Escherichia coli* group, mice were given oral gavage with 1×10^9 bacterial cells on day 0. A total of 100 mM butyrate (But) (Sigma-Aldrich, USA) was dissolved in drinking water in Abs + MTX + But group on day 0 for 30 days. Each group consisted of 6 or 10 mice.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was conducted on Bio-rad CFX Connect platform using the SYBR Fast qPCR Mix (Takara, Japan) to detect the abundance of 16 rRNA gene in fecal bacterial DNA. Gene specific primer sequences were as follows: 16S rRNA (F: CGGTGAATACGTTCCCGG, R: TACGGCTACCTTGTTACGACTT), *B. fragilis* (F: TGATTC CGCATGGTTTCATT, R: CGACCCATAGAGCCTTCATC).

Cultivation of bacteria

Bacteroides fragilis (ATCC 25285) was cultured on brain heart infusion (BHI) blood agar plates (Oxoid, USA) for 48 h at 37°C under anaerobic conditions. *B. fragilis* was harvested from the plates and suspended in sterile PBS. *E. coli* was cultured in Luria-Bertani liquid medium for 16 h at 37°C before harvest. Then, the bacteria were washed with PBS and resuspended in sterile PBS to achieve an OD_{600} = 1, which corresponds to approximately 1×10^9 colony forming units (CFUs) per ml.

Flow cytometry

Spleens were harvested from mice on day 30 after the first injection of MTX. The tissues were cut into small pieces and filtered through a 70 μm cell strainer. The cells were stained with antibodies against the following surface markers: PerCP-CD11b, PE-F4/80 and FITC-CD206. These antibodies were purchased from BD Biosciences. Cell detection were conducted on a flow cytometer (FACSCalibur and Accuri C6, BD Biosciences), the data were analyzed with FlowJo 6.0 and NovoExpress.

Radiological and histological assessment of joint tissues

On day 30, a micro-CT (PerkinElmer, USA) was used to assess the degree of joint injury. Then, mice were sacrificed and joint tissues were harvested. After fixation in 4% paraformaldehyde for 24 h, the tissues were decalcified with EDTA and embedded in paraffin. Finally, 4 μm sections of the joint tissues were prepared and stained with HE.

Metabolomics analysis based on GC-MS

Fecal samples were collected on day 7 after MTX treatment with or without *B. fragilis* gavage. 50 μl 15% phosphoric acid, 100 μl 125 μg/mL isohexanoic acid solution and 400 μl diethyl ether was added to 50 mg feces sample. After homogenate for 1min, the mixture was centrifuged at 12,000 RPM at 4°C for 10 min, and the supernatant was used for short-chain fatty acids (SCFAs) analysis by GC-MS.

Butyrate treatment *in vitro*

Cells isolated from the spleen were stimulated with lipopolysaccharide (LPS, 100 ng/ml) with or without butyrate (100 μM–2 mM) for 24 h, after which cells were collected for flow cytometry.

Statistical analysis

Data were analyzed using GraphPad Prism 8 (GraphPad, La Jolla, CA, USA). Data were depicted as the means ± SEM, and statistical comparisons were conducted using t-test or unpaired one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

Results

MTX therapy alters the composition of gut microbiota in RA patients

In our previous study, we observed that the abundance of *B. fragilis* was decreased in normal *Balb/c* mice after MTX treatment. To evaluate whether MTX influences the composition of intestinal microbiota in arthritis, stool samples were collected from 21 RA patients before and after MTX treatment. 16S rRNA gene amplicon sequencing was conducted to assess the relationship between gut microbiota and clinical response. Unlike the results of animal experiments, the alpha diversity of gut bacteria did not change significantly after MTX treatment in all patients (**Figure 1A**). PCoA and venn diagram showed that the composition of gut microbiota was altered after MTX treatment (**Figures 1B–D**). Clustering heatmap of species abundance showed the changes of 30 bacteria, among which 14 bacteria had increased, such as *Eubacterium ramulus*, and 16 bacteria had decreased, such as *Escherichia coli* (**Figure 1E**). Notably, the content of *B. fragilis* was reduced (**Figure 1E**), which is in agreement with the results in our previous study, confirming that *B. fragilis* is one of gut microbiota that related to the MTX treatment.

Transplantation of *B. fragilis* enhances the effect of MTX in gut microbiota-deficient mice

To further verity the role of *B. fragilis* in the treatment of RA with MTX, the CIA mice were pre-treated (or untreated as controls) with antibiotics for 7 days, followed by MTX injection with or without bacteria (*B. fragilis* or *E. coli*) transplantation (**Figure 2A**). The concentration of stool DNA in the mice treated with antibiotics was significantly lower than that of the mice untreated with antibiotics (**Figure 2B**), indicating that the gut microbiota was effectively reduced. After bacteria gavage, transplantation with *B. fragilis* was able to restore the abundance of *B. fragilis* (**Figure 2C**). Consistent with the above experiment, the disease severity evaluation with the clinical score data showed that MTX treatment could not achieve a satisfactory result in the gut microbiota-deficient mice (**Figure 2D**). Compared to the MTX group, the arthritis degree of the mice in Abs + MTX group was not alleviated effectively (**Figure 2D**). However, the inhibitory effect of MTX on arthritis in antibiotics-treated mice was enhanced after *B. fragilis* gavage, indicated by the delayed development of CIA in the Abs + MTX + *B. fragilis* group, comparable to that in the MTX group (**Figure 2D**). *E. coli* gavage did not yield a similar effect as *B. fragilis*, no difference in clinical score was observed between the PBS and the Abs + MTX + *E. coli* group (**Figure 2D**). In addition, the swelling of joints was not evident in the MTX or the Abs + MTX + *B. fragilis* group on day 30 (**Figure 2E**). HE staining showed that synovial hyperplasia, cartilage injury, lymphocyte infiltration and bone erosion of the knee joint in CIA mice were relieved in the MTX and the Abs + MTX + *B. fragilis* groups (**Figure 2F**). It appears that *B. fragilis* could restore the therapeutic effect of MTX in gut microbiota-deficient mice.

Our previous data suggested that *B. fragilis* treatment can lead to significant alternation in macrophages, which also play a critical role in RA (Quero et al., 2017). Therefore, we investigated the inflammatory status of spleen in mice. MTX and antibiotic treatment did not alter the amount of splenic $CD4^+F4/80^+$ macrophage and $CD11b^+CD206^+$ M2 macrophage in CIA mice, while there was a prominent up-regulation of M2 macrophage in the Abs + MTX + *B. fragilis* group, confirming the role of *B. fragilis* in regulating immune cells in MTX-treated CIA mice (**Figures 3A–D**).

B. fragilis gavage promotes butyrate metabolism in methotrexate-treated mice

Bacteroides produces SCFAs in intestine (Macfarlane and Macfarlane, 2003). One relevant question is whether *B. fragilis* gavage affects the production of SCFAs in MTX-treated mice. Therefore, the feces of MTX-treated mice with/without *B. fragilis* gavage were harvested for SCFAs analysis. We found that the concentration of acetic acid, propionic acid, isobutyric acid, butyric acid (butyrate), isovaleric acid and valeric acid was markedly reduced after MTX administration (**Figures 4A–G**). In addition to butyrate, other SCFAs also decreased in mice of MTX + *B. fragilis* group (**Figures 4A–G**), suggesting *B. fragilis* gavage promoted the production of butyrate. To verify the correlation between the content of *B. fragilis* and

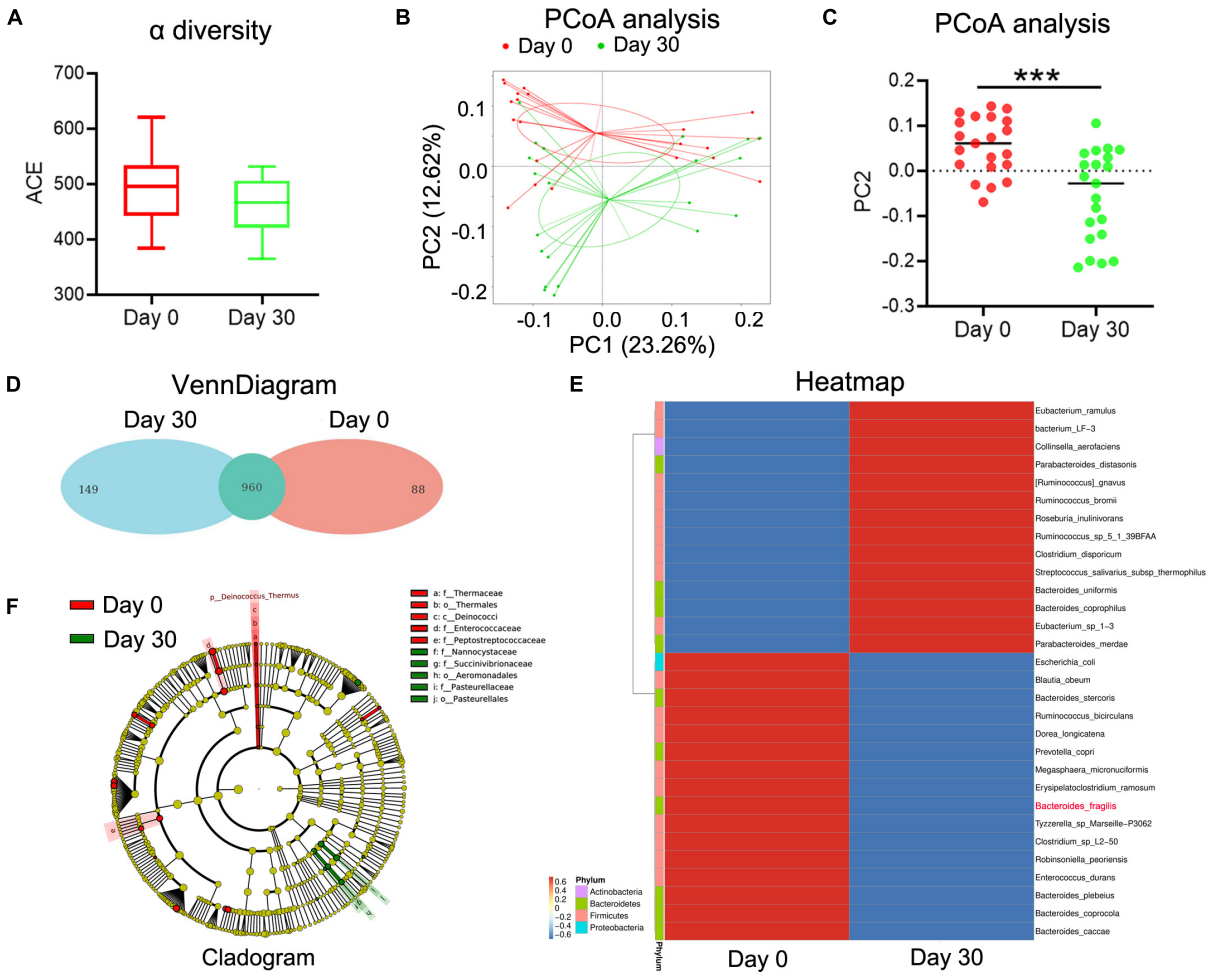

FIGURE 1
The changes of gut microbiota in MTX-treatment patients. Stool samples were collected from 21 RA patients with MTX treatment at day 0 and day 30. Stool bacterial DNA was extracted using the Stool DNA Isolation Kit and detected using 16S rRNA amplicon sequencing. **(A)** ACE analysis to predict species richness among different groups. **(B,C)** PCoA to assess differences between microbial communities. **(D)** Flower figure of VennDiagram to show the common and unique OTUs among different groups. **(E)** Heatmap to present the change of different species. **(F)** Cladogram to analyze different species between groups. ***$p < 0.001$.

the production butyrate, the relative units of *B. fragilis* was detected. Correlation analysis showed that the person r = 0.7823 ($p < 0.01$) (Figure 4H), indicating a positive correlation between content of *B. fragilis* and butyrate. These data reveal that *B. fragilis* gavage could enhance the metabolism of butyrate in MTX-treated mice.

Supplementation of butyrate recovers the effect of MTX in gut microbiota-deficient mice

As butyrate is mainly produced by the gut microbiota (Flint et al., 2015; Forslund et al., 2015), and is an important regulator of immune response (Liu et al., 2018; Silva et al., 2018), we sought to determine the influence of butyrate administration on the MTX treatment in CIA mice with/without pretreatment of antibiotics. Butyrate was provided in drinking water until the end of the experiment (Figure 5A). There was no obvious remission of the arthritis score in CIA mice with butyrate treatment alone (Figure 5B). Moreover, butyrate did not affect the efficacy of MTX in CIA mice untreated with antibiotics (Figure 5B). Nevertheless, the development of arthritis was effectively inhibited in the Abs + MTX + But group compared to that in the Abs + MTX group (Figure 5B). Consistently, butyrate improved the swelling, deformation, cartilage injury, lymphocyte infiltration and bone erosion of joints in the Abs + MTX + But group (Figures 5C–E). Taken together, butyrate can restore the efficacy of MTX in gut microbiota-deficient mice as well as *B. fragilis*.

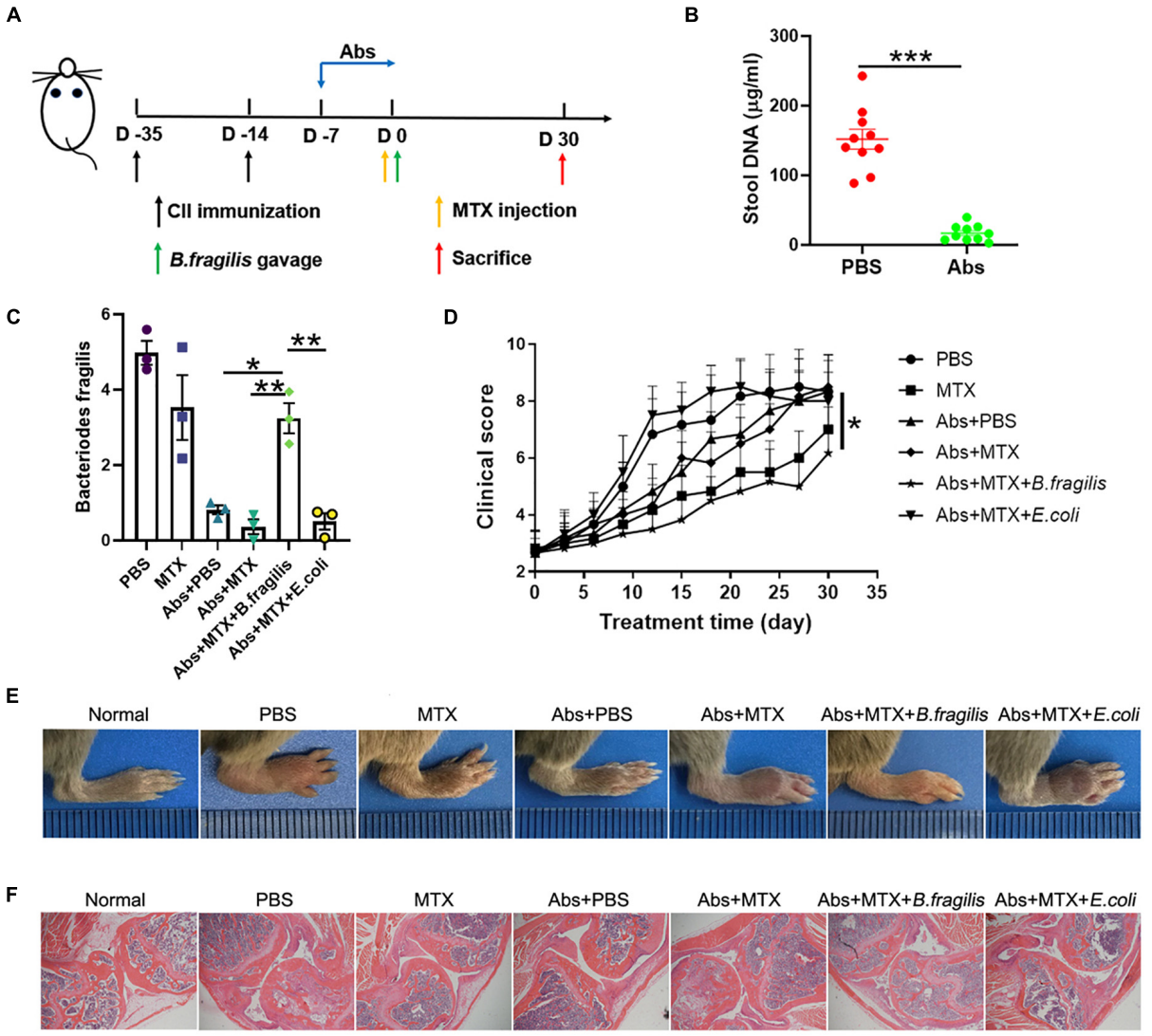

FIGURE 2
The response to MTX was affected by *Bacteroides fragilis* in gut microbiota deficient CIA mice. **(A)** Schematic of treatments. Mice were treated with antibiotics at the onset of arthritis. Mice received *B. fragilis* gavage and MTX injection on day 0. **(B)** The total stool DNA after Abs treatment (n = 10). **(C)** The content of *B. fragilis* after gavage (n = 3). **(D)** Clinical scores of CIA mice (n = 6). **(E)** The photos of hindlimb. **(F)** HE staining of joint tissues (magnification is 40×). Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abs, antibiotics.

We next investigated the impact of butyrate on splenic macrophage. Butyrate promoted the expansion of M2 macrophage in CIA mice with MTX injection (**Figures 6A–D**). In order to test whether butyrate exerts the same effect on lymphocytes *in vitro*, splenic lymphocytes were isolated from CIA mice and stimulated with LPS and butyrate. We found that the percent of M2 macrophage was increased in the presence of butyrate in a dose dependent manner (**Figures 6E, F**). These data indicated that butyrate can regulate the immune response by promoting the development M2 macrophage both *in vivo* and *in vitro*.

Discussion

Gut toxicity and lack of efficacy are still challenging problems in the clinical treatment of RA with MTX. MTX is an antagonist of folate, the folate metabolism pathway also exists in microbiota, the intestinal microbial compositions likely change after MTX treatment (Huang et al., 2020). In addition, MTX-induced mucositis can also lead to bacterial translocation (Ubeda et al., 2010). Previous studies showed that gut microbiota can relieve the toxicity of various drugs, including MTX (Zhou et al., 2018; Huang et al., 2020). Here, with CIA models, we verified the role of gut microbiota in

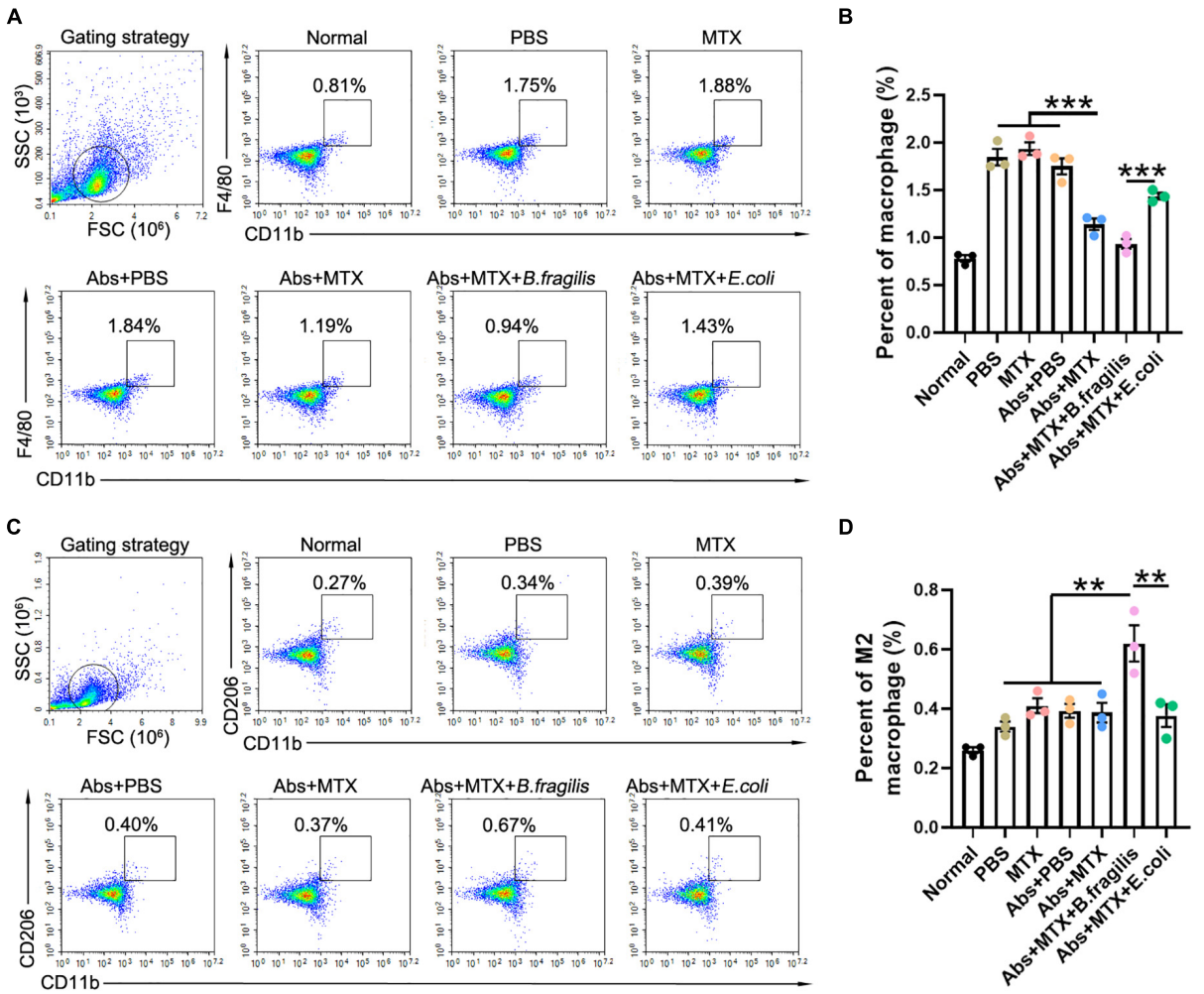

FIGURE 3
Bacteroides fragilis alters the polarization of macrophage. **(A–D)** FACS analysis of splenic cells. Percentages of **(A,B)** macrophages and **(C,D)** M2 macrophages (n = 3). Data are presented as the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$. Abs, antibiotics.

the efficacy of MTX in RA, and further demonstrated that butyrate-regulating *B. fragilis* is an intestinal bacteria related to the efficacy of MTX.

Studies have reported that *B. fragilis* can alleviate MTX-induced intestinal inflammation (Zhou et al., 2018). Since gut microbiota has been proven not only to reduce the intestinal toxicity of drugs, but also to affect their efficacy (Alexander et al., 2017; Weersma et al., 2020), and the detection of gut microbiota of RA patients showed the content of *B. fragilis* was decreased after MTX therapy, we speculated that transplantation of *B. fragilis* may enhance the therapeutic effect of MTX in gut-deficient CIA mice. To test our hypothesis, we conducted *B. fragilis* gavage in MTX-treated mice after removing microbiota with antibiotics. While the arthritis-inhibitory effect of MTX was lowered after the clearance of gut microbiota, supplementation with *B. fragilis* could restore the therapeutic effect to a similar level found in antibiotics-untreated mice, which did not occur with the supplementation of *E. coli*. Anti-inflammatory M2 macrophages are crucial in the pathogenesis of immune-inflammatory disorders (Tardito et al., 2019). Our observation that *B. fragilis* up-regulated the number of M2 macrophage suggested that *B. fragilis* contributed to the therapeutic effects of MTX by regulating the development of anti-inflammatory lymphocytes.

The mechanisms of intestinal microbiota affecting drug efficiency include metabolism, immune regulation, translocation, enzymatic degradation and ecological variation (Panebianco et al., 2018). Gut microbiota metabolizes complex dietary carbohydrates through a large number of enzymes, and degrades dietary fiber to produce organic acids, gases and a large amount of SCFAs (Martin-Gallausiaux et al., 2021). Gut microbiota regulate the function of immune cells through its metabolites SCFAs (Rooks and Garrett, 2016;

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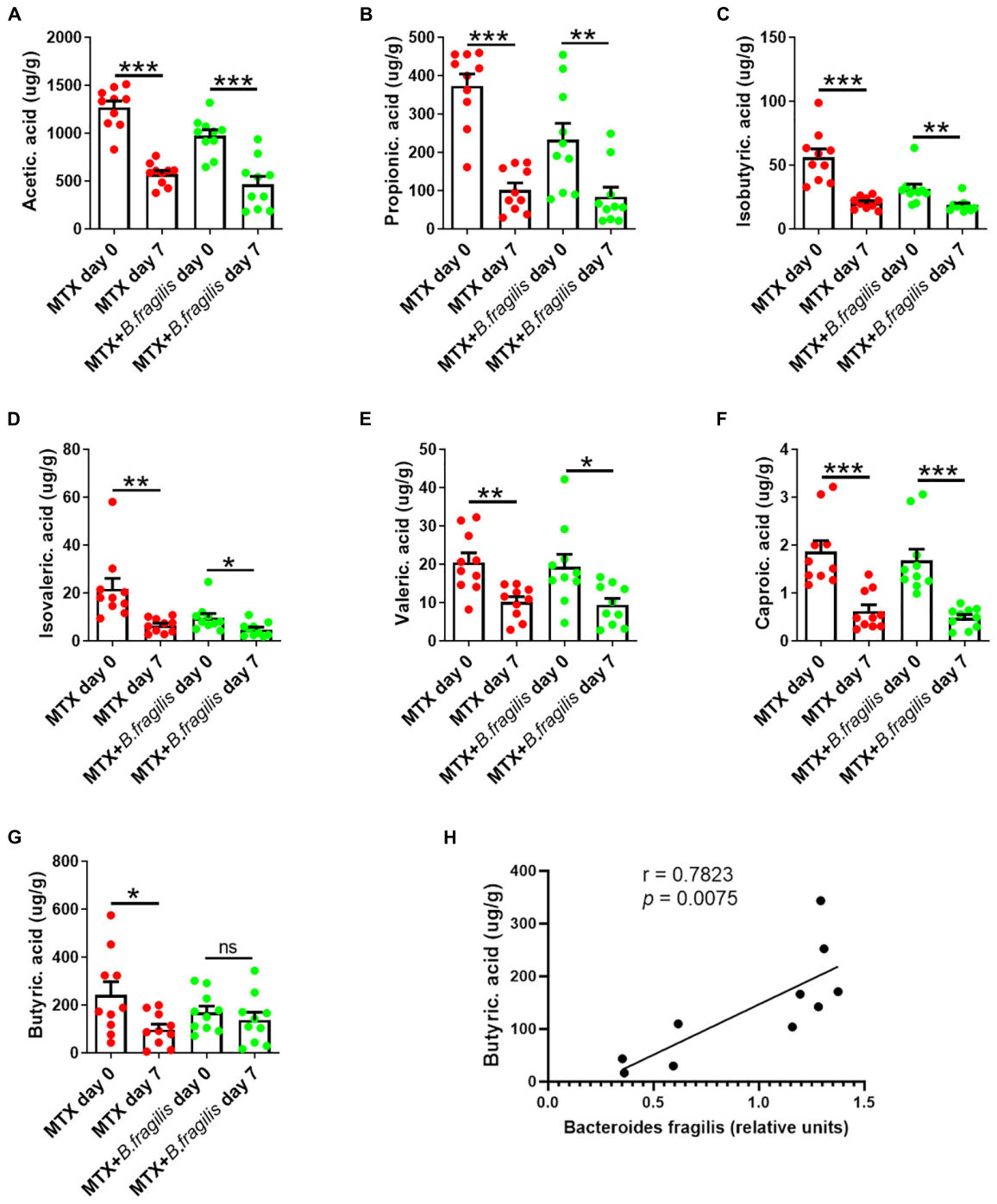

FIGURE 4
Analysis of SCFAs in feces of mice. MTX-treated CIA mice were gavaged with PBS or PBS containing *B. fragilis* for 7 days. Fecal samples were collected for GC-MS analysis of **(A)** acetic acid, **(B)** propionic acid, **(C)** isobutyric acid, **(D)** isovaleric acid, **(E)** valeric acid, **(F)** caproic acid and **(G)** butyric acid (butyrate), (n = 10). **(H)** Correlation analysis between and the relative units of *B. fragilis* and the content of butyrate. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Ratajczak et al., 2019). These biological functions of SCFAs are mediated by SCFA receptors GPR41 and GPR43, expressed on immune cells, adipocytes, and intestinal cells (Tan et al., 2014; Sun et al., 2017). We found that the supplementation of *B. fragilis* prevented the MTX-induced decrease of butyrate, which can limits the autoimmune response (Takahashi et al., 2020). It is possible that butyrate is involved in the beneficial effect of *B. fragilis* on the efficacy of MTX. By adding butyrate

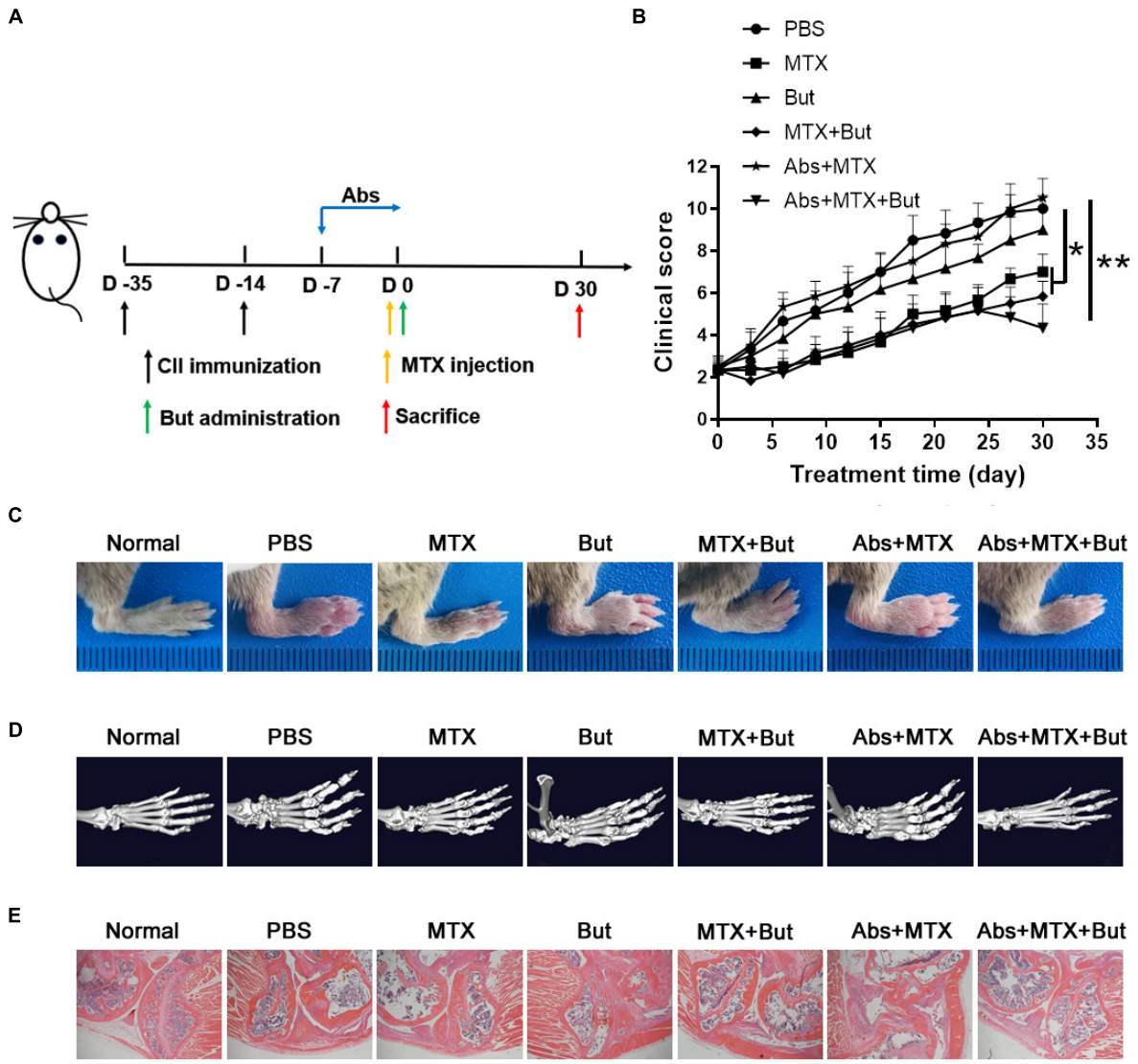

FIGURE 5
Butyrate improves the efficacy of MTX in gut microbiota-deficient mice. **(A)** Schematic of treatments. Mice were treated with antibiotics at the onset of arthritis. Mice received butyrate administration and MTX injection on day 0. **(B)** Clinical scores of CIA mice (n = 6). **(C)** The photos of hindlimb. **(D)** Micro-CT assessment of paws. **(E)** HE staining of joint tissues (magnification is 40×). Data are presented as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$. Abs, antibiotics; But, butyrate.

to the drinking water during MTX treatment, we observed that butyrate restored the therapeutic effect of MTX in gut microbiota-deficient CIA mice, to a similar level achieved with *B. fragilis* gavage. Our *in vivo* and *in vitro* experiments confirmed that butyrate promote the proliferation of M2 macrophage. Macrophage polarization is a complex process of multi-factor interaction, which is regulated by a variety of intracellular signaling molecules and their pathways, including JAK/STAT signaling pathway, PI3K/Akt signaling pathway (Vergadi et al., 2017). Butyrate has been reported to improve inflammation by regulating the signaling pathway PI3K/Akt via GPRs (Pirozzi et al., 2018). Taken together, regulating the host immunity through the alternation in butyrate metabolism is one of the potential ways that that *B. fragilis* plays a role in MTX therapy. Notably, there is no evidence that *B. fragilis* is a producer of butyrate. Considering microbiota transplantation may alter the intestinal microenvironment and nutrient competition, thereby altering the gut microbiota population (Gu et al., 2016), we speculated that *B. fragilis* supplementation resulted in structural changes in gut microbiota and promoted the proliferation of butyrate-produced species, suggesting that more attention should be paid to the dynamics of intestinal microbiome after microbiota transplantation.

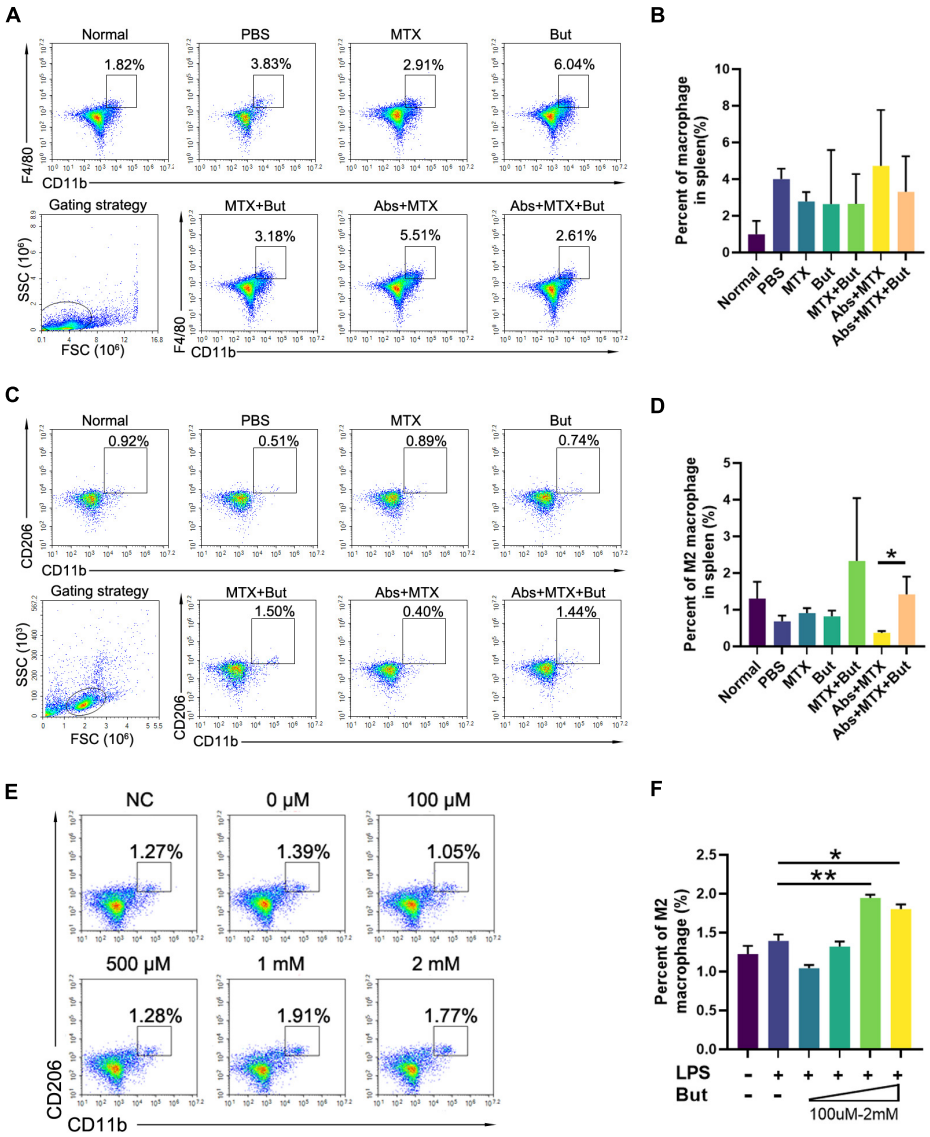

FIGURE 6
The impact of butyrate on macrophage. **(A–D)** FACS analysis of splenic cells. **(A,B)** Macrophages and C,D M2 macrophages (n = 3). **(E,F)** FACS analysis of macrophage *in vitro*. Cells isolated from the spleen were stimulated with lipopolysaccharide (LPS, 100 ng/ml) with or without butyrate (But, 100 μM–2 mM) for 24 h before FACS analysis. The alteration of **(E,F)** M2 macrophages (n = 3) were recorded. Data are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$. Abs, antibiotics; But, butyrate.

Although numerous studies have reported that metabolites of gut microbiota are associated with the progression of arthritis and suggest butyrate as a therapeutic strategy for arthritis, these studies have not specifically explored the relationship between gut microbiota produced-derived metabolites and drug therapy (Kim et al., 2018; Rosser et al., 2020; He et al., 2022; Martinsson et al., 2022). Based on the correlation between intestinal microbiota and drug toxicity, this study evaluated the influence of gut microbiota on drug efficacy, which is helpful to solve the problems of intestinal toxicity and unsatisfactory response of MTX. However, the comparison of 16S rRNA amplicon sequencing analysis results between the animal model and the patient showed that the changes of intestinal microbiota after MTX treatment were not completely similar, suggesting that the animal model could not completely represent the clinical influence of gut microbiota. We need to design reasonable clinical experiments to verify the role of *B. fragilis*. In addition, gut microbiota is a complex system that interacts in human to affect immune response. We have also observed changes in other bacteria in MTX treatment, which suggested other bacteria may also be involved in this effect except *B. fragilis*. Among these altered strains, *Escherichia coli* has been reported to suppress

the development of arthritis in germ-free rats (Kohashi et al., 1986), but its effect on the MTX is not clear. In this study, we found that E transplantation had no significant effect on the therapeutic efficacy of MTX (**Figures 2**, **3**). *Bacteroides uniformis* and *Prevotella copri* have also been shown to be associated with the development of arthritis (Miller et al., 2015; Seifert et al., 2022). The effect of these bacteria on MTX is also worth further investigation.

In summary, we found that MTX treatment was correlated to the abundances of *B. fragilis* in the gut of the RA patients. Next, we demonstrated that *B. fragilis* plays an essential role in the efficacy of MTX by regulating the metabolism of butyrate. These findings advocate for a potential microbial intervention strategy for improving the efficacy of MTX in RA management.

Data availability statement

The data presented in the study are deposited in the jianguoyun repository (https://www.jianguoyun.com/p/DZIBy9MQzYaeCxj66OoEIAA).

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Affiliated Hospital of Zunyi Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care Committee of Sichuan University.

Author contributions

LY contributed to study design. BLZ, CD, BYZ, KL, and HX contributed to data and figures collection. LZ contributed to writing. YT and RZ contributed to literature search. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by grants from the Sichuan Natural Science Foundation Project (No. 2022NSFSC1309), National Natural Science Foundation of China (No. 82203016), National Science and Technology Major Project (No. 2018ZX09733001-001-007 and No. 2018ZX09201018-013), and "Leading Academic of Ten-thousand Talents Program" and 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University. All the funding from these sources were used for data collection and analysis.

Acknowledgments

Thanks are due to Prof. Limei Yu for assistance with the collection of patient fecal samples.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 20 December 2022
DOI 10.3389/fimmu.2022.1072720

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

RECEIVED 17 October 2022
ACCEPTED 30 November 2022
PUBLISHED 20 December 2022

CITATION
Littlefield KM, Schneider JM, Neff CP, Soesanto V, Siebert JC, Nusbacher NM, Moreno-Huizar N, Cartwright IM, Armstrong AJS, Colgen SP, Lozupone CA and Palmer BE (2022) Elevated inflammatory fecal immune factors in men who have sex with men with HIV associate with microbiome composition and gut barrier function. *Front. Immunol.* 13:1072720. doi: 10.3389/fimmu.2022.1072720

Elevated inflammatory fecal immune factors in men who have sex with men with HIV associate with microbiome composition and gut barrier function

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Introduction: People living with HIV infection (PLWH) exhibit elevated levels of gastrointestinal inflammation. Potential causes of this inflammation include HIV infection and associated immune dysfunction, sexual behaviors among men who have sex with men (MSM) and gut microbiome composition.

Methods: To better understand the etiology of gastrointestinal inflammation we examined levels of 28 fecal soluble immune factors (sIFs) and the fecal microbiome in well-defined cohorts of HIV seronegative MSM (MSM-SN), MSM with untreated HIV infection (MSM-HIV) and MSM with HIV on anti-retroviral treatment (MSMART). Additionally, fecal solutes from these participants were used to stimulate T-84 colonic epithelial cells to assess barrier function.

Results: Both MSM cohorts with HIV had elevated levels of fecal calprotectin, a clinically relevant marker of GI inflammation, and nine inflammatory fecal sIFs (GM-CSF, ICAM-1, IL-1β, IL-12/23, IL-15, IL-16, TNF-β, VCAM-1, and VEGF). Interestingly, four sIFs (GM-CSF, ICAM-1, IL-7 and IL-12/23) were significantly elevated in MSM-SN compared to seronegative male non-MSM. Conversely, IL-22 and IL-13, cytokines beneficial to gut health, were decreased in all MSM with HIV and MSM-SN respectively. Importantly, all of these sIFs significantly correlated with calprotectin, suggesting they play a role in GI inflammation. Principal coordinate analysis revealed clustering of fecal sIFs by MSM status and significant associations with microbiome composition. Additionally, fecal solutes from participants in the MSM-HIV cohort significantly decreased colonic transcellular fluid transport *in vitro*, compared to non-MSM-SN, and this decrease associated with overall sIF composition and increased

concentrations of eight inflammatory sIFs in participants with HIV. Lastly, elevated levels of plasma, sCD14 and sCD163, directly correlated with decreased transcellular transport and microbiome composition respectively, indicating that sIFs and the gut microbiome are associated with, and potentially contribute to, bacterial translocation.

Conclusion: Taken together, these data demonstrate that inflammatory sIFs are elevated in MSM, regardless of HIV infection status, and are associated with the gut microbiome and intestinal barrier function.

KEYWORDS

human immunodeficiency virus (HIV), inflammation, men who have sex with men (MSM), gut microbiome, immune factors, cytokines

1 Introduction

Pathogenesis of HIV infection is closely tied with the gastrointestinal (GI) tract because it is a major site of HIV replication. Large numbers of activated CCR5-expressing $CD4^+$ T cells, which are specifically targeted by HIV, reside in the gastrointestinal tract (1). These cells fuel HIV infection in the gut, resulting in profound depletion of T cells in the lamina propria and chronic inflammation (2). Intestinal inflammation can promote the breakdown of the epithelial barrier and bacterial translocation, which in turn leads to systemic immune activation/inflammation (3) that contributes to HIV pathogenesis and disease progression (4). Furthermore, while gut inflammation and impaired barrier function improve with ART, these GI issues persist (5), and have been linked with metabolic (6), and other co-morbidities (7) in people living with HIV (PLWH) on ART. Microbiome differences, such as lower alpha diversity in untreated individuals with low $CD4^+$ T cell counts (8) or ART-treated individuals with low NADIR (9), have been observed in PLWH (10). In addition, bacteria from fecal material of HIV-positive individuals has been shown to induce higher immune activation *in vitro* (11). Furthermore, others have shown that compositional shifts in the fecal microbiome associated with HIV correlated with changes in metabolic function and production of cytokines detectable in plasma samples (12, 13) and mucosal biopsies (14).

In the United States men who have sex with men (MSM) comprise over 60% of new cases of HIV infection annually (15). We and others have shown gut microbiome composition in MSM regardless of HIV infection is highly altered compared to seronegative non-MSM (10, 16), and is characterized by high relative abundance of the bacterial genus *Prevotella* and low *Bacteroides*, as well as many additional differentiating taxa (16, 17). Intestinal microbiome composition has also been associated with the risk of HIV acquisition in MSM (14). Whole fecal bacterial communities isolated from the stools of MSM with and without HIV induce immune activation and increase HIV infection of lamina propria mononuclear cells *in vitro* (11). Additionally, gavage of fecal bacteria from these MSM cohorts into gnotobiotic mice leads to elevated levels of intestinal immune activation compared to non-MSM controls (18). This has been linked in part through particular enriched bacteria in MSM such as *Holdemanella* (19). Because of the importance of gut immune activation for HIV pathogenesis, transmission among MSM, and co-morbidity, in-depth profiling of inflammation in the gut is essential for understanding these processes.

Measurement of soluble immune factors (sIF) present in feces, including chemokines, cytokines, growth factors and other signaling factors, is an attractive method for studying gut immune activation since collection of fecal material is relatively non-invasive. Although such methods have been used to assess GI inflammation in the context of GI diseases (20–23), how they differ with HIV infection, treatment, and MSM status has not been explored nor has whether their levels associate with intestinal microbiome composition or effect barrier function. Here we assessed well-vetted measures of intestinal inflammation, fecal sIFs, microbiome composition and markers of bacterial translocation to characterize and gain mechanistic insights into the relationship between intestinal inflammation and barrier function in HIV infection and in MSM, using stool samples collected from HIV-seronegative MSM (MSM-SN), MSM with HIV infection with (MSM-ART) and without ART treatment (MSM-HIV) and male non-MSM-SN participants. Taken together, our data demonstrate that inflammatory sIFs are elevated in MSM and with HIV infection, associate with gut microbiome composition, and negatively influence intestinal barrier function in HIV.

2 Materials and methods

2.1 Study participants

Participants from the Denver metropolitan area were recruited under study protocol #14-1595 approved by the Colorado Multiple Institutional Review Board (CoMIRB). All participants provided written consent prior to collection of data and samples and were separated into cohorts based on sex, HIV infection and current use of ART, and sexual behavior. All participants on ART underwent treatment for at least 12 consecutive months using a minimum of three separate ART medications prior to study entry and displayed at least six consecutive months of viral suppression. Participants not undergoing ART were either never treated or off treatment for six consecutive months prior to study participation. Use of antibiotics within three months of sample collection, diagnosis with an active gastrointestinal disease, opportunistic/chronic infection or malignancy, and/or prescription of anticoagulant or hypoglycemic medications were exclusionary for this study. The four cohorts of male participants were MSM with HIV, not currently using ART (MSM-HIV: n=15), HIV-positive MSM undergoing ART (MSM-ART: n=13) and HIV-seronegative men who either had sex with men (MSM-SN: n=17) or who did not (non-MSM-SN: n=14). A cohort of female participants with HIV and on ART treatment (F-HIV: n=5) were also included and compared to a matched cohort of women with no history of HIV infection (F-SN: n=5). Analysis of the female cohorts was limited due to the small cohort size. All cohort demographics data are included in Table 1.

2.2 Collection of fecal samples and surveys

Participants collected full stool samples in a commode specimen collector prior to their clinic visit, which were shipped or transported within 48 hours either frozen or cool. Upon delivery, samples were transferred to long-term storage at -80°C. During research visits, participants completed a GI-symptoms questionnaire based on the GSRS (24) comprised of 14 multiple choice questions covering common GI issues experienced within the prior year and 24 hours before stool sample collection, including diarrhea, constipation, bloating, flatulence, vomiting and abdominal pain. Multiple choice answers ranged from "1 – little to no symptoms" to "4 – debilitating symptoms". An aggregate GI Symptoms Score was calculated as the average value across all 14 questions. Participants were also asked to evaluate the consistency of their stool sample at the time of collection by utilizing the visual Bristol stool scale (25). Each participant assigned a subjective score (1-7) that most closely resembled their fecal sample.

2.3 Fecal solute preparation and sIF quantification

Fecal solute preparations were adapted from previously described methods with significant alterations (21, 22). Specifically, an aliquot of two grams of frozen feces was obtained and mixed with 8 mL of saline solution (DPBS, Protease Inhibitor, EDTA, DNase). Samples were homogenized for 1 min and placed on ice for 30 min. Samples were then ultra-centrifuged at 12,000 rpm for 45 min at 5°

TABLE 1 Participant cohort demographics and characteristics.

	Male				Female	
	Non-MSM-SN	MSM-SN	MSM-ART	MSM-HIV	F-SN	F-HIV
N	14	17	13	15	5	5
Race (BA/A/W/O)[a]	0/1/13/0	1/0/17/0	2/0/12/0	2/0/13/0	0/0/4/1	0/0/5/0
Ethnicity (H/NH)[b]	2/12	1/17	0/13	2/13	1/4	2/3
Median age (years)	32 (22-70)	35.5 (27-50)	56[c] (44-65)	36 (23-55)	29 (24-61)	54 (30-63)
Median HIV-1 viral load (RNA copies/mL)	NA	NA	0 (0-20)	62800[d] (159-5.2e5)	NA	20 (0-20)
Median $CD4^+$ T cell count (cells/μL)	NA	NA	648 (177-1114)	577 (201-939)	NA	639 (187-1417)
Median fecal BCA (ug/mL)	881.7	829.8	700.1	763.8	868.6	1025.8

Kruskal-Wallis tests were performed for all demographic characteristics, corrected for multiple comparisons and significant differences are indicated. Units are provided in parentheses in the first column and all information in parentheses in subsequent columns are data ranges.
[a]BA, Black/African American; A, Asian; W, White; O, Other.
[b]H, Hispanic; NH, non-Hispanic.
[c]The median age of MSM-ART is significantly higher compared to all cohorts excluding F-HIV. (Non-MSM-SN: P = 0.0017; MSM-SN: P = 0.0060; MSM-HIV: P=0.0051; F-SN: P=0.020).
[d]P < 0.0001 in comparison to MSM-ART and P=0.0056 in comparison to F-HIV. NA, Not Applicable.

C, and the supernatant was passed through a 0.2 µm filter, aliquoted and stored at -80°C until testing. Total protein levels were assessed using a Bicinchoninic acid (BCA) assay. Standard sandwich ELISAs were used to measure fecal calprotectin (Epitope Diagnostics, San Diego, CA), IL-22 (eBioscience/Thermofisher, Waltham, MA), sCD14 (Hyclone, Uden, Netherlands) and sIgA (BioVendor, Brno, Czech Republic), while multi-plex ELISAs (MesoScale Diagnostics, Rockville, MD) were used to measure multiple analytes simultaneously each following manufacturer's protocols. The following V-Plex MSD (MesoScale Diagnostics, Rockville, MD) kits were utilized: Proinflammatory Panel 1 (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α), Vascular Injury Panel 2 (SAA, CRP, VCAM-1, ICAM-1), and Cytokine Panel 1 (GM-CSF, IL-1α, IL-5, IL-7, IL-12/23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A).

2.4 DNA extraction and sequencing

DNA was extracted from the same fecal samples used in the ELISAs using the standard Power Soil Kit protocol (Qiagen). Extracted bacterial DNA was PCR amplified with barcoded primers targeting the V4 region of 16S rRNA according to the Earth Microbiome Project (EMP) standard protocols (http://www.earthmicrobiome.org). Each PCR product was quantified using PicoGreen (Invitrogen, Carlsbad, CA), and equal amounts of DNA from each sample were pooled and cleaned using the UltraClean PCR Clean-Up Kit (MoBio, Carlsbad, CA). Sequences were generated on three runs using a MiSeq personal sequencer (Illumina, San Diego, CA).

2.5 Sequence data analysis

Raw sequences were quality filtered and assigned to samples based on their barcodes using the default parameters of QIIME version 1.5.0 (26). Sequences were assigned to 97% identity operational taxonomical units (OTU)s by comparing them to a nonredundant reference database of near-full length sequences (Greengenes database) (27), and unassigned sequences were clustered into *de novo* OTUs using UCLUST (28). Since samples contained between 4,694 and 72,828 sequences, analyses were standardized at 4,600 sequences per sample to avoid biases. UniFrac (29) PCoA analyses were conducted using QIIME. Bacterial families and genera in each sample were determined using the RDP classifier retrained on the Greengenes taxonomy (30).

2.6 Fecal sIF and microbiome PCoA analysis

Fecal sIF data was normalized by dividing each individual value by the mean value of that sIF across all samples. All data were entered into a feature table with 17 features to perform analysis using QIIME2 (31). Distances between fecal samples based on their sIF levels were calculated using Canberra distances. PCoA ordination and biplot functionality in QIIME2 visually integrate the sample feature metadata. Microbiome beta diversity was calculated using unweighted UniFrac and plotted by PCoA. A one-sided Mantel test with Pearson correlation was performed to test for a correlation between the two distance matrices from the microbiome data (unweighted UniFrac) and the immune data (Canberra).

2.7 Fecal solute stimulation of intestinal epithelial cells

Human intestinal epithelial T84 cells were grown and maintained in DMEM nutrient mixture F-12 ham (DMEM F-12) media (Gibco, Grand Island, NY) as previously described (32). Cells were plated on permeable transwell inserts (Costar, Cambridge, MA) and grown to confluency and high resistance (>1,000 Ω•cm2). Agonist-stimulated short circuit currents (Isc) were measured in Hank's balanced salt solution (Sigma-Aldrich) on the apical side using an EVOM2 voltohmmeter (World Precision Instruments, Sarasota, FL). Measurements were taken before fecal solutes were added, 30 minutes post-addition of fecal solutes, and then once an hour for four hours. Cl- secretory responses are expressed as a change in short circuit current (ΔIsc) as previously described (33).

2.8 Statistical analysis

Statistical analyses comparing differences between cohorts and correlations for clinical measures, sIF levels, ΔIsc and PC values were performed using GraphPad Prism Version 7 (GraphPad, San Diego, CA). These consist of Kruskal-Wallis with Dunn's corrections and rank Spearman correlation analyses corrected for multiple comparisons with the false discovery rate (FDR) method of Benjamini and Hochberg (34) were used to determine significance of differences between cohorts. Any correlations where $P < 0.05$ after FDR correction was considered statistically significant.

3 Results

3.1 Gastrointestinal symptoms and inflammation are increased in MSM with and without HIV infection

There was no significant difference in the age between cohorts except for the MSM-ART cohort, which was significantly older (Table 1). There was also no significant

difference in the median $CD4^+$ T cell count between participants with HIV with or without ART indicating those in the MSM-HIV cohort were fairly healthy (Table 1). To ensure that stool consistency was not responsible for differences in sIFs, we also measured the total protein concentration of the fecal solutes (total BCA) and no significant difference between cohorts was noted (Table 1).

To evaluate differences in overall GI discomfort between our cohorts the gastrointestinal symptom rating survey (GSRS) (24) was completed by all study participants, and aggregate scores compared. Both MSM-SN and MSM-HIV reported a statistically significant increased GI symptom frequency and severity compared to non-MSM-SN (P=0.031; P=0.029, respectively) (Figure 1A). After providing a stool sample participants were asked to rate the sample's consistency using the Bristol stool scale (25), and MSM-ART participants reported more watery/loose stool consistency than non-MSM-SN participants (P=0.039) (Figure 1B). We then measured fecal calprotectin, a quantitative clinical marker of GI inflammation. Only MSM-HIV participants had statistically elevated levels of calprotectin in their fecal samples compared to non-MSM-SN (P=0.014) (Figure 1C). However, all MSM cohorts had a higher proportion of participants with calprotectin levels over 50 µg/g than the non-MSM-SN cohort (non-MSM-SN: 21.4%, MSM-SN: 44.4%, MSM-ART: 64.3%, MSM-HIV: 66.7%), which can indicate potential GI inflammatory disease (35). Several MSM participants had fecal calprotectin levels greater than 200 µg/g which is strongly associated with GI inflammatory disease (35) (16.7%, 14.3%, and 33.3% of MSM-SN, MSM-ART, and MSM-HIV respectively) while none in the non-MSM-SN cohort had comparable levels. All three of these measures were also examined in F-SN and F-HIV and while the aggregate GI symptom score was significantly higher for F-HIV compared to F-SN (P=0.047) there was no difference in stool consistency (Supplementary Figure 1A). Fecal calprotectin showed a similar pattern as for HIV-positive men but was a smaller cohort and did not reach statistical significance (Supplementary Figure 1A). Based on both reported symptoms and fecal calprotectin we show a more inflammatory GI environment in HIV-positive individuals and in MSM compared to non-MSM and HIV-SN, which prompted further exploration into the specific characteristics of this inflammation.

3.2 Distinct inflammatory MSM fecal sIF profile is exacerbated with HIV infection

Twenty-seven sIFs, excluding calprotectin, were measured from fecal samples using both multiplex and standard ELISAs. Seventeen of these were measurable within the standard ranges of each assay for more than 75% of all samples tested. Ten markers (IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12p70, SSA, IL-5, IL-17A, TNF-α) where less than 75% of participants had detectable levels in their fecal solute were excluded. Of those seventeen, twelve sIFs showed significant differences across cohorts and values for all participants (Figure 2). The five sIFs that were

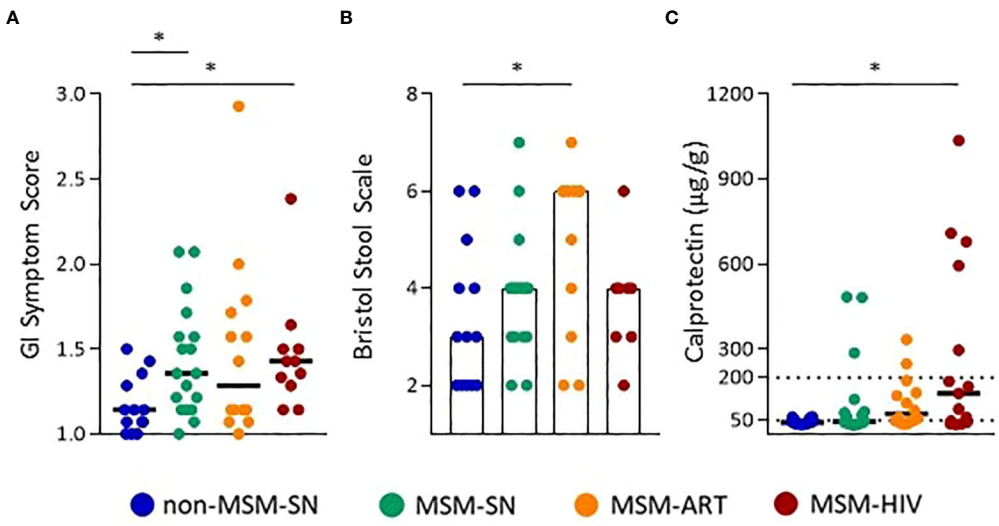

FIGURE 1
Increased GI symptoms and inflammation in MSM with and without HIV infection. **(A)** GI Symptom Scores, and **(B)** Bristol Stool Scale Scores calculated from survey responses. **(C)** Calprotectin levels (µg/g) determined by ELISA. Each point represents data from one participant and are colored based on cohort: non-MSM-SN (dark blue), MSM-SN (green), MSM-ART (orange) and MSM-HIV (red). Black lines/hollow bars represent the median of each cohort. The dotted line at 200 µg/g represents the cutoff for clinically significant fecal calprotectin. Kruskal-Wallis tests were used to determine statistical significance with Dunn's multiple comparisons test where * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

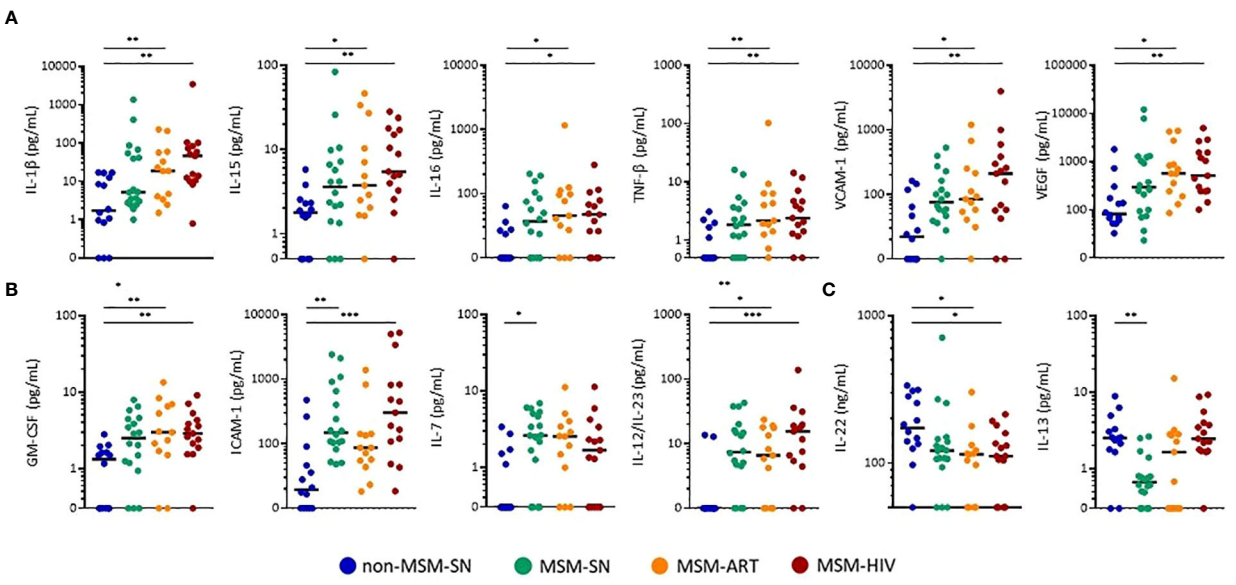

FIGURE 2
Concentrations of fecal sIFs are altered in MSM compared to non-MSM. Levels of **(A)** IL-1β, IL-15, IL-16, TNF-β, VCAM-1, VEGF (pg/mL), **(B)** GM-CSF, ICAM-1, IL-7, IL-12/23 (pg/mL), **(C)** IL-13 (pg/mL), and IL-22 (ng/mL) comparing MSM-SN, MSM-ART, and MSM-HIV cohorts to non-MSM-SN. Each point represents data from one participant and are colored based on cohort: non-MSM-SN (dark blue), MSM-SN (green), MSM-ART (orange) and MSM-HIV (red). Black lines represent the median of each cohort. Kruskal-Wallis tests were used to determine statistical significance with Dunn's multiple comparisons test where *p < 0.05, **p < 0.01, and ***p < 0.001.

detectable but did not have significantly different fecal levels between cohorts were CRP, IL-1α, IL-8, sCD14 and sIgA. Nine (GM-CSF, ICAM-1, IL-1β, IL-12/23, IL-15, IL-16, TNF-β, VCAM-1, and VEGF) were elevated in either cohort of participants with HIV compared to non-MSM-SN (Figures 2A, B). The most striking example of fecal sIF elevation for PLWH was seen in IL-1β, where there were 27-fold (MSM-HIV) and 10.9-fold (MSM-ART) increases compared to the non MSM-SN cohort. Many sIFs were highest in MSM-HIV, though still significantly elevated in MSM-ART with the exception of ICAM-1 which was not significantly higher compared to non-MSM-SN. Interestingly, three sIFs (GM-CSF, ICAM-1 and IL-12/23) were also significantly higher in MSM-SN compared to non-MSM-SN and IL-7 was not elevated in either HIV cohort (Figure 2B). We also compared non-MSM-SN to MSM-SN using a Mann-Whitney T test. In this analysis, seven (GM-CSF, ICAM-1, IL-7, IL-12/23, IL-16, TNF-β and VCAM-1) of the sIFs were significantly elevated while one (IL-13) was lower in HIV-negative MSM compared to HIV-negative non-MSM (Supplementary Table 1). In contrast, IL-22 levels were lower across MSM cohorts compared to non-MSM, most significantly in participants with HIV, and IL-13 was lower in MSM-SN alone (Figure 2C). While fecal CRP, IL-1α, IL-8, sCD14 and sIgA levels were detectable for the majority of participants, there were no significant differences between cohorts (data not shown). A separate comparison of only MSM cohorts was done where both MSM-HIV and MSM-ART were compared to MSM-SN and the only significant difference was elevated IL-13 in MSM-HIV (Supplementary Table 2). There were no other significant differences between the MSM cohorts. Taken together, our data show significant differences in sIF levels for MSM compared to non-MSM that is present regardless of HIV-infection, and further elevated in participants with HIV.

Levels of fecal sIFs were also examined in the cohorts of female participants. Because we were unable to recruit enough male non-MSM with HIV, we examined a small cohort of females with HIV as their microbiome is similar. Significant increases were observed in F-HIV compared to F-SN for IL-15 (p=0.016), IL-16 (p=0.0079), TNF-β (p=0.0079), GM-CSF (p=0.0079), and IL-12/23 (p=0.0079) (Supplementary Figure 1B). Most of these trends align with the observations of fecal sIFs in the male cohorts; however, for IL-1β, ICAM-1 and IL-22 there was no significance between F-HIV and F-SN. Due to the small size of the female cohorts and known gut microbiome differences of MSM (17) the remainder of analyses focused on male participants.

3.3 Fecal sIF frequencies correlate with fecal calprotectin

Fecal calprotectin is a standardly used clinical marker for diagnosis and monitoring of inflammatory gut diseases, so we

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examined associations between fecal calprotectin and sIF levels to better understand their role in GI inflammation. Statistically significant correlations were found between fecal calprotectin levels and IL-1β, IL-8, IL-15, IL-16, GM-CSF, TNF-β, VEGF-A and VCAM-1 (Table 2). The most significant of these correlations was between calprotectin and IL-1β (P=0.0005, r=0.51), an inflammatory cytokine associated with GI disease (36) (Table 2). In fact, most of the fecal sIFs associated with calprotectin have inflammatory properties (IL-1β, GM-CSF, TNF-β, IL-8, IL-16, and VEGF-A) or are involved in trafficking of leukocytes into tissue (VCAM-1). While the significant correlations between calprotectin and inflammatory sIFs indicate these markers are relevant to overall gut inflammation, it is notable that the two cytokines that were decreased in one or all MSM cohorts, IL-13 and IL-22, did not correlate with calprotectin levels. Additionally, none of the fecal sIFs correlated with the aggregate GSRS or Bristol stool scores. The associations between fecal sIF concentrations and fecal calprotectin indicate sIFs contribute to clinically significant GI inflammation.

3.4 Fecal sIF profile is related to microbiome compositional differences

Next, we compared the overall fecal sIF profile to microbiome composition. The evaluation of global differences in sIFs was made by performing a principal coordinate analysis (PCoA) of Canberra distances calculated from sIF profiles after values were normalized (Figure 3A). Non-MSM-SN individuals clustered separately from MSM cohorts across principle coordinate 1 (PC1), which was separated by elevated IL-22 and sIgA for non-MSM-SN and higher levels of ICAM-1, VCAM-1, IL-1β, TNF-β, and GM-CSF for MSM (Figure 3A). Clustering of MSM-ART and MSM-HIV cohorts largely overlapped and were both distinct from non-MSM-SN, while the MSM-SN cohort was more diffuse. We and others have reported that the enteric microbiome of MSM with and without HIV infection is distinctly different than that of non-MSM, in part due to an increase in *Prevotella* and decrease in *Bacteroides* (17). To relate the microbiome composition to fecal cytokine profiles, we generated 16S rRNA sequence data from the same fecal samples. As shown previously (17), MSM microbiome compositions did not clearly cluster by HIV or ART status using PCoA (Figure 3B). Differences in between MSM and non-MSM were again associated with relatively Prevotella rich and Bacteroides poor microbiome composition in the MSM. Comparison of the pairwise distance matrices of the fecal sIF data and unweighted UniFrac values with a mantel test showed a significant relationship (P=0.029) indicating that the microbiome differences explained some of the variation in sIF profiles across fecal samples. We also looked at the abundances of *Prevotella and Bacteroides* individually and found patterns similar to our previous study (17) (Supplementary Figures 2A–C). When these abundances were correlated with fecal sIF concentrations *Prevotella* did positively associate with IL-1β levels (P=0.038, r=0.48) and negatively with IL-22 (P=0.004, r=-0.52) for all participants, but there were no correlations within MSM cohorts (Supplementary Figure 2D).

TABLE 2 Fecal calprotectin correlates with fecal sIF levels.

Correlate	R-value	P-value
IL-1β	0.5111	0.0005
VEGF	0.4692	0.0017
IL-15	0.4502	0.0017
VCAM-1	0.4384	0.0021
IL-16	0.4258	0.0024
GM-CSF	0.4156	0.0028
IL-8	0.4153	0.0024
TNF-β	0.4127	0.0023
IL-12/23	0.3662	0.0075
IL-7	0.3598	0.008
ICAM-1	0.3365	0.013
IL-1α	0.313	0.021

Significant correlations between fecal sIF concentrations and fecal calprotectin. Test results for ranked Spearman correlations with an FDR<0.05 are shown, followed by the corrected p-value.

3.5 Inflammatory sIF composition of MSM with HIV associates with decreased intestinal barrier integrity in colonic epithelial cells

To assess the effects of sIFs on the colonic epithelial barrier we added fecal solutes to the apical portion of confluent T-84 gut epithelial cells at resistance (1000 Ω•cm2). We measured short circuit current (Isc), a measure of apical fluid transport (37), over a 24-hour period of time and found the peak change from the initial Isc occurred at 4 hours (ΔIsc). We found fecal solute from MSM-HIV induced a significantly lower ΔIsc compared to non-MSM-SN (p = 0.033) and MSM-ART also trended lower (Figure 4A). None of the fecal solutes from non-MSM-SN had a negative ΔIsc whereas solutes from 18% and 31% of the MSM-ART and MSM-HIV cohorts respectively decreased Isc compared to the initial reading. We then compared PC1 values of the sIF composition PCoA to ΔIsc using a stratified analysis and found significant associations between PC1 and ΔIsc for all participants (P=0.0003, r=-0.50), participants with HIV (P=0.003, r=-0.59), and MSM-HIV (P=0.011, r=-0.69)

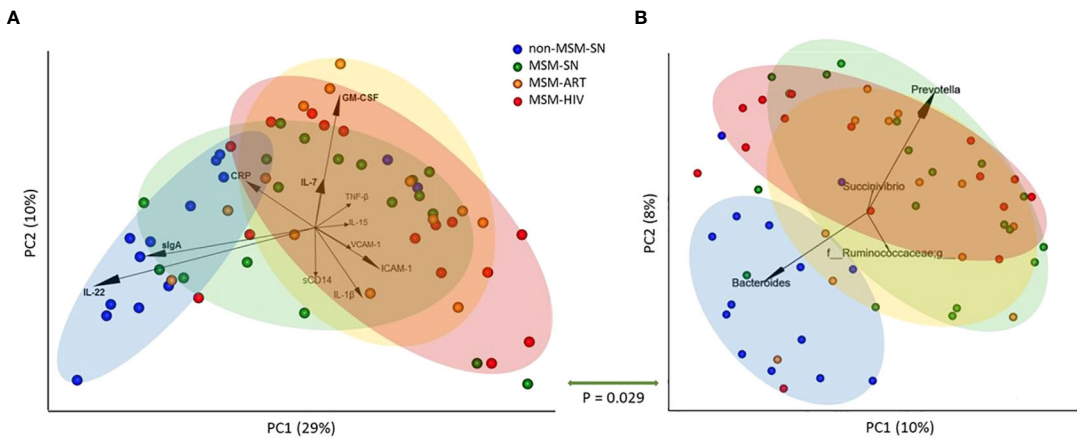

FIGURE 3
Principal coordinate analyses of fecal sIF and microbiome composition segregate by MSM status. **(A)** PCoA plot of fecal sIF composition after normalization across samples. Each point represents one participant's sIF composition where the distance between points is representative of relative similarity – two points closer together are more similar than two further apart. Labeled arrows of the 11 most influential sIFs on this PCoA's distribution are included with corresponding directionality and impact as shown by the arrow's length. **(B)** Unweighted UniFrac PCoA plot of fecal microbiome composition where each point represents one individual's overall microbiome composition. Labeled arrows of the 4 most influential taxa on this PCoA's distribution are included with corresponding directionality and impact as shown by the arrow's length. Points are colored based on cohort: non-MSM-SN (dark blue), MSM-SN (green), MSM-ART (orange) and MSM-HIV (red). Each colored oval encircles the majority of participants in the cohort with corresponding color. The size and location were determined manually and <20% of each cohort falls outside the oval. The P value below the double-sided green arrow between plots is the result of a Mantel test that shows a significant positive correlation between these two matrices.

while there was no correlations in other stratifications by HIV infection status or by cohort (Figures 4B–D). Positive PC1 values, categorized by increased concentration of inflammatory sIFs ICAM-1, VCAM-1, IL-15 IL-1β and TNF-β and decreased IL-22 and sIgA, were associated with decreased gut barrier integrity. There were no correlations found between ΔIsc and microbiome PC1 or levels of individual sIFs for all participants or when stratified by cohort. (Supplementary Data 1). We repeated this analysis stratified based on participants' HIV status and eight of the 12 sIF frequencies significantly correlated with ΔIsc (Figure 4E). The strongest associations among participants with HIV involve IL-15 (P=0.0072, r=-0.63), TNF-β (P=0.0096, r=-0.61) and VCAM-1 (P=0.0099, r=-0.59), and GM-CSF, IL-7, IL-12/23, IL-16 and VEGF-A also negatively associate with ΔIsc (Figure 4E). There were no significant associations among participants without HIV. These findings indicate the reduced apical fluid transport of the gut epithelial barrier is associated with elevated concentrations of inflammatory sIFs in participants with HIV.

3.6 Elevated systemic sCD14 in HIV is associated with fecal sIF induced intestinal barrier dysfunction

Lastly, to examine the associations between barrier function and systemic inflammation, we measured levels of inflammatory sIFs in plasma. Few significant differences between MSM and non-MSM cohorts in plasma sIFs were noted, and many sIF trends are antithetical to sIFs in the feces (Supplementary Table 3). Of note, IL-22 levels in plasma were increased in all MSM cohorts compared to non-MSM, while they were decreased in feces, and of twelve matched sIFs in blood and feces only three positively correlated (Supplementary Table 3). We also measured plasma sCD14 and sCD163 to assess the connections between sIF levels and bacterial translocation (38, 39). Plasma sCD14 was significantly elevated in MSM-HIV (P=0.0050, Median=1760 μg/mL) and trended higher in MSM-ART (P=0.074, Median=1726 μg/mL) compared to non-MSM-SN (Median=1398 μg/mL) (Figure 5A). There was no significant difference in plasma sCD14 between MSM-SN and non-MSM-SN (P>0.99, Median=1376 μg/mL) (Figure 5A). ΔIsc was found to negatively correlate with sCD14 (P=0.018, r=-0.38) for all participants (Supplementary Figure 3A) and the relationship was even stronger for participants living with HIV (P=0.021, r=-0.51) (Figure 5B). There were no significant associations when stratified by cohort or among HIV-seronegative participants. Additionally, there was a much weaker association between plasma sCD14 and fecal sIF PC1 (P=0.07, r=0.29) and no association with fecal microbiome PC1 (P=0.87, r=0.02) (data not shown). Of the 12 fecal sIFs tested there was one significant association between VEGF and plasma sCD14 (P=0.029, r=0.40) (Supplementary Table 4). Plasma sCD163 was also significantly elevated in MSM-HIV (P=0.0003, Median=1166 μg/mL)

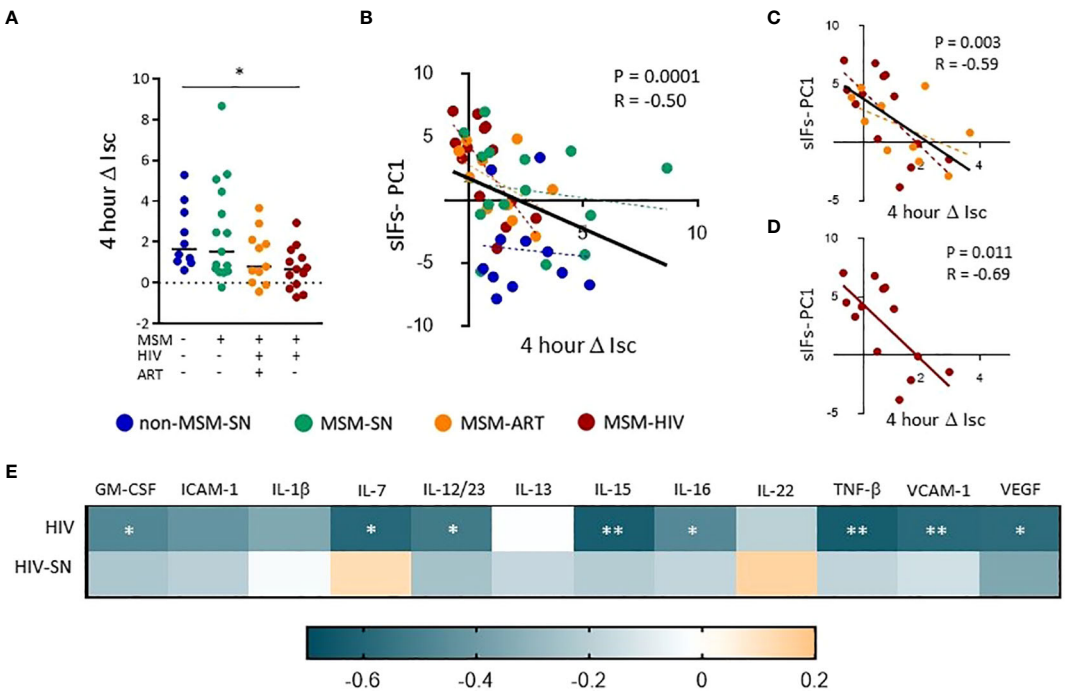

FIGURE 4
Fecal solutes from MSM with HIV infection increase transcellular gut epithelial permeability and associate with sIFs. **(A)** The change in short circuit current (ΔIsc) after 4 hours was calculated using Ohm's law. A Kruskal-Wallis test was performed to determine statistical significance where * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. Correlations between ΔIsc and sIF PC1 values from PCoA from figure 3 for all **(B)**, HIV **(C)**, and HIV-ART **(D)** participants. Each point represents data from one participant and are colored based on cohort: non-MSM-SN (dark blue), MSM-SN (green), MSM-ART (orange) and MSM-HIV (red). Each black line represents the linear regression for all included points, and P- and R-values are associated with this line. Each dotted line represents the linear regression for the cohort with the corresponding color. **(E)** A heat map showing associations between sIF concentrations and 4 hour ΔIsc. Teal indicates a negative R-value whereas orange represents a positive R-value. Rank order spearman correlations were run to determine statistical significance where * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

compared to non-MSM-SN (Median=675 μg/mL), but neither MSM-ART (Median=734 μg/mL) or MSM-SN (Median=794 μg/mL) were significantly higher (Figure 5C). While plasma sCD163 did not have significant associations with sIF composition, barrier function or microbiome composition for all participants (Supplementary Figure 3B), when stratified by HIV status participants with HIV show a significant correlation between sCD163 and gut microbiome composition (P=0.04, r=-0.41) (Figure 5D). There were no significant associations when stratified by cohort. Additionally, there were no significant correlations between individual fecal sIF levels and plasma sCD163 (data not shown). These findings connect systemic inflammation markers to both intestinal barrier function and microbiome composition, both of which are also associated with fecal sIF composition.

4 Discussion

HIV infection has long been associated with gastrointestinal disease. Acquired immune deficiency syndrome (AIDS) was initially classified as a chronic wasting disease because of the severe diarrhea and malabsorption seen in PLWH without ART treatment (40). In recent years, profound depletion of $CD4^+$ T cells in the intestine and increased bacterial translocation from the gut have been strongly associated with HIV disease progression (41, 42), and these findings have sparked renewed interest of the role of the gastrointestinal tract in HIV pathogenesis. One area of particular interest is the intersection between the gut microbiome and inflammation in HIV, in part due to the strong connections between microbiome composition, inflammatory gut diseases and various chronic conditions (43, 44). Our group and others have shown that HIV infection is associated with intestinal microbiome dysbiosis (45, 46) but interestingly, it has only been recently determined that sexual behavior contributes more significantly to alterations in microbiome composition in HIV-infected MSM than HIV infection itself (17). Understanding the interactions between the gut microbiome, inflammation and how these factors influence HIV pathogenesis and transmission following receptive anal intercourse (RAI) is of interest considering the known associations between vaginal dysbiosis and HIV

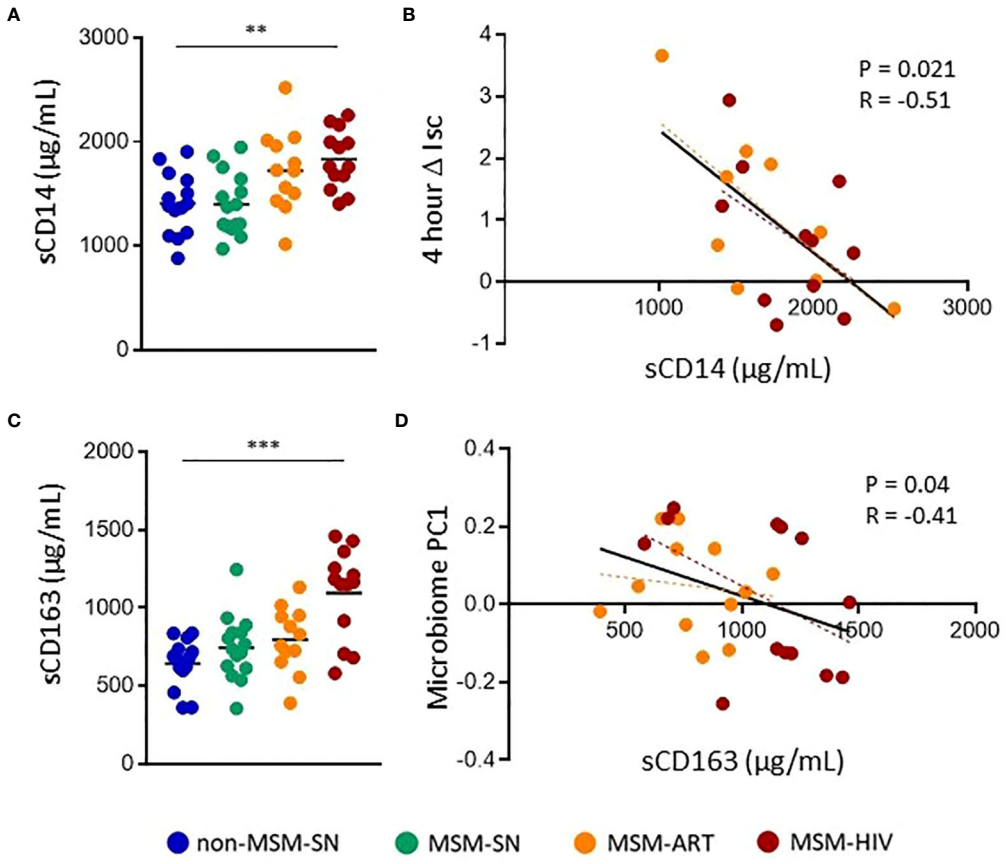

FIGURE 5
Elevated plasma markers of bacterial translocation in MSM-HIV correlate with increased gut epithelial permeability and microbiome composition. **(A)** Plasma sCD14 levels determined by ELISA and separated by cohort. **(B)** Correlations between Δlsc and plasma sCD14 for participants with HIV. **(C)** Plasma sCD163 levels determined by ELISA and separated by cohort. **(D)** Correlations between Microbiome PC1 and plasma sCD163 for participants with HIV. For **(A)** and **(C)**, Kruskal-Wallis tests were used to determine statistical significance between cohorts where * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. For **(B)** and **(D)** rank order spearman correlations were run where * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. Each point represents data from one participant and are colored based on cohort: non-MSM-SN (dark blue), MSM-SN (green), MSM-ART (orange) and MSM-HIV (red). Each black line represents the linear regression for all included points and reported P- and R-values are associated with this line. Each dotted line represents the linear regression for the cohort with the corresponding color.

transmission (47, 48). The relationship between fecal soluble immune factors (sIFs), many of which are important in gut inflammation (36, 49), and the gut microbiome and barrier function has not been previously examined in the context of HIV. Here we show that MSM-SN have higher levels of GI inflammation than non-MSM-SN, and that many of these sIF levels are further elevated in MSM with HIV infection. We also found there was a significant association between the inflammatory composition of fecal sIFs with microbiome composition and associations between individual gut microbiome species and sIF concentrations in a stratified cohort analysis. Lastly, we found both the overall composition of sIFs and increased levels of specific sIFs in MSM with HIV associated with decreased transcellular fluid transport, which in turn strongly associated with measures of bacterial translocation in the plasma of the participants.

We initially assessed gut health and inflammation in our study cohorts using standard clinically validated techniques; GI symptoms questionnaire (24), self-reported Bristol Stool scale (25) and fecal calprotectin (35). As expected, participants with untreated HIV infection reported more GI symptoms compared to non-MSM-SN; however, surprisingly we found that MSM-SN participants also reported more GI symptoms. This could be explained by the higher rate of sexually transmitted infections (STIs) among MSM than non-MSM populations (50), which previously were thought to be the primary cause of increased gut

inflammation in MSM. However, there were no significant correlations between sIF levels and either stool consistency or the average severity of reported GI symptoms. Additionally, the rates of STIs – including *Neisseria gonorrhoeae* and *Treponema pallidum* which cause gonorrhea and syphilis, respectively – have increased in recent years among MSM and may be influenced by increased use of pre-exposure prophylaxis for HIV and associated behavioral changes (51); however, these bacteria were not detected in our microbiome analysis and no viral or parasitic STIs were reported by participants. Another notable finding was that the stool from MSM-ART participants had a looser consistency compared to non-MSM-SN. This aligns with known associations between diarrhea and other GI symptoms with various ART regimens (52). We also measured the levels of fecal calprotectin which is directly related gut inflammation (53). HIV-ART participants were more likely to have elevated levels of gut inflammation than non-MSM-SN, marked by fecal calprotectin between 50μg/g and 200μg/g (35), and fecal calprotectin in ART-naïve participants was significantly higher compared to non-MSM-SN. In fact, many had levels typical of those observed with IBD which is similar to previous findings in HIV infection (54). We were surprised to see that 44% of MSM-SN had fecal calprotectin above 50 μg/g while only 21% of non-MSM-SN participants had comparable levels. Cumulatively, these findings further solidify the connection between MSM, regardless of HIV infection, and GI inflammation and prompted further exploration into how a broad range of fecal sIFs differ with both HIV infection and MSM status.

Analysis of stool sIFs has been previously used to study various inflammatory diseases in the gastrointestinal tract (20–23). These studies found that analysis of fecal sIFs provide a sensitive and noninvasive measurement of gastrointestinal inflammation and provide information on the intestinal environment during or after viral infection (55). Of the 17 sIFs that fell into range, eleven were elevated in MSM with HIV compared to seronegative non-MSM and many of these are associated with inflammatory GI diseases. Both MSM-HIV and MSM-ART had elevated levels of eight inflammatory sIFs (GM-CSF, IL-1β, IL-12/23, IL-15, IL-16, TNF-β, VCAM-1 and VEGF), while ICAM-1 was increased in MSM-HIV compared to non-MSM-SN. Of these, IL-1β, IL-12/23, IL-16 and TNF-β are all elevated in IBD and associated with its pathogenesis (36, 56, 57), IL-15 and GM-CSF are elevated in inflamed intestinal tissues (58, 59), and anti-VEGF therapies have been shown to reduce intestinal inflammation in models of IBD (60). Recently elevated IL-23 in the gut has also been associated with disease severity after infection with SARS-CoV-2, which has a high incidence of GI symptoms (55). Since these sIFs have strong connections to inflammatory diseases and viral infections, it is unsurprising to see elevated fecal levels in PLWH but levels of four sIFs (GM-CSF, ICAM-1, IL-7 and IL-12/23) were similarly elevated in MSM-SN compared to non-MSM-SN. Both GM-CSF and IL-12/23 are pro-inflammatory, while ICAM-1 and IL-7 modulate the trafficking of immune cells into tissues and proliferation of lymphocytes respectively, all functions that support epithelial barrier integrity (59, 61–63). The elevation of these sIFs in MSM-SN is reasonable following increased prevalence of mechanical injuries and pathogenic microbes in the rectum and colon, both of which can negatively affect gut barrier health. Additionally, IL-16, TNF-β and VCAM-1 were also significantly higher in MSM-SN when directly compared to non-MSM-SN indicating GI inflammation, while exacerbated by HIV infection, is elevated in seronegative MSM. Decreased IL-22 was observed across MSM cohorts, most significantly in participants with HIV. This may be functionally significant since IL-22 – produced in the gut by $CD4^+$ T helper subsets and innate lymphoid cells (ILCs) – is known to promote epithelial barrier integrity (64–66). This is particularly important in the context of HIV since IL-22 producing $CD4^+$ T cells are preferentially infected by HIV and both these T cells and ILCs are significantly depleted in the gut even following long-term ART (66–68). Surprisingly, of the twelve sIFs that were detectable in both the blood and feces of these participants only three – CRP, IL-16 and VCAM-1 – had significant direct correlations. Interestingly, while IL-22 was lower in both HIV cohorts compared to non-MSM-SN in feces, these levels were significantly elevated in all MSM cohorts in the blood. While twelve of the seventeen sIF levels positively associated with fecal calprotectin, there were five sIFs, including IL-22, that did not have significant associations. Taken together, this indicates that fecal sIFs measurement reveals immune processes that are specific to the gut, are a valid measurement of inflammation and more sensitive than fecal calprotectin alone. These data provide broader insight into intestinal inflammation than other non-invasive measures and allow for a deeper understanding of the different interactions of the gut microenvironment of MSM.

Evaluation of the differences of fecal sIF profile by PCoA showed clustering of MSM and non-MSM cohorts. This analysis indicated IL-22 levels contributed the most of any sIF to the clustering observed, and in addition to its contribution to epithelial barrier integrity, IL-22 can modulate gut microbiome composition (69, 70). Particularly commensal *Clostridia*, through the production of short-chain fatty acids (71), can increase IL-22 production in both *in vitro* and *in vivo* models (72). Based on the known differences in microbiome composition in MSM and non-MSM (17) and the microbiome's connection to IL-22, we hypothesized that the gut microbiome may contribute to elevated inflammatory sIFs. To assess this, we performed a mantel test to compare pairwise

distances between fecal sIF profiles and gut microbiome composition. However, the association was weak indicating that other factors that we did not measure, such as sexual practices (73, 74), may also contribute to the differences. We also found plasma sCD163 was elevated in MSM-HIV and associated with gut microbiome composition. A marker of macrophage activation and bacterial translocation (39), sCD163 has also been connected to mortality and viral replication in PLWH (75, 76). Recent studies evaluating the microbiome in MSM with HIV infection have also found both altered microbiome and elevated sCD163 in this population, but the cause of this association remains unclear (77). Interestingly, this relationship between sCD163 and microbiome composition is specific to PLWH, indicating the possibility of a mechanism specific to HIV infection. Based on these findings, further investigation of the relationship between the gut microbiome and sIFs in MSM and PLWH is warranted.

Systemic immune activation in HIV is caused in part by translocation of microbial products from the gut (78, 79). To better understand the effect of sIFs on gut barrier integrity we cultured T-84 cells with total fecal solutes and measured ΔIsc – a measure of apical fluid transport. We found that addition of fecal solutes from MSM-HIV to T-84 monolayers significantly lowered levels of apical fluid transport as compared to non-MSM-SN, while MSM-ART trended similarly. Increased apical fluid transport is associated both with improved mucosal hydration and decreased bacterial translocation (37), therefore fecal inflammatory sIFs could contribute to increased bacterial translocation and decreased barrier integrity associated with HIV (78). These data indicate there is a connection between fecal sIF composition, decreased intestinal barrier function and bacterial induced systemic inflammation. While the fecal solutes used also included bacterial metabolites that are thought to contribute to intestinal barrier function (80), we also found negative associations between apical fluid transport and IL-15, TNF-β, VCAM-1, VEGF, GM-CSF, IL-7, IL-12/23 and IL-13 in participants with HIV. Interestingly, no significant association between sIF concentration and resistance to intracellular transport was seen in participants without HIV, suggesting HIV-specific fecal sIF compositions are more disruptive to the gut barrier. Various cytokines in the intestinal lumen, including IL-12, negatively impact intestinal membrane permeability (81) and IL-15 is known to drive epithelial tissue destruction (82), cause inflammation downstream of the antiapoptotic signals it initiates (83) and direct intraepithelial lymphocyte motility in the gut (84). VEGF overexpression can be pathogen-induced and also contributes to barrier permeability (85). T helper subsets, which are preferentially depleted in the gut of PLWH (86), associate with worse outcomes from infections and are also associated with increased GM-CSF (87). Most importantly, we discovered a significant inverse association between our *in vitro* measure of gut permeability and plasma sCD14 (38), a marker of bacterial translocation levels, indicating that disruption of barrier function *in vitro* correlated with *in vivo* levels of bacterial translocation. As plasma sCD14 is associated with mortality in PLWH even with ART (88) and the mechanisms behind microbial translocation in HIV are not completely understood (89), further investigation into the interactions between sIFs and gut barrier function could reveal new therapeutic targets to decrease systemic inflammation and associated co-morbidities (2, 90) in PLWH. Taken together, these data outline the interactions between fecal sIFs, gut barrier function and bacterial translocation and connects our *in vitro* findings to direct *ex vivo* measurements.

Analysis of fecal sIF allows for a deeper understanding of the gut microenvironment not detectable by other low-risk, noninvasive methods. The relevance of fecal sIF analysis is confirmed by strong relationships with fecal calprotectin, while also illuminating unique mechanistic insights into gastrointestinal inflammation in HIV infection. Here we connect fecal sIFs to the overall composition of the gut microbiome and individual bacterial taxa abundance, barrier function and bacterial translocation. More work is needed to determine if microbiome dysbiosis is causal, or if inflammatory conditions allow for increased abundance of opportunistic bacteria. However, this study clearly shows elevated levels of multiple sIFs in the stool of MSM with and without HIV infection. Furthermore, we show relationships between plasma markers of bacterial translocation and fecal sIF and microbiome compositions further supporting the theory that gut dysbiosis contributes to chronic systemic inflammation in HIV.

Data availability statement

The microbiome data presented in the study are deposited in the European Nucleotide Archive repository, accession number PRJEB28485. Additional data may be requested through the University of Colorado Institutional Data Access Committee (contact Crao_Contracts@ucdenver.edu) for researchers who meet the criteria for access to confidential data.

Ethics statement

The studies involving human participants were reviewed and approved by Colorado Multiple Institution Review Board

(COMIRB# 14-1595). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JMS, SC, and BP designed research. JMS, CN, IC, NN, and NM-H performed research. KL, JMS, VS, JCS, AA, CL, and BP analyzed data. KL, JMS, CN, CL, and BP wrote the manuscript. All authors approved the final version of this manuscript to be published.

Funding

This study was funded by the National Institutes of Health (NIH) grants RO1 DK104047 (B.E.P and C.A.L) and RO1 DK108366 (B.E.P and C.A.L), with additional support from NIH/NCATS Colorado CTSA grant UL1 TR002535.

Acknowledgments

Authors acknowledge study participants for providing their time, blood and stool samples.

Conflict of interest

JCS was employed by CytoAnalytics.

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1072720/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 19 January 2023
DOI 10.3389/fimmu.2022.1045901

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

RECEIVED 16 September 2022
ACCEPTED 30 December 2022
PUBLISHED 19 January 2023

CITATION
Liu T, Ning Z, Liu P and Gao H (2023) Cassane diterpenoid ameliorates dextran sulfate sodium-induced experimental colitis by regulating gut microbiota and suppressing tryptophan metabolism. *Front. Immunol.* 13:1045901. doi: 10.3389/fimmu.2022.1045901

Cassane diterpenoid ameliorates dextran sulfate sodium-induced experimental colitis by regulating gut microbiota and suppressing tryptophan metabolism

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Ulcerative colitis (UC) is one form of inflammatory bowel disease (IBD), characterized by chronic relapsing intestinal inflammation. As increasing morbidity of UC and deficiency of conventional therapies, there is an urgent need for attractive treatment. Cassane diterpenoids, the characteristic chemical constituents of *Caesalpinia* genus plants, have been studied extensively owing to various and prominent biological activities. This study attempted to investigate the bioactivity of caesaldekarin e (CA), a cassane diterpenoid isolated from *C. bonduc* in our previous work, on dextran sulfate sodium (DSS)-induced experimental colitis and clarify the function mechanism. The results indicated that CA ameliorated mice colitis by relieving disease symptoms, suppressing inflammatory infiltration and maintaining intestinal barrier integrity. Furthermore, 16S rRNA gene sequencing analysis indicated that CA could improve the gut microbiota imbalance disrupted by DSS and especially restored abundance of *Lactobacillus*. In addition, untargeted metabolomics analysis suggested that CA regulated metabolism and particularly the tryptophan metabolism by inhibiting the upregulation of indoleamine 2,3-dioxygenase 1 (IDO-1). It also been proved in IFN-γ induced RAW264.7 cells. Overall, this study suggests that CA exhibits anti-UC effect through restoring gut microbiota and regulating tryptophan metabolism and has the potential to be a treatment option for UC.

KEYWORDS

colitis, caesaldekarin e, anti-inflammation, gut microbiota, metabolomics, tryptophan metabolism

1 Introduction

Inflammatory bowel disease (IBD), characterized by the chronic and relapsing inflammatory disorder of the gastrointestinal tract, generally begin in young adulthood and last throughout life (1). As the two main clinicopathological subtypes of IBD, ulcerative colitis (UC) and Crohn's disease (CD) show different inflammatory location and histological alterations in the intestinal wall. For the UC, the inflammation is limited to the colon with

fewer complications. While the CD involves the entire gastrointestinal tract, usually accompanied by strictures, abscesses, fistulas as well as other complications (2, 3). The etiology of IBD is complex, and interactions between genetic factor, the host immune system and gut microbiota are thought to underlie the development of IBD (2, 4). From birth to death, the human gastrointestinal tract is colonized by a vast and complex community of bacteria that approximately 10-fold of the total number of cells in the human body. Interactions between bacteria and their hosts can be viewed in terms of a continuum between symbiosis, commensalism and pathogenicity, and the relationship between gut microbiota and their hosts can shift from commensalism to pathogenicity in certain disease states (5). The development of novel analysis techniques, such as shut gun sequencing, metagenomics and next-generation sequencing, allow us to bypass the traditional culture-dependent bias and deepen and broaden our understanding of the composition, diversity, and roles of the gut microbiota in human health and diseases (6). IBD is associated with tremendous changes in the composition of gut microbiota, underlining the importance of the microbiota in disease etiology. Notably, dysbiosis and decreased complexity of the gut microbiota have been observed in CD and UC patients (7–9).

The current treatments for IBD are generally divided into two types: nonbiological therapy and biological therapy. Nonbiological therapies such as corticosteroids, immunomodulators and aminosalicylates, characterized by short half-life, low production cost and high patient's satisfaction from oral administration, have been used for a long time, which can improve clinical symptoms but do not change the overall disease course of IBD (10–12). In addition, these small molecule drugs pose various side effects such as hyperglycemia, hypothalamic-pituitary-adrenal axis suppression, opportunistic infections and osteoporosis for glucocorticoid, leukopenia, increased susceptibility to infection and hepatotoxicity for thiopurines, and headache, nausea and epigastric pain for sulfasalazine (13–15). Biologics are a group of molecules including monoclonal antibodies, recombinant cytokines and specific antagonists of receptors and cytokines that participate in regulating inflammation during immune-mediated process. They are attractive treatment options for patients who poorly or do not respond to small molecule drugs such as steroids or immunosuppressants, or patients suffering from serious adverse reactions of other IBD drugs (16). However, in addition to risks for side effects and patients failing to response, higher manufacturing and quality control costs of biologics impose a burden to economically disadvantaged patients and the healthcare system (17–19). Collectively, the development of safe, effective and economical therapeutics for IBD are urgently needed.

Cassane diterpenoids, the characteristic chemical constituents of medicinal plants of the *Caesalpinia* genus, have attracted considerable interest owing to their significant biological activities including antimalarial, anti-inflammatory, antimicrobial, antitumor, and antioxidant properties (20). Caesalpinin M2, a cassane furanoditerpenoid isolated from the seeds of *C. minax* in our previous work (21), exerted anti-inflammatory effect as a selective glucocorticoid receptor modulator by repressing NF-κB-dependent transcription without inducing glucocorticoid receptor transactivation, providing therapeutic potential in the treatment of inflammatory diseases (22). In this study, caesaldekarin e (CA), a cassane diterpenoid isolated from the seed kernels from *C. bonduc* in our previous work (23), was used to evaluate its anti-inflammatory activity and investigate its effect against DSS-induced experimental colitis.

2 Materials and methods

2.1 Preparation of chemical and reagents

CA was isolated from seed kernels of *Caesalpinia bonduc* in the laboratory and its purity (>95%) was confirmed by HPLC analysis in our previous study. Its structure was determined by a combination of ^{1}H and ^{13}C NMR spectra and comparison with reference. Dextran sulfate sodium (DSS, molecular weight 36-50 kDa) was purchased from Meilunbio (Dalian, China). Sulfasalazine (SASP) was purchased from Shanghai Xinyi Tianping Pharmaceutical Co. Ltd. Other chemicals, solvents and reagents were analytical grade.

2.2 Cell culture

Murine macrophage RAW264.7 were obtained from the Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) in 5% CO_2 at 37 °C.

2.3 Animals

Male BALB/c mice (18-22 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd (License no. SCXK (Liaoning) 2020-0001). Mice were housed under standard conditions (temperature 23 ± 2 °C and 12 h light/dark cycle) and fed with standard chow pellets and water *ad libitum*. Mice were acclimatized for one week prior to the experiments. The experimental procedure was performed according to the guidelines approved by the Institutional Animal Care and Use Committee of Shenyang Pharmaceutical University.

2.4 MTT assay

RAW264.7 cells were cultured in 96-well plates at a density of 1×10^4 overnight, which were subsequently treated with compound CA for 24 h. After removing the medium, MTT was added to the 96-well plate and cultured at 37 °C for 4 h to form formazan. Finally, the formazan was solubilized in DMSO, and the absorbance was detected at 490 nm.

2.5 NO generation assay

RAW264.7 cells were seeded in 96-well plates at a density of 2×10^4 and cultured overnight. Cells were pretreated with CA (3.125, 6.25, 12.5, 25 μM) as indicated concentrations for 2 h, then cells were treated with LPS (0.1 μg/mL) for another 24 h. The culture medium

was collected to determine the nitrite level using Griess reagent according to the instructions (Beyotime Biotechnology, Shanghai, China).

2.6 Induction and treatment of colitis

50 BALB/c mice were randomly divided into five groups: blank control group (Control), DSS-induced UC model group (DSS), CA-treated UC group by intraperitoneal injection (CAip), CA-treated UC group by intragastric administration (CAig) and SASP-treated UC group (SASP). Experimental colitis was induced by administration with distilled water containing 3% DSS (wt/vol) for 7 days, followed by distilled water for the next 2 days. Control group Mice were supplied with distilled water without DSS throughout the experiment. CAig and SASP groups were administered intragastrically with CA (20 mg/kg) or SASP (200 mg/kg) suspended in 0.5% CMC-Na water solution, while the mice in Control, DSS and CAip groups received same volume of 0.5% CMC-Na as vehicle from day 1 to 9. For the CAip group, CA (20 mg/kg, suspended in 0.2 mL saline) was provided daily through intraperitoneal injection for 9 days, and animals in Control, DSS, CAig and SASP groups received 0.2 mL normal saline *via* intraperitoneal injection for 9 days.

2.7 Sample collection

The whole blood sample were collected on the 10^{th} day from the retroorbital venous plexus under ether anesthesia. After standing for 1 h at room temperature, the blood was centrifuged at 3500 rpm for 10 min and the serum was transferred to another tube and stored at -80 °C for subsequent metabolites analysis. Next, Mice were sacrificed and the colon was collected to measure the length between the proximal rectum and the ileocecal junction. Then, colon samples were cut into fragments and about 1 cm of distal colon was used for histological analysis. Mouse feces in colon were transferred into sterile centrifuge tubes and stored at -80 °C after liquid nitrogen freezing. The remaining colon tissues were rinsed with normal saline and subsequently stored in a refrigerator at -80 °C for further analysis.

2.8 Assessment of disease activity index

The body weight, rectal bleeding and fecal consistency were monitored every day. The disease activity index (DAI) was determined from a combination of following parameters: a) body weight loss (0: none, 1: 1-5%, 2: 5-10%, 3: 10-20%, 4: >20%); b) diarrhea (0: normal, 1: soft but formed, 2: very soft, 3: half diarrhea, 4: diarrhea); c) hematochezia (0: none, 2: slight bleeding, 4: serious bleeding).

2.9 Histopathological assessment

The distal colon was cut and fixed in 4% paraformaldehyde, followed by paraffin embedding. Sections (5 μm thick) were stained with hematoxylin and eosin (H&E). The histological damage and inflammation were observed using Eclipse Ci-L microscope (Nikon, Japan).

2.10 Measurement of colonic myeloperoxidase activity and cytokines

The MPO activity of colon tissues were measured by a myeloperoxidase assay kit according to the manufacturer's instructions (Nanjing Jiancheng Biotechnology Company, Nanjing, China). Colon tissues were weighed and homogenized with normal saline on ice and then centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were collected for the measurements of IL-6, TNF-α and IL-1β using commercial ELISA kit (Servicebio, Wuhan, China). Inflammatory cytokines and markers including IL-6, TNF-α, IL-1β and serum amyloid A (SAA) in serum were also evaluated by ELISA kit (Lianke Biotech Co. Ltd., Hangzhou, China).

2.11 Quantitative real-time PCR analysis

Total RNA was extracted from colon tissues using Trizol reagent according to the manufacturer's instructions (Takara, Japan). Total RNA was reversely transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). Real-time PCR was carried out using TB Green Premix Ex Taq II (Takara, Japan) in CFX96 Real-Time PCR Detection System. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin expression. Primer sequences are shown in Table S1.

2.12 Western blot analysis

Colon tissues of the mice and cultured cells were lysed using RIPA buffer with protease inhibitor and incubated 30 min on ice. The homogenate was centrifuged at 12000 rpm for 10 min at 4 °C and the supernatant was collected. The protein concentration was determined with BCA protein assay kit (Beyotime Biotechnology, China). Total proteins (20 μg) for each sample were separated by SDS-PAGE and subsequently transferred to NC membranes. Membranes were blocked with 5% non-fat milk for 2 h at room temperature, then were incubated overnight at 4 °C with iNOS (1:2000), Occludin (1:2000), Claudin-1 (1:1000) and IDO-1 (1:5000) antibodies (Proteintech Group, Inc., China). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Proteintech Group, Inc., China) at room temperature for 1 h. Images were detected by chemiluminescence detection system (Amersham Imager 680, Sweden). The obtained chemiluminescence signals were analyzed with Image J software.

2.13 Fecal DNA extraction and Illumina Miseq sequencing

Fecal genomic DNA was extracted with kit according to the manufacture's instruction. Extracted DNA was used as template to amplify the V3-V4 region of bacterial 16S rRNA gene with the

primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT). After purifying the PCR products, high-throughput sequencing was performed on Illumina MiSeq PE250 system (Novogene, Beijing, China).

2.14 Processing of sequencing data and diversity analysis

The raw 16S rRNA gene sequencing reads were pieced and quality-filtered, followed by removing chimeric sequences to obtain the effective tags for subsequent analysis. Operational taxonomic units (OTUs) were clustered by UPARSE with 97% similarity. Alpha diversity was measured based on the observed OTU number and presented with Chao, Shannon and Simpson indices. Beta diversity was determined by principal coordinate analysis (PCoA) based on the distance matrix. Linear discriminant analysis (LDA) effect size (LEfSe) method was used to identify significantly different biomarkers between groups (LDA score threshold of 4).

2.15 UPLC-MS Global profiling of serum metabolites

The refrigerated serum samples were thawed thoroughly before proceeding. 100 µL of each serum sample from Control, DSS, CAip, CAig and SASP groups was mixed with 400 µL of methanol. After vortex mixing, the samples were incubated for 5 min on ice and centrifuged at 15000 g, 4 °C for 20 min. 400 µL of supernatant was transferred into another clean Eppendorf tube and diluted with LC-MS grade water to contain 53% methanol. The samples were subsequently centrifuged at 15000 g, 4 °C for 20 min, and the supernatant was applied to LC-MS/MS analysis.

QC sample was prepared by mixing equal volume of each serum sample. QC samples were analyzed every 10 runs to monitor the instrument and evaluate the stability and reproducibility of LC-MS system throughout the analysis procedure.

The UHPLC-MS/MS analysis was carried out on a Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive™ HF mass spectrometer (Thermo Fisher, Germany). Each sample was injected into a Hypesil Gold column (100 × 2.1 mm, 1.9 µm, Thermo Fisher) with a flow rate of 0.2 mL/min at 40 °C. The mobile phase for the positive polarity mode consisted of eluent A (0.1% formic acid in water) and eluent B (methanol)), and eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol) for the negative polarity mode. The gradient elution program was set as follows: 0-1.5 min, 2% B; 1.5-3.0 min, 2-100% B; 3.0-10.0 min, 100% B; 10.0-10.1 min, 100-2% B; 10.1-12.0 min, 2% B. For mass spectrometry analysis, the Q Exactive HF mass spectrometer with an electrospray ionization source was used. The parameters employed were as follows: ESI positive and negative mode; mass range, m/z 100-1500 Da; spray voltage, 3.5 kV; capillary temperature, 320 °C; sheath gas flow rate, 35 psi; aux gas flow rate, 10 L/min; S-lens RF level, 60; aux gas heater temperature, 350 °C.

The raw data files obtained from UHPLC-MS/MS were imported into Compound Discoverer 3.1 to perform peak alignment, peak picking and quantitation for each metabolite. The main parameters were as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3, et al. After that, peak intensities were normalized to the total spectral intensity. The normalized date was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. Finally, the peaks were matched with mzCloud, mzVault and MassList database to obtain the accurate qualitative and relative quantitative results.

KEGG database (https://www.genome.jp/kegg/), HMDB database (https://hmdb.ca/metabolites) and LIPID MAPS database (http://www.lipidmaps.org/) were used to annotate identified metabolites. Partial least squares discriminant analysis (PLS-DA) was performed at metaX. Statistical significance (P-value) was calculated by univariate analysis (t-test). The metabolites with variable importance in projection (VIP) > 1, P-value < 0.05, and fold change (FC) > 1.2 or FC < 0.83 were considered as differential metabolites.

2.16 Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted by one-way analysis of variance (ANOVA), followed by Tukey's test using GraphPad Prism 9.0.0. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* Control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* DSS group.

3 Results

3.1 CA exhibited anti-inflammatory effect in macrophages

MTT assay was used to test the cytotoxicity of CA (Figure 1A), and the results indicated that CA did not affect the cell viability even at the dosage of 100 µM (Figure 1B). Then, LPS-stimulated RAW264.7 cells were used to investigate the anti-inflammatory activity of CA. Nitric oxide (NO) is a free radical acting as a cellular signaling molecule, mainly produced by inducible nitric oxide synthase (iNOS), which has been associated with the pathophysiological of inflammation (24, 25). In the present study, LPS stimulation caused dramatic increase of NO in RAW264.7 cells, however, CA treatment significantly inhibited the production of NO in a dose-dependent manner (Figure 1C). ELISA assay revealed that CA dose-dependently inhibited the release of inflammatory cytokines including IL-6, IL-1β and TNF-α induced by LPS (Figures 1D, E, F). Furthermore, CA concentration-dependently inhibited the overexpression of iNOS protein induced by LPS (Figures 1G–H). Overall, CA effectively suppressed inflammatory response in RAW264.7 cells.

3.2 CA administration ameliorated the clinical symptoms of DSS-induced colitis

A UC mouse model was induced through 3% DSS administration in drinking water for 7 days. In the course of experiment, DSS group showed body weight loss, diarrhea and hematochezia. By contrast,

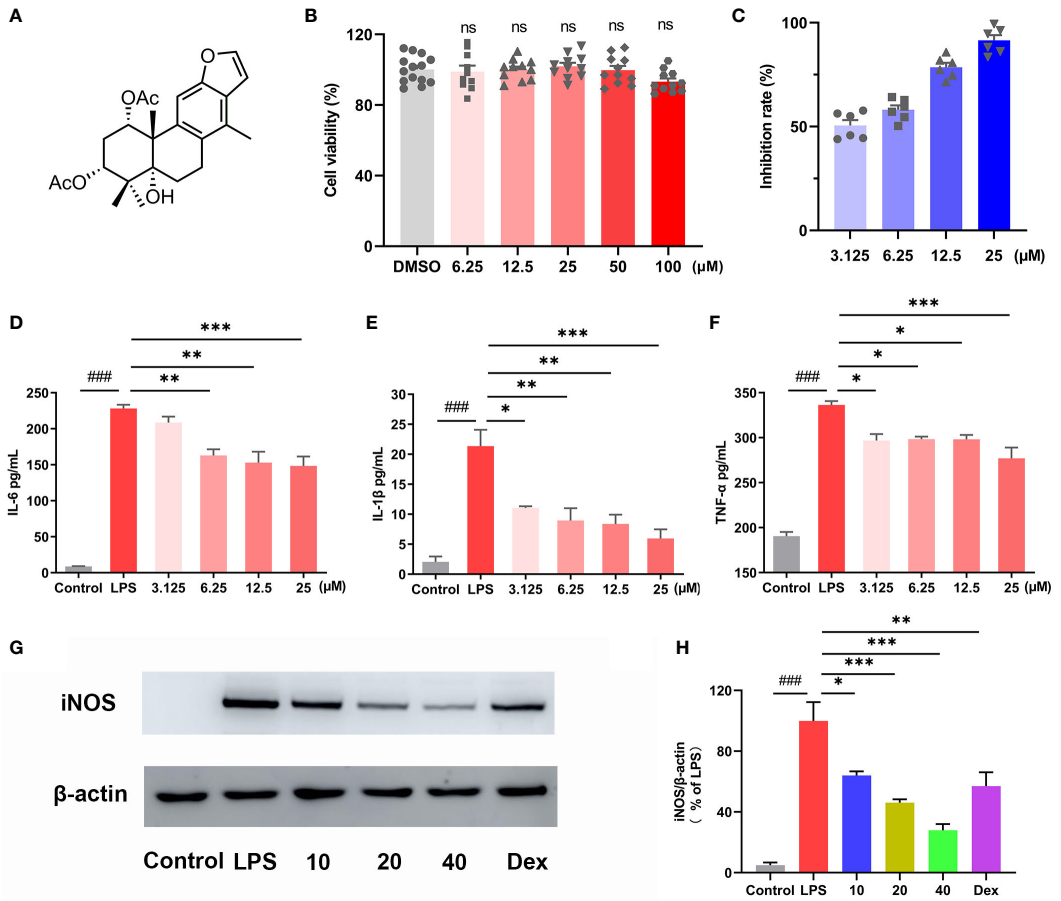

FIGURE 1
CA exhibited anti-inflammatory effect in macrophages. **(A)** Chemical structure of caesaldekarin e (CA). **(B)** RAW264.7 cells were treated with CA for 24 h as indicated concentrations. The cell viability was determined by MTT assay. **(C)** RAW264.7 cells were pretreated with the indicated concentration of CA for 2 h, followed by LPS (0.1 μg/mL) stimulation for another 24 (h) Then, the culture medium was collected to determine the nitrite levels using the Griess reagent. RAW264.7 cells were pretreated with the indicated concentration of CA for 2 h, followed by LPS (0.1 μg/mL) stimulation for another 24 (h) The culture medium was collected to determine the levels of IL-6 **(D)**, IL-1β **(E)** and TNF-α **(F)** by ELISA. **(G)** RAW264.7 cells were pretreated with CA (10, 20, 40 μM) for 2 h and stimulated with LPS (0.1 μg/mL) for another 12 (h) Then, iNOS protein expression was determined by Western blot analysis. **(H)** The relative protein expression of iNOS was normalized to β-actin. Data are expressed as the mean ± SEM of three independent experiments. $^{###}P < 0.001$ *vs.* Control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* LPS group. ns, no significant difference with DMSO treatment.

treatment with CA (CAip and CAig) and SASP alleviated these pathological alterations and decreased DAI scores (Figures 2A, B). The colon length of DSS group was evidently shorter than that of the control group, while DSS-induced colon shortening was improved by treatment with CA and SASP, especially by CA *via* intraperitoneal injection (Figures 2C, D).

3.3 CA suppressed inflammatory infiltration of colon tissue

Histopathological evaluations of colon were conducted by H&E staining and representative results were shown in Figure 2F. In the control group, the colon tissues showed intact mucosa, submucosa, muscular layer and outer membrane. However, the colon from DSS group showed disruption of the epithelial layer, loss of goblet cells, crypts loss and inflammatory cell infiltration. However, CA and SASP treatment had less distortion of epithelium, relative integral crypt structures and fewer infiltration of inflammatory cells.

Neutrophil infiltration leads to remarkable elevation of MPO activity in colon, which is a typical inflammatory marker of colitis (26). As shown in Figure 2E, MPO activity of DSS group significantly higher than that of the control group, while CA and SASP treatment significantly suppressed the elevation of MPO activity. Compared with DSS group, the reductions in CAip, CAig and SASP groups were 70.8%, 55.4% and 61.2%, respectively. Taken together, these results suggested that CA exhibited therapeutic effect on DSS-induced colitis through inhibiting inflammatory infiltration.

3.4 CA decreased the level of inflammatory markers in colon tissue and serum

To investigate the effect of CA on the release of pro-inflammatory cytokines in the colon, colon tissues were collected and the expression of pro-inflammatory cytokines were measured by ELISA and qRT-PCR. As shown in Figure 3, the levels of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α in colon tissue significantly

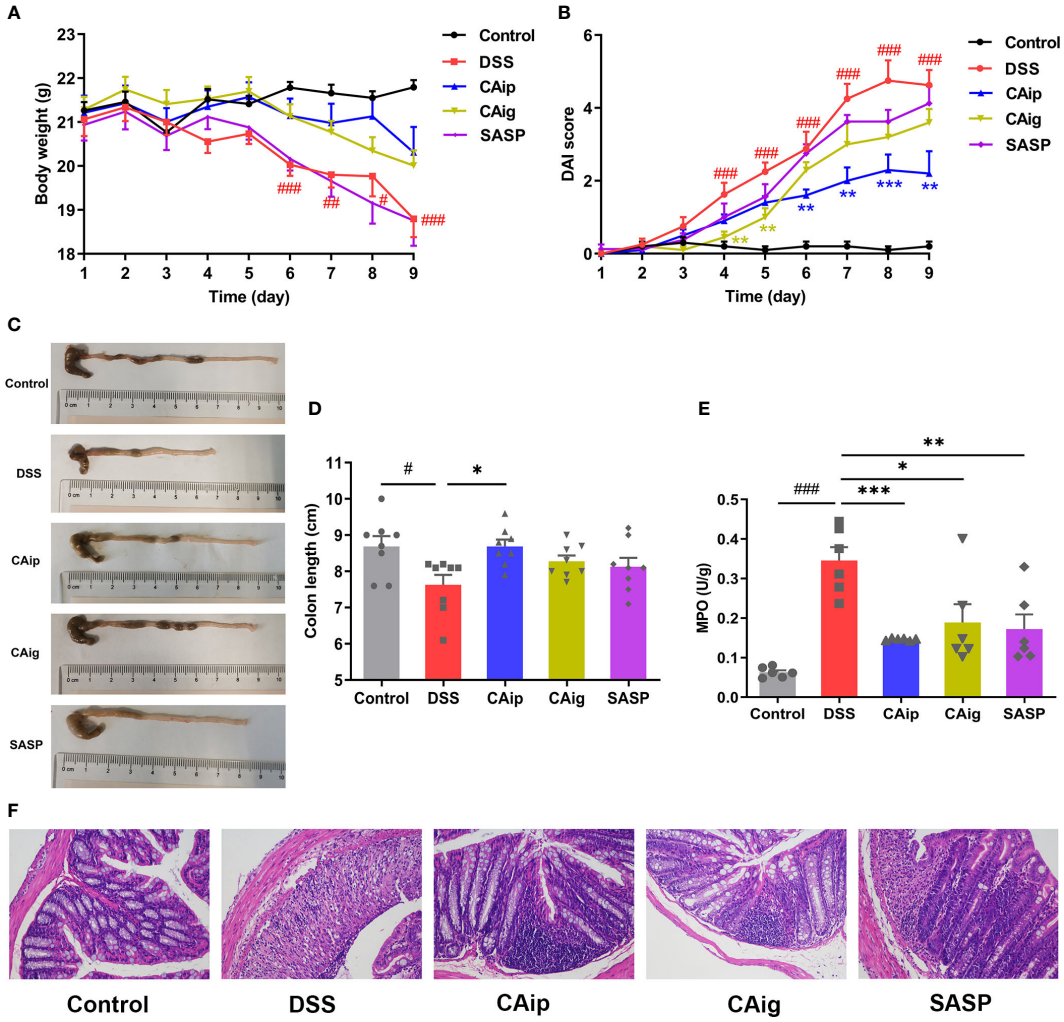

FIGURE 2
CA treatment ameliorated DSS-induced experimental colitis. **(A)** Body weight change. **(B)** Disease activity index (DAI) score. **(C)** Representative pictures of colon appearance and colon length. **(D)** colon length. **(E)** MPO activity. **(F)** Representative microscopic pictures of H&E staining (200 × magnification). **(A, B, D)** Data are presented as the mean ± SEM (n = 8~10). **(E)** Data are presented as the mean ± SEM (n = 6). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* Control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* DSS group.

increased in the DSS group. However, the administration of CA markedly reduced the levels of these cytokines. Notably, the effects of CA for decreasing the level of pro-inflammatory cytokines were superior to SASP. Similarly, DSS caused considerable increase of inflammatory markers including IL-6, IL-1β, TNF-α and SAA in serum, which also been reduced through CA treatment.

3.5 CA improved intestinal barrier by enhancing TJ protein expression

Intestinal barrier integrity is a prerequisite for mucosal functional homeostasis and one of the main causes of several gastrointestinal diseases such as IBD (27). In addition to depending on coordinated proliferation and cell death, barrier function is also determined by tight junction (TJ), the paracellular barrier of intestinal epithelium. The TJ proteins responsible for barrier and passage function consist of 2 families: the TAMP family consisting of occludin, tricellulin and marvelD3, and claudins family (28). To investigate the effect of CA on intestinal barrier function, the expression levels of TJ proteins occludin and claudin-1 were detected by western blot. As shown in Figure 4, compared with control group, occludin and claudin-1 were obviously down-regulated in DSS group, indicating that the TJ structure was disrupted. By contrast, CA treatment markedly enhanced occludin and claudin-1 expression, suggesting that CA could improve intestinal integrity.

3.6 CA altered gut microbiota diversity and composition

To determine whether CA treatment changed the microbiome, 16S rRNA sequencing was performed for fecal samples of each group. We next compared the alpha diversity and beta diversity among different groups. The alpha diversity of a sample reflects the richness and diversity of the microbial community. As shown in Figures 5A–C, compared with the control group, DSS-treated colitis group exhibited extremely significant reduction of diversity (Shannon and Simpson),

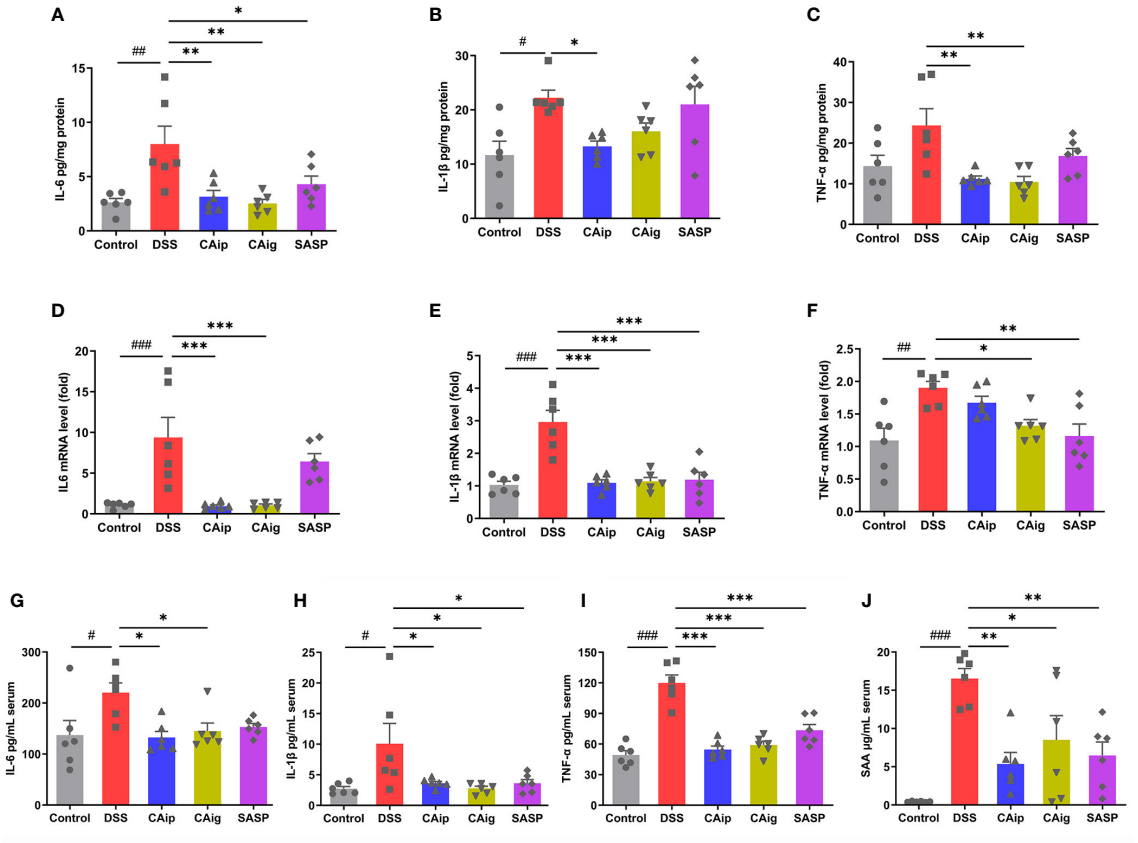

FIGURE 3
Effects of CA on inflammatory cytokines IL-6 **(A)**, IL-1β **(B)** and TNF-α **(C)** by ELISA and mRNA expression of IL-6 **(D)**, IL-1β **(E)** and TNF-α **(F)** by qRT-PCR in colon tissue. Effects of CA on inflammatory markers IL-6 **(G)**, IL-1β **(H)**, TNF-α **(I)** and SAA **(J)** in serum by ELISA. Data are expressed as the mean ± SEM (n = 6). $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ *vs.* Control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* DSS group.

whereas there was no significant difference of microbial species richness (Chao) among the five groups. Notably, CA oral administration and SASP treatment markedly improved the microbial diversity.

The Beta diversity was displayed in Figure 5D. The principal coordinate analysis (PCoA) based on Bray-Curtis distance at OUT level showed overall structure shift of gut microbiota in mice after DSS challenge compared with that of the normal mice. Although CA and SASP treatment could not completely reverse the change of gut microbiota, they still partially mitigate the abnormal gut microbiota in DSS-induced UC mice.

3.7 Gut microbiota composition of UC mice at different level

Histograms were used to illustrate the microbial community structure and show the differences in the relative abundance of major microbiota at different levels. In terms of bacterial composition at the phylum level, all groups possessed similar taxonomic communities mainly composed of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Desulfobacteria. Compared with control group, Firmicutes and Fusobacteria were enriched, while Desulfobacteria and

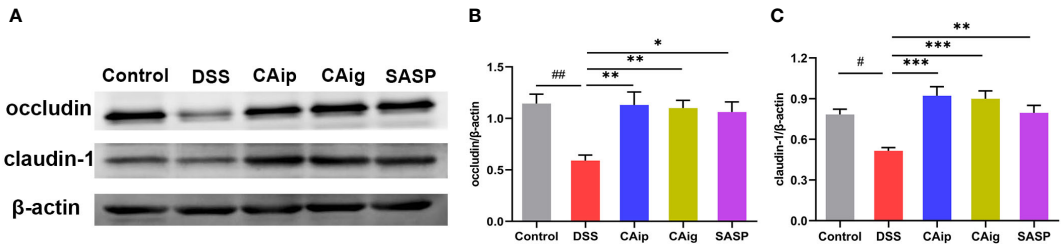

FIGURE 4
CA protected intestinal epithelial barrier by enhancing TJs proteins. Representative western blotting images of occludin and claudin-1 **(A)**, and the relative protein expressions were normalized to β-actin **(B, C)**. Data are shown as the mean ± SEM (n = 5). $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ *vs.* Control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* DSS group.

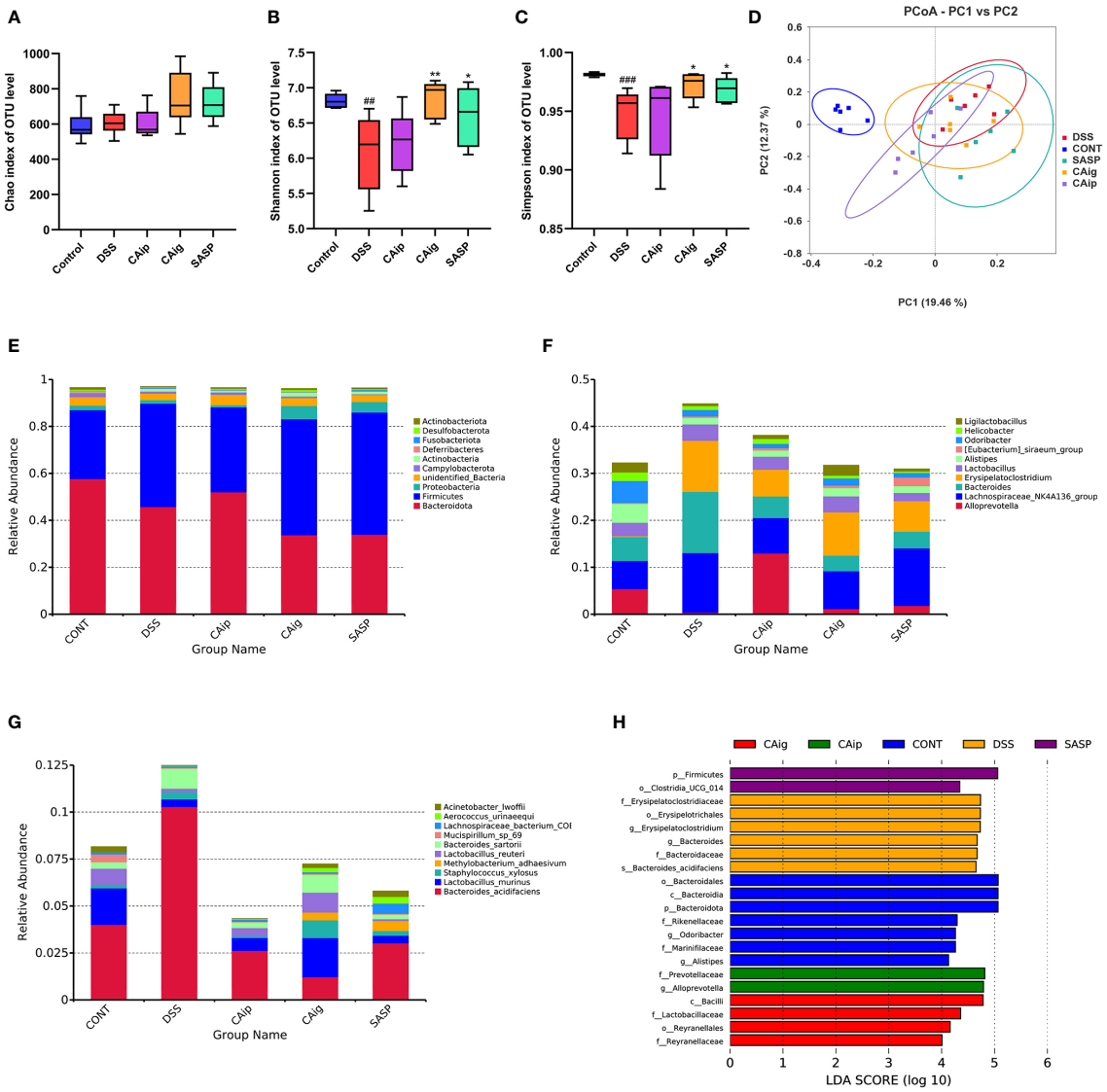

FIGURE 5
CA treatment altered the gut microbiota diversity and composition. Alpha diversity estimated by the Chao **(A)**, Shannon **(B)** and Simpson **(C)** indices of OTU level. **(D)** Principal coordinate analysis (PCoA) using Bray-Curtis metric distances of beta diversity. The relative abundance of fecal microbiota in the top 10 of phylum **(E)**, genus **(F)** and species **(G)** levels. **(H)** The LEfSe analysis. The criterion is log LDA score > 4.0. n = 6 for each group. $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$ vs. Control group, * $P < 0.05$, ** $P < 0.01$ vs. DSS group.

Actinobacteria were reduced in DSS group. However, CAip and CAig treatment could increase the level of Desulfobacteria, and CAig and SASP could increase the level of Actinobacteria (Figure 5E, Supplementary Figure 1). Taxonomic compositions of each group were also compared at the class, order and family levels (Supplementary Figures 2–5). At the genus level, bacterial genera that ranked top ten in relative abundance were analyzed. As shown in Figure 5F and Supplementary Figure 6, the DSS-treated group exhibited significantly increased proportions of *Bacteroides* and *Erysipelatoclostridium*, but decreased proportions of *Alloprevotella*, *Alistipes*, *Odoribacter* and *Ligilactobacillus* compared to the control group. However, CAip and CAig treatment could reduce the proportion of *Bacteroides*, and notably, CAig could increase the abundance of *Ligilactobacillus*. Besides, compared with the control group, the relative abundances of *Lactobacillus murinus* and *Lactobacillus reuteri* were significantly decreased, whereas *Bacteroides sartorii* was obviously increased in the DSS-induced colitis group. However, the disorder of the microbiota community could be partially restored by CAip and CAig treatment (Figure 5G, Supplementary Figure 7).

Linear discriminant analysis effect size (LEFSe) analysis was applied to identification of significant biomarkers and dominant bacterial community that might be responsible for the impact on DSS-induced colitis mice in each group. As shown in Figure 5H, the genus *Erysipelatoclostridium* (the order Erysipelotrichales and the family Erysipelatoclostridiaceae) and *Bacteroides acidifaciens* (the family Bacteroidaceae and the genus *Bacteroides*) were the crucial bacteria leading to gut microbiota dysbiosis in the DSS

group. Nevertheless, Prevotellaceae (the family and the genus *Alloprevotella*) were identified to be the predominant microbiota in CAip group, which might be correlated with its improvement on DSS-induced colitis. Furthermore, Lactobacillaceae (the family and the class Bacilli) and Reyranellaceae (the family and the order Reyranellales) relatively enriched in CAig group, which might be associated with its ameliorating effect on colitis. Taken together, CA treatment, oral administration in particular, significantly altered the gut microbiota diversity and composition.

3.8 Identification of metabolite biomarkers through metabolomics

Partial least squares discrimination analysis (PLS-DA) was employed to evaluate metabolic variations between groups based on the metabolomics data obtained from both ESI+ and ESI- modes. As shown in Figure 6, metabolic phenotype separations were observed between Control *vs.* DSS, DSS *vs.* CAip, DSS *vs.* CAig, and DSS *vs.* SASP groups, indicating that there were remarkable variations in

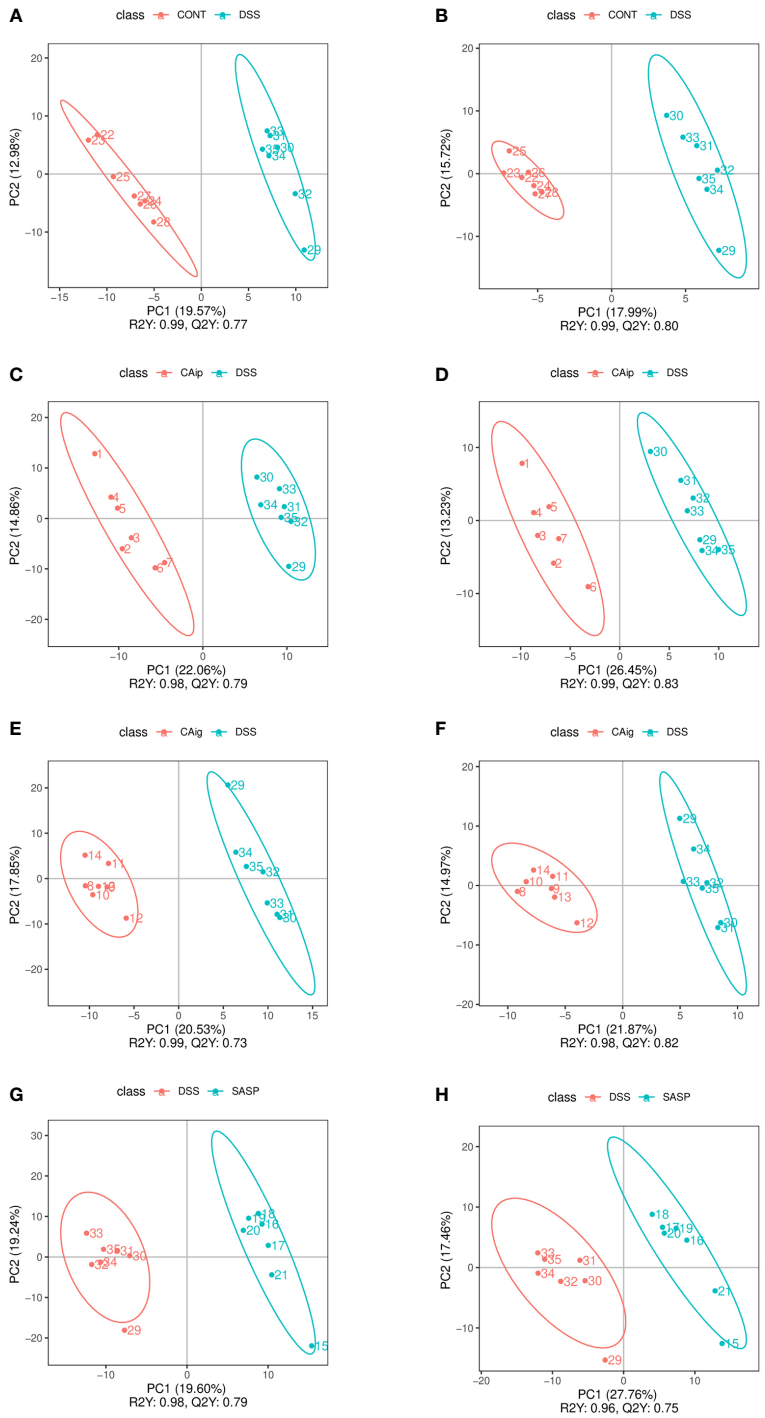

FIGURE 6
PLS-DA analysis of Control *vs.* DSS **(A, B)**, DSS *vs.* CAip **(C, D)**, DSS *vs.* CAig **(E, F)**, and DSS *vs.* SASP **(G, H)** groups in positive ion and negative ion modes.

endogenous metabolites among five groups. As shown in Supplementary Table 2, 80 metabolites in serum were changed obviously in the DSS group compared to those of control group, whereas 30, 31 and 17 metabolites were reversed by CAip, CAig and SASP treatment, respectively. In addition, a considerable number of metabolites showed no significant difference between the Control and DSS groups, but changed obviously after CA or SASP treatment.

Through the KEGG pathway enrichment analysis of serum differential metabolites, potential metabolic pathways were identified to separate the above groups. As shown in Supplementary Figures 8, 9, metabolic pathways that were perturbed in DSS group mainly including neuroactive ligand-receptor interaction, synaptic vesicle cycle, gastric acid secretion, insulin resistance, fatty acid degradation, glutathione metabolism, and tryptophan metabolism. Tryptophan metabolism, phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, aldosterone synthesis and secretion, pyruvate metabolism, and protein digestion and absorption, participated in the therapeutic effect of CA *via* intraperitoneal injection. Tryptophan metabolism, phenylalanine metabolism, pyruvate metabolism, protein digestion and absorption, oxidative phosphorylation, and nicotinate and nicotinamide metabolism were the key metabolic pathways in the CA treatment through oral administration. SASP-mediated metabolic pathways were focused on the glutathione metabolism, nicotinate and nicotinamide metabolism, arachidonic acid metabolism, vitamin digestion and absorption, regulation of lipolysis in adipocytes, and tryptophan metabolism. Collectively, tryptophan metabolism was the common pathway of CA and SASP regulating the metabolites of colitis mice.

3.9 CA regulated tryptophan metabolism *via* inhibiting IDO-1

Tryptophan is an essential amino acid and is metabolized through three major pathways in the intestines: kynurenine pathway in the immune cells and intestinal lining, serotonin pathway in the enterochromaffin cells, and indole pathway in the gut microbiota (29). About 90-95% of dietary tryptophan is metabolized through kynurenine pathway mediated by the rate-limiting enzyme indoleamine 2,3-dioxygenase 1 (IDO-1) in the gut, leading to the production of kynurenine and downstream products such as kynurenic acid, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, xanthurenic acid and quinolinic acid (30). The metabolomics analysis demonstrated that the levels of kynurenine and kynurenic acid in serum significantly increased in DSS group. However, treatment with CA both *via* oral administration and intraperitoneal injection, dramatically reduced the levels of kynurenine and kynurenic acid, and even decreased the levels of other downstream metabolites including N-formylkynurenine, xanthurenic acid and 3-hydroxyanthranilic acid (Supplementary Table 2). We thus investigated whether DSS increased the expression of IDO-1 in colon tissue and CA regulated its expression. The result showed that DSS could induce the upregulation of IDO-1 to some degree but no statistical difference. However, the expression of IDO-1 was significantly reduced by CAip and CAig treatment (Figure 7).

3.10 CA inhibited IDO-1 expression in IFN-γ induced RAW264.7 cell

IDO-1 is expressed in a variety of tissues and cell types, either constitutively or in response to stimulation associated with inflammatory and immune stimuli. Interferon gamma (IFN-γ) is considered to be the most effective IDO-1 inducer in a range of cell types including dendritic cells and macrophages (31, 32). To further confirm the effect of CA on IDO-1 expression, RAW264.7 cells were pretreated with CA for 2 h, followed by IFN-γ (0.2 μg/mL) incubation for 8 h. Western blot analysis showed that IFN-γ stimulation remarkedly increased the expression of IDO-1 in RAW264.7, however, CA treatment significantly inhibited the upregulation of IDO-1 (Figure 8).

4 Discussion

In the present study, we found that treatment with CA both in oral administration and intraperitoneal injection alleviated DSS-induced experimental colitis in mice. Administration with CA maintained the body weight, decreased DAI score, improved colon length and ameliorated inflammatory cell infiltration. The therapeutic effects of CA were attributed to relieving inflammation, improving intestinal barrier, restoring the disrupted gut microbiota, and modulating the metabolites (Figure 9). These results provided insights into the protective effects of CA on colon inflammation.

Organisms used as probiotics are most frequently of the lactic acid bacteria and *Bifidobacterium* species and are included in many functional foods and dietary supplements. The main mechanisms of

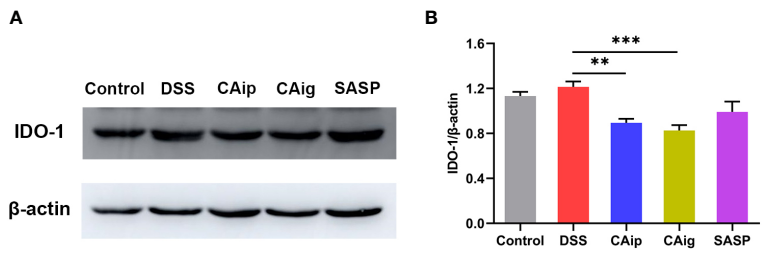

FIGURE 7
Effect of CA on the protein expression of IDO-1 in colon tissue. Representative western blotting image **(A)**, and the relative protein expression was normalized to β-actin **(B)**. Data are shown as the mean ± SEM (n = 5). ** $P < 0.01$, *** $P < 0.001$ *vs.* DSS group.

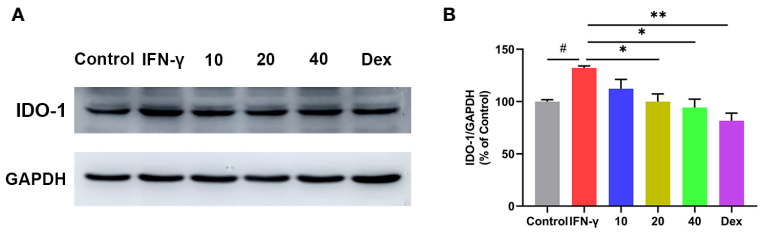

FIGURE 8
Effect of CA on the protein expression of IDO-1 in INF-γ induced RAW264.7 cells. Representative western blotting image **(A)**, and the relative protein expression was normalized to GAPDH **(B)**. Data are shown as the mean ± SEM of three independent experiments. $^{\#}$ $P < 0.05$ *vs.* Control group, * $P < 0.05$, ** $P < 0.01$ *vs.* INF-γ group.

action of probiotics include: 1) colonization and modulation of disordered intestinal microbial communities in children and adult; 2) competitive exclusion of pathogens and bacteriocin production; 3) enzymatic activities and production of volatile fatty acids; 4) cell adhesion and mucin production; 5) modulation of the immune system; and 6) interaction with the brain-gut axis (33, 34). *Lactobacillus* are a major component of symbiotic microbiota of mammals and are one of the most commonly used probiotics (35). Studies suggested that a variety of diseases, including infectious disease, irritable bowel disease, IBD, rheumatoid arthritis, obesity, multiple sclerosis, type 1 diabetes, type 2 diabetes, cancer, and cognitive development and behavior, are correlated with the notable variation of *Lactobacillus* in intestinal abundance. Moreover, probiotic *Lactobacillus* administration play a broader role in the prevention and mitigation of part of aforementioned diseases (36). *L. reuteri*, one species of *Lactobacillus*, possessing features of surviving in low pH and enzyme-rich environment, adhering to epithelium for host-probiotic interaction, competition with pathogenic microorganisms and safety, endows it with great probiotic properties (37). Numerous studies have demonstrated that *L. reuteri* can mitigate experimental colitis by maintaining intestinal immune homeostasis through stimulating dendritic cell maturation and IL-10 production (38, 39), modulating gut microbiota and metabolic disorders (40), increasing mucus thickness and tightening epithelium (41), and decreasing bacterial translocation from mucosa to mesenteric lymph nodes (42, 43). In the present study, we observed that DSS treatment caused significantly reduced abundance of *L. reuteri* compared with the control group. However, the decline was reversed by CA oral administration, suggesting that the increase of *L. reuteri* contributed to the therapeutic effect of CA against DSS-induced colitis. Previous research has shown that *L. murinus* could induce Treg cell expansion, thereby providing resistance against experimental colitis (44). Interestingly, in our current study, the abundance of *L. murinus* disrupted by DSS was also restored through CAig treatment. To further identify the potential biomarkers and dominant bacteria regulated by CA treatment, LEfSe analysis was performed in each group. The family Prevotellaceae and the genus *Alloprevotella* were relatively enriched in CAip group. Consistent with our results, the genus *Alloprevotella* was demonstrated to be lower in DSS-induced experimental colitis, which was generally considered to be short chain fatty acid producer and its abundance was inversely correlated with inflammation (45, 46). Collectively, the alleviative effects of CA on the experimental colitis might be attributed to its gut microbiota modulation activity.

Metabolomics is a technique with advantages of high throughput, satisfactory sensitivity and accuracy for the qualitative and

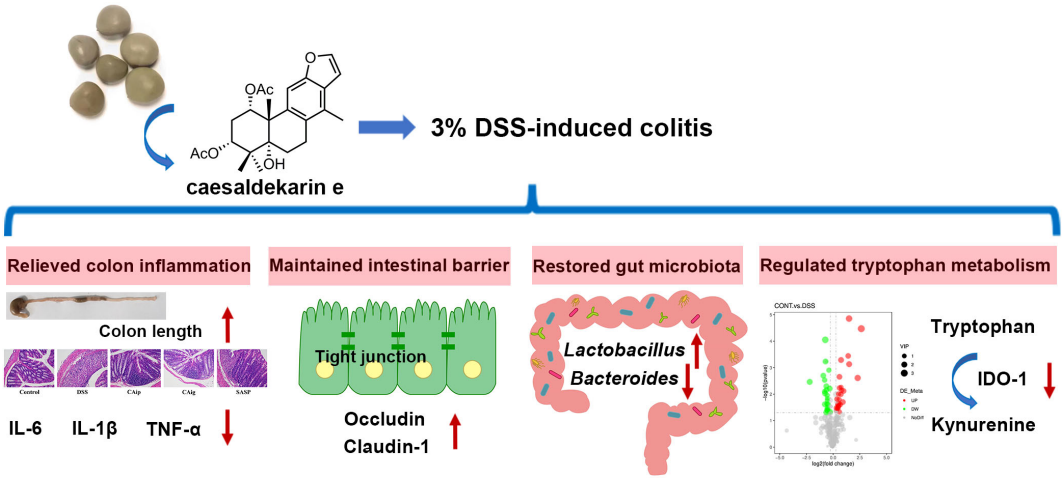

FIGURE 9
Graphical abstract of this article. The up arrow (↑) and down arrow (↓) represent upregulation and downregulation effects of CA, respectively.

quantitative analysis of small molecule metabolites from biological samples including serum, plasma, urine, feces, breath and biopsy samples, which can not only achieve the quantification of change in a single metabolite but also integrate the changes of multiple metabolites through multivariate analysis to gain a holistic comprehension of metabolic profile (47). In view of heterogeneity, complex etiology and easy recurrence of IBD, metabolomics has bounced into IBD in recent years for assessing disease activity, elucidating the underlying mechanisms, predicting the therapeutic response, and monitoring the disease relapse (47, 48). In this study, untargeted metabolomics analysis of serum was conducted in each group to clarify the mechanism of CA on experimental colitis. As expected, we observed that DSS treatment caused remarkable changes of metabolomic profile covering fatty acids, glycerophospholipids, sphingolipids, sterol lipids, eicosanoids, and amino acids, which could be partially reversed by CA or SASP administration. Through the KEGG pathway enrichment analysis for differential metabolites, tryptophan metabolism was considered to be the important pathway affected by colitis and closely associated with the therapeutic effects of CAip and CAig. Consistently, other studies also reported disturbed metabolites associated with tryptophan metabolism in DSS-induced experimental colitis (49, 50). Similar to our findings, studies on IBD patients have reported that, compared with normal subjects, serum concentration of kynurenine and kynurenic acid significantly increased and IDO was overexpressed in IBD patients, and IDO expression was positively associated with disease activity (51, 52). Treatment with CAip and CAig reduced the level of kynurenine, kynurenic acid and other downstream metabolites, and even downregulated the expression of IDO-1 in colitis mice. Furthermore, CA also inhibited the expression of IDO-1 in IFN-γ induced RAW264.7 cells.

Lipids have several major functions in the organism, including as structural components of cell membranes, energy storage, signal molecules, protein recruitment platforms and substrates for protein-lipid modification (53). Numerous studies have shown that there existed significant disorder of lipid metabolism in IBD patients and animal models (54–56). Phosphatidylcholines (PC), the most abundant phospholipid in all mammalian cell types and subcellular organelles, can be digested by phospholipase A2 to produce lysophosphatidylcholine (LPC) (57). The decrease of PC content and increase of LPC/PC ratio were observed in UC patients (56, 58). Consistently, the present study demonstrated that the PC significantly reduced and LPC content increased in DSS group, with respect to controls. However, the PC concentrations were restored by CA treatment especially *via* intraperitoneal injection. Besides, the contents of sphingomyelin (SM) and lysophosphatidylethanolamine (LPE) declined in the DSS group, and similar results were obtained from previous studies on patients with IBD (56, 59, 60). Treatment with CA or SASP increased the levels of SM and LPE downregulated by DSS, and even promoted those showing no difference between the Control and DSS groups. Overall, CA could effectively regulate the metabolic disorder of phospholipid caused by experimental colitis.

In conclusion, current results demonstrated that CA effectively ameliorated DSS-induced experimental colitis. CA could obviously relieve disease symptoms, suppress inflammatory infiltration and restore intestinal barrier integrity. Meanwhile, CA regulated the disturbance of gut microbiota, particularly by increasing the abundance of *Lactobacillus* and decreasing the abundance of *Bacteroides*. Furthermore, the therapeutic effects of CA might be associated with its modulation on tryptophan metabolism, evidenced by CA inhibited the upregulation of IDO-1 in colon tissue of colitis mice and IFN-γ induced RAW264.7 cells.

Data availability statement

The datasets presented in this study can be found in the NCBI Sequence Read Archive (SRA) repository under the project number PRJNA880572.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Shenyang Pharmaceutical University.

Author contributions

HG and TL conceived and designed the experiments. TL, ZN and PL performed the experiments. TL analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (No. 82273824, 31670359), Liao Ning Revitalization Talents Program (No. XLYC1905019) and Natural Science Foundation of Liaoning Province (No. 201602691).

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/fimmu.2022.1045901/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 23 January 2023
DOI 10.3389/fimmu.2023.1072655

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 17 October 2022
ACCEPTED 06 January 2023
PUBLISHED 23 January 2023

CITATION
Moreno E, Ron R and Serrano-Villar S (2023) The microbiota as a modulator of mucosal inflammation and HIV/HPV pathogenesis: From association to causation.
Front. Immunol. 14:1072655.
doi: 10.3389/fimmu.2023.1072655

The microbiota as a modulator of mucosal inflammation and HIV/HPV pathogenesis: From association to causation

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Although the microbiota has largely been associated with the pathogenesis of viral infections, most studies using omics techniques are correlational and hypothesis-generating. The mechanisms affecting the immune responses to viral infections are still being fully understood. Here we focus on the two most important sexually transmitted persistent viruses, HPV and HIV. Sophisticated omics techniques are boosting our ability to understand microbiota-pathogen-host interactions from a functional perspective by surveying the host and bacterial protein and metabolite production using systems biology approaches. However, while these strategies have allowed describing interaction networks to identify potential novel microbiota-associated biomarkers or therapeutic targets to prevent or treat infectious diseases, the analyses are typically based on highly dimensional datasets —thousands of features in small cohorts of patients—. As a result, we are far from getting to their clinical use. Here we provide a broad overview of how the microbiota influences the immune responses to HIV and HPV disease. Furthermore, we highlight experimental approaches to understand better the microbiota-host-virus interactions that might increase our potential to identify biomarkers and therapeutic agents with clinical applications.

KEYWORDS

microbiota, HIV, HPV, inflammation, biomarkers, omics, personalized medicine

1 Introduction

Evolutionary and ecological mechanisms have favored the cooperation of microorganisms that ensure critical functions for host fitness, such as the response against viral infections. The largest fraction of the microbiota resides in close interaction with the mucosa-associated lymphoid tissue (MALT) (1). Therefore, the expectations that the microbiota could exert a clinically relevant impact on viral infections, such as HPV and HIV, are high. The pathogenesis of HPV and HIV infection is intimately associated with the MALT, from the early establishment of infection to their persistence or progression (2–4). For example, HIV infection causes chronic defects in mucosal immunity (5, 6) and translocation of microbial products from the gut to the blood. These changes promote T

cell activation, monocyte activation, and proinflammatory cytokine release (7–10). In HPV, the gut microbiota appears to influence viral persistence, immune responses, the host-mucosal environment, and HPV-related cancer progression (11, 12).

Although omics technologies have allowed us to map the functional alterations produced by viral infections, many studies show correlations, and we lack a granular understanding of the underlying mechanisms for the functional alterations. Omics techniques have allowed linking specific microbiome profiles to certain disease phenotypes (13, 14). The influence of bacterial proteins and metabolites on disease is gaining interest and being more deeply studied (15–21). However, most studies in the field are still correlational and hypothesis-generating. Furthermore, although proteomics and metabolomics are helpful tools to infer pathways and generate hypotheses and they have become increasingly efficient, their results still have limitations and biases and warrant experimental validation. Thus, following the enthusiasm of omics-based studies, the classical approach of designing hypothesis-driven studies focused on digging deeper into particular questions after interrogating highly dimensional datasets, is gaining attention. Here, we review specifically the current concepts on the reciprocal interactions between the microbiota and two persistent viral infections, HIV and HPV. We discuss the opportunities for omics techniques and their limitations in the field, highlight examples of studies aimed at understanding the consequences of the microbiota in HIV and HPV infections, and summarize the experimental approaches that have improved our mechanistic insight.

2 Influence of the microbiota on HIV and HPV infections

Correlations between changes in gut mucosa leading to "dysbiosis" (i.e., alterations in the intestinal microbiota) and viral infections are commonly studied. The commensal microbiota appears to be a significant determinant of the acquisition and replication of some pathogenic viruses. This may include mechanisms not well understood yet, including pathogen growth regulation, competitive metabolic interactions, localization in intestinal niches, and host-immune response induction (22–26). However, as we will review below, there is a clear connection between the microbiota status and the clinical course of HIV and HPV.

2.1 Influence of HIV infection on the microbiota

Acute HIV infection exerts dramatic and perhaps irreversible MALT damage (27, 28). The fact that HIV replication has been associated with a loss of anti-inflammatory bacteria (20, 29) has spurred research into the hypothesis that HIV infection affects the microbiota and that this altered microbiota may contribute to persistent inflammation, increasing the risk of comorbidities. Mechanistically, HIV infection could affect the microbiota by inducing depletion of Th17 cells in MALT, enteropathy, mucosal inflammation, aberrant cytokine production, and intestinal epithelial cell damage (25, 30–34). In the context of HIV infection, microbial-induced immune activation occurs and correlates with markers of intestinal damage, suggesting that the microbiota is a relevant driver of systemic inflammation (35–41). Even the changes appreciated in the oral microbiota of people living with HIV (PLWH), who exhibit an increased prevalence of dental caries and periodontal inflammation, seem to be connected to shifts in systemic immune responses (reviewed in (42)). Specific Lactobacillus species-rich vaginal microbiota have been associated to protection from HIV infection (last reviewed in (43)).

It is now widely accepted that impairment of intestinal integrity and dysbiosis lead to translocation of bacterial derivatives from the gut to the bloodstream, resulting in chronic inflammation. This may occur by immunosuppressive or immunostimulatory mechanisms and *via* various non-mutually exclusive processes, including augmented antigenicity, adjuvanticity, or bystander T-cell activation (44, 45). This fact has been studied before for HIV-associated inflammation, which has been associated with an increase of active microorganisms leading to different pathways related to immune modification (46), These pathways include (i) decreased amino acid catabolism, leading to nutritional deficits (47). (ii) induction of indolamine-2,3-dioxygenase-1 (IDO1) leading to an increased transformation of tryptophan into the immunosuppressive kynurenine derivatives, bacterial translocation, and systemic inflammation, which has been linked with excess mortality risk ,45). (iii) increased butyrate synthesis, which, among other functions, tempers intestinal inflammation (48). and (iv) accumulation of inflammatory molecules, such as arachidonic acid and leukotriene-B4 (49).

Inflammatory biomarkers levels remain increased in PLWH even when ART is started early (50). Chronic inflammation has consistently been associated with an excess risk of comorbidities during treated HIV infection and is suggested as a contributing risk factor (51, 52). Thus, the HIV field has pursued whether the microbiota affects inflammation during treated infection. For example, microbiota metabolic profiles affect HIV inflammation by promoting changes in glutathione metabolism and zeatin biosynthesis, butyrate production, or tryptophan catabolism (46, 49, 50, 53). Furthermore, a well-defined deleterious consequence of HIV infection is bacterial translocation triggering immune activation (54–56). A few sequence-based and ultramicroscopic studies have uncovered a blood bacterial DNA profile in HIV. Following acute SIV infection in macaques, analysis of bacterial DNA isolated from the colon, liver, and mesenteric lymph nodes demonstrated a preference for the phylum Proteobacteria to translocate to these compartments and an increased metabolic activity of Proteobacteria within the colonic lumen (57). In a study in PLWH diagnosed with advanced disease and starting ART, we also found that Proteobacteria was the predominant phylum in the blood, indicating commonalities in the mechanisms by which bacterial translocate from the gut into the bloodstream between SIV and HIV infections. The same study showed that ART initiation in late-presenters attenuated the bacterial signature of untreated HIV infection, characterized by the presence of DNA from commensal bacteria with pathogenic potential (58). Relationships between the translocated microbiome, systemic inflammation, and clinical outcomes were described in a different

study showing increased CD4 T cell counts following one year of ART that were associated with high *Serratia* abundance, innate proinflammatory cytokines and metabolites driving Th17 gene expression signatures, and restoration of mucosal immunity (59).

Current evidence supports investigating therapeutic strategies for immune modulation in HIV. However, so far, no intervention targeting the microbiota of PLWH either by using prebiotics (16, 16, 60), probiotics (61–63), synbiotics (64), rifaximin (65, 66), or even fecal microbiota transplants (67, 68) have convincingly proved to effectively temper inflammation or enhance boost immune recovery following ART initiation. In general, there was lack of standardization in the outcomes assessed (ranging from studies designed to assess T cell changes (60, 64) to exploratory studies evaluating multiple markers of T cell activation (69) or soluble markers of inflammation or bacterial translocation), the duration of the intervention (from weeks (16, 61, 62, 69) up to one year), the disease status (from ART naive patients followed without ART (60) or patients presenting at advanced stages of the disease (64) starting ART to patients under ART-mediated HIV RNA suppression (61–63), and even the dosage and components of the prebiotic or probiotic mixtures (16, 62–64, 69). Therefore, this still represents a field of active research, and has been extensively reviewed elsewhere (70).

2.2 Influence of HPV infection on the microbiota

We know less about the impact of HPV infection on the microbiota epithelial surface integrity, mucosal state, and immune regulation, all factors related to HPV persistence and progression to cancer (46, 71–74). For example, metabolites associated with the vaginal microbiome, including biogenic amines, glutathione, and lipids, have been implicated in HPV persistence (75). It has been described that the microbiota composition can affect all these factors in the context of HPV infection (76–80). A meta-analysis found that *Lactobacillus iners* and non-*Lactobacilli* species dominance in the vaginal microbiota is associated with a higher risk of persistent HPV infection and dysplasia (22) compared to the dominance of *L. iners* and L. *crispatus* (81, 82).

A *Lactobacillus-depleted* microbiome has been associated with a proinflammatory environment that may increase malignant cell proliferation and HPV E6 and E7 oncogene expression (22, 83, 84) and promote coinfections by other pathogens such as *Chlamydia trachomatis* (80). Specifically, it has been shown that HPV down-regulates some innate molecules, such as SLPI, S100A7, elafin, HβD1, and TNFα/LPS that are used by some *Lactobacillus* species as an amino acid source sustaining their growth, in keeping with their decreased abundance in microbiome analyses of HPV infected individuals (80). Even virome alterations are associated with features of the vaginal microbiota and genital inflammation changes related to HPV infection (85).

Some authors have connected the expression of proinflammatory and chemotactic cytokines related to HPV-induced carcinogenesis with an increased presence of *Sneathia* or *Gardnerella* in the vaginal microenvironment (86–90). Furthermore, even though a certain level of inflammation has been described as potentially beneficial to decrease HPV dissemination, several studies have shown specific inflammation markers as related to the progression to a carcinogenic status that could be used as clinical markers to prevent high-grade squamous intraepithelial lesions (46, 86, 90–95) and specific metabolic profiles (96–98). However, most studies in the field are cross-sectional, so it is hard to assess whether the microbiota influences HPV infection or vice versa.

2.3 Microbiota mechanisms with consequences on viral infection

Viruses infecting epithelial cells can profoundly affect the mucosal immune system—the central habitat of the mucosal microbiota—altering the immunological signals required to orchestrate commensal colonization and possibly affecting systemic immune responses and other processes. While from an applied perspective, the gut microbiota functionalities are more relevant for health, most studies have focused on the compositional level, and only fewer studies have focused on the functional consequences. Some microbiota-associated mechanisms possibly influencing the clinical course have been characterized for HIV and HPV (Table 1).

3 Potential and limitations of current approaches for understanding microbiota effects on HIV and HPV infections

Studying the interactions between host factors and pathogens is complex, especially when a third term—a virus— is added to the multifaceted dichotomy of host and microbiota. However, multi-omic techniques have allowed applying systems biology approaches and ecological concepts to analyze host-microbiota interactions during viral infections (130, 131). These approaches have boosted our ability to understand viral infection to the level in which we are starting to appreciate the importance of the commensal bacterial communities on the pathogenesis of diseases that not so long ago were assumed to depend only on the interactions between viruses and human cells. Nevertheless, current omic techniques have several limitations, such as the scarcity of standardized methods to integrate the different omic levels (132). More importantly, the research potential and fascination with the increasingly efficient omics approaches have often relegated hypothesis-driven research to a second position. We believe that, while the microbiome research primarily relying on 16S rRNA gene studies has been crucial to generate hypotheses, the field needs to move towards more mechanistic, hypothesis-driven studies and applied research.

3.1 Multiomic techniques

Technologies such as Next Generation Sequencing (NGS), RNA sequencing (RNA-seq), and mass spectrometry (MS/MS) and all their different variations have already been used to describe the global landscape of viral-host interactions. Typically, gain and loss-of-function studies are performed to study the differential expression

10.3389/fimmu.2023.1072655

TABLE 1 Summary of the major mechanisms by which the microbiota influences HIV and HPV infection.

Pathway/ Function	Virus	Bacteria implicated	Biological Mechanism	Clinical Consequences	References
Regulation of innate immune molecules	HIV	↓ *Lactobacilli* ↓ *Lachnospira* spp. ↓ *Roseburia intestinalis* ↓ Ruminococcaceae	Peptidoglycan signaling Decreased butyrate production Increased local inflammation	Increased HIV transmission	(16, 99–101)
	HPV	↓ *Lactobacilli* ↓ *Bifidobacterium* ↑ *Anaerobes and diversity*	Down-regulation of SLPI, S100A7, elafin, HβD1, TNFα/LPS. Cytokines and chemokines	Enhance antitumor immunity and anti-PD-L1 efficacy. Higher risk of sexually transmitted infections	(102–105)
Tryptophan catabolism	HIV	↑ *Gammaproteobacteria* ↑ *Pseudomonas* spp. ↑ *Bacillus* spp. ↑ *Burkholderia* spp. ↑ *Prevotella* ↑ *Acidaminococcus*	Immunotolerance Barrier failure Angiogenesis IDO1 inhibition ↑ immunosuppressive kynurenine derivatives ↓ Th17 cells Bacterial translocation	Higher risk of non-AIDS comorbidities	(15, 53, 106, 107)
	HPV	*N.f.*	Increased kynurenine derivatives increase oxidative stress	HPV malignant transformation to cancer	(108)
IL-10 signaling pathway	HIV	↑ *Bacteroides fragilis*	Immunotolerance: Polysaccharide A production TLR-2 activation IL-10 expression Systemic immune activation. Inflammation	Periodontitis Higher risk of non-AIDS comorbidities	(53, 109–112)
	HPV	↓ *Lactobacilli*	IL-10 increase breaks the balance with IL-2 leading to Th2 dominance	Immunosuppression state leading to progress of lesions	(113)
Choline metabolism	HIV	↑ Actinobacteria ↓ Bacteroidetes ↑ Firmicutes ↑ Gammaproteobacteria ↑ Clostridium XIVa ↑ *Faecalibacterium* spp.	Endothelial dysfunction Inflammation. TMAO production. Monocyte activation	Increased atherosclerosis and cardiovascular risk	(114–116)
	HPV	-	Aberrant DNA methylation associated with HPV infection.	Cervical tumorigenesis	(117)
Activation of adaptative immunity	HIV	↑ Bifidobacteria	CTL responses Epithelial cell turnover Immunomodulatory strain-dependent effects ↑ Dendritic cell activation ↑ CD8+ T cell priming and accumulation in the tumor microenvironment ↑ Cross-reactivity with tumor antigens	Improved immune recovery under ART	(118, 119)
	HPV	↓ *Lactobacillus dominance* ↑ *Anaerobes and diversity*	Recruitment of immune cells	Bacterial vaginosis (BV)	(120)
Chemotaxis	HIV	↓ *Akkermansia muciniphila*	Host immune regulation ↓ Mucin degradation. Higher systemic inflammation (sCD14, IP10) and intestinal inflammation (fecal calprotectin)	Higher risk of non-AIDS comorbidities	(121, 122)
	HPV	↓ *Lactobacillus dominance* ↑ *Anaerobes and diversity*	Reduction in the viscosity of the cervicovaginal fluid (CVF), due to the production of mucin-degrading enzymes	Breaking the first line of defense against exogenous pathogen colonization.	(120)
Cell proliferation	HIV	↑ *Fusobacterium* spp.	Cell proliferation and oncogenesis: TLR-4 signaling. PPAK1 cascade. Nuclear factor KB induction	Impaired immune recovery after ART	(29, 123)
	HPV	↑ *Lactobacillus inners* ↑ *Gardnerella vaginalis* ↑ *Atopobium vaginae* ↑ *Sneathia*	Persistent coinfection with other bacteria is linked to epigenetic changes, oncogenes expression, non-coding RNA regulations, p53 deregulation, etc. But no direct experimental evidence for bacteria other than *C. thrachomatis* and found LPS from bacteria in exosomes	Association with cervical intraepithelial neoplasia (CIN) to	(124)

(Continued)

TABLE 1 Continued

Pathway/ Function	Virus	Bacteria implicated	Biological Mechanism	Clinical Consequences	References
		↑ *Fusobacterium* ↑ *Chlamydia trachomatis*		carcinoma *in situ* (CIS)	
Inflammation. Antitumoral immunity	HIV	↑ *Lactobacillales*	Enhanced antitumor response: Upregulated IFN-γ, GZMB, and PRF1 expression in CD8+ T-cells	Improved immune recovery after ART	(20, 123, 125)
	HPV	–	The interplay of viral oncoproteins and inflammatory cytokines leads to continuous immune evasion, which promotes the progression of the lesion. Also, increased oxidative stress has been attributed to inflammation	Progression of the initial lesion to malignancy	(126–129)

↑ increased abundance and ↓ decreased abundance.

of DNA or RNA, either of human or bacterial origin, after viral infections. However, since this cannot capture the whole picture of the complex interactions between the virus and the host, mass spectrometry started being used to study the complete proteome, secretome, and metabolome, and even for bacterial identification in clinical microbiology (133). In addition, some studies have used these technologies, even performing integration of some of them (134), to study the role of the microbiome in the inflammation state produced by HIV infection (20) and reviewed in (24, 46, 135) and in pathogenesis and progression to cancer after HPV infection (74, 93, 136).

Improvements in meta-omic techniques have mainly been used to study the totality of the aimed compounds (genes, proteins, and metabolites) in a set of commensal organisms (metagenomics, metaproteomics, and metametabolomics). Currently, sophisticated versions of these methodologies are becoming more commonly used. For example, shallow metagenomics sequencing is being used to obtain strain-level resolution (137). This, together with the development of advanced computational methods (138), is increasing our resolution allowing the identification of novel strains with probiotic potential, an unmet need by previous studies with prebiotics or probiotics in PLWH (39, 64). Other thriving methods include single-cell technologies, which allow isolating, culturing, and characterizing the genomes and transcriptomes of individual microbes in complex communities (139), or tridimensional mapping of the host microbiota interactions within the mucosa, which is advancing our understanding of the microbiota-immune response interactions to the next level (140).

3.2 From hypothesis-generating microbiota studies to hypothesis-driven and applied research

Inside and outside the HIV and HPV fields, the lack of methodological standardization is one of the main limitations in the study of the microbiome and challenges reproducibility (141). For example, a comparison of the clinical impacts of the use of probiotic showed very different results (142) (see Table 2). Although, as discussed before, technologies are improving, and now is possible to perform whole genome shotgun sequencing to enhance the detection of diversity, prediction of genes, and accuracy of bacterial species detection (166). However, it is also important to complement the studies by using omics other than genomics to obtain information at the functional level, although there are also challenges regarding the standardization of these methodologies (136, 167, 168). One of these challenges is the integration of datasets (169), which has led to the proposal of the use of machine learning and artificial intelligence for this task, which also have intrinsic limitations (170).

Omic technologies result in compositional profiles and large taxonomic lists for which we lack culture methods in most cases. 'Culturomics'—a high-throughput culture method— and MALDI-TOF mass spectrometry allow the growth of fastidious bacteria together with the identification of several bacterial species and longer incubation periods. However, these techniques have only allowed us to partially overcome the previously mentioned limitations (171). Furthermore, validation of results obtained from the omic techniques is challenging since, in most of the cases, if validation is performed, only a few of the most statistically significant hits are selected for validation. Even when results are validated, any assumption made or reductionist approach used in the experimental design need to be revisited in order to ensure that the results are physiologically relevant and translation to their clinical use can be performed.

In the case of microbiota studies, omic techniques may often result in compositional profiles and large taxonomic lists for which we lack culture methods in most cases. 'Culturomics'—a high-throughput culture method—allows the growth of fastidious bacteria and more extended incubation periods, and MALDI-TOF mass spectrometry allows the identification of several bacterial species. However, these techniques have only allowed us to partially overcome the previously mentioned limitations (171).

Thus, the previously described shortcomings pose an enormous challenge to unleashing the clinical potential of microbiota role in medicine. If we want to assess the causal-effect relationship better and move towards applied microbiome research, it will be necessary to start with a clinical question and use the most consistent methodology to perform hypothesis-driven research that identifies convincing interactions and confounders. For this, we will need to define first the best hypothesis inspired by a clinical question. Then, from the hypothesis, we should carefully design the experimental approaches (e.g. different omics) and analysis (e.g. network models) and further perform experimental validation, including controls and complementary data

TABLE 2 Summary of the experimental models used to study the effects of microbiota on HIV and HPV infection.

Virus	Experimental model	Mechanism identified	References
HIV	Immune cells stimulation with fecal bacterial communities isolated from HIV patients	Enteric microbiota of untreated HIV-infected subjects induces monocytes and T-cell activation.	(41, 143)
	Immune cells stimulation with LPS from specific bacteria, such as mycobacteria or *Holdemanella*, related to HIV infection	Chemokines and IL-1β released by macrophages. T-cell activation. Macrophages tolerance. Higher frequency of CCR5 +CD4+T cells.	(41, 144–150)
	Effect of fecal microbial transplantation on immunity-related to HIV	Increased Th17 and Th22 cells and reduced CD4+Tcell activation.	(67, 68, 151, 152)
	Study of immune activation after fecal transplant in gnotobiotic mice of feces from HIV-negative vs HIV-positive individuals	Non-significant differences	(143)
	Treatment of infection with extracellular vesicles (EVs) or outer membrane vesicles (OMVs) derived from bacteria such as Lactobacilli or Neisseria meningitidis	Demonstrated direct interaction of EVs with viral proteins	(153–157)
	Characterization *in vitro* of the anti-HIV properties of differentially detected candidates by metabolomics	Dipeptides bind to HIV, acting as antivirals and supporting Prevotella growth.	(158)
HPV	OMVs containing HPV antigens create an antitumor vaccine	OMVs stimulated the expression of dendritic cell maturation markers and interferon-gamma-expressing splenocytes.	(157, 159, 160)
	Quantification of bacterial release from vaginal swabs	Differential results depending on the used swab	(161–163)
	Coinfections of bacteria, protozoan, and viruses and quantification of inflammatory cytokines	Galectin-mediated immunity dysregulation	(164)
	Three-dimensional cervical epithelial cell model to study bacterial vaginosis	Identification of some metabolites acting as inflammatory mediators	(165)

sets (e.g., qPCR to confirm sequencing, immunoblot to confirm proteomic, fluorescence resonance energy transfer to confirm AP-MS data, infection kinetics, results validation in external cohorts, etc).

For example, we recently sought to solve a clinical need using applied microbiome research. We asked whether the microbiome could be harnessed to improve the prevention of anal precancer—a leading neoplasia in PWLH— for which we need better screening tools. After investigating a discovery and a validation cohort of at-risk patients, we discovered twelve proteins, previously reported to be associated with cancer progression, that were overexpressed in the anal bacteria from subjects with precancerous lesions. Since these proteins contribute to succinyl-CoA and cobalamin production, we measured the intracellular bacterial concentrations of these metabolites. We discovered that cobalamin and succinyl-CoA were increased in the anal microbiome of patients with anal precancer and overperformed the reference test—anal cytology—. Furthermore, we validated the findings in an external validation cohort, and we demonstrated greater *in vitro* production of succinyl-CoA and cobalamin in bacteria associated with HSIL or cancer vs. those presumably protective (172). Therefore, starting from a clinical question and integrating data from different omic levels we were able to define a new microbiome-based tool that could help in the prevention of a common cancer in PLWH by discovering two powerful biomarkers of anal precancer that could improve anal cancer prevention.

4 Experimental models commonly used to study the effects of microbiota on HIV and HPV infection

To overcome the limitations mentioned before and demonstrate the mechanisms driving the effects of the microbiota on HIV and HPV infection, hypothesis-driven experimental designs based on the information generated from the omics technologies should be encouraged. Some leading studies using this approach have been performed in HIV and HPV fields and are summarized in Table 2.

Although some improvements are being established in the experimental designs to demonstrate mechanisms led by microbiota components, there are still several limitations. These include a lack of standardization of the methods for obtaining the samples; understanding of differences on the effect of microbiota compartments (such as feces, tissues or EVs) and finding their correct origin (173); or extrapolation of findings in other model organisms, such as rodents (174), to human diseases, that are unrealistic. Furthermore, the *in vivo* models have been helpful in the past in proving the functional consequences of the microbiota. However, the differences between the animal models and the human anatomy, immune system, and genetic background are significant, and the type and mechanisms of interactions of the host with the pathogens are hard to reproduce, even in humanized mice models.

Even more, if we only look at studies based on the human model, we still find difficulties in setting up proper validations and standardizations. For example, a significant challenge for human studies is controlling for confounding factors beyond age, sex, and sexual preferences (175), such as host genetic, diet, life style or presence of other pathologies or infections.

5 Future perspectives

Although in the last decade, we have witnessed remarkable advances in the field of the microbiota in HIV and HPV infections, we still need to improve our understanding of the specific mechanisms by which the microbiota influences HIV and HPV pathogenesis and how effectively modulate the relevant microbiota-host interactions through targeted interventions. The current state-of-the-art suggests that the microbiota could offer relevant clinical applications for HIV and HPV diseases that might prove suitable to stratify the risk of HIV acquisition (reviewed in (176)), helping to the diagnosis of comorbidities (e.g., tuberculosis or anal dysplasia). This field might also advance the therapeutic options for HIV and HPV, including the development of new treatments or adjuvants through probiotics or postbiotics that could lead to more personalized medicine approaches, including targeting chronic inflammation (67), enhancing immune recovery (60), or facilitating HPV clearance (177). However, if we want to translate our current knowledge into clinical applications, we will have to overcome several methodological challenges, such as standardization of the methods to assess the species level and identify unknown microorganisms that represent today a significant fraction of the microbiota. Advancing culturomic approaches, microbiome-imaging techniques, multiomic integration, and validating the findings in hypothesis-driven experimental designs will also help the field to move forward. Finally, we will need to validate the conclusions from translational research in observational or interventional studies designed *ad hoc* to test previously generated hypotheses. While one decade of research has paved the road for investigating clinical applications of the microbiome in HIV and HPV infections, we face the challenge of learning how to harness the microbiome in medicine in the next years.

Author contributions

EM: writing first draft. All authors: revision and writing of manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the Instituto de Salud Carlos III and Fondos FEDER, Acción Estratégica en Salud (PI18/00154, ICI20/00058 and PI21/00041).

Acknowledgments

We thank all the participants and funders who support research in this field.

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 02 February 2023
DOI 10.3389/fimmu.2023.1086078

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 01 November 2022
ACCEPTED 16 January 2023
PUBLISHED 02 February 2023

CITATION
Zhu L-R, Li S-S, Zheng W-Q, Ni W-J, Cai M and Liu H-P (2023) Targeted modulation of gut microbiota by traditional Chinese medicine and natural products for liver disease therapy.
Front. Immunol. 14:1086078.
doi: 10.3389/fimmu.2023.1086078

Targeted modulation of gut microbiota by traditional Chinese medicine and natural products for liver disease therapy

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The gut microbiota not only constitutes intestinal microenvironment homeostasis and human health but also exerts indispensable roles in the occurrence and progression of multiple liver diseases, including alcohol-related liver disease, nonalcoholic fatty liver disease, autoimmune liver disease and liver cancer. Given the therapeutic status of these diseases, their prevention and early therapy are crucial, and the detailed mechanism of gut microbiota in liver disease urgently needs to be explored. Meanwhile, multiple studies have shown that various traditional Chinese medicines, such as Si Miao Formula, Jiangzhi Granules, Liushen Capsules, Chaihu-Shugan Power, Cassiae Semen and Gynostemma, as well as some natural products, including Costunolide, Coprinus comatus polysaccharide, Antarctic krill oil, Oridonin and Berberine, can repair liver injury, improve fatty liver, regulate liver immunity, and even inhibit liver cancer through multiple targets, links, and pathways. Intriguingly, the aforementioned effects demonstrated by these traditional Chinese medicines and natural products have been shown to be closely related to the gut microbiota, directly driving the strategy of traditional Chinese medicines and natural products to regulate the gut microbiota as one of the breakthroughs in the treatment of liver diseases. Based on this, this review comprehensively summarizes and discusses the characteristics, functions and potential mechanisms of these medicines targeting gut microbiota during liver disease treatment. Research on the potential effects on gut microbiota and the regulatory mechanisms of traditional Chinese medicine and natural products provides novel insights and significant references for developing liver disease treatment strategies. In parallel, such explorations will enhance the

comprehension of traditional Chinese medicine and natural products modulating gut microbiota during disease treatment, thus facilitating their clinical investigation and application.

KEYWORDS

liver disease, gut microbiota, traditional Chinese medicine, natural product, therapeutic strategy

1 Introduction

Liver diseases are mainly categorized into alcoholic liver disease (ALD), nonalcoholic liver disease (NAFLD), autoimmune liver disease (AILD), liver injury, liver cancer, etc., according to different etiologies and pathogeneses (1). Epidemiological studies have indicated that liver diseases constitute an important part of global morbidity and mortality and have become a huge economic burden and an urgent public health crisis (2, 3). However, due to the lack of understanding of their pathogenesis, late diagnosis and rapid progression, the clinical therapeutic strategies for liver diseases are still insufficient, which directly leads to unsatisfactory treatment effects (4). Therefore, the clinical treatment and management of liver diseases remains a considerable challenge, and there is an urgent need to deeply explore the mechanism and develop promising therapeutic drugs and strategies on this basis.

Increasing evidence has shown that gut microbes are closely associated with the pathogenesis of liver diseases in general (5, 6). A study found an interaction between gut microbiota and the pathogenesis of NAFLD (7), whereas ALD patients exhibited increased intestinal permeability and excessive gut microbiota overgrowth (8). Short-term probiotic supplementation helps restore the beneficial flora in the gut of ALD patients and effectively improves liver function (9). Moreover, dietary cholesterol was found to induce gut microbiota dysbiosis and metabolite alterations in mice that drive NAFLD-hepatocellular carcinoma (HCC) formation, while both cholesterol suppression and gut microbiota modulation showed potential anti-HCC effects (10). Furthermore, gut commensal-controlled bile acid metabolism increases the number of natural killer T (NKT) cells and is related to antitumor immune surveillance of the liver (11). These findings reflect the nonnegligible role of gut microbes in the regulation of liver disease, indicating that more detailed and in-depth mechanistic exploration will provide valuable clues and directions for liver disease therapy.

At present, clinically effective therapeutic regimens and strategies for liver diseases are far from sufficient, which makes it an urgent issue to explore novel drugs and promising therapeutic strategies to overcome the existing deficiencies. Importantly, such a status also facilitates the gradual emergence of hepatoprotective effects of traditional Chinese medicines (TCMs), such as Si Miao Formula, Jiangzhi Granules, Liushen Capsules, Chaihu-Shugan Power, Cassiae Semen and Gynostemma, as well as some natural products (NPs), including Costunolide, Coprinus comatus polysaccharide, Antarctic krill oil, Oridonin and Berberine, both at the basic exploration and clinical research levels. Among them, some TCMs, such as Huazhi-Rougan Formula, have received increasing attention for their protective effects against NAFLD, and terpenoids are considered to be the main active ingredients (12–14). In addition, many NPs, including fisetin, salidroside and oridonin, have also been proven to modulate various liver injuries by regulating the NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome, and their specific mechanisms remain to be further explored (15). Moreover, TCMs, such as Curcumae rhizome (16) and Xiaoyaosan (17), have shown excellent anticancer activity during liver cancer treatment. These findings suggest that TCMs and NPs can be valuable sources of drugs for liver disease therapy by virtue of their low toxicity, multiple targets and multiple pathways. More delicately, prior studies have found that the therapeutic effects of TCMs and NPs on liver disease are closely related to their regulation of gut microbes. For example, the active ingredient Poria cocos polysaccharides (PCP) downregulates the nuclear factor kappa-B (NF-Kb)/CCL3/CCR1 axis by regulating gut microbes to prevent nonalcoholic steatohepatitis (NASH) (18); Si Miao Formula (19) and Ophiopogon polysaccharide MDG-1 can alleviate NAFLD by inhibiting the gut microbiota and gut-liver axis (20); and oridonin has also been proven to reduce liver injury by altering gut microbiota and promoting the hepatic urea cycle (21). The combination of these studies highlights the great potential of TCMs and NPs in targeting gut microbiota for the treatment of liver diseases.

Based on the crosstalk between TCMs/NPs, the liver and the gut microbiota, this review comprehensively summarizes and explores the effect and mechanism of such substances in liver disease treatment by targeting the gut microbiota (Figure 1). Such exploration provides novel mentality and important references for the establishment of therapeutic strategies for liver diseases and deepens the understanding of these medicines regulating gut microbiota during disease treatment, thus promoting their clinical transformation research and application.

2 Targeting gut microbiota for ALD

Studies have confirmed that the progression of ALD is modulated not only by genetic factors, sex, duration and extent of alcohol abuse but also by some potentially modifiable factors, especially the gut microbiota, which provides insights for the mechanistic exploration and therapy of ALD (22, 23).

Alcohol intake or exposure disrupts the ecological balance of the gut microbiota in many ways, and gut microbiota dysbiosis promotes ALD progression through complex mechanisms (Figure 2). In this

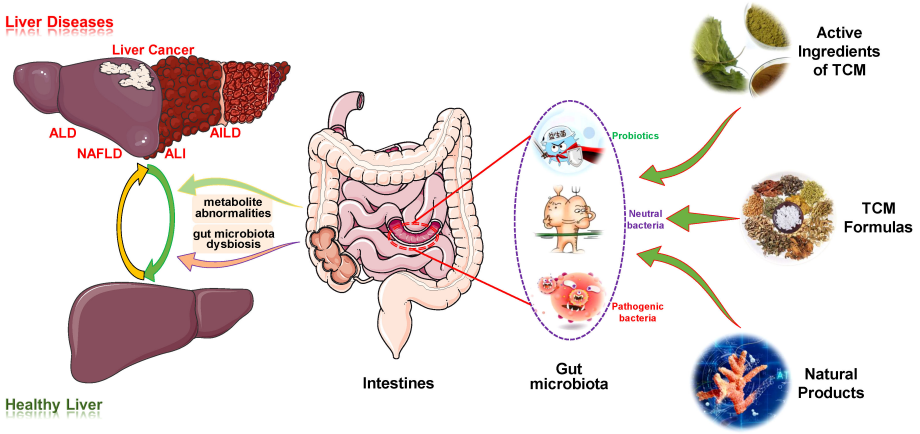

FIGURE 1
Overview of the therapeutic effects of TCM/NPs targeting the regulation of gut microbiota for liver disease treatment. AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ALD, alcoholic liver disease; ALI, acute liver injury; TCM, traditional Chinese medicine.

process, alcohol directly leads to the overgrowth and enrichment of some gut microbiota, such as *Actinobacteria*, *Proteobacteria*, *Enterobacteriaceae*, *Corynebacterium* and *Streptococcus*, while the abundance of other gut microbiota subsequently decreases, such as *Bacteroidetes* and *Akkermansia* genera of the phyla *Verrucomicrobia*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium* and *Roseburia* of the phylum *Firmicutes* (24). The significant reduction in the number of *Lactobacilli* leads to a reduction in the synthesis of saturated long-chain fatty acids, which in turn accelerates hepatic lipid metabolism, oxidative stress, inflammation and fibrosis, thereby attenuating its protective effect on the liver (25). In addition, acute or long-term drinking leads to injury or even death of intestinal epithelial and immune cells, thus disrupting the integrity and barrier function of the gut mucosa (23). These abnormalities promote some enteric

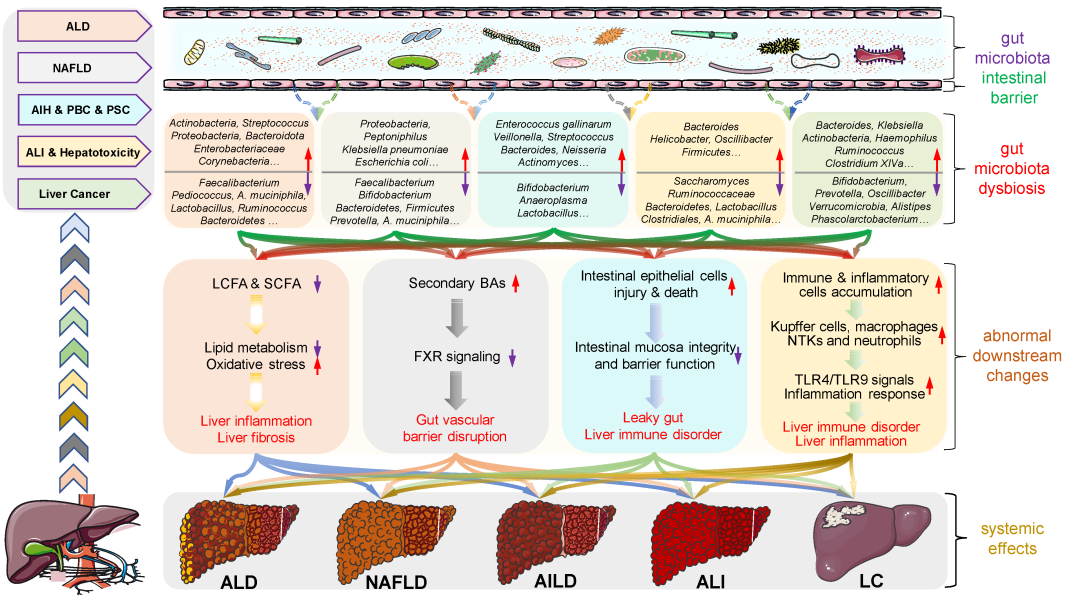

FIGURE 2
Roles of gut microbiota microenvironment alterations in the occurrence and development of liver diseases. There is growing evidence that gut microbiota dysbiosis is closely associated with the development and progression of several liver diseases, such as alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), autoimmune liver disease (AILD), acute liver injury (ALI) and liver cancer (LC). Specifically, alcohol exposure, drugs and other factors induce dysbiosis in the diversity and abundance of gut microbiota species including probiotic, neutrophilic and pathogenic bacteria, as well as abnormal changes in metabolites. These changes subsequently contribute to increased intestinal permeability, intestinal barrier dysfunction, and flora shifts, which in turn accelerate the progression of these diseases through multiple mechanisms, such as the fatty acid metabolic pathway, bile acid (BA) metabolic pathway, inflammatory responses, and immune disorders. These findings reflect the nonnegligible role of gut microbes in the regulation of liver disease and provide valuable clues and directions for the treatment of liver disease. AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ALD, alcoholic liver disease; ALI, acute liver injury; BA, bile acid; FXR, farnesoid X receptor; LC, liver cancer; LCFA, long-chain fatty acid; NAFLD, nonalcoholic fatty liver disease; NTKs, natural killer cells; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SCFA, short-chain fatty acid; TLR, toll-like receptor.

pathogens (especially gram-negative bacteria) and harmful metabolites (lipopolysaccharide (LPS), acetaldehyde, bacterial DNA, and peptidoglycan) to enter the systemic circulation through the gut mucosa, followed by transfer and retention in liver tissue (26). The gut microbiota and harmful substances initiate the downstream immune signal of liver cells through toll-like receptor 4 (TLR4) and other pattern recognition receptors, which not only directly cause an inflammatory response but also enhance the production of inflammatory factors in Kupffer cells/macrophages, thereby aggravating inflammation-induced liver injury (27). Subsequently, inflammatory factors and chemokines gradually accumulate in the hepatic lobules, leading to neutrophil accumulation and further promoting the development of ALD (28).

During ALD treatment, strategies to modulate gut microbiota are gradually being recognized, which is closely related to the nonnegligible effects of TCMs and NPs (Table 1). According to prior research, various TCMs and NPs have been found to affect ALD by regulating the ecological balance and function of the gut microbiota. A study showed that probiotic-fermented *Pueraria lobata (Willd.) Ohwi* significantly reduced the abundance of *Bacteroidetes* and *Akkermansia muciniphila* while increasing the abundance of *Firmicutes* and *Lactobacillus*, which modulated aberrant gut microbiota composition and activated nuclear transcription factor (erythroid-derived 2)-like 2 (Nrf2)-mediated signals, thereby improving lipid accumulation and inflammation and exerting antioxidant effects and ultimately preventing ALD (29). One of the naturally occurring sesquiterpene lactones is costunolide, which has been extensively investigated for a wide range of biological activities. A study found that costunolide treats ALD by modulating oxidative stress and reducing inflammation *in vivo* and *in vitro*, which is inseparable from its impact on the gut microbiota (30). Coprinus Comatus polysaccharide, a polysaccharide extract of the edible mushroom species Coprinus Comatus, has been shown to exert prebiotic-like effects to increase gut microbiota diversity, which might improve adverse changes in gut microbiota caused by alcohol consumption and delay ALD progression (31). With extensive research, an increasing number of TCMs, such as Jianpi Liqi Huoxue Decoction (32), Semen Hoveniae Extract (33), and natural products, including Antarctic Krill Oil (34), have been proven to target the regulation of gut microbiota balance, bile acid metabolism and intestinal permeability to treat ALD. Hence, such medicines should be regarded as a valuable source of medicines to delay ALD progression and even achieve therapeutic effects by regulating the abundance, proportion and distribution of gut microbiota, bile acid metabolism and intestinal permeability.

Although research on the treatment of ALD with these agents targeting gut microbiota has gradually deepened from efficacy observation to mechanism exploration, there are still many deficiencies in the breadth and depth of the current research, such as narrow research scope, unclear target and the lack of clinical trials. Therefore, we need to perfectly integrate existing research with advanced concepts, such as high-throughput screening based on artificial intelligence, proteomics combined with network pharmacology, and research based on clinical phenomena and alterations, which are represented in Table 1. These measures will be conducive to more accurate screening of promising therapeutic targets for ALD, form a wider range of potential TCMs and NPs, and establish a deeper target identification system between these drug candidates and ALD-specific targets, which will provide an important reference and novel direction for research on drugs targeting gut microbiota during ALD therapy.

3 Targeting gut microbiota for NAFLD

NAFLD is a major cause of chronic liver disease worldwide and is prone to developing into liver fibrosis, cirrhosis and even HCC (55). Although the prevalence rate is increasing yearly, there is still a lack of an ideal treatment method, which makes the exploration of in-depth mechanisms and targeted therapeutic strategies necessary (56).

The exploration of enterohepatic circulation has confirmed that gut microbiota dysbiosis is closely related to the progression of NAFLD, which not only manifested in the great changes in the gut microbiota diversity and abundance in NAFLD patients compared with healthy subjects but also reflected that gut microbiota disrupted the inflammatory balance and glucose and lipid metabolism through intestinal metabolites (57), as shown in Figure 2. Specifically, gut microbiota dysbiosis, such as increased abundance of *Proteobacteria* and *Actinobacteria* and decreased numbers of *Bacteroidetes*, *Prevotella and Firmicutes phyla*, reduces the expression of tight junction protein genes, directly leading to the impairment of intestinal barrier function, making harmful microbiota and microorganisms pass through the intestinal barrier, stimulating the immune system and inducing immune cell inflammation, and ultimately accelerating NAFLD and liver fibrosis (58, 59). In addition, a dysfunctional gut microbiota produces various metabolites, such as ethanol, short-chain fatty acids, LPS, bile acids, choline, and ammonia (60). The ethanol produced by *Klebsiella pneumoniae* and *Escherichia coli* under anaerobic conditions can increase the activity of the cytochrome P450 2E1 enzyme, resulting in an increase in reactive oxygen species and free radicals, thus leading to oxidative damage and necrosis of liver cells (61). Meanwhile, the accumulated ethanol also stimulates the NF-κB signal to induce tissue damage by impairing intestinal barrier function, leading to an increased LPS concentration in the portal vein and entry into the enterohepatic circulation. LPS not only stimulates Kupffer cells and hepatic stellate cells and induces steatohepatitis but also promotes the release of TNF-α from hepatocyte macrophages and subsequent insulin resistance, accelerating the development of NAFLD (62). Moreover, studies have found that the differences in bile acids may also affect the dynamics of portal circulation, thus influencing hepatic fat accumulation and the progression of NAFLD (63, 64), while choline deficiency is also associated with the reduced production of very low-density lipoprotein in the liver, which leads to intrahepatic triglyceride accumulation as well as the occurrence of NAFLD (65). In addition, ammonia, a marker of hepatic encephalopathy, is thought to contribute to the pathogenesis of NAFLD through different pathways (66). Hence, strategies targeting the gut microbiota to improve or treat NAFLD will provide novel clues and directions for disease treatment.

According to recent reports, numerous TCMs, including Si Miao Formula [composed of Phellodendron chinense Schneid, Atractylodes lancea (Thunb.), Coix lacryma-jobi L. var. mayuen (Roman) Stapf and Achyranthes bidentata Bl], Jiang Zhi Granules

10.3389/fimmu.2023.1086078

TABLE 1 Research overview of TCMs and NPs targeting gut microbiota for liver disease therapy.

Disease Type	TCM/NP Name	Study Phase	Main Ingredients	Gut Bacterial Alterations	Possible Mechanism	Reference
ALD	Probiotic-fermented Pueraria lobata (Willd.) Ohwi	Preclinical	Polyphenols, Flavonoids, Lactic acids	*Firmicutes/Lactobacillus*↑ *Bacteroidota/Akkermansia*↓	Restore gut microbiota composition, Nrf2 signaling (+), lipid accumulation and inflammation↓, antioxidant defense↑.	(29)
	Costunolide	Preclinical	Costunolide	*Firmicutes/Actinobacteria*↑ *Bacteroidetes/ Proteobacteria*↓	Regulate gut microbiota capacities, LPS-TLR4-NF-κB signaling pathway (-), inflammation and oxidative stress↓.	(30)
	Coprinus comatus polysaccharide	Preclinical	Coprinus comatus, polysaccharide	*Firmicutes/Muribaculacea/ Verrucomicrobia/ Lactobacillus*↑ *Rikenellaceae*↓	Increase gut microbiota diversity, gut epithelium barrier integrity damaged (-), SCFA and hepatic gluconeogenesis, insulin resistance and immune responses↓, hepatic inflammation and oxidative stress↓.	(31)
	Jianpi Liqi Huoxue Decoction	Real World Study	Rhizoma Atractylodis Macrocephatae, Puerariae Lobatae Radix, Radix Paeoniae Alba, etc.	Reverses the abnormal ERIC-PCR fingerprinting of gut microbiota (lacks of specific gut microbials)	Regulates gut microbial abundance/diversity, gut epithelial mucosal permeability↓, endotoxin leakage↓, reduction of fatty liver and liver injury.	(32)
	Semen Hoveniae Extract	Preclinical	Dihydromyricetin, dihydroquercetin, Quercetin, etc.	*Akkermansia/ Verrucomicrobia phylum/ Lactobacillus/Alloprevotella/ Parabacteroides/ Bacteroides*↑, *Helicobacter*↓	Alters gut microbial abundance, gut tight junction proteins↑, gut epithelium barrier integrity damaged (-), gut leakiness and gut-derived endotoxin absorption↓, inflammatory responses↓, attenuates hepatic steatosis and NAFLD.	(33)
	Antarctic Krill Oil	Phase II (NCT02089165)	N-3 PUFAs, Astaxanthin, Phospholipids, etc.	*Clostridium IV/Actinomyces*↑, *Anaerovorax/ Methanobrevibacter/ Psychrobacter*↓	Regulates gut microbial abundance/diversity, regulate BAs metabolism, BA level↓, gut-hepatic FXR/FGF15/ FGFR4 axis (+), intrahepatic cholestasis-induced hepatic injury↓, attenuates hepatic steatosis and NAFLD.	(34)
NAFLD	Si Miao Formula	Real World Study	Phellodendron chinense Schneid, Atractylodes lancea (Thunb.), Coix lacryma-jobi L. var. mayuen (Roman) Stapf, Achyranthes bidentata BI	*Akkermansia muciniphila*↑	Rebalances gut microbiota composition, gut barrier function↑, lipids metabolism and pro-inflammatory cytokines↓, attenuates hepatic steatosis and NAFLD.	(19)
	Jiangzhi Granules	Phase II (ChiCTR2000034583)	Gynostemma pentaphyllum (Thunb.) Makino, Polygonum cuspidatum Sieb. et Zucc., Salvia miltiorrhiza Bunge.,	*S24_7/Lachnospiraceae*↑, *Desulfovibrionaceae*↓	Rebalances gut microbiota composition, lipopolysaccharide biosynthesis and sulfur metabolism	(35)

(Continued)

TABLE 1 Continued

Disease Type	TCM/NP Name	Study Phase	Main Ingredients	Gut Bacterial Alterations	Possible Mechanism	Reference
			Artemisia capillaris Thunb., Nelumbo nucifera Gaert.		pathway↓, hepatic inflammation level and lipid metabolism↓, improves hepatic steatosis, function and insulin resistance, ameliorates NAFLD.	
	Cassiae Semen	Preclinical	Cassiae Semen extract	*Firmicutes/Bacteroidetes/ Dehalobacterium/ Oscillospira/ Coprococcus/Ruminococcus*↑, *Erwinia/Klebsiella/ Morganella/Trabulsiella/ Proteobacteria*↓	Rebalances gut microbiota composition, gut mucosal protein expression↑, gut mucosal barrier injury↓, endogenous endotoxemia and lipid accumulation↓, liver injury and inflammation↓, ameliorates NAFLD.	(36)
	Berberine	Phase II (NCT04049396)	Berberine	Bifidobacterium/ Bacteroidetes/Firmicutes↑	Reconstructs gut microbiota composition, tight junction proteins↑, regulates BA deconjugation, transformation, and gut barrier function↑, bile acid/FXR signaling pathway (+), lipid metabolism/NF-κB activation (-), liver inflammation and oxidative stress↓, ameliorates NAFLD.	(37–39)
	Gynostemma	Phase II (NCT05118698)	Gypenosides	*Relative abundance of Bacteroides*↑, *Relative abundance of Fissicatena/Akkermansia*↓	Enhances gut microbiota diversity, gut microbiota disorder↓, gut/liver lipid metabolism/ insulin resistance↓, gut and liver lesion↓, liver steatosis and lobular inflammation↓, ameliorates NAFLD.	(40)
	MDG-1	Preclinical	Polysaccharide derived from Ophiopogon japonicus	*Akkermansia muciniphila*↑	Increases gut microbial community abundance/diversity, gut barrier function↑, liver lipid accumulation, steatosis and chronic inflammation↓, attenuates NAFLD.	(41)
	Psyllium husk	Preclinical	Psyllium husk	*Sutterella/Faecalibacterium/ Coprobacillus/ Parabacteroides*↑	Alters gut microbial community, bile acid/FXR signaling pathway (+), serum LPS level↓, hepatic lipid metabolism and NAFLD↓.	(42)
AILD	Liquiritin	Preclinical	Liquiritin	*Bacillus* sp. *46/Veillonella* sp. *31 and* sp. *48/Bacteroides* sp. *22 and* sp. *57/ Clostridium* sp. *51*↓	Lack of mechanism exploration.	(43)
	Liushen Capsules	Real World Study	Muschus, Artificial Bezoar	*Bifidobacteria/ Lactobacillus*↑, *Proteobacteria/Veillonella/ Prevotella/Neisseria/ Actinomyces*↓	Lack of mechanism exploration.	(44)

(Continued)

TABLE 1 Continued

Disease Type	TCM/NP Name	Study Phase	Main Ingredients	Gut Bacterial Alterations	Possible Mechanism	Reference
	Chaihu-Shugan Power	Phase II (NCT03018821)	Bupleurum Falcatum	*Anaeroplasma genus*↑, *Enterobacteriaceae/ Staphylococcaceae/ Veillonella genus*↓	Rebalances gut microbiota composition, NLRP3 inflammasome pathway (-), fat accumulation/ inflammatory factor expression↓, chronic metabolic inflammation/AILD/ NAFLD↓.	(45)
	GS	Preclinical	Flavonoids, Saponins, Alkaloids	*Lactobacillus/ Bifidobacterium*↑, *Streptococcus/Escherichia Shigella/Veillonella/ Enterococcus*↓	Rebalances gut microbiota composition, improves gut microenvironment, Nrf2 signaling pathway (+), antioxidant activity↑, ameliorates AILD.	(46)
ALI	Oridonin	Preclinical	Oridonin	*Bacteroides vulgatus*↑	Enriches gut *Bacteroides vulgatus*, *Bacteroides vulgatus*-urea cycle-Nrf2 axis (+) anti-inflammatory and antioxidative effects↑ balances redox homeostasis against APAP-induced ALI.	(21)
	VTE	Phase II (NCT05052515)	Dihydromyricetin, myricetin, kaempferol, etc.	*Eubacterium_fissicatena group/ Ruminococcaceae_UCG-014*↑, *Alistipes/Oscillibacter/ Helicobacter*↓	Decreases gut microbiota abundances, tight junction proteins expression↑, intestinal permeability↑ and gut leaky↓, inflammatory response, oxidative stress and abnormal lipid metabolism↓, ameliorates CCl_4-induced ALI.	(47)
	Zhizichi Decoction	Real World Study	Gardeniae Fructus, Semen Sojae Praeparatum	*Lactobacillus/Romboutsia/ Akkermansia/Prevotella*↑ *Enterococcus/Parasutterella*↓	Rebalances the gut dysbiosis, butyrate and SCFAs production↑, Keap-Nrf2 signaling pathway (+), oxidative stress↓, host defense against ALI↑.	(48)
	Ganshuang Granules	Early Phase 1 (NCT05523648)	Radix Codonopsis, Bupleurum Falcatum, Salviae Miltiorrhiza	*Lactobacillus/Akkermansia*↑, *Allobaculum*↓	Rebalances the gut dysbiosis, intestinal permeability↓, oxidative stress, inflammatory and hepatic fibrosis↓, ameliorates CCl_4-induced ALI.	(49)
LC	Shaoyao Ruangan Mixture	Real World Study	Herba Hedyotidis, Scutellariae Barbatae Herba, Paridis Rhizoma, etc.	*Bacteroides*↓	*Bacteroides abundance*↓, IL-10 levels↓, ameliorates unresectable liver cancer.	(50)
	Jiawei Xiaoyao Powder	Real World Study	Radix Angelicae Sinensis, Radix Bupleurum, Radix Codonopsis Pilosulae, Radix Paeoniae Alba, etc.	*Firmicutes/ Lachnospiraceae*↑, *Bacteroidetes/Proteobacteria/ Bacteroidaceae/Oscillibacter*↓	Regulates gut microbiota Composition and diversity, affects 11 differential metabolites biosynthesis, anti-inflammatory and immunomodulatory effect↑.	(51)
	Panax ginseng					(52)

(Continued)

TABLE 1 Continued

Disease Type	TCM/NP Name	Study Phase	Main Ingredients	Gut Bacterial Alterations	Possible Mechanism	Reference
		Phase 1 (NCT03775837)	Ginsenosides, Golysaccharides	*Coprococcus/ Dehalobacterium/ Anaerotruncus/ Ruminococcus*↑, *Bacteroides/Arthromitus/ Prevotella*↓	Regulates gut microbiota composition and diversity, SCFAs and secondary BAs biosynthesis↑, chronic inflammatory response↓.	
	Safflower yellow	Preclinical	Safflower yellow	*Alloprevotella/ Ruminococcus/ Barnesiella/Bacteroides Ersipelotrichaceae incertae sedis*↓	Regulates gut microbiota composition, $CD8^+$ T-cell and Gr-1^+ macrophage mediated immune suppression↑, TNF-α and NF-κB-mediated inflammation↓, regulates tumor immune microenvironment.	(53)
	Zn (II)-curcumin solid dispersion	Preclinical	Polyvinylpyrrolidone (PVP-k30)-based solid dispersion of Zn (II)-curcumin	*Bacteroidetes/Barnesiella/ Paraprevotella/Prevotella*↑, *Firmicutes/Lachnospiraceae/ Clostridium XIVa/ Oscillibacter*↓	Modulates gut microbiota composition, propionate, SCFA and NKT production↑, liver cancer growth↓, immunotherapy response and efficacy↑.	(54)

AILD, autoimmune liver disease; ALI, acute liver injury; BA, bile acid; CCl_4, carbon tetrachloride; FGF15, fibroblast growth factor 15; FGFR4, fibroblast growth factor receptor 4; FXR, farnesoid x receptor; GS, ginseng and the seeds of Zizyphus jujuba var. spinosa; IL-10, interleukin-10; LC, liver cancer; LPS, lipopolysaccharide; MDG-1, an ophiopogon polysaccharide; N-3 PUFAs, omega-3 polyunsaturated fatty acids; NAFLD, nonalcoholic liver disease; NF-κB, nuclear factor kappa-B; NKT, natural killer T cells; NP, natural product; Nrf2, nuclear transcription factor (erythroid-derived 2)-like 2; SCFA, short-chain fatty acid; TCM, traditional Chinese medicine; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; VTE, Ampelopsis grossedentata.
(+) represents the activation of the signal/signaling pathway; (-) represents the suppression of the signal/signaling pathway; "↑" indicates an increase in content, level, or expression; "↓" indicates a decrease in content, level, or expression.
The therapeutic effects of traditional Chinese medicine (TCM) and natural products (NPs) on liver diseases are expected to be inextricably linked to the targeted regulation of gut microbial dysbiosis. Among them, it has been shown that various TCMs, such as Si Miao Formula, Jianpi Liqi Huoxue Decoction, Zhizichi Decoction, Jiangzhi Granules, Panax ginseng and Gynostemma, can affect multiple liver diseases, including alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), autoimmune liver disease (AILD), acute liver injury (ALI) and liver cancer (LC). Meanwhile, a variety of active ingredients of TCM, such as Berberine, Gynostemma saponin, Ophiopogon polysaccharide MDG-1, Semen hoveniae extract, and Safflower yellow, can also be used to prevent and treat liver diseases by regulating gut microbial dysbiosis. Furthermore, some NPs, such as Vine tea extract, Costunolide, Antarctic krill oil, and Oridonin, may alleviate or mitigate liver disease by inhibiting the gut microbiota and the gut-liver axis and promoting hepatic urea cycling, among other mechanisms. The table highlights the great potential of TCM and NPs targeting the gut microbiota during the treatment of liver disease.

[A TCM prescription consisting of Gynostemma pentaphyllum (Thunb.) Makino, Polygonum cuspidatum Sieb. et Zucc., Salvia miltiorrhiza Bunge., Artemisia capillaris Thunb., and Nelumbo nucifera Gaert.], and Cassiae Semen, as well as NPs, such as Berberine and Gynostemma, have been reported to improve NAFLD by regulating the gut microbiota, which provides a promising strategy and direction for NAFLD research and therapy (19, 35, 41, 67). A study found that the classic TCM formula, Si Miao Formula, can significantly alter the composition of the gut microbiota, specifically increasing the proportion of *Akkermansia muciniphila*, which can regulate the expression of genes involved in fat synthesis (e.g., decrease liver sterol regulatory element binding protein expression) and different inflammatory markers (e.g., decrease the expression of interleukin-1β (IL-1β) and IL-6 and the activity of alanine transaminase and myeloperoxidase) (19). These actions positively affect the intestinal barrier function of mice and hepatic fat metabolism to reverse the formation of NAFLD. When exploring the therapeutic effect of Cassiae Semen on NAFLD, Cassiae Semen significantly increased the abundance of *Firmicutes* and *Bacteroidetes* while reducing the number of *Proteobacteria*, thereby alleviating gut microbiota dysbiosis. The restored gut microbiota increases the expression of tight junction proteins in intestinal mucosa, such as zonula occludens (ZO-1) and occludin-1, which will repair the damaged gut barrier and reduce metabolic endotoxemia, ultimately improving lipid accumulation, liver injury, inflammation and even NAFLD (36). The well-known alkaloid active ingredient of NPs, berberine, was shown not only to restore the abundance of *Bifidobacterium*, *Bacteroidetes* and *Firmicutes* to reconstruct the gut microbiota composition and increase tight junction proteins, thus enhancing gut barrier function to ameliorate liver inflammation and oxidative stress (37, 38). Meanwhile, the restored gut microbiota also regulates bile acid deconjugation and transformation to promote the expression of intestinal farnesoid X receptor (FXR) and fibroblast growth factor 15 (FGF15) and further inhibits lipogenesis and NF-κB activation in the liver, thereby activating bile acid/FXR signaling to improve hepatic lipid metabolism (39). These findings suggest that Berberine regulates gut dysbiosis and may be a valuable strategy for the treatment of NAFLD. Furthermore, MDG-1, an Ophiopogon polysaccharide, was shown to inhibit NAFLD by regulating the abundance of *Akkermansia muciniphila* (41). It was found that PCP can prevent the occurrence of NAFLD, which may be related to the regulation of gut microbiota and inhibition of the NF-κB/CCL3/CCR1 axis (18). In addition to the aforementioned medicines, Jiangzhi Granules and Psyllium husk, as shown in Table 1, also showed good anti-NAFLD effects by modulating the gut microbiota (35, 40, 42).

An increasing number of TCMs and NPs have been found to alleviate NAFLD *in vitro* and *in vivo*, which is inseparable from their regulatory effects on gut microbiota. These findings not only indicate that targeting the gut microbiota is a therapeutic strategy with great potential to improve NAFLD but also provide important references for the treatment of NAFLD with these drugs based on gut microbiota. However, although multiple TCMs and NPs have shown promising therapeutic effects at both the cellular and animal levels, research on their transformation and clinical application is seriously insufficient, which is represented in Table 1. Meanwhile, we have found that most studies depended on 16S rRNA sequencing analysis, but it can provide only limited analytical value for mechanistic studies. Therefore, alternative methods relevant to human disease models and *in vivo* sterile animal model systems, such as metabolomics and macrometabolic transcriptome analysis, need to be further developed to evaluate microbial functions and their effects on host cells and thus explore mechanisms in depth. Additionally, nonhuman primate (NHP) models and animal models obtained from gut stem cell cultures are of great value to examine those hypotheses derived from clinical observations and for the formation of mechanistic and conceptual conclusions, which will accelerate their clinical translation and application. Nevertheless, the relevance of these findings to initial clinical observations must be confirmed.

4 Targeting gut microbiota for AILD

AILD mainly includes autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). It is generally believed that multiple factors, such as heredity, immunity, inflammation, infection, and dysbacteriosis, contribute to the progression of AILD. However, as the complex etiology and pathogenesis of the disease have not yet been fully elucidated, there is currently a lack of specific diagnostic criteria and safe and effective drugs (68). Such a situation urgently requires researchers to conduct in-depth exploration to elucidate the pathogenesis of AILD, identify promising therapeutic targets, and provide a powerful impetus for screening ideal drugs and formulating effective therapeutic strategies.

As shown in Figure 2, various lines of evidence have linked gut microbiota dysbiosis with barrier autoimmunity and beyond, especially in the setting of AILD (69, 70). The detection of 16S rDNA sequencing in AIH patients showed that the abundance of *Enterococcus gallinarum* in liver tissue was significantly higher than that in healthy individuals. Moreover, the number of *Bifidobacterium* and *Lactobacillus* in their feces was significantly reduced, which induced a decrease in the proportion of *Bifidobacterium* to *Enterococcus* (71). These alterations lead to intestinal mucosal damage and increased blood endotoxin levels in patients, thus inducing the immune tolerance damage mechanism and exacerbating liver injury in AIH (72). Meanwhile, liver injury of concanavalin A (ConA)-induced AIH can be significantly alleviated, suggesting that the targeted regulation of gut microbiota is beneficial to AIH therapy. Moreover, a prospective randomized controlled clinical study found that the diversity of gut microbiota in PBC patients was significantly lower than that of healthy controls (P = 0.03), and 6 months of ursodeoxycholic acid (UDCA) treatment significantly increased the diversity and abundance of gut microbiota, thereby alleviating PBC progression, which indicates a nonnegligible role for modulating gut microbiota in PBC treatment (73). Furthermore, a study on PSC found a unique correlation between gut microbiota and bile acid, which may be involved in the pathogenesis of PSC by affecting bile acid metabolism and the gut microenvironment; meanwhile, the number of *Veillonella* genera increases with the severity of PSC, suggesting the important role of gut microbiota and its metabolites in the prevention and treatment of PSC (74, 75). These results emphasize the nonnegligible role of gut microbiota dysbiosis in the occurrence and progression of AILD through different pathways or mechanisms, indicating that targeted regulation of the abundance and diversity of gut microbiota may provide new directions and clues for future research and treatment of the disease.

In view of the unignorable or noticeable roles of various gut microbiota in the progression of AILD, it is of great significance to explore TCMs and NPs targeting gut microbiota for AILD research and treatment, as shown in Table 1. Liquiritin, the active ingredient extracted from licorice, has been found to significantly inhibit the growth of multiple pathogenic bacteria, such as *Bacillus* sp. *46*, *Veillonella* sp. *31* and sp. *48*, *Bacteroides* sp. *22* and sp. *57* and *Clostridium* sp. *51*, while it has little impact on the growth of commensal probiotics (such as *Lactobacillus* and *Bifidobacterium*), which provides valuable evidence for the potential activity of this herb against gut microbiota during AILD treatment (43). A Chinese medicine called "Liushen Capsule", produced by Lei Yun Shang Pharmaceutical Group Co., Ltd. with Muschus and Artificial Bezoar as the main ingredients was found to significantly alter the diversity and distribution of gut microbiota in healthy volunteers. Specifically, Liushen Capsule significantly increases the abundance of intestinal anaerobic bacteria (such as *Bifidobacterium* and *Lactobacillus*) while reducing the abundance of some intestinal opportunistic pathogenic microbiota (such as *Proteus*, *Veillonella*, *Prevotella*, *Neisseria* and *Actinomyces*) (44). Meanwhile, both *Bifidobacterium* and *Lactobacillus* were found to be significantly reduced in multiple types of AILD, while opportunistic pathogens were significantly elevated. Thus, Liushen Capsule may be considered a promising drug for the treatment of AILD by targeting the regulation of gut microbiota. In exploring the therapeutic efficacy of Chaihu-Shugan Power, a TCM with Bupleurum Falcatum as its main ingredient, in the treatment of NAFLD. Research showed that the drug significantly reduced the abundance of multiple opportunistic pathogenic bacteria, such as the *Enterobacteriaceae*, *Staphylococcaceae* and *Veillonella* genera, and increased the abundance of the *Anaeroplasma* genus, which shows the potential of Chaihu-Shugan Power targeting gut microbiota dysbiosis during the treatment of AILD (45). In addition, the extract of ginseng and the seeds of Zizyphus jujuba var. spinosa (GS) significantly increased the relative abundance of *Lactobacillus* and *Bifidobacterium* and decreased *Streptococcus*, Escherich*ia coli-Shigella*, *Veillonella* and *Enterococcus* in rats, suggesting that GS extract may be a promising AILD therapeutic drug by balancing the structure and diversity of gut microbiota (46).

Presently, research on gut microbiota-focused AILD treatment has progressed but has mainly concentrated on the exploration of antibiotic applications. Therefore, targeting the gut microbiota to explore novel and promising AILD therapeutic strategies remains an

urgent clinical issue. Notably, research on the regulation of gut microbiota dysbiosis in diseases by TCMs and NPs is well underway, which will provide a novel direction for targeting the gut microbiota to explore promising drugs and potential therapeutic strategies for AILD. Even though it is highly expected, the existing studies have the following inadequacies: 1. More studies are still at the efficacy observation stage and fail to address the in-depth mechanism; 2. Research results are more based on the exploration of fecal 16S rDNA sequencing rather than on the gut microbiota. However, the composition or function of the fecal microbiome is different from that of the gut microbiota, which should be taken into account. Therefore, further investigations should be designed to clarify the specific types and targets of bacteria located in the gut, to elucidate the detailed mechanisms by which the gut microbiota regulates AILD and to provide an impetus for large-scale screening and clinical studies of promising TCMs and NPs.

5 Targeting gut microbiota for acute liver injury

Various factors, such as drugs, toxicants, and viral infections, produce hepatotoxicity and lead to ALI, which severely damages liver function and inevitably damages human health (76). Prior surveys have shown that ALI frequently occurs year by year and is directly responsible for approximately 3% of global mortality (77, 78). Therefore, preventing and eliminating ALI has become an urgent issue to be solved globally. At present, epigenetics, oxidative stress, inflammatory immunity and other pathological mechanisms have been confirmed to be widely involved in the abnormal activities of hepatocytes and inflammatory immune cells and metabolism-induced ALI, and promising targets and potential therapeutic strategies have also been hotly discussed (79). Meanwhile, the role of gut microbiota in ALI has become increasingly prominent and has gradually attracted attention (80). However, the contribution of the existing studies on the gut microbiota to ALI has not been well characterized, which directly leads to the ineffectiveness of strategies targeting the gut microbiota for disease therapy. In view of this, in-depth exploration of the impact of gut microbiota on ALI will provide new ideas and directions for the treatment of such diseases.

Drug hepatotoxicity is the major cause of clinical ALI in many countries, among which acetaminophen (APAP) is widely studied. 16S rRNA sequencing showed that excessive APAP significantly changed the composition and diversity of gut microbiota, including increasing the ratio of *Firmicutes*/*Bacteroidetes* and reducing the abundance of *Proteobacteria*, *Roseburia*, *Lactobacillus*, *Akkermansia muciniphila* and *Saccharomyces cerevisiae* (81). The increase in the proportion of *Firmicutes*/*Bacteroidetes* exacerbates liver inflammation and immune disorders, while the decrease in the abundance of *Saccharomyces cerevisiae* leads to the accumulation of the gut microbial metabolite 1-phenyl-1,2-propanedione, which participates in APAP-induced ALI by depleting hepatic glutathione levels (82). The reduction in the proportion of gut *Lactobacillus* promotes oxidative stress and inflammatory responses (83), while *Akkermansia muciniphila* has been shown to modulate immune and metabolic functions (84). In addition, ALI caused by commonly used medications, such as tacrine and diclofenac, has also been proven to be closely related to gut microbial dysbiosis (85, 86). These results indicate that gut microbial dysbiosis is an important factor in promoting drug-induced hepatotoxicity and even ALI. Carbon tetrachloride (CCl_4) is one of the most common toxicants causing ALI. Integrating 16S rRNA sequencing and LC–MS metabonomic analysis, CCl_4 caused the dysbiosis of 32 specific gut microbes in 10 phyla, such as significantly reduced levels of *Firmicutes*, *Clostridiales* and *Lactobacillus* and an increased percentage of *Bacteroides*. The reduction in the abundance of gut *Lactobacillus* promotes CCl_4-induced liver oxidative stress and the inflammatory response (87), while the elevated ratio of *Firmicutes*/*Bacteroidetes* aggravates liver inflammation and immune disorders75. Meanwhile, *Clostridiales* is significantly positively correlated with 3-hydroxybutyric acid, which has been proven to reduce inflammation and liver injury (88). Moreover, ALI caused by toxicants, such as D-galactosamine and cisplatin, has also been proven to be closely related to gut microbial dysbiosis (82, 86). The aforementioned findings suggest that gut microbial dysbiosis accelerates toxicant-induced ALI (Figure 2). Based on these studies, it is of great clinical value and practical significance to target gut microbes to reduce hepatotoxicity and ALI caused by various factors.

In view of the multicomponent, multitarget, multipath and mild effects of TCMs and NPs, their rational use has greater advantages in reducing liver toxicity and even treating ALI. Recently, many studies have demonstrated the great potential of such drugs in this field, which is inseparable from their targeted regulation of gut microbiota, as shown in Table 1. Oridonin, a liver protective agent derived from *Rabdosia rubescens*, is believed to reduce APAP-induced hepatotoxicity and ALI by regulating the *Bacteroides vulgatus*-urea cycle-Nrf2 axis (21). A study on the extract of Ampelopsis grossedentata (VTE) indicated that it could alleviate CCl_4-induced hepatotoxicity and ALI by restoring gut microbiota dysbiosis in mice. Specifically, VTE significantly reduced the content of harmful gut microbiota, such as *Helicobacter* and *Oscillibacter*, and increased the abundance of beneficial gut microbiota, such as *Ruminococcaceae_UCG-014* and *Eubacterium_fissicatena*_group. The gradually restored gut microbiota not only reduces liver inflammation and oxidative stress but also enhances the intestinal barrier by promoting the expression of zonula ZO-1, Occludin-1, and Mucin-1 in intestinal tissues and ultimately achieves the effect of reducing hepatotoxicity and ALI (47). When the gut microbiota was depleted, the disappearance of VTE efficacy verified its targeted regulatory mechanism on the gut microbiota. A TCM composed of Gardeniae Fructus and Semen Sojae Praeparatum, named Zhizichi Decoction, was found to reduce liver injury by regulating the gut microbiota population, promoting butyric acid production and activating the antioxidant reaction (48). In addition, Ganshuang Granules [composed of Chinese herbs such as Radix Codonopsis, Bupleurum Falcatum and Salviae Miltiorrhiza] can rebalance the gut microbiota and reduce intestinal permeability, thereby reducing oxidative stress and inflammation and ultimately ameliorating CCl_4-induced hepatotoxicity and ALI (49). At present, the potential roles of these agents based on gut microbiota in alleviating hepatotoxicity and ALI have been constantly explored, which not only provides a promising target for the prevention and treatment of the disease but also deepens

the awareness of the important research value of TCMs and NPs in disease treatment. More importantly, it offers a novel idea and direction for the establishment of hepatotoxicity and ALI therapeutic strategies by targeting gut microbiota dysbiosis.

Although such medicines targeting gut microbiota dysbiosis have great potential in the prevention and treatment of hepatotoxicity and ALI, there are still some deficiencies and challenges, including the following: 1. Most of the existing studies focus on preset animal experiments while ignoring the characteristics of hepatotoxicity and ALI, which are difficult to observe at the early stage and are progressing rapidly. In other words, it is a huge challenge to consider both mild effects and rapid effects. 2. Hepatotoxicity and ALI are heterogeneous due to different influencing factors, which forces us to choose broad-spectrum or specific treatments. 3. How to avoid the hepatotoxicity of some drugs with different properties while paying attention to their liver protective effect. The above issues provide a direction for subsequent research. Regardless of how difficult the road ahead is, therapeutic strategies based on gut microbiota to regulate hepatotoxicity and ALI will be further developed in the future with the continuous deepening of basic research and the extensive development of clinical research, and the role of these potential drugs will be more clearly clarified.

6 Targeting gut microbiota for liver cancer

Liver cancer is one of the most common malignant tumors in the world, and its morbidity and mortality increase each year (89). Although surgical ablation combined with novel targeted drugs, such as sorafenib and atezolizumab, has brought some light to liver cancer patients, the limited types and efficacy of drugs still cannot meet the urgent clinical needs since the pathogenesis of the disease is complex and has not yet been clarified, and most patients are diagnosed at an advanced stage (90). Therefore, it is urgent to elucidate the pathogenesis, explore more potential targets, discover promising drugs, and establish effective targeted therapy strategies for liver cancer treatment.

During the process of exploring potential mechanisms and promising targets, multiple studies have focused on the indispensable role of gut microbiota dysbiosis in promoting liver cancer progression (Figure 2). Based on the fecal gut microbiome analysis of HCC patients, it was found that the diversity and abundance of gut microbiota were significantly abnormal, mainly manifested in the increase in *Bacteroides* and *Ruminococcus* and the decrease in *Bifidobacterium* (91). In contrast to patients with liver cirrhosis, early-stage HCC patients had more intestinal *Actinobacteria*, *Bacteroides*, *Klebsiella* and *Haemophilus*, while *Verrucomicrobia*, *Alistipes*, *Phascolarctobacterium* and *Ruminococcus* decreased significantly (92). These differences indicated that gut microbiota diversity may be a noninvasive biomarker of HCC and demonstrate an integral role in HCC development. Meanwhile, integrating the microbiome and transcriptome found that *Bacteroidetes*, *Lachnospiracea incertaesedis* and *Clostridium XIVa* were enriched in HCC patients, and their changes in the tumor immune microenvironment through serum bile acids may be important factors associated with liver cancer burden and poor clinical outcomes (93). Gut microbiota omics analysis partially explains the pathogenesis of liver cancer and shows the potential to predict its clinical outcome. Subsequently, *C. scindens* and other *Clostridium enterica* species were shown to utilize bile acid as a messenger to control the accumulation of chemokine-dependent hepatic NKT cells and antitumor immunity in the liver, thus protecting against both primary and metastatic liver cancer (11). This study establishes the relationship between gut microbes, their metabolites and liver cancer, which provides new ideas for future liver cancer treatment. Based on the aforementioned explorations, the gut microbiota should be widely recognized as a valuable and potential therapeutic target in the process of liver cancer research and treatment, and corresponding targeted drug screening, research and the establishment of therapeutic strategies should be emphasized.

TCM and NPs have attracted increasing attention in the treatment of liver cancer, which is inseparable from their targeted modulation of gut microbiota (Table 1). In the therapeutic exploration of primary liver cancer (PLC), a TCM preparation produced by Zhejiang Cancer Hospital called Shaoyao Ruangan Mixture (SRM) [composed of 19 Chinese herbs including Herba Hedyotidis, Scutellariae Barbatae Herba, Paridis Rhizoma, Tetrastigma hemsleyanum Diels et Gilg, etc.], could significantly reduce the abundance of Bacteroides in the intestine, which was positively correlated with elevated IL-10 levels and liver cancer development (50). SRM modulates *Bacteroides* to treat PLC, providing an important reference for targeting gut microbiota by TCMs for liver cancer treatment. Meanwhile, fecal microbiology combined with 16S rDNA analysis showed that Jiawei Xiaoyao Powder [consisting of Chinese herbs such as Radix Angelicae Sinensis, Radix Bupleurum, Radix Codonopsis Pilosulae, Radix Paeoniae Alba and Radix Paeoniae Lactiflora] significantly altered the composition of gut microbiota and affected the biosynthesis of 11 differential metabolites, such as primary bile acids and interferon-γ, in liver cancer rats, thus achieving the goal of adjuvant therapy for liver cancer (51). This study provides favorable evidence that TCMs and NPs target the gut microbiota for the treatment of liver cancer. In addition, various TCMs and NPs, such as Panax ginseng (52), Safflower yellow (53) and Zn(II)-curcumin solid dispersion (54), showed valuable therapeutic effects on liver cancer, which is inseparable from their targeted modulation of gut microbiota. Based on these findings, TCMs and NPs targeting gut microbiota are promising therapeutic strategies for liver cancer, while in-depth mechanistic and translational studies need to be further explored.

Currently, research on TCMs and NPs targeting gut microbiota for the treatment of liver cancer is in full swing, and the contribution of multiomics studies with high-throughput screening is outstanding. However, the existing studies still have many limitations, mainly including the following: 1. Most studies have focused on exploring the effects of these medicines on the gut microbiota *in vitro*, ignoring the complexity of the real environment of the organism; 2. Most studies have focused on the changes in gut microbiota after the inhibitory effects of those agents on liver cancer but neglected direct evidence of their targeting of gut microbiota; 3. Insufficient sample size directly led to different individuals showing high variability in the composition and abundance of gut microbiota, which limited the

generalizability of conclusions regarding gut microbiota. Based on the present situation, subsequent studies should focus on 1. In-depth exploration of pharmacological mechanisms; 2. Research targeting specific gut microbiota; 3. Larger sample sizes are needed to overcome such variability and draw meaningful conclusions; 4. Exploration oriented to the metabolic processes and metabolites of TCMs and NPs.

7 Concluding remarks and perspectives

The gut microbiota is essential for maintaining body metabolism and health, while dysbiosis plays a vital role during the occurrence and progression of various diseases, including liver disease (94, 95). Therefore, regulating the gut microbiota to maintain it in a relatively stable state, including gut microbiota diversity, distribution and metabolic stability, has great potential and clinical research value in the treatment of various types of liver diseases. Meanwhile, we should realize that the current research mostly emphasizes the correlation between gut microbiota dysbiosis and disease, as well as disease outcome and gut microbiota alterations, which is far from sufficient. The importance of the gut microbiota is increasingly prominent. However, we must recognize that current research has emphasized the correlation between gut microbiota dysbiosis and disease and the correlation between disease outcome and gut microbiota alteration, which is insufficient. Hence, future research should give more attention to 1. Exploring in depth the detailed mechanisms by which gut microbiota dysbiosis directly contributes to disease pathogenesis; 2. How to develop precision medicine by accurately regulating gut microbiota to implement disease-specific treatments. Based on these findings, it is first necessary to perform in-depth information exploration of bioinformatics resources such as gut microbiomics and metabolomics. Subsequently, the acquired precise information will be explored and validated mechanistically in specific cellular and organoid models. Eventually, the clinical effects will be explored through bacterial colonization and the achievements will be translated. These lines of thought are quite helpful for establishing therapeutic strategies targeting the gut microbiota.

TCMs and NPs provide a huge source for the research and discovery of new drugs by virtue of their obvious anti-inflammatory, antioxidant, liver protective and other effects and have attracted widespread attention. As reported by many researchers, such drugs have played a beneficial role in regulating liver diseases such as ALD, NAFLD, AILD, hepatotoxicity, and liver cancer, which are inseparable from the regulation of gut microbiota dysbiosis (96). However, the majority of existing studies remain at the stage of preliminary pharmacodynamic validation, which makes the development of such drugs and the establishment of therapeutic strategies inadequate. Therefore, future research should emphasize the following: 1. To adequately integrate technical solutions such as artificial intelligence, omics and high-throughput screening to screen and expand the fingerprint profiles and databases of TCMs and NPs to provide resources for the discovery of more drugs; 2. To develop diverse therapeutic routes to meet the broad spectrum of TCMs and NPs to regulate gut microbiota for disease treatment; 3. To advance the isolation techniques of TCMs and NPs together with the improvement of formulation technology and preparation procedures to provide the basis for targeted regulation of gut microbiota therapy rather than wide scattering to cure diseases.

Author contributions

W-JN, MC and H-PL made substantial contributions to the conception or design of the work. L-RZ, S-SL and W-QZ contributed to the acquisition, analysis, and interpretation of literature for the work. L-RZ, W-JN, MC and H-PL drafted the work or revised it critically for important intellectual content. W-JN, MC and H-PL final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81803602, 82204805), the Natural Science Foundation of Anhui Province (No. 1708085QH207), the Fundamental Research Funds for the Central Universities (No. WK9110000018), the Research Foundation of Anhui Medical University (No. 2022xkj113) and the Research Foundation of Anhui Children's Hospital (No. 21etyy003).

Acknowledgments

We thank American Journal Experts (AJE) for their guidance on the grammar and language of this article.

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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frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 08 March 2023
DOI 10.3389/fimmu.2023.1080043

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 25 October 2022
ACCEPTED 13 February 2023
PUBLISHED 08 March 2023

CITATION
Ancona G, Alagna L, Alteri C, Palomba E, Tonizzo A, Pastena A, Muscatello A, Gori A and Bandera A (2023) Gut and airway microbiota dysbiosis and their role in COVID-19 and long-COVID.
Front. Immunol. 14:1080043.
doi: 10.3389/fimmu.2023.1080043

Gut and airway microbiota dysbiosis and their role in COVID-19 and long-COVID

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The gut microbiota plays a crucial role in human health and disease. Gut dysbiosis is known to be associated with increased susceptibility to respiratory diseases and modifications in the immune response and homeostasis of the lungs (the so-called gut-lung axis). Furthermore, recent studies have highlighted the possible role of dysbiosis in neurological disturbances, introducing the notion of the "gut-brain axis." During the last 2 years, several studies have described the presence of gut dysbiosis during coronavirus disease 2019 (COVID-19) and its relationship with disease severity, SARS-CoV-2 gastrointestinal replication, and immune inflammation. Moreover, the possible persistence of gut dysbiosis after disease resolution may be linked to long-COVID syndrome and particularly to its neurological manifestations. We reviewed recent evidence on the association between dysbiosis and COVID-19, investigating the possible epidemiologic confounding factors like age, location, sex, sample size, the severity of disease, comorbidities, therapy, and vaccination status on gut and airway microbial dysbiosis in selected studies on both COVID-19 and long-COVID. Moreover, we analyzed the confounding factors strictly related to microbiota, specifically diet investigation and previous use of antibiotics/probiotics, and the methodology used to study the microbiota (α- and β-diversity parameters and relative abundance tools). Of note, only a few studies focused on longitudinal analyses, especially for long-term observation in long-COVID. Lastly, there is a lack of knowledge regarding the role of microbiota transplantation and other therapeutic approaches and their possible impact on disease progression and severity. Preliminary data seem to suggest that gut and airway dysbiosis might play a role in COVID-19 and in long-COVID neurological symptoms. Indeed, the development and interpretation of these data could have important implications for future preventive and therapeutic strategies.

KEYWORDS

microbiota, microbiome, gut-brain-axis, gut-lung-axis, dysbiosis, COVID-19, long Covid, SARS-CoV-2

1 Introduction

1.1 COVID-19, long-COVID, and gastrointestinal disease during SARS-CoV-2 infection

Coronavirus disease 2019 (COVID-19) is a highly contagious infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, a novel RNA beta-coronavirus, with more than 663 million cases and 6.71 million deaths worldwide documented until 20 January 2023 (1). COVID-19 is mainly a respiratory illness, ranging from asymptomatic, mild-moderate, severe, and critical illness (2), especially affecting elderly subjects with underlying medical conditions (3).

After COVID-19, some patients may experience persistent symptoms or other conditions that are colloquially referred to as long-COVID. The *Centers for Disease Control and Prevention* have defined post-COVID conditions as new, returning, or ongoing symptoms that people experience ≥4 weeks after being infected with SARS-CoV-2 (4). The prevalence of these conditions varies widely from 5% to 80%, and the most frequently reported symptoms are fatigue, cough, shortness of breath, and chest pain (2, 5). Furthermore, half of the patients report persistent neurological symptoms at 6 months, the most frequent being "brain fog" and cognitive changes, described in up to one-third of subjects (6).

With regard to the gastrointestinal (GI) tract involvement, early reports from Wuhan showed that 2% to 10% of patients with acute COVID-19 had GI symptoms including nausea and diarrhea (7), but more recent metaanalyses reported a higher prevalence, up to 20% of patients (8). SARS-CoV-2 virus has been detected in anal swabs and stool samples in almost 50% of patients with COVID-19, suggesting that the digestive tract might be an extrapulmonary site for virus replication and activity (9), through ACE2 receptors binding with spike protein-S.

1.2 Gut microbiota and its role in health and disease

The human gut microbiota harbors up to 10^{14} resident microorganisms, including bacteria, archaea, viruses, fungi, and other eucaryotes, with bacteria being the most abundant microorganisms at the gut level. The most represented phyla at the gut level are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, and *Fusobacteria* (10). An increase in bacteria has been documented from duodenum to colon, with a decrease in facultative anaerobic *Bacilli* (*Firmicutes*) and Enterobacterales (*Proteobacteria*) taxa and an increase in obligate anaerobic bacteria, especially *Bacteroidia* (*Bacteroidetes*) and *Clostridia* (*Firmicutes*) classes (11, 12).

Gut microbiota is crucial for several functions, such as energy extraction from the diet, vitamin and short-chain fat acids (SCFAs) production, and immunomodulation, with the regulation of TH17 and T reg balance (13–15). A complex equilibrium exists among prebiotics, like microbiota accessible carbohydrates (MAC), probiotics, and postbiotics, like their products, SCFAs (16, 17), with involvement of several networks between gut microbiota and other body sites through axes (i.e., gut-lung, gut-liver, gut-brain axis), influencing processes in health and disease.

An unbalance of the crucial homeostasis between *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* phyla (Figure 1) is often associated with a change in the numbers of microbes and/or diversity of the microbiota; such a condition is defined as dysbiosis (18). Recently, a new definition of dysbiosis has been suggested, based on a model represented in several diseases, defined by the increase in facultative anaerobic bacteria, like *Bacilli* class and Enterobacterales order, and a parallel decrease in obligate anaerobic bacteria, such as propionate and butyrate-producing bacteria (BPBs) (11).

Gut microbiota dysbiosis can have a role in several disease models affecting the lung, brain, liver, and heart (19).

In the last decade, research on lung microbiota and its pathogenetic link to pulmonary conditions has significantly improved. Previously, the lung has been considered a sterile organ; however, numerous studies have demonstrated the presence of bacterial DNA in the lower respiratory tract in healthy individuals. The lung microbiota of healthy subjects is characterized by the presence of differentiated ecological niches belonging to *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla and *Prevotella*, *Streptococcus*, *Veillonella*, *Fusobacterium*, and *Haemophilus* genera (20). Its balance is the result of acquisition and clearance (Figure 1). Many other factors contribute to this complex mechanism, such as the immune system (innate and adaptive immune recognition, secretory IgA), in addition to various exogenous components such as diet, environmental biodiversity, and drug treatments, in particular antibiotics (21).

Chronic respiratory diseases are often characterized by an imbalance between microbial immigration and elimination in the lung. Moreover, the presence of chronic inflammation results in the alteration of physicochemical proprieties that facilitate the growth of select species in the microbial community, such as microorganisms from the *Proteobacteria* phylum, that are linked to a proinflammatory state (22). It is important to emphasize that lung and gut microbiota are in close communication with each other through the circulation of soluble metabolites (i.e., peptidoglycan or LPS) transported by the blood (21). These peptides are recognized by host cells that express pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs). The interplay between lung and gut microbiota, defined as the gut-lung axis, has been demonstrated in different animal models (23–26).

Further studies are needed to better understand the complex gut-lung interplay and characterize the gut microbial metabolites (i.e., indole derivative, niacin, polyamines, urolithin, and pyruvic acid) that act as immunomodulants and might have a possible impact on respiratory health (27, 28).

Another captivating field of microbiota studies is related to its connection with the brain through the so-called gut-brain axis, which is thought to be a bidirectional system. On one side, there is the involvement of microbiota-derived metabolites on the blood–brain barrier like SCFAs, tryptophan, and linoleic acid metabolites

10.3389/fimmu.2023.1080043

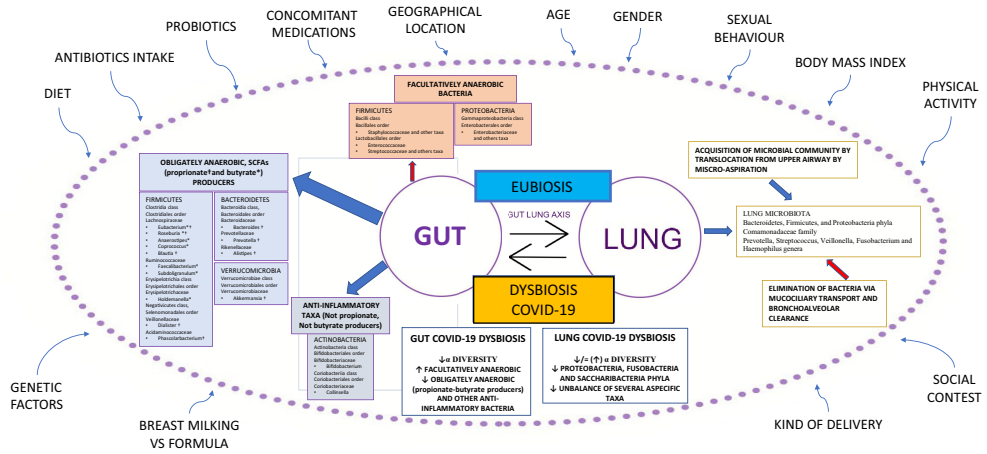

FIGURE 1
Gut-lung axis microbiota in COVID-19. This figure shows a summary of the gut-lung axis and its alterations during COVID-19. Left: the gut microbiota taxa obligately anaerobic short-chain fatty acids (propionate and butyrate) producers and anti-inflammatory taxa, not propionate and butyrate producers. Upper: facultatively anaerobic bacteria. Right: the homeostasis of the lung microbiota, resulting from acquisition (blue arrow) and elimination (red arrow) clearance. Bottom: the most significant alterations detected in gut and lung microbiota during COVID-19. All around, the confounding factors are strictly related to Microbiota. BMI: Body Mass Index; ° indicates propionate-producing bacteria, * indicates butyrate-producing bacteria, Upward arrows "↑": increase; Downward arrows "↓": decrease.

as well as cytokines produced at the gut level; on the other side, the brain controls gut activity through the neuroendocrine and parasympathetic systems (i.e., regulation of intestinal permeability through the vagus nerve) (29). Such connections have been studied in animal models: physiological aging affects gut microbiota in mouse models through cognitive frailty (30).

Gut microbiota dysbiosis seems to play a role in several neurodegenerative and psychiatric disorders (31), as well as in other neurological conditions (32). For example, damage to the GI barrier is a possible pathological pattern for depression disorders; moreover, increased LPS and microbiota-cytokine production seems to be related to Alzheimer's disease (29).

The relationship between gut microbiota and the brain could be deeper and more complex: alteration of the hypothalamic "master clock" could impact the diurnal environmental fluctuations and lead to dysbiosis-related metabolic disorders like obesity and/or diabetes (33). Furthermore, gut dysbiosis could determine sleep disturbances (sleep loss, alteration of circadian rhythm), eventually leading to fatigue (34). Following this hypothesis, the gut microbiota, which is mostly influenced by diet, could represent a link between the immune and endocrine systems through brain function and the host metabolism (35).

High-fat food intake can indeed damage the GI barrier, affecting both the "intestinal epithelial barrier" (characterized by the mucus layer and the epithelial cells) (36) and the "gut vascular barrier," regulated by the expression of plasmalemma vesicle-associated protein-1 (PV1). This condition, known as "leaky gut," can favor microbial translocation to the liver (37), leading to hepatic and systemic disease.

Finally, another example of the role of dysbiosis in disease has been studied in the cardiological setting, where the increased production of trimethylamine (and its metabolite-liver trimethylamine-N-oxide) by gut microbiota has been linked to the development of cardiovascular disease (29).

2 Gut microbiota dysbiosis in acute COVID-19

2.1 Study characteristics and confounding factors

We identified 22 studies on gut microbiota in COVID-19 patients published in a 2-year window period between 03 January 2020 and 03 January 2022 (Table 1A).

To critically revise the studies, we first considered all the variables potentially influencing the final observations: study design, location, material source, microbial technology used, sample size, and patient characteristics—age, body mass index (BMI), gender, sexual behaviors, COVID-19 severity index, comorbidities, recent previous use of antibiotics/probiotics, diet, and lifestyle.

The cross-sectional study design was the most common. Less than half of studies (45%) had a longitudinal/prospective design, 20% of which focused on long-COVID-19.

The study location was a critical factor: most studies (19/22, 86%) were set in Asia (18 in China, one in South Korea), and three of 22 (14%) in Europe; no other geographic regions were represented.

TABLE 1A Selected studies on gut microbiota and COVID-19.

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
Yu et al. (38)	China	Cross-sectional study Anal swab Nanopore-targeted sequencing technology	-2 hospitalized patients are "critical" -Age: 65 and 78 years old -Men: 100% -BMI: no data During ABT and antiviral ongoing lesser comorbidities	No data	**COVID-19 vs. controls** **Relative abundance comparison** ↑ *Actinobacteria* and *Firmicutes* phyla ↑ *Corynebacterium* and *Ruthenibacterium* genera ↓ *Bifidobacterium*, *Lactobacillus*, and *Eubacterium*	The immunologic decline for both patients First report with a link between immune disorders and gut microbiota
Tang et al. (39)	China	Cross-sectional study Stool samples qPCR 10 taxa	-57 hospitalized patients (general, 20 patients; severe, 19 patients; critical disease, 18 patients) -Age (median): 59, 66, 68 -Men: 40%, 47%, 66% -BMI: no data A total of 50.9%, 5.3%, and 12.3% of patients received antibiotics, antifungal drugs, and probiotics, respectively Many patients have more comorbidities especially in critical (hypertension) ones	No data	**Comparison among COVID-19 subgroups** **Relative abundance comparison** ↑ In *Enterococcus* genus/*Enterobacteriaceae* family ratio Ec/E ratio (in critical patients) ↓ *Bifidobacterium*, *Lactobacillus*, *Faecalibacterium prausnitzii*, *Clostridium butyricum*, *Clostridium leptum*, and *Eubaterium rectale*	Correlation between butyrate-producing bacteria (BPBs) and inflammatory markers (PCR, WBC, lymphocyte ratio, neutrophil ratio, IL-6) The first study used the Ec/E ratio to predict death in critically ill patients. The reduction of *Enterobacteriaceae* could be explained using antimicrobial agents active vs. gram-negative.
Zuo et al. (40)	China	Cross-sectional study (15 patients) Prospective study subgroup (5/15) Fecal samples Shotgun metagenomic sequencing	-15 hospitalized patients (2 mild, 8 moderate, 3 severe, 2 critical COVID-19) -Age (median): 55 -Men: 46.6% -BMI: no data 6/15 patient comorbidities (46.7% had stool positivity for SARS-CoV-2. Only 1 patient had diarrhea at presentation)	No data	**Relative abundance comparison** **Patients with high SARS-CoV-2 infectivity:** ↑ *Collinsella aerofaciens*, *Collinsella tanakaei*, *Streptococcus infantis*, *Morganella morganii* **Patients with low-to-none SARS-CoV-2 infectivity:** ↑ *Parabacteroides merdae*, *Bacteroides stercoris*, *Alistipes onderdonkii*, and *Lachnospiraceae bacterium* **Longitudinal arm**: all patients showed substantial variations in fecal microbiome composition regardless of the presence of fecal viral infectivity (confirmed by the longitudinal subgroup)	Patients with low-to-none SARS-COV-2 intestinal replications had a higher abundance of SCFA-producing bacteria Patients with high viral intestinal infectivity have shown an abundance of opportunistic pathogens and higher functional pathways involved in nucleotide metabolism, carbohydrate metabolism, and amino acid biosynthesis
Gu et al. (41)	China	Cross-sectional study Fecal samples V3–V4 of the 16S rRNA gene	-30 hospitalized patients with COVID-19 (15 general, 15 severe COVID-19) 24 hospitalized patients with H1N1	**α-Diversity** ↓ Shannon diversity Index ↓ Chao-1 diversity Index in COVID-19 and H1N1 patients compared to healthy controls **β-Diversity**	**LEfSe analysis** **COVID-19 subgroup vs. controls** ↓ *Ruminococcaceae* family, *Fusicatenibacter*, *Anaerostipes*, *Agathobacter*, unclassified *Lachnospiraceae*, and *Eubacterium halli* belong to the *Lacnospiraceae* family)	COVID-19-related genera: *Streptococcus*, *Rothia*, *Veillonella*, *Erysipelatoclostridium*, and *Actinomyces* Control group-related genera: *Romboustia*, *Faecalibacterium*, *Fusicatenibacter*, and *Eubacterium halli* group

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			30 matched healthy controls -Age (median): 55 vs. 53 -Men: 56% vs. 56% -BMI: 24.6 vs. 22.9 In the COVID-19 group, 33.3% had at least 1 coexisting medical condition; hypertension (30.0%); 16.7% patients with diarrhea All subjects who received antibiotics, probiotics, or both within 4 weeks before enrollment were excluded.	No differences between general and severe COVID-19 patients β-Diversity separation according to Bray–Curtis between COVID-19 and controls (and H1N1 subgroup) No β-diversity separation according to the severity index	↑ *Streptococcus* genus No differences between general and severe COVID-19 patients	*Agathobacter*, *Fusicatenibacter*, *Roseburia*, and *Ruminococcaceae* −Correlated with CRP, PCT, or D-dimer levels CRP and D-dimer levels + correlated with COVID-19-enriched bacteria Significant depletion of BPB in the COVID-19 cohort
Tao et al. (42)	China	Cross-sectional Fecal samples V4 of the 16S rRNA gene	-26 patients COVID-19 33 influenza patients 40 controls Clinical information not shown	**α-Diversity** ↓Chao-1 **β-Diversity** The Unifrac-weighted separation between COVID-19 and controls	**LEfSe analysis** **COVID-19 vs. controls** ↑ *Streptococcus*, *Clostridium*, *Lactobacillus*, and *Bifidobacterium* genera ↓ *Bacteroides*, *Roseburia*, *Faecalibacterium Coprococcus*, and *Parabacteroides*	+Correlation IL-18 and gut marker and *Peptostreptococcus*, *Fusobacterium*, and *Citrobacter* taxa −Correlation between *Bilophila* and *Citrobacter* genera and disease severity *Streptococcus* genus
Zuo et al. (9)	China	A prospective study (short-term, from admission until discharge) Fecal samples Shotgun metagenomic sequencing	-15 hospitalized patients with COVID-19 (1 mild, 9 moderate, 3 severe, 2 critical) 6 hospitalized patients with pneumonia 15 healthy individuals -Age: 55 median (COVID-19), 48-year-old median controls -Men: 47% (COVID-19) vs. 60% (controls) -BMI: no data 40% of patients with COVID-19 had comorbidities, especially hypertension, hyperlipidemia, and diabetes mellitus (only 1 patient had diarrhea) Antibiotic used: amox/clav, cephalosporin, tetracyclin 7/15 patients ABT naïve	**α-Diversity** No data **β-Diversity** Bray–Curtis dissimilarity between antibiotic-naive patients, patients who received antibiotics, and controls	**Relative abundance comparison** **Antibiotic-naïve subgroup vs. controls:** ↑ *Actinomyces viscosus*, *Clostridium hathewayi*, and *Bacteroides nordii* ↓ *Eubacterium ventriosum* **In the antibiotic subgroup:** ↓ *Eubacteriaceae* and *Ruminococcaceae families* ↓ *Blautia, Eubacterium*, *Faecalibacterium*, *Roseburia*, and *Coprococcus* genera ↓ *Dorea formicigenerans*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Ruminococcus obeum*, *Lachnospiraceae bacterium*, and *Eubacterium ventriosum* species 23 bacterial taxa were found to be significantly associated with COVID-19 disease severity: ↑ *Erysipelotrichia* class ↑ *Erysipelotrichales* order ↑ *Erysipelotrichaceae* family ↑ *Coprobacillus*, *Enterobacter* genera ↑ *Clostridium ramosum*, *Clostridium hathewai*, *Erysipelotrichaceae noname*, *Actinomyces odontolyticus*, *Erysipelotrichaceae bacterium*, *Enterobacter cloaceae*, *Parabacteroides* unclassified, and *Alistipes indistinctus* species ↓ *Dorea*, *Roseburia*, and *Faecalibacteirum* genera ↓ *Bifidobacterium pseudocatenulatum*, *Dorea longicatena*,	*Clostridium ramosum* and *Clostridium hathewayi* were +associated with COVID-19 disease severity. *Alistipes onderdonkii* and *Faecalibacterium prausnitzii* showed a −correlation with COVID-19 severity 14 Bacterial species associated with a fecal viral load of SARS-CoV-2: -*Bacteroides dorei*, *Bacteroides theteiotaomicron*, *Bacteroides massilinesis*, and *Bacteroides ovatus* showed significant −correlation with fecal SARS-CoV-2 load *Erysipelotrichaceae* bacterium showed the strongest +correlation with fecal SARS-CoV-2 load Antibiotic treatment in patients with a more heterogeneous microbiome configuration In antibiotic-naive patients with COVID-19 ↑ opportunistic pathogens ↓ multiple bacterial species, which are symbionts beneficial COVID-19 condition the strongest factor on gut microbiota followed by hyperlipidemia, pneumoniae, and antibiotics Gut dysbiosis persistence over time regardless of clearance of SARS-COV-2

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			Antiviral therapy: LPV/RTV; ribavirin, INFbeta-1b		*Bacteroides ovatus*, *Anaerostipes hadrus*, *Lachnospiraceae* bacterium, *Faecalibacterium prausnitzii*, and *Alistipes onderdonkii*	The link between gut dysbiosis and the expression of ACE2: possible role of *Firmicutes* members to upregulate ACE2-R expression; possible role of *Bacteroidetes* members to downregulate ACE2-R expression
Yeoh et al. (43)	China	2 Hospital cross-sectional study Longitudinal arm subgroup 30 days after virological clearance Fecal samples Shotgun metagenomic sequencing	100 Hospitalized patients with COVID-19 (mild, 45; moderate, 45; severe, 5; critical, 3) 78 controls -Age: 36 vs. 45 years old -Men: 53% vs 42% -BMI: no data ABT: 34 patients Antivirals: 46 patients prior to stool collection (LPV/RTV, ribavirin, oseltamivir) Comorbidities: hypertension, hyperlipidemia, diabetes, and heart conditions (17% diarrhea at admission) For control hypertension	**α-Diversity** No significant differences in species richness and Shannon diversity between COVID-19 and controls **β-Diversity** Separation among COVID-19 with antibiotics, without antibiotics, and controls After virological cure, gut microbiota remained significantly distinct at 30 days (more dissimilar composition in patients who had received antibiotics)	**Relative abundance comparison** **COVID-19 vs. controls** ↑ *Bacteroidetes* phylum *Ruminococcus gnavus*, *Ruminococcus torques*, *Bacteroides dorei* species ↓ *Actinobacteria* phylum ↓ *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, and *Eubacterium rectale* species After antibiotic effects evaluation: ↑ *Parabcteroides* genus ↑ *Sutterella wadsworthensis*, *Bacteroides caccae* species ↓ *Adlercreutzia equolifaciens*, *Dorea formicigenerans*, *Clostridium leptum* species	–Correlation in *Faecalibacterium prausnitzii* and *Bifidobacterium bifidum* with severity +Correlation in CXCL10, IL-10, TNF-α, AST, GGT CRP, LDH, NT-proBNP, and erythrocyte sedimentation rate with microbiota composition Microbiota distribution was associated with COVID-19 and antibiotics but not with stool SARS-CoV-2 viral replication, antiviral, corticosteroids, and pomp inhibitor use. Continuum PCA visualization of a gut microbial composition according to severity index disease Postulated that gut microbiota was associated with the magnitude of immune response to COVID-19
Mazzarelli et al. (44)	Italy	Cross-sectional monocenter study Anal swab V2, V4, V8, and V3–6, 7–9 of the 16S gene	-15 hospitalized inpatients (9 in the ward w-COVID-19, 6 intensive cure unit, i-COVID-19) 8 hospitalized inpatient controls (3 in the intensive care unit, 5 on the floor) Severity: not possible stratification; all patients (including controls) pneumonia -Age: 67 (ward), 70 (ICU), 69 controls -Men: 55%, 50%, and 62%, respectively, in wards, ICU, and controls -BMI: no data ABT: 55%, 50%, and 37%, respectively, in the ward, ICU, and controls	**α-Diversity** ↓ Chao-1 Trend ↓ Shannon diversity index **β-Diversity** According to Bray–Curtis distinct patterns among the 3 groups	**Relative abundance comparison** **w-COVID-19 vs. controls** ↑ *Proteobacteria* phylum ↑ *Peptostreptococcaceae*, *Enterobacteriaceae*, *Staphylococcaceae*, *Vibrionaceae*, *Aerococcaceae*, *Dermabacteraceae* families, *Actinobacteria* taxa ↓ *Spirochaetes* and *Fusobacteria* phyla ↓ *Nitrospiraceae*, *Propionibacteriaceae*, *Aeromonadaceae*, *Moraxellaceae*, and *Mycoplasmataceae* families **w-COVID-19 vs. i-COVID-19:** ↑ *Carnobacteriaceae*, *Peptobacteriaceae*, *Moritellaceae*, *Selenomonadaceae*, *Micromonosporaceae*, and *Coriobacteriaceae* families ↓ *Staphylococcaceae*, *Microbacteriaceae*, *Micrococcaceae*, *Pseudonocardiaceae* families; *Erysipelotrichales* taxa **i-COVID-19 vs. CTRL:** ↑ *Staphylococcaceae*, *Aerococcaceae*, *Dermabacteraceae*, *Erysipelotrichaceae*, *Microbacteriaceae*, *Mycobacteriaceae*, *Pseudonocardiaceae*, *Brevibacteriaceae* families; *Actinobacteria* taxa ↓ *Carnobacteriaceae*, *Coriobacteriaceae*, and *Mycoplasmataceae* families	High levels of ferritin detected in i-COVID-19 patients in comparison to w-COVID-19 ↓ Of SCFA-producing bacteria A distinct profile can be distinguished between i-COVID-19 and w-COVID-19 with the latter being closer to CTRL.

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			48% antibiotics 1 or 2 days before the anal swab			
Liu et al. (45)	China	Prospective, interventional, single-centered pilot study on fecal microbial transplantation (FMT) Fecal samples before and after 1 week of FMT 16S sequencing	11 COVID-19 patients 1-month after a hospital discharge form -Age: 50 average -Men: 6/11 (54%) -BMI: no data 10 patients non-severe, 1 patient severe No antibiotics or an anti-inflammatory drug for 2 weeks prior to the treatment 5 out of 11 patients suffered from GI	**α-Diversity** -6 months ↑ Chao-1 after TMT No differences with other indexes (Shannon, Simpson, observed, OUT num) **β-Diversity** No data	**Relative abundance comparison** **Before vs after 1 week of FMT** ↓ *Proteobacteria* ↑ *Actinobacteria* ↑ *Bifidobacterium*, *Faecalibacterium*, and *Collinsella* genera	FMT effect on B lymphocytes ↓ naïve B cells, ↑ memory B cells, and non-switched B cells Alleviated GI symptoms were observed after FMT. First intervention study with FMT in a COVID-19 setting
Xu et al. (46)	China	Prospective study 35 days after symptomatic resolution Throat samples and anal swabs V4 region of bacterial 16 S rRNA gene	-35 COVID-19 patients, 19 healthy controls 10 non-COVID-19 patients with other diseases 34/35 COVID-19 patients with mild symptoms -Age: 47 average -Men: 57% -BMI: no data ABT: 13/35 37%, essentially fluoroquinolones 1 patient receiving steroids 14 patients receiving oseltamivir or INF Comorbidities: 16/35, with hypertension, more representative	**α-Diversity** ↓ Richness (observed) and Evenness (Pielou's evenness) indexes from types I to III during the early phase of COVID-19 **β-Diversity**: according to Bray–Curtis, 3 microbial community types were identified (types I–III)	**Dirichlet multinomial mixture (DMM) clustering: comparison among groups** Type I: *Bacteroides* genus and several known butyrate-producing bacteria: *Faecalibacterium*, *Roseburia*, *Blautia*, and *Coprococcus* genera; 1 opportunistic pathogenic bacterium *Finegoldia* genus Type II: *Neisseria*, *Actinomyces*, and others Type III: *Pseudomonas* genus members -A shift of the gut microbiome from the lower-diversity community type (II or III) toward a higher-diversity type (I or II) was observed over time in 7/10 patients who had anal swab tests at different timepoints -Clear trend of increased bacterial diversity and the relative abundance of *Bacteroides* and *Faecalibacterium* from early to late stages of COVID-19 like restoration of gut microbiota	Respiratory microbiome: α-Diversity decreased from type I to type IV. Except for the duration of COVID-19, the upper respiratory and gut microbial community divergence seemed not to be associated with age, gender, antibiotics use, and detection of SARS-CoV-2 RNA (the use of antibiotics could emphasize both dysbioses) The shift of microbiome community types over time appeared to match between the throat and the gut in 6/8 patients –Correlation α-diversity with serum LPS Dysbiosis of the upper airways seems to appear early and worse compared to the gut, due to a different resilience status in association with a high permeability among organs due to inflammation.
Ren et al. (47)	China	Cross-sectional study -Fecal samples and tongue-coating samples V3–V5 region of the 16S rRNA gene	The discovery cohort: CPs: 24 fecal samples 48 tongue-coating samples HCs: 48 fecal samples 100 tongue-coating samples -Age: 48 years old, 48 years old for controls	**α-Diversity** ↓ Observed richness and evenness/diversity index (Shannon index) **β-Diversity** PCoA separation among groups	**Relative abundance comparison** **Comparison between COVID-19 and controls** ↓ *Pseudobutyrivibrio*, *Ruminococcaceae* uncultured, *Blautia*, *Faecalobacterium*, *Bacteroides*, *Akkermansia*, *Lachnospiraceae incertae sedis*, and *Bifidobacterium* taxa ↑ *Streptococcus* and *Enterococcus* genera The article described 5 reduced genera (*Faecalibacterium*,	Oral microbiome alterations: α-Diversity → Shannon index and Simpson index significantly decreased in the CPs vs. HCs β-Diversity → Significant distinction of oral microbial communities between both groups

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			-Men: 28% vs. 8% -BMI: not calculated Severity index not calculated: probably mild No clinical information about comorbidities		*Lachnospira* genera, and others) and 5 increased genera (not specified)	
Chen et al. (48)	China	Prospective study: 6 months follow-up Fecal samples V3–V4 of the 16S rRNA gene	-30 patients subdivided post-convalescence phase using the median Chao-1 cutoff 259 in low α-diversity (N = 15), high α-diversity (N = 15) -Acute phase (from illness onset to viral clearance) -Convalescence (from viral clearance to 2 weeks after hospital discharge) -Post-convalescence (6 months after hospital discharge) 30 control patients -Age: 53 -Men: 63% -BMI: 24 33.3% severe illness	**α-Diversity** ↓ Richness Chao-1 Index in the acute phase compared to controls Richness was not restored to normal levels after 6-month recovery (trend toward controls) **β-Diversity** A Bray–Curtis analysis separation between COVID-19 and controls	Abundance relative analysis was not performed	Patients with reduced post-convalescence richness had higher levels of CRP as well as a higher occurrence of ICU admission and HFNC during the acute phase. In post-convalescence, low richness was associated with reduced FVC, FEV1, inspiratory vital capacity, and total lung capacity. Post-convalescence patients with lower microbial richness had worse pulmonary functions. Patients with lower richness at 6 months had an illness severity during the acute phase with a strong link between inflammatory response and COVID-19 gut microbiota dysbiosis.
Gaibani et al. (49)	Italy	Cross-sectional multicentered study Fecal samples V3–V4 of the 16S rRNA gene	-69 COVID-19 control patients: healthy age-gender-therapy and hospitalization-related confounder-matched (like exposure to antibiotics 2 weeks before: 69%) Italians For a subanalysis, a non-COVID-19 in ICU controls matched for age, gender, antibiotics, and other factors -Age: 73 -Men: 55% -BMI: 24 median (16% with obesity); 22–27 IQR 77% presented with moderate/severe	**α-Diversity** ↓ Evenness index (inv.Simpson index) **β-Diversity** According to Bray–Curtis, the a significant separation between COVID-19 patients and healthy controls. Note: gut microbiota profiles of COVID-19 patients showed no segregation by age, sex, antibiotic intake in the 2 weeks prior to fecal sampling, length of hospital stay, the time interval between fecal sampling, length of hospital stay, the time interval between fecal sampling and hospital admission, and outcome (death/discharge).	**LefSe analysis** **COVID-19 patients vs. controls** ↑ *Enterococcaceae*, *Coriobacteriaceae*, *Lactobacillaceae*, *Veillonellaceae*, *Porphyromonadaceae*, *Staphylococcaceae*, and *Eysipelotrichaceae* families ↑ *Enterococcus*, *Lactobacillus*, *Collinsella*, *Staphylococcus*, *Akkermansia*, *Parabacteroides*, *Actinomyces*, *Serratia*, *Lactococcus*, *Phascolarbacterium*, *Odoribacter*, *Acidaminococcus*, and *Methanobrevibacter* genera ↓ *Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Prevotellaceae*, and *Clostridaceae* families ↓ *Prevotella*, *Bacteroides*, *Faecalibacterium*, *Coprococcus*, *Blautia*, *Ruminococcus*, *Erwinia*, *Oxalobacter Roseburia*, *Anaerofustis*, *Lachnospira*, *Scardovia*, *Anaerofilum*, *Dialister*, *Oscillospira*, *Holdemania*, *Cloacibacillus*, and *Cristensenella* genera Note: sequences assigned to *Enterococcus* were *E. faecium* (8.4%) along with *E. hirae* (5.5%), *E. faecalis* (1.8%), and *E. villorum* (1.1%) ↑ *Enterococcus* in ICU patients and those developing BSI.	The severity of COVID-19-related dysbiosis is strongly associated with the development of BSI and ICU admission The percentage of patients who developed E-BSI was significantly higher during the COVID-19 pandemic than in the previous 3 years. Due to the severity of the clinical setting of the population, they could not exclude previous antibiotic intake before ICU admission, but controls were matched also for this parameter After an intragroup comparison between patients ICU admitted vs. patients COVID-19 no-ICU admitted, they did not see α-diversity differences but only a β-diversity separation among groups (including ICU controls). Both COVID-19 subgroups (ICU and no-ICU) expressed high levels of *Enterococcus*

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			pneumonia during hospitalization: 33% severe respiratory failure, 23% ICU, and 14% mechanically ventilated Hydroxychloroquine, low-molecular-weight heparin (LMWH): 88.4% Tocilizumab: 36% DRV; DRV/Cobi: 4.4%, 7.2% Several comorbidities: hypertension, 63%; COPD, 22%; diabetes, 17%; and others		↑ *Streptococcus*, *Oscillospira*, *Blautia*, and other *Ruminococcaceae*, *Lachnospiraceae*, and *Clostridiales* taxa in patients who had not entered the ICU and those who had not developed BSI	species compared to ICU controls No-ICU COVID-19 had an overrepresentation of *Ruminococcus*, *Oscillospira*, *Dorea*, and *Coprococcus*. ICU controls had an overrepresentation of *Enterobacteriaceae* (in particular, *Klebsiella* species)
Zhou et al. (50)	China	Cross-sectional study -Fecal samples -Shotgun metagenomic sequencing	-187 COVID-19 patients (127 patients with fever and 60 patients with no fever). All moderate COVID-19 -Age: 39 median (37 in the fever subgroup vs. 48 in the no-fever subgroup) -Men: 34% (36% vs. 31%) -ABT: No data Several comorbidities especially hypertension	**α-Diversity** ↓ In patients with fever with a strong trend according to Chao-1 (not significant according to Shannon) **β-Diversity** According to Bray–Curtis, different compositions in the gut microbiota between the 2 groups	**LEfSe analysis** **Fever group vs. no-fever group** ↑ *Ascomycota* phylum (fungal) ↑ *Saccharomyces* (fungal) and *Enterococcus* genera ↑ *Enterococcus faecalis*, *Citrobacter freundii*, *Citrobacter* unclassified, *Haemophilus parainfluenzae*, and *Saccharomyces cerevisiae* species ↓ *Bacteroidetes* phylum ↓ *Anaerostipes*, *Prevotella*, *Parabacteroides*, *Phascolarbacterium*, *Eggerthella* genera ↓ *Bacteroides cellulolyticus*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides xylanisolvens*, *Eubacterium ramulus*, and *Erysipelotrichacae bacterium*	Patients with fever: more pathogens, and lack butyrate-producing species. 5 epitopes were enriched in the fever group. Some of these were +correlated with clinical indices (IL-6, WBC, neutrophils, CRP, D-dimer, and LDH). 4 of the 5 epitopes were all +correlated with *E. faecalis* (↑ in the fever group). Same background, although during ABT treatment and with no available diet investigation information
Kim et al. (51)	South Korea	Prospective monocenter study 2 time points: from positive to negative virological cure Fecal samples V3–V4 of the 16S rRNA gene	12 out-patients Longitudinal analysis from positive (infected state) to negative virological test (recovered state) 36 controls Asymptomatic infection or mild COVID-19 -Age: 26 -Men: 66% -BMI: 23 No medicines and/or antibiotics and/or probiotics ongoing Few comorbidities but gastrointestinal tract	**α-Diversity** ↑ Evenness index in the recovered state (Pielou's evenness) (the trend for Shannon; not for richness indexes like faith and observed) trend toward controls **β-Diversity** Differences for quantitative indexes Bray–Curtis and weighted Unifrac (respectively phylogenetic and no-phylogenetic measures). No differences for qualitative indexes Jaccard and unweighted Unifrac (respectively no-phylogenetic and phylogenetic measures) trend toward controls	**Relative abundance comparison** **Infected state vs. recovered state** ↓ *Bacteroidetes, Bacteroidia*, *Bacteroidales*; *Bacteroidaceae*, *Marinifilaceae*, and *Tannerellaceae* families ↑ *Actinomycetals* order, *Actinomyces* order **COVID-19 vs. controls** ↓ SCFA-producing bacteria and *Bacteroides*, *Butyricimonas*, and *Odoribacter* taxa and members of *Lachnospiraceae* and *Ruminococcaeae* families	↑ *Firmicutes*/*Bacteroidetes* ratio in an infected state, in the absence of antimicrobial therapy and without obese patients +Correlation between *Escherichia*/*Shigella*, *Citrobacter*, *Collinsella*, and *Bifidobacterium* and COVID-19

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			involvement (reflux esophagitis, irritable bowel disease, fatty liver)			
Zhou et al. (52)	China	Cross-sectional prospective study of recovered COVID-19 healthcare workers (HCWs) after 3 months Fecal samples V3–V4 of the 16S rRNA gene	-15 HCWs, 14 controls 80% had at least 1 long COVID-19-related symptom (especially cough and fatigue) -Age: 29 medians vs. 37 controls -Men: 20% vs. 35% in controls -BMI: 22 vs. 24 2 recovered HCWs with hypertension; no comorbidities in the controls Excluded patients with previous antibiotics and/or probiotics within 3 months before enrolment (no information on lifestyle/diet)	**α-Diversity** **HCWs vs. controls** ↓ with Shannon (and not sign with other indexes) **β-Diversity** According to Bray–Curtis, a significant difference in the fecal microbiota between recovered HCWs and HCs	**Relative abundance comparison** **HCWs vs. controls** ↑ *Actinobacteria* phylum ↑ *Escherichia*, *Flavonifractor*, and *Intestinibacter* genera ↑ *Esherichia* unclassified, *Intestinibacter bartlett*, *Clostridium aldenense*, *Clostridium bolteae*, *Flavonifractor plautii*, and *Clostridium ramosum* species ↓ *Lachnospiraceae*, *Desulfovibrionaceae* families ↓ *Faecalibacterium*, *Roseburia*, *Fusicatenibacter*, *Ruminococcus*, *Clostridium XVIII*, *Dorea*, *Butyricicoccus*, *Romboutsia*, *Intestinimonas* and *Bilophila* genera ↓ *Faecalibacterium prausnitzii*, *Roseburia inulinivorans*, *Fusicatenibacter saccharivorans*, *Ruminococcus bromii*, *Blautia faecis*, *Butyricicoccus pullicaecorum*, and *Intestinimonas butyriciproducens* species	–Correlation between *Faecalibacterium prausnitzii* and chest tightness after activity –Correlation between *Intestinimonas butyriproducens* and cough +Correlation between *Escherichia* unclassified and fatigue, chest tightness after activity, and myalgia +Correlation between *Intestinobacter bartlettii* and anorexia and fatigue Compared with HCs, the fecal microbiota of recovered HCWs at 3 months after discharge exhibited decreased bacterial diversity
Moreira-Rosario et al. (53)	Portugal	Multicenter cross-sectional study Fecal samples V3–V4 of the 16S rRNA gene	-115 COVID-19 patients Severity index: 19 mild, 37 moderate, 59 severe Location: 14 ambulatory, 40 wards, 61 ICU -Age: 68 median -Men: 63% -BMI: not shown, percentage of overweight or obese: 65% Comorbidities: hypertension, diabetes, and other ABT: 38% during the last 6 months	**α-Diversity** Decrease trend for α-diversity Shannon index (diversity index) from mild to severe. **β-Diversity** No data	**Relative abundance comparison** **Mild COVID-19 vs. moderate COVID-19 and mild COVID-19 vs. sever COVID-19:** Decrease tendency from mild to moderate and from moderate to severe for: *Bifidobacteriaceae* (*Bifidobacterium* genus) and *Coriobacteriaceae* (*Collinsella* genus) taxa with significant differences ↓ for *Lachnospiraceae* family (*Roseburia* and *Lachnospira* genera) ↑ *Ralstonia* genus (*Proteobacteria* phylum) with COVID-19 severity score index *Firmicutes*/*Bacteroidetes* ratio has decreased through severity increase	In a multivariate analysis, the Shannon index and CRP were associated with COVID-19 severity, with cut-off values of 2.25 and 96.8 ml/L. RNA viral replication: no associations were found for SARS-CoV-2 replication and COVID-19 severity Patients with lower Shannon diversity displayed SARS-CoV-2 fecal replications 4 features: ↓ *Firmicutes*/*Bacteroidetes* ratio; ↑ *Proteobacteria* phylum; ↓ butyrate-producing bacteria from *Lachnospiraceae* family (*Roseburia* and *Lachnospira* genera) ↓ *Actinobacteria* essentially *Bifidobacteria* (*Collinsella*)
Wu et al. (54)	China	Longitudinal study for both (oral and fecal districts) during hospitalization from positive to	-53 COVID-19 patients divided into 2 subgroups: non-severe COVID-19 (mild-moderate) and severe group (sever–critical)	**α-Diversity** ↓ Faith in severe COVID-19 and non-severe COVID-19 subgroups compared to controls (with increased gradient among groups from severe to non-severe to controls)	**LEfSe analysis** **Comparison between COVID-19 and controls** ↓ *Blautia*, *Coprococcus*, and *Collinsella* genera ↓ *Bacteroides caccae*, *Bacteroides coprophilous*, *Blautia obeum*, *Clostridium colinum* species ↑ *Streptococcus*, *Weisella*, *Enterococcus*, *Rothia*, *Lactobacillus*,	*Granulicatella* and *Rothia* increased in both districts investigated (oral and gut) of COVID-19 patients. At the gut level, SARS-CoV-2 replication: +Correlation to *P. copri* and *E. dolichum* –Correlation to other taxa like *S. anginosus*,

(Continued)

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
		negative virological cure Fecal samples and throat swabs V3–V4 of the 16S rRNA gene	73 controls Also, throat analyses Clinical features not shown	**β-Diversity** Separation among 3 groups (severe COVID-19, non-severe COVID-19 and controls) according to unweighted Unifrac	*Actinomyces*, and *Granulicatella* genera ↑ *Clostridium citroniae*, *Bifidobacterium longum*, *Rothia mucilaginosa* species	*Dialister*, *Alistipes*, *Ruminococcus*, *C. citronieae*, *Bifidobacterium*, *Haemophylus*, and *H. parainfluenzae* taxa SARS-CoV-2 infection associated with oral microbiome alterations In β-diversity: distinguishing ongoing antibiotics: both subgroups (with and without antibiotics) displayed different clusters compared to controls (but not between subgroups)
He et al. (55)	China	Longitudinal study until 3 months follow-up Fecal samples Multi-omics profiling (metaproteomics, glycoproteomics, metabolomics, lipidomics)	-13 COVID-19 patients with different severity index disease (7 mild, 5 moderates, 1 severe) 21 controls -Age 27 median but 2 patients < 3 years old (1 patient 1 year old, 1 patient 10 months), 1 patient 5 years old; controls 43 years old -Male 77%; controls 57% -BMI 24 with 2 obese patients and 1 underweight Comorbidities: 1 diabetic patient, 2 patients with sinusitis or rhinitis; several patients with gastrointestinal disorders and anorexia	**α-Diversity** No data **β-Diversity** Multiomics profiling confirmed the separation between COVID-19 and controls	**Relative abundance from the metaproteomic approach: COVID-19 vs. controls** ↓ *Lachnospiraceae* family (*Lachnoclostridium*, *Ruminococcus*, *Butyrivibio*, *Dorea*, *Blautia*, and *Tyzerella* genera) ↑ *Bacteroides* genus	Feature of this study: enrichment of gut bacteria-related deleterious metabolites as well as altered host and bacterial lipids.
Li et al. (56)	China	Cross-sectional study Fecal samples Shotgun metagenomic sequencing	-37 COVID-19 and 10 controls in the discovery cohort 10 COVID-19 and 9 controls in the validation cohort (controls matched for age, gender, and BMI. No antibiotics and/or probiotics 4 weeks before enrollment) According to the severity index: 7 mild, 29 moderate, 8 severe, and 3 critical (patients from both cohorts)	**α-Diversity** **Comparison between COVID-19 and controls** ↓ Number of species In the intragroup COVID-19 analysis according to the severity index: ↓ Evenness and Pielou indexes in mild type vs. controls **β-Diversity** Bray–Curtis separation	**Relative abundance comparison** **COVID-19 vs. controls** ↑ *Bacteroidetes* phylum and ↑ *Bifidobacterium longum*, *Streptococcus thermophilus*, and other taxa (note that several patients received probiotics, which include: *Bifibacterium longum Streptococcus thermophilus*, and *Lactobacillus bulgaricus*) ↑ *Bacteroides stercoris*, *Bacteroides vulgatus*, *Bacteroides massiliensis*, *Bifidobacterium longum*, *Streptococcus thermophilus*, *Lachospiraceae* bacterium, *Prevotella bivia*, *Erysipelotrichaceae* bacterium (2 variants) ↓ *Firmicutes* phylum ↓ *Candidatus saccharibacteria* taxa and *Corionacteriaceae* family ↓ *Ruminococcus*, *Dorea*, and *Adlercreutzia* genera ↓ *Clostridium nexile*, *Streptococcus salivarius*, *Coprococcus catus*, *Eubacterium hallii*, *Enterobacter aerogenes*, and *Adlercrutzia equolifaciens*	–Correlation between COVID-19 severity and *Rosebura* and *Megasphaer* genera –Correlation between COVID-19 severity and *Roseburia inulinivorans*, *Bacteroides faecis*, *Bifidobacterium bifidum*, *Parabacteroides goldsteinii*, *Lachnospiraceae* bacterium, and *Megasphaera* species +Correlation between *Paraprevotella*, *Lachnospiraceae*, *Erysipelotrichaceae* taxa, and COVID-19 severity +Correlation between *Paraprevotella* species, *Streptococcus thermophilus*, *Clostridium ramosum*, and *Bifidobacterium animalis*

(Continued)

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			-Age: 44-year-old patients and 37-year-old controls in discovery cohort; 56-year-old patients and 46-year-old controls in validation cohort -Men: 51% COVID-19 vs. 70% controls in the discovery cohort; 50% vs. 55% in the validation cohort BMI: 23 vs. 21 in the discovery cohort; 23 vs. 23 in the validation cohort ABT: 32% in the discovery cohort; 60% in the validation cohort Antiretroviral: 0% in the discovery cohort; 100% in the validation cohort Probiotic during hospitalization: 0% in the discovery cohort: 50% in the validation cohort			
Liu et al. (57)	China	A prospective, multicentered pilot study with a 6-month follow-up after hospital discharge (after virological clearance) Fecal samples shotgun metagenomic sequencing	-68 patients (from 106 enrolled) followed up from admission to 6 months 68 non-COVID controls Post-acute COVID-19 symptoms (PACS): at least 1 persistent symptom 4 weeks after clearance → $N = 50/68$ at 6 months Severity of COVID-19: most patients had mild to moderate severity of COVID-19 (81.1%) -Age, 48 years old -Men: 47% -BMI: no data Comorbidities (45%): hypertension is the most	**α-Diversity** Longitudinal comparison from baseline to 6 months and vs. controls ↓ Shannon diversity and Chao-1 richness at 6 months compared to controls ↓ Shannon diversity and richness at admission in patients who developed PACS compared to controls **β-Diversity** Separation among groups: basal COVID-19 naïve antibiotic patients (and overall), longitudinal time points (1 month and 6 months with essential overlap), and controls No differences between COVID-19-naïve antibiotic patients and antibiotic patient subgroups during follow-up	**LEfSe analysis** **Longitudinal COVID-19 subgroups vs controls** ↓ *Ruminococcus* and *Bifidobacterium* (at 1 and 6 months compared with controls) and other taxa. When the effect of antibiotics was examined at baseline and at 6 months, overall gut microbiota composition was similar between antibiotic-naïve and antibiotic-treated patients. Whereas the overall gut microbiota composition was distinct at 1 month PACS analysis: patients who referred at least 1 COVID-19 symptom at 6 months (76%) maintained a different gut microbiota composition characterized by: ↑ *Ruminococcus gnavus*, *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, *Lachnospiraceae* bacterium oral taxon, *Bacteroides xylanisolvens*, *Parabacteroides distasonis*, *Clostridium innocuum*, *Flavonifractor plautii*, *Lactobacillus delbrueckii*, Erysipelatoclostridium *ramosum*, *Morganella morganii*, *Lactobacillus acidophilus*, *Streptococcus lutetiensis* ↓ *Faecalibacterium prausniztii*, *Collinsella aerofaciens*, *Eubacterium rectale*, *Blautia obeum*, *Ruminococcus torques*, *Ruminococcus bicirculans*, *Roseburia faecis*, *Adlecreutzia*	The first study to demonstrate persistent gut dysbiosis at 6 months after recovery from COVID-19 and the link between altered gut microbiota and common lingering symptoms. Specific gut microbiome profiles were associated with the presence of PACS and with different PACS symptoms +Correlation between PACS patients with respiratory symptoms and opportunistic pathogens +Correlation between the abundance of nosocomial pathogens with neuropsychiatric symptoms and fatigue −Correlation between the relative abundance of multiple bacterial species beneficial to host immunity and the presence of PACS at 6 months −Associations of walking distance test with pathogenic bacteria species +Correlation between walking distance and

(Continued)

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TABLE 1A Continued

ID	Country	Study characteristics	Population characteristics	α/β-Diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
			common comorbidity followed by type 2 diabetes mellitus ABT 23% but analyses on antibiotic-naïve patients Antiviral: 52% LPV/RTV, 28%RBV, 36% INF, 5% remdesivir Symptoms 6 months: fatigue, poor memory, hair loss, anxiety, difficulty sleeping They documented dietary records during the time of hospitalization Exclusion criteria for non-COVID-19 controls were the use of antibiotics in the past 6 months, the use of laxatives or antidiarrheal drugs in the past 3 months, and recent dietary changes		*equolifaciens*, *Coprococcus comes*, *Dorea longicatena*, *Firmicutes* bacterium CAG-83, *Agathobaculum butyriciproducens*, *Dorea formicigenerans*, *Eubacterium* sp CAG-251, *Roseburia inulinivorans*, *Ruthenibacrerium lactatiformans*, *Gemigger formicilis*, *Enterococcus avium*, *Roseburia hominis*, *Ruminococcus lactaris*	several short-chain fatty acids and butyrate producers. No significant correlations between viral load and PACS development.
Ng et al. (58)	China	Prospective observational study Fecal samples Shotgun metagenomic sequencing	-138 adults who have received 2 doses of either the inactivated vaccines (CoronaVac; *n* = 37) or the mRNA vaccine (BNT162b2; *n* = 101) -Age; 47 years -Men: 32.6% -BMI 38.4% were classified as OWOB (i.e., BMI ≥ 23). It is a study to determine whether baseline gut microbiome composition was associated with the immune response to COVID-19 vaccines	**α-Diversity** ↓ At 1 month after the second dose of vaccination compared with baseline samples in both vaccine groups **β-Diversity** Shift at 1 month after the second dose of vaccination compared with baseline samples in both vaccine groups	At the species level: ↑ *Bacteroides caccae* in CoronaVac vaccinees ↑ *Bacteroides caccae* and *Alistipes shahii* in BNT162b2 vaccinees ↓ Common bacterial species including *Adlercreutzia equolifaciens*, *Asaccharobacter celatus*, *Blautia obeum*, *Blautia wexlerae*, *Dorea formicigenerans*, *Dorea longicatena*, *Coprococcus comes*, *Streptococcus vestibularis*, *Collinsella aerofaciens*, and *Ruminococcus obeum* CAG 39 were observed in both vaccine groups ↓ *Actinobacteria* and *Firmicutes* Note: None of the participants reported significant dietary changes during the study period. Among 72 randomly selected participants, no significant changes in detailed dietary intake were recorded at baseline and 1 month after the second dose of vaccination Note: BNT162b2 → Comirnaty	CoronaVac vaccinees: -21/37 (56.8%) showed sVNT (surrogate virus neutralization test) lower than 60% (low responders). Distinct baseline gut microbiome from those with sVNT higher than 60% (high responders). *Bifidobacterium adolescentis* was enriched in high responders while *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, and *Ruminococcus gnavus* were more abundant in the low responder. BNT162b2 vaccinees: Similar to CoronaVac, low responders had a persistently low level of *Actinobacteria*, particularly *B. adolescentis*. 4 specific bacteria in the baseline gut microbiome, including *Eubacterium rectale*, *Roseburia faecis*, and 2 *Bacteroides* species, *B. thetaiotaomicron*, and *Bacteroides* sp. OM05-12 were significantly increased in the highest-tier responders with the top 25% of sVNT level

ICU, Intensive Care Unit; BSI, bloodstream infections; OWOB, overweight and obese; sVNP, surrogate virus neutralization test; UPLC-MS, ultra-performance Liquid chromatography-mass spectrometry; HCWs, healthcare workers; HCs, healthy controls; SCFAs, short-chain fat acids; BPBs, butyrate-producing bacteria; upward arrows "↑", increase; downward arrows "↓", decrease. In correlation and other findings, "–" and "+" means respectively negative and positive correlation.

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Lifestyle and diet were not analyzed, even though both factors are crucial elements in shaping microbial core composition (32, 59, 60).

The material source was a fecal sample in 19/22 (86%) studies, while three of 22 (14%) were based on anal swab analysis. Most studies (12/22) used next-generation sequencing (NGS) technology through ribosomal-S16-DNA hypervariable region sequencing (V4 or V3–V4 regions preferred) to analyze microbiota; shotgun metagenomic sequencing was used in seven of 22 studies, whereas one study used multi-omics methodologies (55), one study nanopore technology (38), and another used quantitative PCR (39).

Regarding patients' characteristics, all studies included both men and women, but no studies considered sexual behavior, although its impact on microbiota core is known in several disease models (61, 62). Only one-third of studies (seven of 22) included BMI data, and control groups, when included, were often matched for BMI. Fifty percent of the subjects in the studies, 50% were aged 50 or younger.

The small sample size was a limit reported by several authors, with a total number of enrolled subjects below 40 in almost two-thirds of studies 13/21 (62%). The COVID-19 severity index was reported by most studies, with high heterogeneity in the works analyzed.

Scarce data were available on comorbidities and concomitant medications; hypertension was the most commonly reported, followed by diabetes.

No data were generally reported on COVID-19 vaccine status for subjects enrolled after the introduction of the vaccine; only one study investigated the microbiota changes in two groups of patients vaccinated with two different vaccines (58). During hospitalization, both antibiotics and/or antiretroviral treatments and probiotics were administered in several studies; however, these data were not critically investigated in most published studies.

2.2 Microbiota analysis

After assessing the possible confounding factors, we compared the gut microbiota features according to two ecological measures, α-diversity and β-diversity, in association with relative abundance results.

In humans, α-diversity measures the level of diversity within individual samples; it includes several indexes gathered in two groups: richness indexes (Faith index, Observed and Chao-1 index) and evenness indexes (Shannon index, Peliou's evenness, Simpson, and inverse Simpson indexes) (63, 64).

In parallel to other disease models, α-diversity at the gut level, more frequently described with richness indexes (like Chao-1), resulted in a global reduction in all COVID-19 patients compared to controls (see details in Table 1A). An interesting study observed this reduction already in the acute phase of the disease (48). On the contrary, Yeoh et al. (43) did not report alterations in α-diversity indexes, even though they enrolled most COVID-19 patients with a mild or moderate severity index (90% of patients).

In a Korean longitudinal analysis performed on patients who were asymptomatic or affected by the mild disease, an increase in α-diversity (Peliou's evenness) was observed in the recovered subgroups compared to infected patients (51). Interestingly, Xu et al. (46) observed a trend toward increased bacterial diversity from the early to late stages of COVID-19 in a 35-day longitudinal analysis of inpatients with mild disease. Furthermore, the same study described an interesting synchronous restoration of microbiota in both gut and upper airways, suggesting a possible role of the gut-lung axis.

Moreira-Rosario et al. (53) described a reduced α-diversity gradient trend (Shannon index) from mild to severe COVID-19 patients, and Chen et al. (48) showed how richness was not restored to a normal level even after 6 months in 30 COVID-19 patients (one-third with severe disease), although a trend toward healthy controls was noticed.

β-Diversity measures the level of diversity (or dissimilarity) between samples, mostly by using a Permanova analysis (65, 66). All the studies showed a difference between COVID-19 patients and controls, in general, and according to different severity index categories.

Mazzarelli et al. (44) have shown a difference in β-diversity among patients hospitalized in regular wards compared to ICU patients and hospitalized no-COVID-19 controls, although no data on prior antibiotic intake was gathered. Regarding this aspect, two studies (9, 43) compared microbiota composition in COVID-19 patient subgroups (with and without antibiotics) with healthy controls, confirming a separation among groups, with high heterogeneity revealed in the antibiotic subgroup.

Regarding relative abundance analysis, several studies described a significant reduction in *Firmicutes* members, especially for BPBs (both *Lachnospiraceae* and *Ruminococcaeae* families, mostly *Faecalibacterium prausnitzii*) in COVID-19 patients compared to no-COVID controls, while discordant data have been reported about *Erysipelotrichaceae* and *Veillonellaceae* taxa.

Conversely, several facultative anaerobic bacteria like members of the *Bacilli* class, resulted in increased growth, mostly in the *Enterococcaceae* family as well as *Streptococcaceae* and *Lactobacillaceae* (Table 1). Contrasting data have been described regarding the *Bacteroidetes* phylum during COVID-19, with some works reporting an increase in *Bacteroidetes* phylum with a consequent reduction of the *Firmicutes*/*Bacteroidetes* ratio (53) as opposed to other studies reporting a reduction in taxa belonging to this phylum. Other factors, like diet and/or antibiotics, could play a role in these findings, highlighting the importance of assess for confounding factors when considering the study results.

Reduction in the *Actinobacteria* phylum, including the *Bifidobacterium* genus and *Collinsella* genus (recently associated with SARS-CoV-2-ACE2 binding inhibition), represents another significant finding in COVID-19 studies (67). The *Bifidobacterium* genus was found to be increased only in three studies (notably, in one study, a probiotic including this taxon was administered (56)), while the *Collinsella* genus resulted was increased in a few other studies (40, 45, 49); the reason for this last difference is not clear. *Proteobacteria* resulted increased in almost all studies performed on COVID-19 patients, although some authors have described an increase in *Enterococcaceae*/*Enterobacteriaceae* ratio (39), probably linked to the use of antibiotics. Finally, the *Akkermansia*

genus (*Verrucomicrobia*), a propionate-producing bacterium genus with anti-inflammatory features, resulted in reduced COVID-19 (but not in all studies). To note, the severity of COVID-19 disease seems to emphasize differences in the relative abundance of gut microbiota, although most studies included asymptomatic/mild/moderate categories.

3 Airway microbiota dysbiosis in acute COVID-19

We analyzed 13 studies on airway microbiota changes during SARS-CoV-2 infection, mostly comparing COVID-19 patients with healthy subjects and/or patients with different respiratory diseases (Table 1B).

Nasopharyngeal swabs were the most studied material, with the exception of three studies analyzing samples from the lower respiratory tract, such as bronchoalveolar lavage fluid and endotracheal aspirate. Bacterial communities were prevalently mapped by amplification of 16S gene hypervariable regions, with only a few studies employing genome sequencing. Eighty percent of the studies were set in China or Europe (five studies each). No data on possible confounding factors such as diet, BMI, relevant comorbidity, and antibiotic/antiviral consumption were investigated.

Overall, patients with SARS-CoV-2 infection showed diminished diversity in airway microbiota composition, by means of Shannon, Simpson, and Chao-1 indexes, when compared to both healthy subjects (46, 69, 70, 75, 77–79) and patients with community-acquired pneumonia (70).

A similar reduction in diversity measures is reported in critically ill COVID-19 patients, as opposed to subject with milder symptoms, other coronavirus infections, and healthy subjects (69). Interestingly, a reduction in diversity and greater difference at principal coordinate analysis (PCoA) is observed in patients needing mechanical ventilation compared to non-intubated patients regardless of SARS-CoV-2 infection (75). Such data suggest that COVID-19 impacts airway microbiota diversity mostly in severe infections, and this imbalance is strongly biased by other confounding factors such as intubation.

Of note, a number of the report showed no significant differences between COVID-19 patients and the control group in both bacterial richness and diversity/evenness indexes (observed species, Shannon index, and inverse Simpson index) (68, 71, 76). These findings can be partially explained by the heterogeneous population included in the studies and by the different methods used to sequence bacterial communities and assess diversity.

Curiously, Rosas-Salazar et al. (74) observed higher overall α-diversity in SARS-CoV-2-infected subjects compared to healthy controls, with no significant differences in any of the measured β-diversity.

COVID-19 severity correlates to α-diversity in oropharyngeal samples at the first time point, with lower diversity associated with higher disease severity (79). However, no significant association between high versus low SARS-CoV-2 viral load and any of the α-diversity or β-diversity metrics was observed (74).

In the studies analyzed, the airway microbiota of healthy individuals is characterized by the predominance of *Bacteroidetes* and *Comamonadaceae* taxa (46, 68), and no specific microbiota pattern has been found in COVID-19 patients. However, some peculiar alterations in relative composition have been observed.

Reduced abundance in *Proteobacteria* and *Fusobacteria* phyla is reported in subjects with SARS-CoV-2 infection as compared to controls, and decreased oropharyngeal *Proteobacteria* and *Actinobacteria* phyla correlate with greater disease severity (71, 79). At the genus level, patients with more severe diseases have significantly lower relative abundances of *Haemophilus*, *Actinomyces*, and *Neisseria*, all of which are abundant in the normal oropharyngeal microbiome (74, 79). Interestingly, *Fusobacterium periodonticum* is less represented in COVID-19 patients, negatively correlating with the severity of symptoms (71). A possible explanation is that these bacteria could modulate sialic acid metabolism and regulate ACE expression, impacting SARS-CoV-2 binding to the epithelium of the respiratory tract, as shown for other intestinal microorganisms (71, 80).

Conversely, COVID-19 patients show a high abundance of *Saccharibacteria* (formerly known as TM7), *Streptococcus mitis* group, *Streptococcus bovis* group, and *Rothia mucilaginosa* taxa (46, 72, 73), the latter often associated with cancer and bacteremia (81).

Significant changes among operational taxonomic unit (OTU) abundances are also reported, with decreased complexity of coabundance networks in severe COVID-19. OTUs associated with higher disease severity are members of the genus *Prevotella* and *Veillonella*. Particularly, it has been postulated that *Prevotella* spp. can worsen disease progression by activating immune signaling pathways that modulate inflammation (73).

Critically ill COVID-19 patients display a complete depletion of *Bifidobacterium* and *Clostridium* genera, with the presence of *Salmonella*, *Scardovia*, *Serratia*, and *Pectobacteriaceae* taxa. In these subjects, there is also a relative abundance of the *Pseudomonaceae* family, known to be associated with pathogenic conditions such as severe acute respiratory syndromes (69). Another characteristic of the airway microbiota in severe COVID-19 patients is low diversity and more richness in non-fermenting bacteria like *Acinetobacter*, *Pelomonas*, *Ralstonia*, and *Sphingomonas* genera. As mentioned before, these changes might be attributed to intubation and mechanical ventilation rather than COVID-19 pneumonia per se (75).

Interestingly, similar characteristics of an imbalanced microbiota with an enrichment of proinflammatory *Enterobacteriaceae* are found in patients with other respiratory diseases (46).

To date, there is scarce data coming from longitudinal studies on airway microbiota in SARS-CoV-2 infection. Analyzing throat swabs from 64 patients, 35 of which with confirmed infection, Xu et al. (46) postulated that a peculiar microbial community might represent the progressive imbalance of the respiratory microbiota. Interestingly, even though over half COVID-19 patients analyzed maintained relatively stable microbiome community types, 70% of the subjects experienced a gradual decrease of microbial diversity,

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TABLE 1B Selected studies on airway microbiota and COVID-19.

ID	Country	Study characteristics	Population characteristics	α/β-diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
De Maio et al. (69)	Italy	Cross-sectional study Nasopharyngeal swab Amplification V1–V2–V3 regions of the bacterial 16S rRNA	40 patients; 18 with confirmed SARS-CoV-2 infection, 22 HCs	No difference (observed species, Shannon index, and inverse Simpson)	Most sequences in all samples (98% in both SARS-CoV-2 and HCs) belonged to 5 phyla: *Firmicutes* (42% and 51%, respectively), *Bacteroidetes* (25% and 20%, respectively), *Proteobacteria* (18% and 16%, respectively), Actinobacteria (8% and 6%, respectively), and *Fusobacteria* (5% and 5%, respectively)	
Rueca et al. (70)	Italy	Cross-sectional study Nasal and oropharyngeal swabs Amplification V1–V2–V3 regions of the bacterial 16S rRNA	39 patients, 21 with confirmed SARS-CoV-2 infection; 8 affected by a different human coronavirus (HKU, NL63, and OC43); 10 HCs Disease severity: critically ill (ICU) vs. paucisymptomatic (Pauci)	Chao-1 decreased SARS-CoV-2 ICU as compared to SARS-CoV-2 Pauci patients, other HCoVs and HCs Shannon index decreased in SARS-CoV-2 ICU patients compared to HCs and SARS-CoV-2 Pauci patients	At the phylum level: -*Deinococcus Thermus* was present only in controls as compared to SARS-CoV-2 ICU patients, SARS-CoV-2 Pauci, or other HCoV patients - *Candidatus Saccharibacteria* (TM7) was strongly increased in negative controls and SARS-CoV-2 Pauci patients as compared to SARS-CoV-2 ICU patients and Other HCoV patients At the family level: - *Alicyclobacillaceae*, *Chromobacteriaceae*, *Deinococcacaee*, *Hydrogenophilaceae*, *Thermoanaerobacteraceae*, *Sporomusaceae*, and *Thermoanaerobacterales* family III. Incertae Sedis were exclusive microorganisms detected in neg control patients -*Pectobacteriaceae* were exclusive to SARS-CoV-2 ICU patients At the lower taxonomic level: -*Johnsonella*, *Tepidiphilus*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Thermosinus*, and *Variovorax* were exclusive to neg control patients -*Salmonella*, *Scardovia*, *Serratia*, and unk_*Pseudomonadaceae* were included exclusively in SARS-CoV-2 ICU patients	SARS-CoV-2 ICU patients displayed a complete depletion of *Bifidobacterium* and *Clostridium* The presence of *Moraxellacaea* spp. was observed exclusively in SARS-CoV-2 Pauci patients The presence of *Pseudomonaceae* was found exclusively in SARS-CoV-2 ICU
Shen et al. (71)	China	Cross-sectional study BALF RNA extraction, reverse-transcripted, amplified	53 patients, 8 with confirmed SARS-CoV-2 infection; 25 with CAP, and 20 healthy controls	Significative lower in patients with pneumonia (both COVID-19 and CAP)	3 types of microbiotas: -Type I dominated by the possible pathogens -Type II were mostly environmental organisms (contamination) -Type III mainly commensal species	
Nardelli et al. (72)	Italy	Cross-sectional study Nasopharyngeal swab Amplification V1–V2–V3 regions of the bacterial 16S rRNA	38 patients, 18 with confirmed SARS-CoV-2 infection; 20 HCs	No difference (Chao-1: $p = 0.28$, Shannon: $p = 0.27$, and Simpson: $p = 0.32$)	5 phyla prevalent in both HCs and COVID-19: - *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* In COVID-19: -Significant lower abundance of *Proteobacteria* and *Fusobacteria* -At the genus level, reduced *Leptotrichia*, *Fusobacterium*, and *Haemophilus*	Negative correlation between the relative abundance of *Fusobacterium periodonticum* and the severity of the patient's symptoms
Budding et al. (73)	The Netherlands	Cross-sectional study Throat swab Differentiation of species by length polymorphisms of the 16S-23S rDNA region combined with phylum-specific sequence	135 patients, 46 with confirmed SARS-CoV-2 infection, 89 HCs	No data	A cluster of 77 samples with a similar microbiota composition (both HCs and COVID-19) with a high abundance of *Haemophilus parainfluenzae*, *Neisseria cinerea*, *Streptococcus mitis* group, *Streptococcus bovis* group, *Leptotrichia buccalis*, and *Rothia mucilaginosa*	

(Continued)

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TABLE 1B Continued

ID	Country	Study characteristics	Population characteristics	α/β-diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
		polymorphisms of the 16S rDNA				
Ventero et al. (74)	Spain	Cross-sectional study Nasopharyngeal swab Amplification V1–V2–V3 regions of the bacterial 16S rRNA	74 patients, 56 with confirmed SARS-CoV-2 infection; 18 HCs	No data	Most abundant phylum: -*Firmicutes* (52.9% ± 4.0%) -*Bacteroidota* (22.1% ± 6.1%) -*Proteobacteria* (12.7% ± 7.3%) -*Actinobacteria* (5.4% ± 0.6%) At the genus level: -*Streptococcus* (25.2% ± 2.0%) -*Prevotella* (16.2% ± 5.7%) -*Veillonella* (14.4% ± 2.2%) -*Haemophilus* (5.23% ± 4.78%) -*Moraxella* (3.2% ± 3.6%) OTUs: -*Bacteroidota* (18) -*Firmicutes* (25)	The most common genera among the OTUs found exclusively on COVID-19-positive patients were *Prevotella* (13), followed by *Leptotrichia* (4) and *Streptococcus* Among the OTUs positively associated with COVID-19 severity, 3 were classified as members of the genus *Prevotella*, and 1 to a closely related genus, *Alloprevotella*
Rosas-Salazar et al. (75)	USA	Cross-sectional study Nasal swab Amplification V1–V2–V3 regions of the bacterial 16S rRNA	59 patients, 38 with confirmed SARS-CoV-2 infection; 21 HCs	Higher α-diversity in SARS-CoV-2 No differences in any of the measured β-diversity metrics between groups	HCs: -*Staphylococcus* (41.56%), *Corynebacterium*_1 (28.09%), *Moraxella* (8.48%), *Dolosigranulum* (3.56%), and *Neisseria* unclassified (1.98%) COVID-19: -*Corynebacterium*_1 (33.66%), *Staphylococcus* (29.34%), *Dolosigranulum* (5.29%), *Peptoniphilus* (3.91%), and *Lawsonella* (3.22%) COVID-19 with high viral load: -*Corynebacterium*_1 (35.69%), *Staphylococcus* (28.83%), *Peptoniphilus* (6.67%%), *Anaerococcus* (4.79%%), and *Bacteroides* (3.83%) COVID-19 with low viral load -*Corynebacterium*_1 (41.44%), *Staphylococcus* (20.75%), *Dolosigranulum* (12.30%), *Lawsonella* (4.50%), and *Peptoniphilus* (2.76%).	No correlation between SARS-CoV-2 viral load and diversity measures
Miao et al. (76)	China	Cross-sectional study BALF, ETA RNA and DNA extraction, reverse transcription, and use of DNA libraries	50 airway samples from 323 patients with confirmed SARS-CoV-2 infection	α-Diversity of critically severe COVID-19 patients is lower than non-intubated patients but similar to intubated non-COVID-19 group PCoA analysis: the greatest difference between non-intubated patients versus the other 2 groups with intubation	Higher relative abundance in COVID-19: -*Acinetobacter*, *Klebsiella*, *Pelomonas*, *Ralstonia*, and *Sphingomonas* Lower relative abundance in COVID-19: -*Actinomyces*, *Haemophilus*, *Neisseria*, *Prevotella*, *Streptococcus*, and *Veillonella*	
Braun et al. (77)	Israel	Cross-sectional study Nasopharyngeal swab Amplification V1–V2–V3 regions of the bacterial 16S rRNA	33 patients with confirmed or suspected SARS-CoV-2 infection	No difference in α-diversity (faith's phylogenetic diversity, Shannon) and evenness	No cluster identified	

(Continued)

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TABLE 1B Continued

ID	Country	Study characteristics	Population characteristics	α/β-diversity	Microbiome modifications: relative abundance analyses	Correlations and other findings
				(Wilcoxon rank sum test) Unweighted Unifrac-based PCoA: no clustering by COVID-19 test results		
Zhang et al. (78)	China	Cross-sectional study Nasopharyngeal swab and sputum RNA isolation, reverse transcription with N6 random primers after adaptor ligation with T4 ligase and library amplification, sequencing	187 patients, 62 with confirmed SARS-CoV-2 infection; 125 HCs	Shannon diversity index in sputum samples is significantly lower in COVID-19 cases	31 species in nasopharyngeal samples and 178 species in sputum samples with different abundance between COVID-19 and non-COVID-19 cases Most species less abundant in COVID-19 cases	
Mostafa et al. (79)	China	Cross-sectional study Nasopharyngeal swab cDNA sequencing for sequencing poly (A) RNA full-length transcripts	50 patients; 40 with confirmed SARS-CoV-2 infection; 10 with suspected SARS-CoV-2 infection Each patient was assigned a 4-point severity index according to the clinical presentation	Lower diversity in COVID-19 (Shannon diversity index, Chao-1 richness estimate, Simpson diversity)	*Propionibacteriaceae* are proportionately more abundant in COVID-19 *Corynebacterium accolens* decreased in COVID-19	
Merenstein et al. (80)	USA	Longitudinal study Oropharyngeal, nasopharyngeal, ETA, BALF Amplification V1–V2–V3 regions of the bacterial 16S rRNA	83 patients with confirmed SARS-CoV-2 infection; 42 HCs	Lower diversity in COVID-19	Upper airway microbiota comparison between COVID and HCs: -COVID-19 patients lower abundance of *Proteobacteria*, a greater abundance of *Bacteroidetes* Association with disease severity: -Different microbiota between COVID-19 patients with moderate/severe (WHO 4–6) and critical/fatal outcomes (WHO 7–10) -Decreased oropharyngeal *Proteobacteria* and *Actinobacteria* correlated with greater WHO score over the course of hospitalization -At the genus level, patients with more severe disease had significantly lower relative abundances of *Hemophilus*, *Actinomyces*, and *Neisseria*, all of which are abundant in the normal oropharyngeal microbiome	α-Diversity in oropharyngeal samples at the first time point correlated with COVID-19 severity, with lower diversity associated with higher severity The rate of change in oropharyngeal bacterial community structure was significantly greater in COVID-19 than in non-COVID subjects
Xu et al. (46)	China	Longitudinal study Throat swab Amplification V1–V2–V3 regions of the bacterial 16S rRNA	64 patients, 35 with confirmed SARS-CoV-2 infection, 10 with other diseases	Decrease in α-diversity, significantly lower richness and evenness in COVID-19	HCs: -Prevalence of genus *Bacteroides* and unclassified *Comamonadaceae* COVID-19, 4 community types, with a progressive imbalance of microbiota: -Type 1: *Alloprevotella* -Type 2: *Porphyromonas*, *Neisseria*, *Fusobacterium*, and unclassified *Bacteroidales* -Type 3: *Pseudomonas* -Type 4: *Saccharibacteria incertae sedis*, *Rothia*, and unclassified *Actinomycetales*	Among 22 COVID-19 adults who had specimens at 2 or more timepoints, over half (12, 54.5%) maintained a relatively stable microbiome community types

ICU, Intensive Care Unit; HCs, healthy controls; BALF, bronchoalveolar lavage fluid; CAP, community acquired pneumonia; OTU, operational taxonomic unit; ETA, endotracheal aspirate.

with the enrichment of opportunistic pathogenic bacteria such as *Saccharibacteria* and *Rothia* and a reduction of *Alloprevotella*. This shift toward dysbiosis shows how impaired homeostasis of inflammation pathways, a hallmark of the advanced stage of SARS-CoV-2 infection, affects microbial communities and can represent a biomarker of disease progression.

4 Microbiota dysbiosis in long-COVID

4.1 Microbiota changes in long-COVID

Few studies tried to investigate α-diversity alterations during long-COVID: in this setting, Zhuo et al. (52) reported a reduced Shannon index in a 15-patient cohort, followed up for 3 months with at least one persistent COVID-19 symptom. Coherently with these findings, in a 6-month follow-up, Liu et al. (57) have confirmed in long-COVID patients both a persistently reduced α-diversity (Shannon and Chao-1 indexes) and different gut microbiota clusters compared to controls. Notably, the subgroup who had COVID-19 at baseline without developing long-COVID did not show the same dysbiosis pattern. Reduced BPBs were reported in both COVID-19 subgroups compared to controls, but only in the long-COVID subgroup the microbial composition was different compared to controls at 6-month follow-up (Table 1A). Interestingly, the authors found no correlation between viral load in the gut and respiratory levels and long-COVID development at 6 months, nor did they find any effect of previous antibiotic intake. On the contrary, in the long-COVID subgroup, increased fecal relative abundance of opportunistic pathogens was positively associated with fatigue, respiratory and neuropsychiatric symptoms, while decreased other anti-inflammatory/BPB taxa was negatively correlated with long-COVID at 6 months. Coherently, Zhuo et al. (52) described both a negative correlation between some taxa (*Faecalibacterium prausnitzii*, *Intestinimonas butyriproducens*) and chronic respiratory symptoms as well as a positive correlation between *Proteobacteria* members and long-COVID symptoms.

4.2 Microbiota role in neurological and pulmonary symptoms

Persistent dysbiosis in long-COVID and its pathogenic role still need to be studied in humans, while rodent and non-human primate animal models of COVID-19 already showed long-term changes in both lung and gut microbiome (82, 83). The influence of gut microbiota on neurological symptoms, *via* the gut-brain axis, has been investigated in the animal model since the early decades of the new millennium. In murine models, Bercik at al. suggested that gut microbiota could influence the behavior of mice (84). Recently, Carloni et al. identified a closing in the choroid plexus vascular barrier during gut inflammation, suggesting a link between intestinal inflammation and neurologic/psychiatric symptoms, like a deficit in short-term memory and anxiety-like behavior (85). Moreover, a recent review summarized three different arms of inflammation for the gut-brain axis in a non-COVID-19 setting, where the systemic humoral pathway, cellular immune pathway, and neuronal pathway are involved (86). By translating these inflammatory patterns to the long-COVID setting, where gut dysbiosis persists at least after 6 months of follow-up, we can conclude that this microbial imbalance plays a role in maintaining both a chronic inflammatory status at the gut level and favoring the development of neurological/neuropsychiatric symptoms, as seen in the animal models mentioned above. However, it is not clear which immunologic pathway is dominant during long-COVID. It is plausible that several factors could coexist in the same disease model: (a) reduction in BPBs leading the butyrate loss linked to neuropsychiatric disorders (87); (b) development of the cytokine release syndrome during COVID-19, in particular with increased kynurenine:tryptophan ratio, already linked to depression syndrome (88); and (c) changes in L-DOPA production, regulated by ACE2 activation at the gut level (89).

There is still a lack of evidence on the role of microbiota dysbiosis in respiratory symptoms during long-COVID. Shortness of breath, frequently experienced by subjects after recovery from primary SARS-CoV-2 infection, could represent a clinical manifestation of the fibrosis secondary to chronic inflammation of lung parenchyma, leading to reduced total lung capacity. Such a condition is already linked to gut dysbiosis in non-COVID patients, as described in a recent review (90).

5 Relationship between gut dysbiosis, fecal SARS-CoV-2 replication, and immune-inflammation in COVID-19

It is well known that some microbial species can modulate ACE2 receptor expression and/or prevent SARS-CoV-2-ACE2 binding (67). Moreover, some studies found that the gut microbiota composition of COVID-19 patients, especially during hospitalization, is correlated with plasma concentrations of several cytokines, chemokines, and inflammation markers, suggesting that the gut microbiota could play a role in modulating host immune response and potentially influence disease severity and outcomes (43).

Interestingly, Zhuo et al. (50) studied α-diversity in a COVID-19 cohort stratified according to the presence of fever, discovering that COVID-19 patients with fever have shown a trend in reduced Chao-1 index compared to patients without fever, and similarly a β-diversity separation measured with Bray–Curtis. A negative correlation between PBPs and both inflammatory markers (9, 39, 43) and viral gut SARS-CoV-2 replication (40) was reported, despite the presence of GI disease and/or virological clearance. Interestingly, Zuo et al. (9) have discovered a negative correlation between *Bacteroides* taxa and fecal SARS-CoV-2 load and a positive correlation between *Erysipelotrichaceae* taxa and fecal SARS-CoV-2 replication. In contrast, Moreira-Rosario et al. (53) failed to see an association between fecal RNA viral replication and COVID-19 severity.

Wu et al. (46) reported a positive correlation between fecal SARS-CoV-2 replication and *P. copri*, *E. dolichum* taxa and a negative correlation between SARS-CoV-2 replication and other taxa like *Streptococcus*, *Dialister*, *Alistipes*, *Ruminococcus*, *Clostridium*, *Bifidobacterium*, and *Haemophylus* genera.

Finally, a longitudinal interventional study implementing fecal microbiota transplantation (FMT) in COVID-19 (45) described modulation of both gut microbiota core and peripheral lymphocyte subsets, with an increase in healthy taxa associated with a reduction in peripheral naïve B cells and an increase in memory B cells.

Data coming from clinical trials enrolling COVID-19 patients analyzing other possible drugs modulating gut microbiota, such as probiotics, are still scarce and not conclusive (91).

6 Conclusion

Microbiota homeostasis plays a role in human health and disease, and that applies to SARS-CoV-2 infection as well. During the last 2 years, several studies reported dysbiosis in COVID-19 patients for both gut and lung microbial composition. The main microbiota alterations that have been observed during COVID-19 were (a) significant reduction in α-diversity, already during the early phase of the disease and especially at the gut level, with a gradient from mild to severe clinical categories; (b) different β-diversity composition of microbiota core, characterized by a profile with higher facultative anaerobic bacteria and lower obligate anaerobic bacteria; and (c) possible connections between gut dysbiosis and peripheral inflammation markers, such as cytokines.

Data from longitudinal analyses currently available do not clearly show whether gut dysbiosis in COVID-19 ends with a complete functional restoration or if it does persist, posing the physiopathological premises for long-COVID. Indeed, a prolonged alteration of gut microbiota following the primary infection could contribute to causing some of the neurological and respiratory symptoms reported *via* the gut-brain and gut-lung axis. Further longitudinal studies are needed to characterize these conditions and assess the impact of prior comorbidity on the natural history of dysbiosis in SARS-CoV-2 infection.

Moreover, a knowledge gap regarding the role of FMT and other therapeutic approaches emerged, reinforcing the necessity for new evidence on the interaction of microbiota with host immunity. Such information is paramount to developing microbiota interventions aimed at improving COVID-19 and long-COVID outcomes.

Author contributions

Conceptualization: GA, LA, EP, and AB. Data analysis: GA, LA, EP, AT, and AP. Editing and supervision: AM, CA, AG, and AB. All authors have read and agreed to the published version of the manuscript.

Funding

This study was partially funded by the Italian Ministry of Health —Current Research IRCCS, the Fondazione Cariplo 2021-4236 LLC Network project, the Fondazione Bolton Hope Onlus "PREP-COVID" project, and the Associazione Nazionale per la Lotta contro l'AIDS (ANLAIDS). The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication. All authors declare no other competing interests.

Acknowledgments

We would like to acknowledge all the nurses, doctors, and clinical trial staff of the Infectious Diseases Unit of Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan.

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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